Méthodes analytiques pour le dosage des agents anticancéreux dans différentes matrices

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"Méthodes analytiques pour le dosage des agents anticancéreux dans différentes matrices"

Thèse
présentée à la Faculté des Sciences
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pour accéder à la fonction de privat-docent
par

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« Les machines un jour pourront résoudre tous les problèmes,
mais jamais aucune d'entre elles ne pourra en poser un. »
« Si vous ne pouvez expliquer un concept à un enfant de six ans,
c'est que vous ne le comprenez pas complètement. »

A. Einstein

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Table des matières

Résumé du travail de thèse (FR)

Parallèlement au vieillissement de la population, le nombre de patients présentant un cancer et sous traitement chimique ne cesse d'augmenter. Même si de nouvelles molécules médicamenteuses prometteuses souvent issues des thérapies dites ciblées sont développées, il n'en demeure pas moins que les agents anticancéreux conventionnels (petites molécules) constituent, actuellement, les chimiothérapies les plus prescrites. En raison de leur grande toxicité souvent associée à une faible stabilité chimique, la mise au point de méthodes capables de les qualifier et de les quantifier constitue un véritable challenge et explique le faible nombre de données scientifiques publiées dans ce domaine.

Ce travail de thèse s'intéresse à l'analyse des agents anticancéreux conventionnels en lien direct avec la reconstitution quotidienne des chimiothérapies en pharmacie hospitalière et s'articule autour de deux axes : la qualité des chimiothérapies produites et l'impact de ces agents hautement toxique sur l'Homme et l'environnement.

Une attention toute particulière a été portée sur l'exposition du personnel hospitalier via le développement d'une méthode analytique capable de détecter des traces d'agents anticancéreux sur des surfaces. Cette méthode LC-MS a permis l'évaluation des contaminations de surface dans les pharmacies de 24 hôpitaux suisses ainsi que sur les parois de flacons d'agents anticancéreux commercialisés sur le territoire helvétique. Des études ont pu être également menées sur l'efficacité de différentes stratégies visant à décontaminer les surfaces par le biais de cette méthode LC-MS.

L'évaluation de la contamination des effluents hospitaliers par certains agents anticancéreux a également été conduite incluant une méthodologie de priorisation pour le suivi de molécules médicamenteuses (y compris les agents anticancéreux) ainsi qu'une méthode ICP-MS détectant les traces de dérivés du platine dans les effluents.

Finalement, le développement de méthodes analytiques capables de renseigner la qualité des chimiothérapies produites par la pharmacie dans le cadre de contrôle qualité et d'étude de stabilité a été effectué.

Résumé du travail de thèse (EN)

In recent years, an increase of the chemotherapies administered, together with the growth of the number of patients with cancer, is clearly observed. Although promising new drug substances, often derived from targeted therapies, have been developed, conventional antineoplastic drugs (small molecules) are currently the most prescribed chemotherapies. Given their high toxicity often associated with a low chemical stability, the development of methods allowing their identification and quantification is a real challenge; which can explain the few of scientific data published in this field.

The aim of this work is the analysis of conventional antineoplastic agents in correlation with the daily chemotherapies preparation in hospital pharmacies and covers two different aspects: the quality of produced chemotherapies and the impact of these highly toxic agents on human people and environment.

A particular attention is paid on the exposure of healthcare professionals via the development of an analytical method allowing the detection of antineoplastic drugs traces on different surfaces. This LC-MS method permitted the evaluation of surface contaminations in pharmacies of 24 Swiss hospitals and on vials containing different antineoplastic drugs available on the Swiss market. Studies on the efficacy of different strategies for the surface decontamination were achieved through this LC-MS method.

The evaluation of hospital wastewaters contamination by antineoplastic drugs were also performed including a prioritization methodology for the monitoring of pharmaceutical ingredients (with antineoplastic drugs) and an ICP-MS method allowing the determination of platinum derivatives compounds in wastewaters.

Finally, the development of analytical methods that can inform the quality of chemotherapies produced by a hospital pharmacy for the quality control and stability studies was carried out.

Activités d'enseignement

2 h: **<u>Analyse et pharmacopées</u>**
Adressé aux étudiants en 3ème année à l'Ecole de Pharmacie Genève-Lausanne

Ce cours est consacré à l'application des techniques analytiques dans le contrôle qualité des formulations pharmaceutiques dans le respect des pharmacopées nationales et internationales en vigueur.

10 h : **<u>Les agents anticancéreux : manipulations et aspects analytiques</u>**
Programme doctoral proposé par la Section des Sciences Pharmaceutiques de l'Université de Genève

Ce cours se propose dans un premier temps de faire connaître la problématique des agents anticancéreux de leur préparation à leur élimination. Puis, une attention particulière est donnée aux différents outils analytiques utilisés pour la mise en œuvre de mesures permettant de limiter/contrôler au maximum la toxicité de telles molécules et de s'assurer de la qualité des chimiothérapies produites en milieu hospitalier.

Détails du cours intitulé « agents anticancéreux : manipulations et aspects analytiques » :

2 h : Les agents anticancéreux conventionnels et biologiques : propriétés physico-chimiques
Cette première partie est consacrée aux différentes classes de molécules (conventionnelles et biologiques) utilisées dans le traitement de cancers. Les propriétés toxiques et physico-chimiques de ces molécules sont discutées.

1 h : Préparation des agents anticancéreux en vue d'une administration au patient
Cette seconde partie est dédiée aux différentes formulations d'agents thérapeutiques mis sur le marché. Le flux de ces formulations en milieu hospitalier est décrit de l'étape de reconstitution à l'administration au patient. Les recommandations lors de la manipulation de ces formulations sont abordées.

2 h : Exposition aux agents anticancéreux : cycle de vie des agents anticancéreux
Au cours de cette partie, l'exposition du personnel soignant ou tout autre être vivant (environnement) en contact avec les agents anticancéreux est décrite sur la base d'études publiées. Le comportement de ces substances dans l'environnement est abordé. Les méthodes analytiques utilisées pour rechercher ces molécules et leurs produits associés sous forme de trace sont reportées en discutant de leurs avantages et de leurs limitations.

2 x 2h : Contrôle qualité des agents anticancéreux
- Cas des molécules conventionnelles : cette partie est consacrée aux différentes méthodes analytiques séparatives et non séparatives mises en œuvre lors du contrôle qualité de formulations produites à base d'agents anticancéreux conventionnels (principe, avantages et limitations) ;
- Cas des molécules biologiques : la problématique analytique des agents biologiques est soulevée. L'utilisation de méthodes analytiques orthogonales est décrite.

1h : Stabilité des agents anticancéreux
Les différentes sources d'informations sur la stabilité des agents anticancéreux en solutions sont décrites. Les outils aussi bien bibliographiques (ouvrages spécialisés), informatiques (logiciels) et analytiques (étude de stabilité) sont détaillés.

Avant propos

En 2014, l'Organisation Mondiale de la Santé (OMS) a déclaré que le cancer était une cause majeure de décès dans le monde (8.2 millions de décès en 2012) et que le nombre de cas de cancers par an devrait augmenter de 14 millions en 2012 à 22 millions au cours de ces deux prochaines décennies. Ce pronostic accablant se traduit par une consommation croissante d'agents anticancéreux qui constituent avec la chirurgie et/ou la radiothérapie les principaux traitements contre le cancer. Deux classes de molécules de différentes générations se distinguent au sein de l'arsenal des chimiothérapies : les molécules dites conventionnelles et les molécules issues de la thérapie ciblée. La chimiothérapie conventionnelle trouve son origine dans les armes chimiques développées au début du XXème siècle. Une des premières familles d'agents anticancéreux, encore administrée aujourd'hui, est issue de l'Ypérite ou gaz moutarde employé sur les champs de bataille de la 1ère guerre mondiale. Un effet secondaire observé de ce gaz, à savoir une myélosuppression, constitue le point de départ du développement des premiers agents anticancéreux dirigés contre les leucémies. Fort de ces recherches, les Etats-Unis lancent plusieurs programmes nationaux, après la seconde guerre mondiale, dont les objectifs sont le développement de nouvelles molécules pour le traitement de cancers. S'ensuit alors une course qui durera 20 ans (jusque dans les années 60) vers la découverte de nouveaux agents anticancéreux comme les antifolates (fin des années 1940), les analogues puriques et pyrimidiques (années 1950) ou les antibiotiques (années 1960). La majeure partie des molécules anticancéreuses administrées aujourd'hui a été développée durant cette période. La guerre contre le cancer connait une seconde offensive avec l'apparition des thérapies ciblées dans les années 1990. Toutefois, malgré la découverte prometteuse de ces agents qui agissent comme des armes de précision, les armes de destruction massive que constituent les molécules anticancéreuses conventionnelles restent les molécules de première ligne pour le traitement médicamenteux d'un grand nombre de cancers.

La haute réactivité chimique des agents anticancéreux conventionnels est responsable non seulement de leur action cytotoxique mais constitue aussi leur principal inconvénient. En effet, associée à une forte toxicité, cette réactivité chimique constitue un véritable obstacle à la mise au point de formulations

pharmaceutiques stables conduisant à l'effet thérapeutique recherché. Administrée dans la majeure partie des cas par voie injectable, la stratégie employée est de proposer une forme pharmaceutique intermédiaire comme les formes sèches lyophilisées ou les solutions fortement concentrées dissoutes dans un solvant compatible. Une étape de « reconstitution » qui permet de transformer la formulation intermédiaire en formulation prête à être administrée au patient est alors indispensable. Cette étape est généralement effectuée extemporanément par le personnel hospitalier (pharmacie centrale ou infirmière). La sécurité et la qualité qui entoure la manipulation de ces molécules hautement toxiques revêtent un caractère primordial pour l'homme et l'environnement.

Comme pour les autres molécules médicamenteuses, les agents anticancéreux ne dérogent pas à la règle et font l'objet de méthodes analytiques dans de nombreux domaines allant de la conception du médicament (ex : découverte de la molécule, essais cliniques…) à son élimination (biotransformation, taux d'abattement dans les stations d'épuration…) en passant par le suivi thérapeutique et le contrôle qualité pharmaceutique. Mais encore une fois, leur instabilité chimique (et/ou leur toxicité) rend le développement de méthodes d'analyses délicat.

L'intérêt porté par le laboratoire de Contrôle Qualité de la Pharmacie des Hôpitaux Universitaires de Genève (HUG) pour l'analyse des agents anticancéreux est en lien direct avec la reconstitution quotidienne de chimiothérapies à la pharmacie (une centaine de préparations par jour). Nos domaines de recherche s'articulent autour de deux axes : la sécurité des personnes en contact avec les agents anticancéreux et la qualité des chimiothérapies. Une attention toute particulière a été portée sur l'exposition du personnel hospitalier via le développement de méthodes analytiques capables de détecter des traces d'agents anticancéreux sur des surfaces (estimation du taux de contamination, étude sur les moyens possibles de décontamination). L'évaluation de la contamination des effluents hospitaliers par certains agents anticancéreux a également été conduite. Finalement, le développement de méthodes analytiques capables de renseigner la qualité des chimiothérapies produites par la pharmacie dans le cadre de contrôle qualité et d'étude de stabilité a été effectué.

Présentation de la thèse

La thèse présentée se divise en plusieurs parties. La première (Chapitre 1) présente le cycle de vie d'une chimiothérapie en milieu hospitalier : de sa fabrication à son élimination dans les effluents hospitaliers en portant une attention particulière sur l'impact des agents anticancéreux conventionnels sur l'Homme et l'environnement. Le chapitre 2 est consacré à la bibliographie des différentes méthodes analytiques publiées dans la littérature pour l'analyse des agents anticancéreux conventionnels en fonction des différentes familles de molécules et du domaine d'application. Les chapitres 3, 4 et 5 reportent les méthodes développées au cours de ce travail de thèse ainsi que leur application selon la matrice considérée (formulations pharmaceutiques, échantillons de surfaces et effluents). La dernière partie (Chapitre 6) comprend les conclusions et les perspectives de cette thèse.

Les principaux sujets abordés au cours de ce travail sont reportés ci-dessous :

✧ L'analyse des agents anticancéreux

Le cœur de ce travail de thèse est l'analyse des agents anticancéreux. Une bibliographie qui a fait l'objet de deux publications (article Ia en 2011 et article Ib en 2017) est entièrement consacrée aux différentes méthodes analytiques publiées pour la détermination des différentes classes d'agents anticancéreux conventionnels.

✧ Le développement de méthodes analytiques pour le dosage d'agents anticancéreux dans différents domaines

- Contrôle qualité des chimiothérapies reconstituées : une méthode MEKC-UV a été développée pour l'analyse des chimiothérapies reconstituées contenant les dérivés du platine (article II) et une méthode LC-MS a permis d'évaluer la stabilité des formulations de busulfan reconstituées à la pharmacie des HUG (article III) ;

- Evaluation des contaminations de surface : une méthode de prélèvement de surface couplée à un dosage par LC-MS a été développée et validée pour la

quantification de 10 agents anticancéreux conventionnels (articles IV et V). Cette méthode a été appliquée pour l'évaluation des contaminations de surface dans les pharmacies hospitalières de 24 hôpitaux suisses (article VI) ainsi que pour l'évaluation de la contamination par des agents anticancéreux sur les parois de flacons commercialisés sur le territoire helvétique (article VII). La méthode développée a également permis d'étudier l'efficacité de différentes stratégies visant à une décontamination de surface dans des conditions expérimentales (article VIII) et réelles (article IX) ;

- Evaluation de la contamination par des agents anticancéreux d'effluents hospitaliers : dans un premier temps une méthodologie de priorisation pour le suivi de molécules médicamenteuses (y compris les agents anticancéreux) dans les effluents hospitaliers a été conduite (article X) puis une méthode ICP-MS a permis de détecter des traces de dérivés du platine dans les effluents hospitaliers (article XI).

Communications scientifiques

Ce travail de thèse a fait l'objet d'articles publiés dans des revues scientifiques ainsi que de présentations sous forme de conférences orales et d'affiches dans le cadre de congrès.

Articles publiés

Ia. Analysis of anticancer drugs: A review.

S. Nussbaumer, P. Bonnabry, J.-L. Veuthey, S. Fleury-Souverain. *Talanta* 2011 ; 85 : 2265-2289

Ib. Antineoplastic drugs and their analysis: a state-of-the-art review.

N. Guichard, D. Guillarme, P. Bonnabry, S. Fleury-Souverain. *Analyst* 2017; 142: 2273-2321

II. Quality control of pharmaceutical formulations containing cisplatin, carboplatin and oxaliplatin by micellar and microemulsion electrokinetic chromatography (MEKC, MEEKC).

S. Nussbaumer, S. Fleury-Souverain, J. Schappler, S. Rudaz, J.-L. Veuthey, P. Bonnabry. *Journal of Pharmaceutical and Biomedical Analysis* 2011 ; 55 : 253-258

III. Stability determination of busulfan diluted solutions in polypropylene containers by UHPLC-MS

N. Guichard, P. Bonnabry, S. Rudaz, S. Fleury-Souverain. *American Journal of Health-System Pharmacy*. Accepted

IV. Simultaneous quantification of ten cytotoxic drugs by a validated LC-ESI-MS/MS method.

S. Nussbaumer, S. Fleury-Souverain, P. Antinori, F. Sadeghipour, D.F. Hochstrasser, P. Bonnabry, J.-L. Veuthey, L. Geiser. *Analytical and Bioanalytical Chemistry* 2010; 398: 3033-3042

V. **Wipe sampling procedure coupled to LC-MS/MS analysis for the simultaneous determination of 10 cytotoxic drugs on different surfaces.**
S. Nussbaumer, L. Geiser, F. Sadeghipour, D. Hochstrasser, P. Bonnabry, J.-L. Veuthey, S. Fleury-Souverain. *Analytical and Bioanalytical Chemistry* 2012 ; 402 : 2499-2509

VI. **Evaluation of chemical contamination of surfaces during the preparation of chemotherapies in 24 hospital pharmacies.**
S. Fleury-Souverain, M. Mattiuzzo, F. Mehl, S. Nussbaumer, L. Bouchoud, L. Falaschi, M. Gex-Fabri, S. Rudaz, F. Sadeghipour, P. Bonnabry. *European Journal of Hospital Pharmacy* 2015; 22: 333-341

VII. **Determination of the external contamination and cross-contamination by cytotoxic drugs on the surfaces of vials available on the Swiss market.**
S. Fleury-Souverain, S. Nussbaumer, M. Mattiuzzo, P. Bonnabry. *Journal of Oncology Pharmacy Practice* 2014 ; 20 : 100-111

VIII. **Evaluation of decontamination efficacy of cleaning solutions on stainless steel and glass surfaces contaminated by 10 antineoplastic agents.**
T. Queruau Lamerie, S. Nussbaumer, B. Decaudin, S. Fleury-Souverain, J.-F. Goossens, P. Bonnabry, P. Odou. *The Annals of Occupational Hygiene* 2013 ; 57 : 456-469

IX. **Efficacy of two cleaning solutions for the decontamination of 10 antineoplastic agents in the biosafety cabinets of a hospital pharmacy.**
M. Anastasi, S. Rudaz, T. Queruau Lamerie, P. Odou, P. Bonnabry, S. Fleury-Souverain. *The Annals of Occupational Hygiene* 2015 ; 59 ; 895-908

X. **Priorization methodology for the monitoring of active pharmaceutical ingredients in hospital effluents.**
S. Daouk, N. Chèvre, N. Vernaz, P. Bonnabry, P. Dayer, Y. Daali, S. Fleury-Souverain. *Journal of Environmental Management* 2015 ; 160 : 324-332

XI. Dynamics of active pharmaceutical ingredients loads in a Swiss university hospital wastewaters and prediction of the related environmental risk for the aquatic ecosystems.

S. Daouk, N. Chèvre, N. Vernaz, C. Widmer, Y. Daali, S. Fleury-Souverain. *Science of the Total Environment* 2016 ; 547 : 244-253

Présentations orales

- **Chemical contamination during preparation of chemotherapies: overview in Swiss hospital pharmacies**

  M. Mattiuzzo, S. Nussbaumer, F. Sadeghipour, S. Fleury-Souverain, P. Bonnabry, 1er Congrès Suisse des pharmaciens, 30 novembre-1er décembre 2011, Interlaken (Suisse)

- **Workshop: Final preparation controls**

  P. Bourget, S. Fleury-Souverain, P. Odou, 14èmes journées du GERPAC, 5-7 octobre 2011, Presqu'île de Giens (France)

- **Etat des lieux de la contamination cytotoxique au sein d'une unité de reconstitution**

  S. Nussbaumer, M. Mattiuzzo, L. Geiser, F. Sadeghipour, S. Fleury-Souverain, P. Bonnabry, 14èmes journées du GERPAC, 5-7 octobre 2011, Presqu'île de Giens (France)

- **Evaluation de l'efficacité d'agents de décontamination de surface sur 10 agents antinéoplastiques**

  T. Queruau Lamerie, S. Nussbaumer, P. Odou, J.F. Goossens, S. Fleury-Souverain, P. Bonnabry, 14èmes journées du GERPAC, 5-7 octobre 2011, Presqu'île de Giens (France)

- **Simultaneous quantification of 10 cytotoxic drugs by a validated LC-ESI-MS/MS method**

  S. Nussbaumer, S. Fleury-Souverain, P. Antonori, F. Sadeghipour, P. Bonnabry, J.-L. Veuthey, L. Geiser, Drug Analysis 2010 , 21-24 septembre 2010, Antwerp (Belgique)

Affiches

- **Evaluation d'un dispositif pour le contrôle qualité des chimiothérapies au sein de l'unité de production.**
  S. Senhaji, A Exquis, L. Carrez, L. Falaschi, S. Fleury-Souverain, P. Bonnabry. GSASA, 24-27 novembre 2015, Zurich (Suisse)

- **Evaluation de la contamination chimique externe de flacons de cytotoxiques disponibles sur le marché suisse**
  S. Fleury-Souverain, S. Nussbaumer, M. Mattiuzzo, P. Bonnabry, 17èmes Journées Franco-Suisse de Pharmacie Hospitalière, 15-16 mars 2012, Lons-Le-Saulnier (France)
  2ème Prix

- **Analyses de traces de substances cytotoxiques sur des surfaces**
  S. Nussbaumer, S. Fleury-Souverain, M. Mattiuzzo, F. Sadeghipour, L. Geiser, J.-L. Veuthey, P. Bonnabry, 1er Congrès Suisse des pharmaciens, 30 novembre-1er décembre 2011, Interlaken (Suisse)
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- **Contamination chimique lors de la préparation des chimiothérapies: état des lieux dans différentes pharmacies d'hôpitaux Suisses**
  M. Mattiuzzo, S. Nussbaumer, F. Sadeghipour, S. Fleury-Souverain, P. Bonnabry, 1er Congrès Suisse des pharmaciens, 30 novembre-1er décembre 2011, Interlaken (Suisse)

Abréviations

AD	Détection ampérométrique
ADN	Acide désoxyribonucléique
ARN	Acide ribonucléique
ASHP	American Society of Health-System Pharmacists
ATP	Adénosine triphosphate
BCNU	Carmustine
CCM	Chromatographie sur couche mince
CCNU	Lomustine
CE	Electrophorèse capillaire
CEP	Concentration environnementale prédite
CMR	Cancérogènes, mutagènes ou toxiques pour la reproduction
DMA	Diméthylacétamide
EC	Electrochimie
ECD	Détecteur par capture d'électrons
ECL	Electrochémiluminescence
EGFR	Récepteurs du facteur de croissance épidermidique
ELSD	Détecteur évaporatif à diffusion de la lumière
FFP	Filtering facepiece particules
FIA	Flow injection analysis
FD	Fluorescence
GC	Chromatographie gazeuse
HEPA	High efficiency particulate air
HILIC	Chromatographie à interaction hydrophile
ICP-MS	Spectrométrie de masse à plasma induit
ISOPP	International Society of Oncology Pharmacy Practioners
IR	Infrarouge
LC	Chromatographie liquide
LIF	Fluorescence induite par laser
LLE	Extraction liquide-liquide
LOD	Limite de détection
LOQ	Limite de quantification
MECK	Electrophorèse capillaire électrocinétique micellaire

MEECK	Chromatographie électrocinétique en microémulsion
MS	Spectrométrie de masse
MNNG	N-méthyl-N'-nitro-N-nitrosoguanidine
MNU	N-méthyl-N-nitrosourée
mTOR	Mammalian target of rapamycine
NACE	Electrophorèse capillaire en milieux non aqueux
NaCl	Chlorure de sodium
NaOH	Hydroxyde de sodium
NIOSH	National Institute for Occupational Safety and Health
NPD	Détecteur azote-phosphore
OMS	Organisation Mondiale de la Santé
OSHA	Occupational Safety and Health Administration
PP	Précipitation des protéines
PSM	Poste de sécurité microbiologique
PVA	Polyalcool de vinyle
SDS	Sodium dodécyl sulfate
SPE	Extraction sur support solide
SUVA	Caisse nationale suisse d'assurance en cas d'accidents
TK	Tyrosine kinase
TPMT	Thiopurine méthyltransférase
UV	Ultraviolet
VEGFR	Récepteurs du facteur de croissance de l'endothélium vasculaire
5FU	5-fluorouracile

Chapitre 1 : Introduction

Actuellement, plus d'une centaine de substances médicamenteuses possédant une action anticancéreuse composent l'arsenal chimique des oncologues. Deux types d'agents anticancéreux peuvent être distingués. Les molécules dites « conventionnelles » qui ont vu le jour, pour la plupart, il y a plusieurs décennies (de 10 à 60 ans) et les molécules issues des thérapies ciblées apparues au cours de ces vingt dernières années. Même si l'apparition de ces agents de plus récente génération apporte un nouvel espoir dans le combat contre le cancer, en pointant sélectivement le cœur des mécanismes de l'oncogenèse, il n'en demeure pas moins que les molécules conventionnelles restent, actuellement, les armes lourdes de premières lignes. Seules ces dernières sont considérées dans ce travail.

Ce chapitre introduit le cycle de vie des agents anticancéreux conventionnels au sein d'une structure de pharmacie hospitalière. Dans un premier temps, la fabrication des chimiothérapies est présentée en mettant l'accent sur les notions de sécurité liées à leur manipulation et leur impact sur l'Homme et l'environnement. Ensuite, le contrôle qualité des chimiothérapies ainsi que leur stabilité sont abordées.

1. La préparation des chimiothérapies

1.1. Classification des agents anticancéreux

Les agents anticancéreux sont des molécules présentant une toxicité qui suscite un intérêt tout particulier en ce qui concerne leur manipulation par l'homme et leur impact sur l'environnement. En parallèle de l'élaboration de mesures ou de recommandations visant à réduire le risque lié à l'exposition de telles molécules, plusieurs classifications/marquage des agents anticancéreux en fonction de leur degré de dangerosité ont vu le jour. Il faut savoir que les agents anticancéreux sont marqués au fer rouge comme médicaments CMR à savoir possédant des propriétés cancérogènes (C), mutagènes (M) ou toxiques pour la reproduction (R). Le Centre International de Recherche sur le Cancer affine la classification en fonction de l'effet cancérogène de la molécule sur la base d'études animales et l'observation d'apparition de tumeurs secondaires chez des patients traités. Les groupes suivants

(qui comprennent toutes substances et pas seulement les agents anticancéreux) sont alors distingués :

- Groupe 1 : Cancérogène pour l'homme (exemples : Busulfan, Etoposide, Thiotépa)
- Groupe 2A : Cancérogène probable pour l'homme (exemples : azacitidine, cisplatine, procarbazine)
- Groupe 2B : Cancerogène possible pour l'homme (exemples : amsacrine, dacarbazine, bléomycine)
- Groupe 3 : ne peut être classé sur son caractère cancérogène pour l'homme (exemples : méthotrexate, 6-mercaptopurine, vincristine)
- Groupe 4 : probablement non cancérogène pour l'homme.

1.2. Naissance des recommandations

Un des premiers questionnements sur le risque lié à la manipulation des agents cytotoxiques est abordé en 1970 avec une lettre à l'éditeur d'un collaborateur du service d'hématologie d'un hôpital pédiatrique (Oakland, Californie, Etats-Unis) qui manipule et administre des chimiothérapies injectables aux patients (1). Quelques années plus tard, plusieurs études démontrent l'exposition du personnel soignant à des agents anticancéreux (2-6). Dès lors différentes recommandations visant à garantir la sécurité des manipulations de médicaments dangereux apparaissent dans plusieurs pays. Parmi les premiers à éditer un tel rapport figure l'OSHA (Occupational Safety and Health Administration), dont la mission première est la protection de la santé des travailleurs aux Etats-Unis (7). Leurs recommandations, publiées en 1986, décrivent les équipements et les procédures à utiliser pour réduire toute exposition du personnel de santé aux agents anticancéreux. D'autres textes conducteurs voient le jour par la suite comme celui du NIOSH (National Institute for Occupationnal Safety and Health) en 2004 (8) qui fera l'objet de compléments d'information par l'ASHP (American Society of Health-System Pharmacists) en 2006 (9). Les Etats-Unis ne sont pas les seuls à s'inquiéter pour les manipulateurs d'agents anticancéreux : la Norvège, la Suède, l'Angleterre, l'Espagne établissent eux aussi des recommandations. Des associations de professionnels ont également édités des recommandations dont celle de l'ISOPP (International Society of Oncology Pharmacy Practioners) éditées en 2007 (10). La Suisse ne fait pas figure

d'exception. Les recommandations prévalant sur le territoire Helvétique sont issues de la Caisse nationale suisse d'assurance en cas d'accidents (la SUVA) qui a la charge de surveiller l'application de l'Ordonnance sur la Prévention des Accidents. La première édition est publiée en 1987 après consultation de la Société Suisse d'Oncologie et du Groupe Suisse de Recherche Clinique sur le Cancer. La version actuelle intitulée « Sécurité dans l'emploi des cytostatiques » a pour lignes directives la mise en place de mesures de protection techniques, organisationnelles et personnelles avec pour objectif de minimiser les risques liés à l'utilisation des agents anticancéreux (11).

1.3. Sécurisation de la procédure de préparation

Toutes les recommandations s'accordent sur le confinement de la préparation qui passe à la fois par l'utilisation d'une enceinte adaptée et la centralisation de cette manipulation. Les mesures de protection personnelle comme l'utilisation de gants adaptés ainsi que des mesures plus opérationnelles comme l'emploi de systèmes de transfert fermés font partie des dispositions incontournables dans la manipulation des chimiothérapies.

1.3.1 Sécurité organisationnelle

La centralisation des chimiothérapies est indubitablement la première mesure de sécurité liée à leur préparation. Elle consiste à confier la préparation des chimiothérapies à une seule structure bénéficiant des compétences et des moyens nécessaires à la réalisation de cette mission à risque. L'objectif final est alors d'assurer la qualité (microbiologique et physico-chimique) de la formulation tout en protégeant les opérateurs et l'environnement vis-à-vis d'une exposition à des agents anticancéreux. Dans ces conditions, le personnel soignant des unités de soin est déchargé de cette tache pour laquelle il ne dispose pas véritablement de conditions sures et de compétences adéquates pour la réaliser. Ces structures spécialisées sont pour la plupart les pharmacies d'hôpitaux. La centralisation, fortement prônée par toutes les recommandations, se veut également une stratégie économique permettant de mutualiser les ressources (humaines et matérielles) (12, 13). Toutefois, il est important de préciser que la centralisation permet de réduire les

risques liés à la préparation des chimiothérapies (qualité de la formulation produite et exposition du personnel) que si elle est accompagnée de mesures complémentaires comme une formation initiale et continue des opérateurs ainsi qu'une mise en place de procédures permettant de sécuriser l'étape de préparation. Dans ces procédures, se côtoient différents aspects de la préparation des chimiothérapies comme l'environnement (PSM et/ou isolateurs dans des zones à atmosphère contrôlée), les équipements de protection personnelle (gants, masque, blouse, lunettes…) et les techniques de travail.

1.3.2. Postes de sécurité microbiologique et isolateurs

Les premières recommandations s'appuient sur l'utilisation d'enceintes sécurisées pour la manipulation des agents anticancéreux. Il est généralement préconisé un poste de sécurité microbiologique (PSM) de classe II type B2, un PSM de classe III ou encore un isolateur (9, 14). Il s'agit d'enceintes ventilées visant à protéger l'environnement et l'opérateur d'agents toxiques. En ce qui concerne les PSM, il est important de préciser que leur conception première vise à protéger l'environnement ainsi que l'opérateur d'agents biologiques pathogènes. En effet, une des premières enceintes de protection microbiologique a été construite par Robert Kohr au début du XXème siècle pour lui permettre de travailler en sécurité avec les germes responsables de la tuberculose, de l'anthrax et du choléra. Ce n'est que dans les années 1950 qu'apparaissent les premiers PSM sur le marché et depuis ils ne cessent d'évoluer parallèlement aux progrès de la microbiotechnologie. Actuellement, les PSM sont classés en fonction de leur performance et du niveau de protection biologique atteint (norme européenne EN 12469). Leurs principales caractéristiques sont reportées dans le tableau 1.

A l'instar des PSM de classe I, les PSM de classe II sont des enceintes ouvertes partiellement sur le devant qui possèdent un flux d'air dirigé vers l'intérieur du poste (flux entrant). La principale différence réside dans l'écoulement d'un flux d'air supplémentaire unidirectionnel descendant maintenant une dépression dans l'espace de travail et donc empêchant la sortie de polluant vers l'opérateur. La présence d'un ventilateur externe permettant l'évacuation de l'air vers l'environnement extérieur à travers un système de conduits définit les PSM de Classe II type B. En outre, dans le

cas d'un PSM classe II type B2, l'air émanant des flux entrants et descendants est totalement évacué via des filtres HEPA (High Efficiency Particulate Air) vers les conduits extérieurs, contrairement à un PSM Classe II type B1 où 30% de l'air est recyclé vers l'espace de travail sous forme de flux descendants (via un filtre).

Tableau 1: Principales caractéristiques des PSM

PSM Classe	Caractéristiques
PSM Classe I	• Ouverture frontale • Flux entrant uniquement • 100% air évacué filtré • 1 filtre HEPA sur la sortie d'air
PSM Classe II	• Ouverture frontale • Flux entrant et flux descendant • Type IIA : 70 % air évacué filtré • Type IIB : 100% air évacué filtré • 2 à 3 filtres HEPA
PSM Classe III	• Enceinte entièrement fermée – Manchons/gants • Air entrant filtré et évacuation de l'air contaminé sur filtre(s) • 100% air évacué filtré • 2 à 3 filtres HEPA

Les PSM de classe III sont des enceintes entièrement closes équipées de manchons et de gants qui permettent à l'opérateur de manipuler à l'intérieur du PSM. Ainsi la protection de l'opérateur est améliorée. L'air pénètre dans l'enceinte après filtration sur HEPA en flux unidirectionnel et est complètement évacué après filtration. Ainsi ces systèmes fonctionnent généralement en dépression (pression négative) et le matériel transite par des sas d'entrée (décontamination manuelle) et de sortie. Apparus dans les années 1980, les isolateurs sont également des enceintes complètement closes mais ils se distinguent des PSM de par le fait que ces enceintes peuvent être décontaminées en utilisant des méthodes reproductibles et validées (autrement dit une stérilisation par gaz). La « décontamination » des PSM de classe III se fait manuellement lors de l'ouverture de l'enceinte. Les isolateurs évoluent en général en pression positive et le matériel est introduit dans l'enceinte via des sas où une stérilisation par gaz a lieu. S'il apparaît évident que l'utilisation d'une enceinte close est préférable pour la sécurité du produit et de l'opérateur par rapport à une enceinte partiellement ouverte, il en va autrement du choix du type d'enceinte close. Deux critères sont alors à prendre en considération : la qualité microbiologique du produit et la protection de l'opérateur et de l'environnement vis-à-

vis des agents anticancéreux. Les bonnes pratiques de fabrication aseptiques se tournent indéniablement vers des systèmes à pression positive (air contaminé n'a pas accès à l'espace de travail). Par contre, la manipulation d'agents toxiques est préférable dans des enceintes à pression négative permettant de les confiner dans un espace réduit. Or tout le dilemme réside dans le fait que les chimiothérapies sont des préparations stériles hautement toxiques ; ce qui est sujet à discussion et ne permet pas de trancher clairement. Parmi les utilisateurs de PSM de classe II, figurent les Etats-Unis, le Canada et l'Allemagne. Les britanniques et les scandinaves utilisent préférentiellement des isolateurs. Quand à la Suisse, les principaux hôpitaux disposent de PSM de classe III et d'isolateurs (11).

1.3.3. Sécurité personnelle

La première barrière de protection entre la personne qui manipule et l'agent cytotoxique est le gant. Si le choix des gants est plus ou moins bien établi pour la manipulation d'agents chimiques conventionnels, il en va autrement pour les agents anticancéreux. En effet, d'une manière générale, la famille d'agents chimiques manipulée conditionne le type de gants à utiliser. Par exemple, les gants PVA (polyalcool de vinyle) sont couramment employés pour la manipulation des hydrocarbures tandis que les gants de type butyle offrent une protection élevée vis-à-vis des acides forts. Etant donné l'éventail relativement large des propriétés physico-chimiques des anticancéreux, il est délicat de définir un type de gants en particulier pour leur manipulation. Ces 30 dernières années, plusieurs études ont testé la perméation de différents gants vis-à-vis de plusieurs agents anticancéreux (15-21). D'une manière générale, les résultats obtenus démontrent que les matériaux comme le latex, le nitrile ou encore le néoprène offrent une meilleure protection que le vinyle. En outre, tous s'accordent sur le fait que l'épaisseur du gant est un élément déterminant dans la protection et que deux paires de gants sont fortement recommandés avec des changements toutes les 30 minutes. Il est également démontré que la perméation est également fonction des propriétés physico-chimiques des molécules testées. Ainsi, les molécules fortement lipophiles comme la carmustine diffusent plus facilement à travers les gants. Toutefois, il est important de préciser que les modes opératoires utilisés divergent entre les études reportées sur plusieurs points comme le type de molécules anticancéreuses, les concentrations, la

partie du gant (paume, doigt ou manchette) et la température testées. Même si ces études se veulent mimer au mieux la réalité, il est difficile d'obtenir toujours les mêmes conditions. Des normes visant à harmoniser les méthodes d'essais des gants sont apparues dans les années 2000. En Europe, la norme EN16523-1 qui concerne la détermination de la résistance des matériaux aux produits chimiques potentiellement dangereux en cas de contact continu est entrée en vigueur en 2015 et remplace la norme EN374-3 de 2004 (22). Depuis 2005, aux Etats-Unis, la norme ASTM D6978-05 s'applique à poser les pratiques standards pour la détermination de la perméation des gants médicaux par des agents anticancéreux (23). La norme européenne revêt un caractère très général car elle concerne aussi bien tous les matériaux de protection comme les vêtements, les gants et les chaussures que tous les produits chimiques. L'évaluation établie par la norme européenne consiste à mesurer le flux de perméation d'un matériau entre un milieu contenant un produit chimique et un milieu collecteur gazeux ou liquide à 23°C dans une cellule de perméation clairement définie. Des prélèvements du compartiment collecteur sont effectués régulièrement et analysés (par une technique non définie). Une valeur limite de 1.0 voire 0.1 $\mu g/cm^2/min$ est fixée. Quant à la norme américaine, elle se veut plus spécifique car elle ne s'applique qu'aux gants et qu'aux agents anticancéreux. L'évaluation repose sur la mesure du flux de perméation à 35°C de 7 molécules anticancéreuses contenues dans un compartiment donneur à des concentrations données (auxquelles peuvent s'ajouter d'autres molécules anticancéreuses à des concentrations définies) à travers le gant à tester (la partie la plus fine : paume et manchette) vers un compartiment collecteur (solution aqueuse) où des prélèvements réguliers sont effectués en vue d'une analyse quantitative. La valeur limite est 100 à 10 fois plus faible que celle donnée par la norme européenne puisqu'elle est de l'ordre de 0.01 $\mu g/cm^2/min$ Les utilisateurs disposent donc de deux normes qui peuvent orienter leur choix de gants pour la manipulation des agents cytotoxiques. Toutefois, il convient de signaler que les gants utilisés lors de la préparation des chimiothérapies ont également pour objectif la protection du produit. Les gants doivent donc être compatibles avec la fabrication de formulations stériles c'est-à-dire stériles eux-mêmes, apyrogènes et ne pas apporter de particules. Ils doivent être également adaptés aux enceintes (isolateurs ou PSM) utilisées. Il ne faut pas oublier également que ces gants peuvent être en contact avec des solutions désinfectantes à base de solutions alcooliques (éthanol ou isopropanol) susceptibles de diminuer

leur résistance chimique. Même si l'étude de Capron et al. (15) qui comprend un prétraitement alcoolique des gants (immersion des gants dans une solution alcoolique pendant 15 min suivi d'une période de séchage) n'a pas montrée de différence significative dans la perméation des gants par 17 agents cytotoxiques, il est difficile de conclure que l'alcool n'a pas d'influence sur la perméation des agents cytotoxiques à travers les gants car le traitement est préalable au test et non simultané comme dans la réalité. C'est pourquoi, des recommandations déconseillent de décontaminer les gants avec de l'alcool (10).

Les recommandations suisses concernant les gants utilisés lors de la préparation de chimiothérapies mentionnent des gants en latex ou nitrile de 0.2 mm d'épaisseur minimum non poudrés (formulation injectable stérile et dispersion des particules de cytotoxiques dans l'air) avec un changement toutes les 30 minutes (11). Une double paire est conseillée lors de la manipulation d'agents avec un haut degré de pénétration (carmustine, thiotépa et cyclophosphamide).

Etant donné le caractère aseptique obligatoire de la préparation des chimiothérapies, le port d'un masque par l'opérateur est indispensable. Ce dernier a pour fonction principale de protéger la préparation de germes émanant de l'opérateur. Dans ces conditions, un masque de type chirurgical semble tout à fait adapté. Toutefois, lorsque la manipulation concerne des agents toxiques, le masque constitue aussi une barrière de protection pour l'opérateur en limitant l'exposition des voies respiratoires à des particules d'agents anticancéreux potentiellement présentes dans l'air. Il est clair qu'il s'agit d'une mesure qui s'ajoute à celles liées au confinement de la préparation (enceinte et système de transfert clos) et qui permet de protéger l'opérateur contre des éclaboussures ou d'aérosols d'agents anticancéreux formés par accident. Dans ces conditions, les masques chirurgicaux n'offrent qu'une maigre protection. Un masque de protection respiratoire est alors préféré. Ces masques revêtent une double protection : pour le produit contre une contamination microbienne et pour le porteur contre les risques liés à l'inhalation d'un air pollué (poussières et aérosols). Ces masques respiratoires sont classés principalement en fonction de leur effet protecteur contre des particules de taille maximale de 0.6 µm et sa fuite totale (étanchéité joint facial) (24). Une efficacité de 95 et 98% ainsi qu'une fuite totale inférieure à 8 et 2% caractérisent les masques FFP2 et FFP3 (filtering

facepiece particules). En règle générale, les masques respiratoires sont recommandés lorsque la préparation des chimiothérapies s'effectue hors enceinte (hors PSM et hors isolateurs) et lors de travaux de nettoyage et d'entretien des enceintes (y compris lors de nettoyage d'une dispersion accidentelle de cytotoxiques) (10, 11). Le port du masque chirurgical est quant à lui suffisant lors de préparation au sein d'un PSM ou d'un isolateur.

Le port de lunettes de protection permet de parer une éventuelle projection de solutions à base d'agent anticancéreux. Le port de lunette est préconisé lorsqu'un risque de projection existe ou encore dans des conditions particulières comme la préparation hors enceinte et lors de phases de nettoyage (11).

Il n'en demeure pas moins que le principe de précaution prévaut et, que dans bon nombre de sites, le port de masques respiratoires et de lunettes fait partie de l'équipement des opérateurs qui préparent les chimiothérapies.

En ce qui concerne les vêtements, les recommandations émises par les Bonnes Pratiques de Fabrication (25) s'appuient sur l'utilisation de vêtements protecteurs stériles ne devant libérer ni fibre ni particule et retenir les particules émises par l'opérateur. Les recommandations s'accordent sur le port de blouses de type polypropylène recouvert de polyéthylène (10, 11, 14). Elles s'appuient sur les travaux de Harrison et Kloos qui démontrent que ce type de blouses offre la meilleure résistance aux projections de solutions de 15 cytostatiques parmi les 6 différents types de blouses testées (26).

1.3.4. Techniques de travail

Toutes les techniques et/ou matériel utilisés pour la préparation des chimiothérapies doivent permettre une réduction voire une élimination de la dispersion d'agents cytotoxiques dans l'environnement. En effet, l'étape de préparation des chimiothérapies qui peut se résumer en une solubilisation et/ou dilution d'une spécialité pharmaceutique puis à un transfert dans un dispositif d'administration peut s'accompagner de la formation de gouttelettes et/ou d'aérosols d'agents anticancéreux inhérente aux multiples piquages et à l'augmentation de pression dans

les contenants (27-29). Des mesures simples comme l'utilisation de matériel muni de connections de type Luer Lock qui évitent toute séparation impromptue entre flacon et aiguille (ou autres dispositifs) ou encore d'aiguilles larges pour réduire les hautes pressions au piquage sont incontournables. Depuis plus de 15 ans, les opérateurs disposent de systèmes de transfert « clos » pour préparer les chimiothérapies. Ces dispositifs ont pour mission d'éviter tout contact entre l'air et la solution d'agents anticancéreux. La notion de système clos est clairement définie par les recommandations américaines (9, 14) en l'assimilant à un dispositif de transfert qui empêche mécaniquement le passage de contaminants de l'environnement à l'intérieur du système et la dissémination de médicaments dangereux ou de vapeurs à l'extérieur du système. Plusieurs études ont observé une moindre contamination de l'environnement par des agents anticancéreux en utilisant des systèmes clos lors de leur manipulation (30-38). Une diminution de la contamination principalement mise en évidence par l'analyse directe de prélèvement sur les surfaces et par une diminution voire une absence d'anticancéreux dans les urines des opérateurs utilisant les systèmes de transfert clos a été démontrée (33, 36, 38). Si Trans et al. n'ont pas pu démontrer une réduction significative de la contamination de surface en utilisant un système clos par rapport aux techniques conventionnelles, ils ont en revanche mis en exergue une importante diminution de la contamination des gants des opérateurs utilisant le système clos (39). Les marqueurs anticancéreux employés dans la plupart des études sont le cyclophosphamide, l'ifosfamide et le 5FU. Vyas et al. ont utilisé en plus du 5FU, l'épirubicine et 3 dérivés du platine (cisplatine, carboplatin et oxaliplatine) avec les mêmes résultats en faveur du système clos (37).

1.3.5. Nettoyage et décontamination

Malgré toutes les précautions mises en place, la contamination de surface par des agents anticancéreux est difficilement inévitable et l'implémentation de procédures de décontamination chimique revêt un caractère indispensable dans la préparation des chimiothérapies pour restreindre au maximum l'exposition et d'éventuelles contaminations croisées. Si les recommandations sont unanimes quant à la mise en place d'étapes de décontamination chimique dans le processus de préparations des chimiothérapies, le type d'agents de décontamination préconisés n'est pas clairement mentionné. En effet, les recommandations internationales proposent un

agent « désactivant si disponible » (14), des désinfectants et détergents (10). En ce qui concerne les recommandations suisses, elles restent vagues en ce qui concerne le nettoyage sans aucune mention de produits particuliers (11). Il faut remonter au début des années 1980, pour voir les premières études sur l'utilisation de méthodes chimiques de nettoyage sur les agents anticancéreux avec pour principale motivation le traitement des déchets contaminés (40-42). Toutefois, les méthodes proposées reposent sur l'emploi de puissants oxydants et acides incompatibles avec une utilisation régulière par le personnel hospitalier. En outre, une autre problématique est à prendre en considération à savoir la formation de résidus toxiques via la dégradation chimique d'agents anticancéreux (41, 43). Depuis, deux méthodes de décontamination chimique des surfaces souillées par des agents anticancéreux se distinguent : l'utilisation d'hypochlorite de sodium (44-48) et l'utilisation de détergents (49-53). La plupart des études mettent en évidence la grande efficacité de décontamination chimique de l'hypochlorite de sodium sur une une large variété de molécules anticancéreuses comme le paclitaxel (47), les oxazophosphorines (45, 46, 48), le melphalan (46), les anthracyclines (44, 45). Toutefois, son action corrosive en limite fortement son utilisation par les opérateurs hospitaliers sur les surfaces de travail s'il n'est pas neutralisé par un autre agent. C'est pourquoi une décontamination chimique en deux étapes dont la première consiste à appliquer l'hypochlorite de sodium et la deuxième le thiosulfate de sodium (afin de neutraliser l'hypochlorite de sodium) est généralement appliquée (45, 48). L'emploi d'un agent de décontamination moins agressif comme les détergents émerge comme une alternative intéressante. Même si leur efficacité est légèrement moindre par rapport à l'hypochlorite de sodium, l'innocuité des détergents vis-à-vis du matériel et des opérateurs ainsi que l'absence de formation de produits de dégradation potentiellement toxiques en font des produits de décontamination chimique particulièrement adaptés pour le nettoyage régulier des sites de préparation des chimiothérapies (54). L'efficacité de lingettes imprégnées de détergents pour décontaminer les parois externes de flacons d'agents anticancéreux afin de réduire les risques d'exposition et de contaminations croisées a été également démontrée (48).

1.4. Exposition du personnel

Depuis ces quarante dernières années, l'exposition du personnel hospitalier aux agents anticancéreux suscite un intérêt croissant. L'exposition aux agents anticancéreux des personnes les manipulant se fait par les voies respiratoires (formation d'aérosol sous forme liquide ou solide), cutanées (contact directe avec la peau) et digestives (ingestion de matériel contaminé ou contact avec mains contaminées) (14). Si les effets aigus sont aujourd'hui relativement bien connus en fonction des agents anticancéreux (céphalées, nausées, irritation…), il en va autrement pour les effets chroniques. En effet, il est très délicat d'établir un lien direct entre une exposition continue aux agents anticancéreux et des effets toxiques comme la prévalence des cancers chez l'homme. Le même raisonnement est tenu en ce qui concerne l'impact des agents anticancéreux sur l'environnement. Le manque de données clairement établies explique en grande partie l'absence de valeurs limites pour les taux d'exposition aux agents anticancéreux dans l'air, l'eau et les surfaces. En revanche, l'influence défavorable des agents anticancéreux sur la reproduction n'est plus à démontrer (55). Ainsi, des dispositions légales ont été mises en place en Suisse régissant la manipulation d'agents dangereux par les femmes enceintes et qui allaitent (56, 57). Dans tous les cas, le principe de précautions prévaut avec comme règle générale une exposition la plus faible possible.

Deux catégories de mesures de l'exposition aux agents anticancéreux sont préconisées par la plupart des recommandations : l'analyse d'échantillons biologiques prélevés sur les personnes en contact avec les agents anticancéreux ou l'analyse d'échantillons issus de l'environnement (surface, air et eau). La mesure du taux de contamination peut se faire de plusieurs manières :

- Une seule fois : l'auteur de l'étude dispose alors d'une photographie à un moment t du taux de contamination du ou des sites testés ;
- Deux fois : « un avant » et « un après » lors de la mise en place d'un nouvel élément (technique ou organisationnel) ;
- Plus régulièrement : l'auteur possède alors un film permettant de suivre l'évolution au cours du temps du taux de contamination et cette mesure peut alors s'insérer dans un processus d'assurance qualité.

1.4.1. Mesure biologique

Le monitoring biologique peut se faire selon trois types d'analyses : les tests de mutagénicité urinaire, les analyses cytogénétiques et l'analyse d'agents anticancéreux dans les fluides biologiques des personnes exposées.

Les tests de mutagénicité urinaire ont été les premiers tests employés pour mettre en évidence l'exposition du personnel hospitalier aux agents anticancéreux de par leur ancienneté mais aussi de par leur mise en œuvre simple. Le test d'Ames, qui figure parmi les tests les plus courants, consiste à mettre en contact l'urine de l'opérateur avec des souches bactériennes (nécessitant de l'histidine exogène pour proliférer) qui peuvent alors synthétiser de l'histidine en présence d'agent mutagène. Un autre test repose sur la détection de thioéther dans l'urine, métabolite de certains agents anticancéreux (alkylants). Les résultats obtenus par ces tests sont souvent contradictoires et discutables étant donnée leur absence de sélectivité et leur faible sensibilité (58).

L'objectif des analyses cytogénétiques est la détection d'une modification du matériel génétique des personnes exposées. L'analyse d'aberrations chromosomiques, les échanges de chromatides sœurs et les tests des micronoyaux sont les plus courants. Malgré leur haute sensibilité, ces tests présentent l'inconvénient d'être plus difficiles à mettre en œuvre (échantillons sanguins). En outre, le rapport entre une mutation et l'incidence de développer un cancer n'est pas clairement établi (58).

L'analyse d'agents anticancéreux dans les fluides biologiques des personnes exposées constitue une autre approche de la mesure de l'exposition à ces subtances toxiques. Des méthodes analytiques hautement sélectives et sensibles sont indispensables pour la détermination de traces d'agents anticancéreux dans les matrices complexes que représentent les fluides biologiques. Jusqu'à la fin des années 1990-début des années 2000, la GC (GC-MS, GC-ECD, GC-NPD) constituait la principale technique utilisée (59) avec comme principal inconvénient une étape préalable de dérivatisation et purification indispensable (agents de dérivatisation couramment employé : l'anhydride trifluoroacétique), souvent longue et laborieuse. La majeure partie des études publiées a mis en évidence des taux

urinaires de cyclophophosphamide sur une période de 24h de l'ordre du µg/L (60-62). Aujourd'hui, la LC- MS est devenue la technique de choix grâce non seulement à ses hautes sensibilité et sélectivité mais aussi en s'affranchissant de l'étape de dérivatisation. Comme pour toutes les analyses de médicaments dans des matrices biologiques, des techniques de préparations d'échantillons visant à purifier et concentrer les analytes comme la LLE et la SPE font partie intégrante du processus analytique. Des méthodes combinant la SPE à la LC-MS ont permis de déceler des anthracyclines (63) et des oxazophosphorines (64) dans l'urine du personnel hospitalier à des teneurs inférieures au µg/L. D'autres techniques analytiques dont le choix est dicté par la nature des agents anticancéreux recherchés peuvent être également utilisées pour la recherche de traces dans les fluides biologiques. Par exemple, une méthode SPE-LC-FD a permis de quantifier l'épirubicine et la doxorubicine dans l'urine du personnel infirmier à des teneurs de l'ordre du µg/L (65). La voltammétrie absorptive a été employée avec succès pour la détermination du platine dans les urines et le sang du personnel hospitalier exposé, à des concentrations de l'ordre du ng/L (66-68).

Il est important d'indiquer que l'analyse d'agents anticancéreux dans les échantillons biologiques nécessite de prendre en compte leur pharmacocinétique propre. En effet, selon les agents anticancéreux et la matrice biologique concernée, l'analyte recherché n'est pas forcément la molécule native mais le(s) métabolite(s) pas toujours identifié(s). Il peut être cité l'exemple du cyclophosphamide. En effet, ce dernier, largement employé comme biomarqueur, n'est pas la substance la plus pertinente du fait de son activation hépatique en métabolites actifs et de sa faible excrétion urinaire sous forme inchangé (inférieur à 20% de la dose administrée) (58). C'est pourquoi l'exposition du personnel hospitalier au 5FU a été monitoré par l'analyse de son métabolite l'α-fluoro-β-alanine dans l'urine par SPE-LC-MS (69).

Certes, la présence d'agents anticancéreux détectés dans les prélèvements biologiques du personnel hospitalier permet de démontrer une exposition. Mais l'impact sur l'organisme est actuellement délicat à formuler d'un point de vue purement éthique mais aussi d'un point de vue toxicologique. Il n'existe pas de valeur seuil si ce n'est que le bon sens suggère que seul l'absence d'agents

anticancéreux dans les prélèvements biologiques est admissible et sans risque pour la santé du personnel hospitalier.

1.4.2. Mesure environnementale

L'autre stratégie appliquée pour la mesure du taux d'exposition aux agents anticancéreux est l'analyse d'échantillons prélevés dans l'environnement. Ces dernières années, une attention particulière a été portée sur la présence des anticancéreux dans l'environnement comme en témoigne le nombre croissant d'études effectuées (Figure 1). Leur grande toxicité et leur consommation croissante conjuguées à leur détection dans l'environnement rendue possible par l'avènement et la vulgarisation de techniques analytiques hautement sensibles et sélectives (comme la LC-MS ou l'ICP-MS) en constituent les principales raisons. Les prélèvements sont effectués sur les surfaces, dans l'air ou dans les eaux (avant et après traitement).

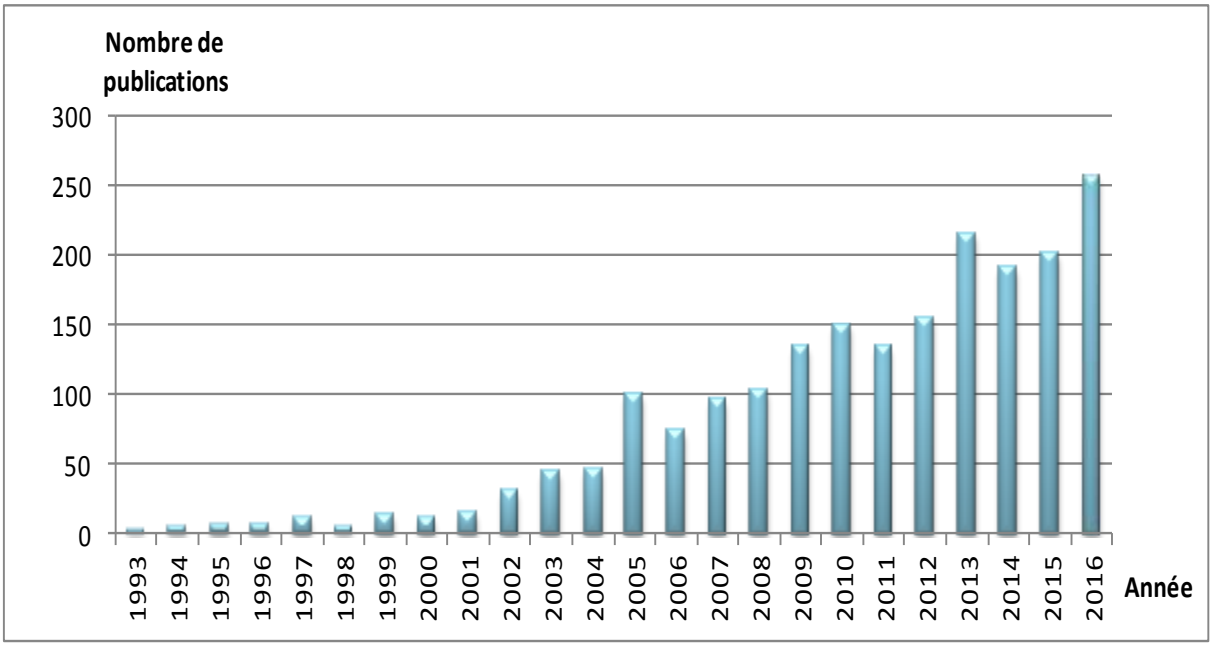

Figure 1 : Nombre d'articles publiés sur les anticancéreux et l'environnement depuis 1993

1.4.2.1. Mesure de la contamination de surfaces

1.4.2.1.1. Surfaces de travail

Les prélèvements peuvent être obtenus en essuyant des surfaces à l'aide d'un papier absorbant imprégné puis plongé dans un solvant pour permettre la désorption d'agents anticancéreux potentiellement présents. Un aliquot du solvant de désorption est finalement analysé (70-75). Il s'agit alors de prélèvements de surface. Plusieurs études ont démontré une contamination de surface par des agents anticancéreux dans les pharmacies hospitalières (75-85) et les unités de soins (76, 78, 82, 83, 85-87). Etant donné que de faibles concentrations de composés sont recherchées, la LC-MS figure parmi les techniques analytiques de choix. Un seul agent anticancéreux peut être utilisé comme marqueur en l'image du cyclophosphamide qui est à la fois fréquemment prescrit et administré à haute dose (83, 87). Toutefois, étant donné que la préparation des chimiothérapies concerne une grande variété de molécules anticancéreuses, plusieurs marqueurs peuvent être définis afin de refléter au mieux la contamination réelle (70-72, 74, 76, 78-82, 84-86). Dans ces conditions, deux approches analytiques sont possibles. La première consiste à développer une seule méthode dite «générique » : les conditions opératoires de l'analyse incluant les étapes de préparation d'échantillon (prélèvement et désorption), de séparation et de détection des analytes sont les mêmes pour tous les marqueurs. Il est alors à noter que le développement d'une méthode « générique » peut être un véritable challenge analytique au vu de la large gamme de propriétés physico-chimiques des anticancéreux. Par exemple, Dal Bello et al. ont développé une méthode LC-MS/MS qui permet la détermination de 9 agents anticancéreux contenu dans des prélèvements de surface (71). L'autre approche repose sur le développement de plusieurs méthodes analytiques (faisant appel généralement à des techniques différentes) en fonction des marqueurs recherchés. Shmaus et al. utilisent la GC-MS pour l'analyse de l'ifosfamide, du cyclophosphamide et du 5-fluorouracil et la voltamétrie pour l'analyse des dérivés du platine (75). Les performances analytiques diffèrent d'une méthode à l'autre mais en règle générale des sensibilités inférieures au ng/cm^2 sont obtenues. Une méthode LC-UV caractérisée par de moins bonnes sensibilités (LOQ comprises entre 0.5 et 300 ng/cm^2) a permis de mettre en évidence

des contaminations par du cyclophosphamide, du 5-fluorouracile et du paclitaxel sur des surfaces de travail dans deux hôpitaux (85).

Comme mentionné précedemment, aucune valeur limite d'exposition aux agents anticancéreux n'est aujourd'hui clairement établie. Toutefois, plusieurs auteurs proposent des valeurs basées sur la détermination de centile provenant de l'analyse de prélèvements de surface effectués dans des situations réelles. Ces valeurs dépendent à la fois de la localisation du prélèvement et de l'agent anticancéreux (88, 89). Lorsque des valeurs inférieures à ces limites sont obtenues, les contaminations de surface sont considérées comme négligeables tandis que des valeurs supérieures impliquent qu'une amélioration des procédures de travail doit être entreprise. Plusieurs études sur les contaminations de surface par des agents anticancéreux en milieu hospitalier ont été conduites : en Allemagne (89), en Suède (88, 90), au Canada (76, 84, 87), en France (80), au Japon (83), en Hollande (91), aux USA (92), au Portugal (85), en République Tchèque (81), en Italie (93, 94) et en Suisse (95). Dans la plupart des cas, les résultats obtenus mettent en avant des contaminations de surface très diverses pouvant aller de quelques pg/cm^2 à plusieurs centaines de ng/cm^2 avec comme tendance des contaminations de surfaces qui s'atténuent de manière concentrique avec l'éloignement de l'épicentre de la préparation des chimiothérapies (plan de travail du PSM ou de l'isolateur). Même si l'association entre les taux de contamination observés et les pratiques quotidiennes du personnel hospitalier est délicate, quelques études parviennent à démontrer certains corollaires. Parmi ces derniers, figure le lien proportionnel entre le nombre de préparations de chimiothérapies produites et le taux de contamination (79, 81, 83). Odraska et al. ont également relevé que le taux de contamination dépendait du niveau de formation des opérateurs (81). Des contaminations de surface moindres ont également été observées lors de l'amélioration de techniques de travail comme l'implémentation de système de tranfert clos pour la préparation (34), de nouvelles méthodes de décontamination (54), lors de la mise en place de nouvelles mesures organisationnelles (84) ou de nouvelles techniques comme l'installation d'un automate de préparation des chimiothérapies (96). La mesure régulière du taux de contamination de surface au sein d'un même site a permis de s'assurer du respect et de l'effet des recommandations au sein du site (76, 93).

1.4.2.1.1. Surface des flacons

En dépit de toutes les recommandations mises en place, des contaminations des sites de reconstitution des chimiothérapies sont généralement observées. Les principales sources de contamination peuvent être définies comme la préparation en elle-même (formation de gouttes résiduelles ou d'aérosol) et les accidents (bris de flacon). Une autre source a également été identifiée en l'image des flacons d'agents anticancéreux fournis par les fabricants industriels. En effet, ces dernières années, plusieurs études ont mis en évidence une contamination chimique sur les parois externes des flacons commercialisés au Canada (53, 97), aux USA (98), en Suède (99, 100), au Royaume-Uni (101), en Allemagne (102), en Belgique (103), au Japon (104, 105), en France (106) et en Suisse (79). Les principaux marqueurs utilisés sont le cyclophosphamide, l'ifosfamide, les dérivés du platine et le 5 FU. Les oxazophosphorines sont analysées par LC-MS (53, 99) ou par GC-MS (97, 98, 101, 102, 106) avec des limites de détection comprises entre 0.02 à plusieurs ng/échantillon. Des méthodes LC-UV (98, 103) et GC-MS (102) ont permis de déterminer le 5FU à des concentrations de l'ordre de quelques ng/mL. Quant aux platines, différentes techniques analytiques sont utilisées comme la polarographie (100), la voltammétrie absorptive (102), l'ICP-MS (101) et la LC-MS après dérivatisation (104, 107) avec des LOD inférieures au ng/échantillon. En règle générale, la méthode de prélèvement correspond à celle utilisée pour la mise en évidence des contaminations de surface (par essuyage puis désorption). Toutefois dans le travail de Favier et al., la procédure analytique employée repose sur 3 étapes : immersion des flacons d'agents anticancéreux dans l'eau, extraction par un solvant organique puis analyse par chromatographie liquide et gazeuse (106). Les contaminations observées sont comprises entre quelques ng/flacon à plusieurs centaines de ng/flacon mais des contaminations supérieures au µg/flacon ont été détectées sur des flacons de cyclophosphamide (98), de 5FU (99) et d'étoposide (79). Il est interessant de mentionner que dans la plupart des études, seul l'agent anticancéreux contenu dans le flacon est recherché sur les parois exterieures. Toutefois, Hedmer et al. ont décelé la présence de traces d'ifosfamide sur des flacons contenant du cyclophosphamide commercialisés en Suède (99). Des contaminations croisées avec d'autres agents anticancéreux ont également été observées sur des flacons disponibles sur le marché helvétique (79). Une

contamination chimique des flacons commerciaux constitue un point de départ pour la contamination des surfaces et de l'air par des agents anticancéreux. En effet, la manipulation de flacons contaminés conduit à une dispersion de la contamination et donc accroît l'exposition humaine et environnementale. Parmi les solutions visant à réduire cette source de contamination, figurent la mise en place de méthode de décontamination (voir paragraphe 2.1.3.5) préalable à la reconstitution des chimiothérapies (53, 98) mais aussi de système de protection des flacons (film plastique sur les flacons après conditionnement) par l'industrie pharmaceutique (79, 102).

1.4.2.2 Mesure de la contamination de l'air

L'air peut également faire l'objet d'une analyse afin de mesurer une éventuelle contamination de l'environnement par des agents anticancéreux. La présence dans l'air d'aérosols contenant des agents anticancéreux peut être considérée comme une conséquence des surpressions possibles au sein des flacons lors de la préparation des chimiothérapies. Dans le cas des PSM de classe II munis d'une ouverture sur le devant, les aérosols produits peuvent s'échapper dans l'air ambiant. Au sein des enceintes fermées, la dispersion de la contamination ne peut se faire qu'en cas de disfonctionnement ou d'ouverture du système lors d'entretien. L'analyse de l'air consiste, dans un premier temps, à aspirer un certain volume d'air à travers un support qui retient les composés d'intérêt. Ensuite, ces composés sont désorbés du support et analysés. Jusqu'à la fin des années 1990, le principal support utilisé pour la recherche d'agents anticancéreux dans l'air est le filtre en Téflon, cellulose ou fibre de verre (59). Or plusieurs études démontrent que les agents anticancéreux présents, en particulier le cyclophosphamide, dans l'air peuvent se trouver effectivement sous forme de particules solides mais aussi à l'état gazeux (78, 108). De ce fait, un materiel permettant la capture de l'agent anticancéreux sous forme de gaz comme un absorbant doit être utilisé (109). L'utilisation concommitante de filtre et d'absorbant comme matériels de prélèvement ont permis de développer des méthodes d'analyses du cyclophosphamide (74, 110, 111) mais aussi d'autres agents anticancéreux (74) présents dans l'air. Les limites de détection obtenues après une analyse par LC-MS des prélèvements d'air sont inférieures à 1 ng/m^3. A l'instar des contaminations de surface, il n'existe pas de valeurs limites pour les taux

de contamination d'air et le principe de précaution indiquant un taux le plus bas possible prévaut également.

1.4.2.3. Mesure de la contamination de l'eau

Le patient contribue, lui aussi, à la présence d'agents anticancéreux dans l'environnement en contaminant les effluents par ses excreta. Dans l'environnement aquatique, ces substances toxiques représentent un rique potentiel pour la santé des écosystèmes et par conséquent pour l'homme (112). En 2006, l'Agence Européenne des Médicaments recommande une évaluation du risque environnemental pour toute nouvelle substance médicamenteuse développée (113). Toutefois, les agents anticancéreux conventionnels, présents sur le marché depuis plusieurs décennies pour la plupart, ne sont pas concernés par de telles mesures et les informations liées à leur impact sur l'environnement restent minces. Deux approches sont généralement employées pour déterminer le taux de contamination de micropolluants (parmi lesquels figurent les agents anticancéreux) dans l'environnement aquatique. La première, purement théorique, consiste à estimer une concentration de la molécule concernée dans les eaux de surface (concentration environnementale prédite ou CEP) sur la base de sa consommation et de son métabolisme (pourcentage de molécule intacte excrétée) et de paramètres liés au devenir de cette molécule dans les systèmes d'évacuation (dilution et taux d'abattement en station de traitement). Cette approche théorique trouve vite ses limites en raison, là encore, du manque de données. Plusieurs études ont reportés des CEP pour plusieurs agents anticancéreux dans différents pays (114-117). L'autre approche a pour objectif de mesurer pratiquement le taux d'agents anticancéreux présents dans l'environnement aquatique comme les effluents domestiques et hospitaliers ou les eaux de surface. Cette approche permet de renseigner le taux de contamination réelle et de pallier le peu de données disponibles. La majeure partie des travaux effectués sur la détermination des agents anticancéreux sont répertoriés dans la review de Kosjek et Heath publiée en 2011 (118). A l'instar des autres analyses utilisées pour l'évaluation de la contamination par des agents anticancéreux, la technique principalement employée jusqu'à la fin des années 1990 est la GC pour laisser place ensuite à la LC-MS. De plus, comme pour les contaminations de surface, plusieurs agents sont recherchés en même temps et le

besoin de disposer d'une méthode dite « générique » se fait sentir. Aux problèmes liés à la séparation et à la détection de molécules disparates viennent s'ajouter ceux liés à la préparation d'échantillon dans le cas d'analyses d'effluents. En effet, les analytes sont présents à l'état de traces dans des matrices complexes (comme dans le cas d'analyses d'échantillons biologiques). Une méthode de préparation visant à purifier (éliminer le maximum d'interférents) tout en permettant une concentration des différents analytes est alors indispensable. La SPE apparaît comme la technique la plus adaptée étant donné que de grands volumes d'échantillons sont disponibles (contrairement aux fluides biologiques). Le choix du type de support est dicté par la nature des analytes. Lorsqu'une large gamme de molécules aux propriétés physico-chimiques différentes est concernée, des supports universels capables d'effectuer des intéractions polaires et apolaires sont employés (118-120). Afin de soulager la préparation d'échantillons, des méthodes automatisant l'extraction sur support solide en utilisant un système à commutation de colonne (support d'extraction sous format de colonne) (121) ou à l'aide d'un robot (122) ont été également développées. Avec l'avènement de la MS hautement sensible et sélective, il est même possible de réduire la préparation d'échantillon à une simple dilution et injection directe dans le système LC-MS. En effet, Gomez-Canela et al. ont développé une méthode permettant d'injecter 100 µL d'effluent (dilué avec 20% de méthanol dans un système LC-MS (123). Dans ces conditions, la sensibilité obtenue est démontrée suffisante pour la détermination de traces de cyclophosphamide et d'épirubicine (LOD de 3.1 et 85 ng/L, respectivement) même si elle est moins bonne que celle obtenue en SPE-LC-MS (facteur 10 et 40). En règle générale, les concentrations détectées dans l'environnement aquatique sont généralement de l'ordre du ng/L ou en-dessous des limites de détection des méthodes développées (118). Toutefois, quelques études ont décelé des concentrations plus importantes (de l'ordre du µg/L) d'agents anticancéreux dans les effluents. Mahnik et al ont développé une méthode CE-UV comprenant une étape préalable d'extraction sur support solide permettant un facteur de concentration de l'ordre de 500 pour la détermination de 5FU dans les effluents hospitaliers avec une LOD de 1.7 µg/L (124).

La majeure partie des études publiées concernent la recherche d'agents anticancéreux sous forme inchangés dans les milieux aquatiques sans véritablement prendre en compte les produits issus de la biotransformation de l'écosystème ni les

métabolites malgré leur toxicité potentielle (112, 114). Peu d'études incluent la détermination de métabolites dans la mesure de la contamination des eaux (122, 125). Etant donné que les métabolites sont généralement des composés plus polaires que les substances mères, les méthodes analytiques appliquées doivent être adaptées. Kovalova et al. ont développé une méthode LC-MS basée sur l'utilisation d'un support HILIC qui a permis la détermination de trois anticancéreux polaires et de leurs métabolites dans l'eau (125). Pour les anticancéreux dérivés du platine, les techniques analytiques employées se basent sur la détection de l'élément platine indépendemment de son incorporation dans une structure organique (ICP-MS ou voltamétrie) (58, 126, 127). Le recours en amont d'un système séparatif peut permettre d'identifier la molécule contenant l'élément platine avant la détection par ICP (126). Kominkova et al. ont développé une méthode basée sur l'injection directe d'échantillons aqueux directement dans un détecteur électrochimique pour l'analyse des dérivés du platine (ciplatine, carboplatine et oxaliplatine) mais aussi plus largement les chlorures de platine (métabolisme/dégradation des agents anticancéreux et autres sources) (128).

2. Contrôle qualité des chimiothérapies

Au vu de leur prescription basée essentiellement sur la surface corporelle du patient, les chimiothérapies sont généralement préparées individuellement : une dose pour un patient. Ainsi, ces formulations magistrales ne sont pas soumises à un contrôle qualité obligatoire du produit fini. Toutefois, le caractére toxique des agents anticancéreux associé à leur marge thérapeutique étroite font des chimiothérapies des formulations à haut risque. Toute erreur médicamenteuse liée aux chimiothérapies peut avoir des conséquences dramatiques d'autant plus que la population de patients concernée par ce type de traitement est fragile. Les agents anticancéreux sont considérés comme la deuxième famille de molécules avec des erreurs de médications léthales, après les agents psychoactifs/analgésiques (129). Des taux d'erreurs de médication concernant les chimiothérapies compris entre 0.6 et 20% ont été publiés (130-134). La majeure partie des erreurs de médication sont détectées avant l'administration aux patients et surviennent essentiellement lors de la prescription ou de l'administration. Afin de réduire ce type d'erreurs, plusieurs recommandations ont été émises dont celles de l'ASHP (135). Quant aux erreurs

liées à la préparation incombant à la pharmacie, elles sont plus rares. Ranchon et al. estiment que les erreurs pharmaceutiques (75% sont liées à la fabrication de la chimiothérapie) représentent 8% des erreurs totales et surviennent à la fréquence de 1.6/1000 chimiotherapies préparées (133).

La plupart des mesures préconisées visent une automatisation/informatisation des procédures conjuguées à l'implémentation de contrôles. En règle générale, les pharmacies hospitalières appliquent ces deux stratégies complémentaires pour réduire les erreurs. L'automatisation de la préparation permet également de réduire les coûts de fabrication (136) ainsi que l'exposition du personnel aux agents anticancéreux (96). Si l'implémentation de contrôles en cours de préparation (contrôle visuel, double contrôle, contrôle gravimétrique) est devenue monnaie courante en pharmacie hospitalière, le contrôle de la chimiothérapie produite est, quant à lui, moins fréquent. En effet, le contrôle qualité de la chimiothérapie doit s'insérer dans le circuit du médicament et pour ce faire doit remplir trois critères souvent difficiles à appliquer pour les pharmacies hospitalières :

- Il doit être rapide (courts délais entre prescription et administration)
- Il doit être simple à effectuer (exécution possible par des personnes sans compétences analytiques);
- Il doit être sécurisé (protection des opérateurs et de l'environnement).

Deux catégories de techniques analytiques peuvent alors être employées : les techniques non-séparatives et les techniques séparatives. Le choix des techniques est dicté principalement par les moyens et les compétences dont dispose la pharmacie hospitalière.

2.1. Techniques non-séparatives

Les techniques analytiques non-séparatives utilisées pour le contrôle qualité des chimiothérapies produites reposent essentiellement sur des techniques spectroscopiques comme l'UV/Visible, l'infrarouge (IR) et le Raman.

Dans tous les cas, il s'agit de techniques relativement simples d'utilisation qui consistent à obtenir un spectre de la chimiothérapie et de le comparer

(qualitativement et quantitativement) à des spectres de référence. Le spectre obtenu lors de l'analyse correspond à l'ensemble des signaux émis par les différents groupements fonctionnels de tous les constituants présents dans la formulation comme la molécule active, les excipients et le contenant. Une des premières techniques utilisées pour l'analyse qualitative et quantitative des chimiothérapies est la spectrophotométrie UV. D'un point de vue pratique, l'échantillon prélevé de la chimiothérapie est injecté dans un chromatographe liquide constitué d'un injecteur automatique couplé directement à un détecteur UV/DAD via une pompe LC. Dans ces conditions, le volume des échantillons analysés est inférieur au mL. Havard et al. ont développé plusieurs méthodes reposant sur l'injection directe dans le flux (FIA) de chimiothérapies contenant les dérivés du platine ou le 5FU (137). Etant donné la grande gamme de concentration des agents anticancéreux dans les formulations, l'injecteur automatique est également utilisé pour effectuer les dilutions appropriées des échantillons avant injection. Toutefois, il faut noter que l'UV souffre d'une sélectivité relativement modeste et qu'elle ne permet pas à elle seule une identification sans ambiguité de molécules possédant des structures similaires ou proches. C'est pourquoi, d'autres techniques analytiques sont utilisées en complément, comme l'IR ou le Raman. L'utilisation concomitante de la spectrophotométrie UV/Visible et IR a été initiée et est toujours employée en œnologie afin de caractériser les vins produits (138, 139). Ces dernières années, l'UV/Vis couplée à l'IR a été appliquée au contrôle analytique des chimiothérapies (140, 141). L'équipement utilisé combine un spectrophotomètre UV/Visible qui est dédié à l'analyse quantitative (dosage à une longueur d'onde) et un spectrophotomètre IR permettant l'analyse qualitatitive (basée sur les spectres enregistrés en 3 régions de longueurs d'ondes) ainsi qu'un injecteur automatique (1.2 mL d'échantillons sont prélevés et transférés dans les deux cellules). Dziopa et al. ont utilisé avec succès cet équipement pour le contrôle qualité de chimiothérapies contenant 14 agents anticancéreux différents produites en pharmacie hospitalière sur une période de 2 ans soit plus de 9'000 formulations (140). Ces principaux avantages sont un temps d'analyse rapide (90s par échantillon) et une simplicité d'utilisation. Cette étude a également soulevé les problèmes du prélèvement de plus de 1 mL de la chimiothérapie rendant l'analyse de formulations de petit volume impossible (conservation de la dose prescrite) ainsi que la nécessité impérieuse de disposer de références (qualitative et quantitative) pour chaque spécialité utilisée lors

de la reconstitution (excipient/composition différente). Bazin et al., qui ont utilisé le même système pour l'analyse de plus de 20'000 chimiothérapies produites sur une période identique, soulignent les difficultés rencontrées pour l'analyse d'anticancéreux de structures proches ou possédant peu de groupements chromophores comme les anthracyclines ou les oxazophosphorines (141). Un équipement, considéré comme le successeur de l'UV-IR, associe cette fois la spectroscopie RAMAN à l'analyse UV/Vis avec le même type d'injecteur automatique. Si les limitations inhérentes au grand volume d'échantillon et aux spectres de références de chaque spécialité demeurent, le temps d'analyse rapide est conservé et la plus haute sélectivité conférée par le Raman permet l'analyse de molécules difficilement identifiables par IR (142, 143).

La spectroscopie Raman, seule, a également fait l'objet de plusieurs études dans le cadre de son application à l'analyse de chimiothérapies. Son intérêt repose non seulement sur sa grande sélectivité, comme indiqué précèdemment, mais aussi sur l'absence de prélèvement d'échantillon (non intrusif). Ainsi, les risques d'exposition liés à l'échantillonage (prélèvement et dilution) sont abolis (144). Toutefois, même si le RAMAN s'est démontré prometteur comme technique analytique pour le contrôle des chimiothérapies produites (144-146), son utilisation en routine seul reste rare en raison de la complexité de l'instrumentation et plus particulièrement du traitement des données (présence d'interférents : contenants et excipients). Actuellement, le RAMAN trouve une application pour le contrôle qualité des chimiothérapies en routine lorsqu'il est utilisé couplé à la spectroscopie UV (utilisation d'un système clé en main intrusif).

2.2. Techniques séparatives

Deux grandes techniques séparatives sont généralement employées pour effectuer un contrôle qualité des chimiothérapies produites : il s'agit de techniques chromatographiques. La première, d'un point de vue historique repose sur la chromatographie sur couche mince à haute performance. Le grand choix de phases stationnaires et de phases mobiles lui confère une grande sélectivité adaptée à l'analyse de la plus grande partie des différentes familles d'agents anticancéreux. L'emploi de faible volume d'échantillons (quelques µL) constitue également un

avantage non négligeable. En outre, plusieurs analyses peuvent être effectuées simultanément avec un temps total compris généralement entre 30 et 60 minutes. Des méthodes CCM ont été développées pour l'analyses d'agents anticancéreux contenus dans des chimiothérapies produites en milieu hospitalier comme le busulfan (147) ou le cyclophosphamide (148) dans des gélules ou des poches pour perfusion. En raison de l'absence de groupements chromophores, les deux agents anticancéreux ont été révélés grace à une étape de dérivatisation. Le facteur de rétention des composés obtenus est comparé à celui des substances de référence pour l'identification et les surfaces des pics obtenus par densitométrie renseignent le dosage des molécules détectées. Bourget et al. ont développé plus d'une dizaines de méthodes CCM pour l'analyse de 24 agents anticancéreux et les ont appliquées avec succès lors du contrôle qualité de plus de 23'000 chimiothérapies produites (149). Dans ces conditions, des composés ayant des structures voisines comme les analogues des bases pyrimidiques (5-FU, gemcitabine et cytarabine) ont pu être clairement séparés et donc analysés sans ambiguité. Les principaux inconvénients de la CCM sont :

- le fait qu'il s'agit d'un système ouvert (pas de barrière entre le toxique et l'environnement/opérateur) ;
- la nécessité de nombreuses interventions humaines (malgré une automatisation partielle du système) ;
- le recours potentiel à une étape de dérivatisation (souvent longue) nécessaire pour la révélation des composés présentant une faible absorption dans l'UV.

La deuxième technique chromatographique employée pour le contrôle qualité des chimiothérapies est la LC. Bénéficiant également d'une grande sélectivité de par les nombreuses phases stationnaires et mobiles disponibles, elle se prête particulièrement bien à l'analyse des chimiothérapies. A l'instar de la CCM, de faibles volumes de chimiothérapies sont nécessaires (quelques µL) en LC. Fonctionnant en système fermé et entièrement automatisée, elle jouit d'une plus grande popularité que la CCM. Une méthode LC-UV reposant sur l'utilisation de 2 colonnes chromatographiques C18 a permis l'analyse de plus de 7000 chimiothérapies produites contenant un des 5 agents anticancéreux suivants: gemcitabine, 5FU, docétaxel, paclitaxel et oxaliplatine (150). Une phase mobile avec une forte proportion d'eau est utilisée pour l'analyse des composés polaires (5FU, gemcitabine

et oxaliplatine) sur une des deux colonnes tandis qu'une phase mobile principalement organique est appliquée sur la deuxième colonne pour l'élution des composés apolaires (taxanes). Des temps d'analyses inférieurs à 5 minutes sont obtenus. Plusieurs études ont utilisé des méthodes LC-UV lorsque l'analyse par FIA-UV était rendue impossible par la présence d'excipients interférents ou par l'analyse d'agents anticancéreux de structures similaires (137, 151). La génération de déchêts de solvants contaminés constitue la principale limitation de la LC.

Une autre technique séparative peut être également employée pour le contrôle qualité des chimiothérapies : la CE. S'agissant d'un système fermé, automatisé et caractérisé par une faible consommation de solvants, la CE peut se révéler particulièrement interessante pour le contrôle qualité des chimiothérapies. Si sa faible sensibilité constitue un désavantage indéniable lors de l'analyse de traces (environnement, biologie), dans le cas des formulations pharmaceutiques où les concentrations des analytes sont plus conséquentes, elle trouve parfaitement sa place. Moins populaire que la LC, l'utilisation de la CE pour le contrôle qualité de chimiothérapies reste occasionnelle. Une méthode CE-UV a été développée pour l'analyse de trois composés dérivés du platine contenu dans des formulations pharmaceutique et a été appliquée avec succès au contrôle qualité des chimiothérapies produites (152).

En règle générale, les méthodes séparatives offrent une sécurité analytique supérieure à celle procurée par les méthodes non séparatives. En effet, la séparation permet de s'affranchir des problèmes éventuels liés à la présence d'interférents commes les excipients qui changent d'une formulation à l'autre et renseigne plus sélectivement l'identification de la molécule d'intérêt. En outre, les méthodes séparatives peuvent également être employées lors d'étude de stabilité du fait de leur sélectivité ; ce qui n'est pas le cas des méthodes non-séparatives. Toutefois, un bémol peut être porté sur les méthodes séparatives qui requièrent un niveau minimum de compétence des opérateurs non seulement pour le développement/validation de la méthode mais aussi pour l'analyse de routine.

3. Stabilité des chimiothérapies

Comme déjà indiqué précédemment, les agents anticancéreux ne sont pas tout à fait des molécules médicamenteuses comme les autres de par leur toxicité propre et par leur instabilité physico-chimique associée. La différence se poursuit au sein des formulations commercialisées d'agents anticancéreux. En effet, contrairement aux autres médicaments qui sont pour la plupart commercialisés dans un véhicule et à une concentration compatible avec une administration directe, les spécialités d'agents anticancéreux (sous forme injectables) doivent subir un traitement intermédiaire (solubilisation et/ou dilution) avant d'être administrés au patient (voir chapitre 2.1). Dans la majeure partie des cas, le temps entre la préparation de la chimiothérapie par la pharmacie hospitalière et l'administration se réduit à 1 ou 2 heures. Toutefois, pour des raisons logistiques et de coûts, des temps supérieurs peuvent être enregistrés. En outre, des temps d'administration sur plusieurs heures ne sont pas rares lors de traitements chimiothérapeutiques. Ainsi un voyant lumineux orange s'allume et la notion de stabilité de la chimiothérapie reconstituée se pose (certains agents anticancéreux comme l'azacytidine ont des durées de stabilité inférieures à 1 heure après reconstitution). Les données de stabilité des formulations reconstituées éditées par les fabricants sont généralement limitées à 24 heures pour des raisons purement bactériologiques. Il se peut toutefois que des dates de stabilité relativement courtes soient également émises car les études de stabilité physico-chimiques n'ont été conduites que sur un bref laps de temps indépendamment de la stabilité chimique réelle de la formulation reconstituée.

Dans ces conditions, les pharmacies hospitalières, avec le souhait de produire une chimiothérapie de qualité afin de garantir la sécurité des patients, partent à la recherche de données de stabilité soit en conduisant elles-mêmes des études de stabilité (pour autant qu'elles disposent des moyens nécéssaires) soit en se basant sur des données de stabilité issues de la littérature scientifique. Dans ce dernier cas, plusieurs points sont à considérer :

- les données publiées doivent être établies dans les mêmes conditions que celles de la pharmacie requêrante (même molécule à la même concentration dans le même véhicule, y compris les excipients, avec le même contenant et dans les mêmes conditions de stockage) ;

- les données doivent être issues d'études respectant les critères et recommendations des études de stabilité.

Les recommandations ICH, et plus particulièrement la Q1A sur les « Etudes de stabilité de nouvelles substances et formulations », font office de référence internationale dans le domaine des études de stabilité (153). Ces recommandations jettent les grandes lignes des conditions dans lesquelles doivent être effectuées les études de stabilité. Elles s'appliquent à toutes les molécules médicamenteuses et pas seulement aux agents anticancéreux. C'est pourquoi, un groupe de professionnels européens ont édité en 2012, un guide pratique pour la réalisation d'études de stabilité de chimiothérapies (154). Ce guide met l'accent sur un certain nombre de points comme par exemple les différents tests à effectuer en accord avec les recommandations ICH pour les petites molécules anticancéreuses conventionnelles et les anticorps monoclonaux. La mise en place de limites en rapport avec la molécule concernée fait également l'objet d'une discussion. En effet, en raison des marges thérapeutiques étroites des agents anticancéreux et de la possible toxicité des produits de dégradation, une limite de 5% par rapport à la concentration initiale est préconisée comme valeur de stabilité contrairement à la valeur de 10% traditionnellement employée dans le cas des molécules médicamenteuses en général.

Le cœur d'une étude de stabilité est la méthode analytique utilisée. Il est fortement conseillé de recourir à une méthode séparative capable de distinguer la molécule mère des produits de dégradation et des excipients éventuels. Cette methode doit faire l'objet d'une validation analytique et être certifiée « indicatrice de stabilité » en procédant à des tests de dégradation forcée. En effet, si les données inhérentes aux impuretés sont relativement bien renseignées (pharmacopées), celles issues des voies de dégradation des molécules médicamenteuses sont peu courantes. Par conséquent les produits de dégradation sont rarement identifiés. Les tests de dégradation forcée consistent dans un premier temps à traiter la molécule médicamenteuse avec des solutions diluées acides, basiques ou oxydantes/réductrices à une température plus ou moins élevée afin d'obtenir des produits de dégradation éventuels sans être trop drastique (une dégradation de l'ordre de 20-30% de la molécule d'intérêt est généralement recherchée) afin d'éviter

une altération des produits de dégradation eux-mêmes. Puis ces composés sont analysés par la méthode dévelopée. La méthode est jugée « indicatrice de stabilité » si les produits de dégradation n'interfèrent pas avec l'élution du composé d'intérêt (en plus des autres critères de validation). La méthode est alors validée et peut être utilisée pour l'étude de stabilité.

La Société Française de Pharmacie Oncologique (SFPO) en partenariat avec la Société Européenne de Pharmacie Oncologique (ESOP) a publié des recommandations quant à la stabilité des formulations d'agents anticancéreux reconstitués (155, 156). Une liste de données de stabilité est dressée pour chaque molécule sur la base des publications étudiées par les deux sociétés de pharmacie oncologique en spécifiant la concentration de la molécule, le contenant, le véhicule et les conditions de conservation. De telles informations constituent un support pour les pharmacies hospitalières.

La majeure partie des méthodes indicatrices de stabilité publiées utilisent la LC-UV et ont été développées pour la plupart il y a plus de 20 ans. Ces méthodes sont caractérisées par l'utilisation de colonnes chromatographiques (phase inverse) longues (250 mm) et, par conséquent, des temps d'analyse variant d'une dizaine de minutes à plus de 30 minutes. De tels temps d'analyse peuvent se révéler inadaptés pour des molécules anticancéreuses fortement instables et le recours à des méthodes « plus rapides » devient indispensable. L'azacitidine avec une stabilité en solution aqueuse de l'ordre de 30 min à 25°C est une des molécules anticancéreuses les plus instables en solution. Une méthode LC-UV indicatrice de stabilité avec un temps d'analyse plus court (inférieur à 5 min) a été développé pour évaluer la stabilité de l'azacytidine reconstituée dans de l'eau stérile froide (157). Il ne faut pas oublier également que tous les agents anticancéreux ne sont pas UV absorbants (aux concentrations présentes dans les formulations reconstituées). Le busulfan qui ne possède aucun groupement chromophore (chaine linéaire saturée), est généralement dérivé par le sodium diethyldithiocarbamate avant analyse lors des études de stabilité des formulations reconstituées (158-160). Dans ces conditions, il doit être clairement démontré que l'étape de dérivatisation n'a pas d'influence sur la dégradation de la molécule d'intérêt. Si l'utilisation de la LC-MS est devenue courante dans l'analyse des agents anticancéreux dans les matrices biologiques ou

environnementales (recherche de traces), il en va tout autrement dans les études de stabilité. Les principales raisons sont les fortes concentrations des agents anticancéreux dans les formulations reconstituées (de l'ordre du mg/mL) et que ces études sont généralement conduites par des pharmacies hospitalières qui utilisent pour leur étude de stabilité, l'équipement dédié au contrôle qualité des formulations produites sur site (fortes concentrations) à savoir la LC-UV dans la plupart du temps. Quelques études reportent l'utilisation de la MS dans le cadre d'études de stabilité de formulations d'agents anticancéreux reconstituées avec pour objectif principal l'identification des produits de dégradation (161, 162).

Dans certains cas, l'administration concomitante d'un agent anticancéreux avec une autre molécule active est requise et, donc, l'étude de stabilité doit porter sur les deux molécules. C'est le cas, par exemple, des oxazophosphorines auxquelles est souvent ajouté le sodium-2-mercaptoéthane-sulfonate (Mesna) qui agit comme chimioprotecteur (détoxication des métabolites). Deux études ont évalué la stabilité du Mesna et de l'ifosfamide (163) et du mesna et du cyclophosphamide (164) par LC-UV.

4. Conclusion

Découvertes, pour la plupart, il y plusieurs dizaines d'années, les molécules anticancéreuses conventionnelles sont toujours administrées comme traitement chimiothérapeutique dans la majeure partie des cancers. Leur grande réactivité chimique associée à leur haute toxicité fait de l'analyse des agents anticancéreux un véritable challenge. Les méthodes développées pour l'analyse d'agents anticancéreux suivent la chronologie des progrès effectués dans le domaine des techniques analytiques. Deux grandes périodes peuvent être distinguées. La première correspond plus ou moins à la deuxième moitié du XXème siècle. Durant cette période, la majeure partie des méthodes analytiques développées pour le dosage d'agents anticancéreux reposent sur des techniques comme la LC-UV ou la GC et s'inscrivent dans le cadre de contrôle qualité de la molécule anticancéreuse (profile d'impuretés) et dans le cadre de suivi thérapeutique (adaptation des doses). Puis, l'intérêt suscité par l'impact de ces molécules hautement toxique sur l'homme

et l'environnement avec l'avènement de techniques hautement sensibles et sélectives comme la LC-MS ouvrent l'ère de l'analyse de traces des agents anticancéreux. En effet, la plupart des méthodes analytiques liées aux agents anticancéreux publiées depuis le début des années 2000 utilisent la LC-MS. Même si ces méthodes trouvent bon nombre d'applications dans l'analyse de matrices biologiques (suivi thérapeutique, exposition du personnel soignant), une grande partie concerne l'analyse d'échantillons de surface ou d'effluents dans le cadre d'études visant à évaluer la contamination de l'environnement par des agents anticancéreux. La détection de traces, toujours plus infimes obtenues grâce à ces techniques analytiques toujours plus performantes, d'agents anticancéreux dans l'organisme des personnes ayant été en contact avec ces molécules (autres que les patients) ou dans l'environnement soulève de nombreuses discussions. En effet, aujourd'hui, aucune norme claire, basée sur des études toxicologiques et environnementales, n'existe sur laquelle les autorités sanitaires et scientifiques peuvent s'appuyer pour protéger l'individu et l'environnement. C'est pourquoi, le principe de précaution prévaut et le risque d'exposition doit être réduit au minimum.

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Chapitre 2 : Revues de la littérature

Deux articles parus en 2011 (article Ia) et 2017 (article Ib) reportent les différentes méthodes d'analyses d'agents anticancéreux conventionnels publiées dans la littérature scientifique ces dernières décennies. La plupart des méthodes développées s'applique à la détermination d'agents anticancéreux dans des matrices biologiques dans le cadre de suivis thérapeutiques ou encore d'études d'exposition à ces agents hautement toxiques. Si les analyses effectuées dans les laboratoires d'analyse médicale reposent indubitablement sur des techniques de type immunoessais, la LC-MS ainsi que l'électrophorèse capillaire démontrent également un réel potentiel pour l'analyse d'agents anticancéreux en s'affranchissant des problèmes de sélectivité (réactions croisées avec les métabolites ou substances apparentées) inhérents aux analyses immunologiques et en offrant une certaine flexibilité analytique (il devient alors possible d'analyser simultanément différentes molécules). De ce fait, l'ensemble de ce travail de thèse s'est inspiré de ces deux bibliographies et repose sur l'emploi de ces deux techniques séparatives à disposition pour le dosage des agents anticancéreux conventionnels dans des matrices simples comme les formulations pharmaceutiques (Chapitre 3) ou les prélèvements de surfaces (Chapitre 4) et dans des matrices plus complexes comme les effluents hospitaliers (Chapitre 5).

Article I*a*

« Analysis of anticancer drugs : a review »

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Review

Analysis of anticancer drugs: A review

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ABSTRACT

In the last decades, the number of patients receiving chemotherapy has considerably increased. Given the toxicity of cytotoxic agents to humans (not only for patients but also for healthcare professionals), the development of reliable analytical methods to analyse these compounds became necessary. From the discovery of new substances to patient administration, all pharmaceutical fields are concerned with the analysis of cytotoxic drugs. In this review, the use of methods to analyse cytotoxic agents in various matrices, such as pharmaceutical formulations and biological and environmental samples, is discussed. Thus, an overview of reported analytical methods for the determination of the most commonly used anticancer drugs is given.

Contents

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1. Introduction

Cancer is a disease in which the control of growth is lost in one or more cells, leading either to a solid mass of cells known as a tumour or to a liquid cancer (i.e. blood or bone marrow-related cancer). It is one of the leading causes of death throughout the world, in which the main treatments involve surgery, chemotherapy, and/or radiotherapy [1]. Chemotherapy involves the use of low-molecular-weight drugs to selectively destroy tumour cells or at least limit their proliferation. Disadvantages of many cytotoxic agents include bone marrow suppression, gastrointestinal tract lesions, hair loss, nausea, and the development of clinical resistance. These side effects occur because cytotoxic agents act on both tumour cells and healthy cells [2]. The use of chemotherapy began in the 1940s with nitrogen mustards, which are extremely powerful alkylating agents, and antimetabolites. Since the early success of these initial treatments, a large number of additional anticancer drugs have been developed [1].

Anticancer drugs can be classified according to their mechanism of action, such as DNA-interactive agents, antimetabolites, antitubulin agents, molecular targeting agents, hormones, monoclonal antibodies and other biological agents [2]. In this review, the most commonly used anticancer drugs (i.e. classical cytotoxic agents) are discussed.

- *Antimetabolites* are one of the oldest families of anticancer drugs whose mechanism of action is based on the interaction with essential biosynthesis pathways. Structural analogues of pyrimidine or purine are incorporated into cell components to disrupt the synthesis of nucleic acids. 5-Fluorouracil and mercaptopurine are typical pyrimidine and purine analogues, respectively. Other antimetabolites, such as methotrexate, interfere with essential enzymatic processes of metabolism.
- *DNA interactive agents* constitute one of the largest and most important anticancer drug families, acting through a variety of mechanisms:
  - Alkylating agents lead to the alkylation of DNA bases in either the minor or major grooves. For example: dacarbazine, procarbazine and temozolomide.
  - Cross-linking agents function by binding to DNA resulting to an intra-strand or inter-strand cross-linking of DNA. Platinum complexes (e.g., cisplatin, carboplatin, oxaliplatin) and nitrogen mustards (e.g., cyclophosphamide, ifosfamide) are the two main groups of this anticancer drug sub-family. Nitrosurea compounds, busulfan and thiotepa are also cross-linking agents.
  - Intercalating agents act by binding between base pairs. The family include anthracyclines (e.g., doxorubicin, epirubicin), mitoxantrone and actinomycin-D.
  - Topoisomerase inhibitors include irinotecan and etoposide compounds. These drugs inhibit the responsible enzymes for the cleavage, annealing, and topological state of DNA.
  - DNA-cleaving agents such as bleomycin interact with DNA and cause strand scission at the binding site.
- *Antitubulin agents* interfere with microtubule dynamics (i.e., spindle formation or disassembly), block division of the nucleus and lead to cell death. The main members of this family include taxanes and vinca alkaloids [2].

Today, with the increase in cancer incidence, treatments containing cytotoxic drugs are widely used. Due to the aging (and increasingly cancer-susceptible) population and the arrival of new treatments, the demand for pharmacy cancer services is expected to more than double over the next 10 years [3]. Even if more selective therapies are developed (e.g., antibodies or molecular targeting agents), treatment schemes will continue to be associated with classical cytotoxic agents.

Consequently, the need for analytical methods to determine anticancer drugs is of outmost importance. The first developed methods for the analysis of cytotoxic compounds are based on the use of liquid chromatography with UV detection (LC-UV). These methods exhibited satisfactory quantitative performance for the analysis of samples containing high concentrations of target drugs (i.e. development of pharmaceutical formulations, stability studies...). However, in the case of samples with low amount of cytotoxics (i.e. biological or environmental analysis), a sample preparation step allowing a pre-concentration of target compounds had to be applied before the LC-UV analysis. In the 1990s, the high selectivity and sensitivity of mass spectrometry revolutionized the whole analytical procedure by simplifying and reducing the sample preparation step. Today, LC–MS is undoubtedly one of the techniques of choice for the analysis of anticancer drugs with very attractive analytical performance. Limit of detection (LOD) in the order of $ng\,mL^{-1}$ are frequently obtained. Other detection systems were coupled to LC such as fluorimetry, evaporative light scattering detector (ELSD) or electrochemical detection (ECD). Furthermore, analytical techniques were also published to determine anticancer drugs such as capillary electrophoresis coupled to UV-detection (CE-UV), amperometric detection or to laser-induced fluorescence (CE-LIF), gas chromatography–mass spectrometry (GC–MS), Raman spectroscopy, infrared spectrometry (IR).

In the first part of this paper, the need for analytical methods allowing the determination of these cytotoxic drugs in various media, such as pharmaceutical formulations, biological matrices and environmental samples, is discussed. In the second part, an overview of the different analytical methods is given according to specific cytotoxic agents.

2. Analysis of cytotoxic drugs: generality

2.1. Analysis of cytotoxic agents in pharmaceutical formulations

From the production of cytotoxic bulk until chemotherapy in a patient, analytical methods are necessary for (i) quality control of bulk and commercialised formulations, (ii) quality control of diluted formulations before patient administration and (iii) studies on formulations regarding compatibility and stability.

2.1.1. Quality control of bulk and formulations

For bulk and pharmaceutical formulations, a valuable method for quality control should be able to simultaneously determine the parent drug and its impurities and degradation products. Quality control, valuable for all pharmaceuticals, must be in agreement with pharmaceutical regulations. Usually, separation techniques offering great selectivity, such as LC or CE, are used. Among the most commonly used detection systems, MS can be considered

the technique of choice. Its high selectivity and sensitivity allows the detection of very low concentrations of impurities or degradation products. For example, Jerremalm et al. studied the stability of oxaliplatin in the presence of chloride and identified a new transformation product (monochloro–monooxalato complex) by LC–MS/MS [4]. However, UV spectrophotometry coupled to a separation technique is used routinely, but the sensitivity of the method must be sufficient for degradation or impurity profile studies. For example, Mallikarjuna Rao et al. developed a stability-indicating LC-UV method for determination of docetaxel in pharmaceutical formulations [5]. LC-UV was also used in studies of the chemical stability of teniposide [6] and etoposide [7] in different formulations.

2.1.2. Quality control of prepared formulation before patient administration

Before administration to the patient, commercialised formulations in the form of freeze-dried powder or high concentrations of drug, are dissolved and/or diluted with sodium chloride (NaCl, 0.9%) or glucose (5%) to obtain the final individualised quantity of drug prescribed by a physician in an appropriate concentration. Stability of these diluted cytotoxic formulations is often limited (or unknown), and they are most often prepared a short time before patient administration by a nurse in the care unit or in a specialised unit at the hospital pharmacy. Even if pharmaceutical regulations do not require a final control of each individualised cytotoxic preparation, analysis can be applied to ensure correct drug concentration and to reduce medication errors and their consequences for patients with increased risk of morbidity and mortality [8].

Different strategies, usually applied by the hospital pharmacy, are used to control the prepared formulation before patient administration. In most cases, these methods allow approximate information on the concentration to be obtained and the cytotoxic substance contained in the reconstituted formulation to be identified. Given the high number of cytotoxic preparations per day and the very short time between prescription, preparation and administration, simple and fast techniques are usually preferred to conventional methods, which are often more expensive and less easy-to-handle. One approach consists of flow injection analysis (FIA) with UV-diode array detection (DAD). As shown by Delmas et al., 80% of cytotoxic preparations (corresponding to 21 different cytotoxic drugs) were successfully determined in a centralised preparation unit in less than 3.5 min [8]. However, due to the absence of separation before detection, the presence of excipients in the formulation can interfere with FIA-UV/DAD analysis, and compounds with similar structures cannot be distinguished.

Quality control of cytotoxic drugs was also performed by coupling Fourier transform infrared (FTIR) spectroscopy and UV spectrophotometry [9,10], which increased the selectivity of the method in comparison to single UV. Identification of the drug compound, excipients and drug concentration was thus achieved in a short analysis time without sample preparation. As for FIA-UV/DAD, additives in cytotoxic formulations or cross-contamination in the analytical system can perturb analyses. Moreover, to the author's knowledge, including quantitative performance with complete validation for quality control of cytotoxic agents has not yet been described with this approach.

Another, more selective technique for quality control of cytotoxic formulations might be Raman spectroscopy. It is a non-destructive and rapid method for identifying and quantifying active drugs and excipients in pharmaceutical formulations [11,12]. Additionally, this analysis is possible without sampling, providing excellent protection for technicians. As for the FTIR and UV/DAD techniques, to the author's knowledge, information on quantitative performance for Raman in cytotoxic formulations has not yet been reported in the literature.

In conclusion, when establishing quality control of cytotoxic drugs in a daily routine before patient administration, generic FIA-UV/DAD assays, FTIR and UV/DAD techniques or Raman spectrometry present interesting approaches in terms of time and simplicity. Nevertheless, the lack of selectivity and quantitative data are the main drawbacks of these techniques.

2.1.3. Formulation studies

Various studies have been performed on the attributes of cytotoxic drugs contained in formulations, including compatibility or stability. The compatibility of cytotoxic drugs with container materials is very important to avoid adsorption or degradation of the active compound, which both have negative consequences for patient treatment [13]. In the 1980s, stability data of antitumor agents in glass and plastic containers [14] or in totally implanted drug delivery systems [15] were established, and a review of stability data for cytotoxic agents was published in 1992 [16]. In these studies, LC-UV was the most commonly used analytical technique.

For new compounds and formulations, stability-indicating methods allowing separation of active compounds and degradation products are required to establish conservation guidelines for each cytotoxic drug in different containers. In the review of Benizri et al., several stability studies were evaluated, antineoplastic agents with sufficient chemical and physical stability were selected for home-based therapy, and a standardisation of anticancer drug stability data was proposed [17].

2.2. Analysis of cytotoxic agents in biological samples

Most of the reported methods were intended for cytotoxic drug quantification in biological matrices, fundamental studies of new drugs, pharmacokinetic (PK) and pharmacodynamic (PD) studies, therapeutic drug monitoring (TDM) or biomonitoring for occupational exposure.

2.2.1. Development of new drugs and formulations

The interaction between drugs and DNA is among the most important aspects of biological studies in drug discovery and pharmaceutical development processes. A review on different techniques used to study anticancer drug–DNA interaction has been published and included the following techniques: DNA-footprinting, nuclear magnetic resonance (NMR), MS, spectrophotometric methods, FTIR and Raman spectroscopy, molecular modelling techniques, and CE [18]. Furthermore, electrochemical approaches can provide new insight into rational drug design and would lead to further understanding of the interaction mechanism between anticancer drugs and DNA [18]. PK and PD studies were frequently the reason for the development of new analytical methods to determine cytotoxic agents in biological samples (e.g., urine, serum, plasma, intracellular matrix, tissues). For example, a recently reported LC–MS/MS method for docetaxel in plasma was found to have better performance than previously reported methods in terms of sensitivity, and it appeared to be a promising method for a large clinical pharmacology study [19].

2.2.2. Therapeutic drug monitoring

TDM for chemotherapy agents is not currently used routinely, mainly due to the lack of established therapeutic concentration ranges. Combinations of different chemotherapies make the identification of a target concentration difficult, as the concentration–effect relationship depends on the different treatments [20]. However, TDM has the potential to improve the clinical use of some drugs and to reduce the severe side effects of chemotherapy. For example, Rousseau et al. reported different possibilities and requirements for TDM [21]. Most commonly, TDM is performed for methotrexate [2]. Reviews on drug monitoring

were already published in 1985 by Eksborg and Ehrsson [22], and hyphenated techniques in anticancer drug monitoring (e.g., GC–MS, LC–MS and CE-MS) were published by Guetens et al. in 2002 [23,24].

2.2.3. Biomonitoring of exposed healthcare professionals

Cytotoxic drugs have been recognised as hazardous for healthcare professionals since the 1970s [25], and different studies have shown how occupational exposure to antineoplastic drugs is associated with a potential cancer risk [26–29]. However, a direct relationship between exposure to cytotoxic contamination and harmful effects is difficult to establish, and no maximal acceptable amount for these drugs has been set by regulation offices until now. Biomonitoring requires very sensitive and selective methods for trace analysis of cytotoxic drugs in urine or blood samples. Moreover, validated and standardised methods are lacking for cytotoxic agent monitoring in biological samples of healthcare professionals [30,31]. The concentration of cytotoxic drugs in biological samples from healthcare professionals, which are exposed to these compounds, is usually lower than for biological samples from patients receiving formulations with drug amount in the order of mg. Even if drug levels are usually lower in urine than in blood samples, urine samples are preferred for practical reasons. That is why methods used for the analysis of cytotoxic drug in samples of healthcare professionals have to exhibit a sufficient sensibility to allow reliable quantification of these compounds. GC–MS and LC–MS are the most commonly used [32,33], but according to the analytes, other techniques may also be interesting (for example, inductively coupled plasma-mass spectrometry (ICP-MS) or voltammetry for platinum compounds [34,35]). Most reported studies have found cytotoxic drugs in the urine or blood of healthcare professionals despite safety standards for handling these compounds [36–39]. According to precautionary principles, exposure should therefore be kept to the lowest possible levels [40].

2.3. Analysis of cytotoxic agents in environmental samples

2.3.1. Surface and air contamination

A complete review of analytical methods used for environmental monitoring of antineoplastic agents was published in 2003 by Turci et al. [36]. Analytical methods for the quantification of one or two model cytotoxic agents and generic methods for the determination of several drugs have been developed. When using marker compounds, wipe samples have been obtained by compound-specific wiping procedures followed by adapted analytical techniques (e.g., voltammetry for platinum drugs [41]). Such methods for marker compounds presented very good quantitative performance regarding detection limits and estimated potential surface contamination [41–46]. However, a wide range of chemotherapy formulations with different drugs and different preparation procedures are usually produced in hospital units. Therefore, to get an overview of several contaminants, multi-compound methods are required with generic wiping procedures. For sufficient selectivity and sensitivity, LC–MS/MS is one of the analytical approaches of choice [47–53].

2.3.2. Wastewater

After administration of anticancer drugs to patients, considerable amounts of cytotoxic agents are eliminated in the urine and thereby reach the wastewater system. Due to their potential toxicity to humans and the environment, analysis of cytotoxic drugs and their metabolites is also needed in hospital effluents and wastewater samples. Various analytical techniques can be used for this purpose, including ICP-MS for platinum compounds [54], CE-UV for fluorouracil [55], LC with fluorescence detection for anthracyclines [56] and LC–MS/MS for antimetabolites [57] and other cytotoxic agents [58,59].

3. Overview of analytical methods for specific cytotoxic drugs

In this Section, analytical methods for each cytotoxic drug are discussed. Only the most commonly used cytotoxic agents, i.e., antimetabolites, DNA interactive agents and antitubulin agents, are considered in this paper.

3.1. Antimetabolites

Analysis of pyrimidine analogues, purine analogues and other antimetabolites are described in this section. The chemical structures of antimetabolites are shown in Fig. 1, and published analytical methods for determination of these compounds in pharmaceutical formulations, biological and environmental samples are reported in Table 1.

3.1.1. Pyrimidine analogues

3.1.1.1. 5-Fluorouracil, tegafur, capecitabine. 5-Fluorouracil (5-FU) is a widely used cytotoxic agent for the treatment of breast tumours and cancers of the gastrointestinal tract, including advanced colorectal cancer. It is also effective for certain skin cancers by topical administration. The main side effects include myelosuppression and mucositis [2]. Tegafur and capecitabine are metabolised to 5-FU and are given orally for metastatic colorectal cancer.

Few stability-indicating LC-UV methods for stability studies of 5-FU in pharmaceutical dosage forms containing various additives [60,61] and in rat caecal tissues [62] have been developed with good quantitative performance in terms of accuracy and precision. Simple sample preparation including centrifugation and dilution was performed and an LOQ of 500 ng mL^{-1} was achieved for 5-FU in rat caecal tissues [62]. However, 5-FU was observed to be degraded under alkaline conditions, while only negligible degradation was observed in acidic, neutral, oxidative and photolytic conditions. Drug combinations of 5-FU and doxorubicin were also successfully determined by LC-UV in injection solutions and biological samples [63]. A complete separation between doxorubicin and methyl hydroxybenzoate, used as a preservative, was obtained.

Generally, published methods for the analysis of tegafur and capecitabine allowed a simultaneous separation and quantification of 5-FU [64,65]. Zero-crossing first-derivative spectrometry [64] and CE-UV with large-volume sample stacking (LVSS) were successfully used for the determination of 5-FU and its prodrug (tegafur) in pharmaceutical formulations [65]. This method is characterised by a short analysis time (less than 3 min) and high selectivity and sensitivity. Without the LVSS procedure, limits of detection (LOD) were 600 ng mL^{-1} and 771 ng mL^{-1} for 5-FU and tegafur in standard solutions, respectively. With the LVSS procedure, however, sensitivity was significantly improved (LODs of 5-FU and tegafur were decreased to 7.9 ng mL^{-1} and 6.5 ng mL^{-1}, respectively). Sensitised chemiluminescence based on potassium permanganate oxidation in the presence of formaldehyde has also been used for the determination of 5-FU in pharmaceuticals and biological fluids [66] and presented an LOD of 30.0 ng mL^{-1} and a calibration range from 100 ng mL^{-1} to 80.0 μg mL^{-1}. Serum samples were prepared by protein precipitation with trichloroacetic acid and standard addition method was used to avoid matrix effects. LC-UV methods have also been reported for impurity profile studies [67], and analysis of bulk products, pharmaceutical formulations [68] and capsules [69] of capecitabine. For capecitabine in standard solutions, these methods have shown LODs and LOQs about 80.0 and 300 ng mL^{-1}, respectively.

Table 1
Analytical methods for antimetabolites.

Compound	Matrix	Analytical technique	References
Azacitidine	Pharmaceutical formulation	LC-UV, spectrophotometry	[93,138–141]
	Biological samples	LC-UV	[142]
	Biological samples	LC–MS/MS	[137]
Azathioprine	Pharmaceutical formulation	^{1}H NMR	[144]
	Pharmaceutical formulation	CE-UV	[145]
	Bulk drug	UHPLC-UV	[143]
	Biological samples	Second derivative spectra method	[465]
	Biological samples	LC-UV	[149–152]
	Biological samples	LC–MS/MS	[148]
	Residues for cleaning validation	LC-UV	[155]
	Chemical degradation	LC-UV	[154]
	Environmental samples	LC-UV	[156]
	Sewage Water	LC–MS/MS	[58]
Capecitabine	Pharmaceutical formulation	LC-UV	[67–69]
	Biological samples	CE-UV	[79]
	Biological samples	Review	[24]
	Biological samples	LC-UV	[80–82]
	Biological samples	LC–MS	[83]
	Biological samples	LC–MS/MS	[84–88]
Cladribine	Biological samples	Spectrofluorimetry	[466]
	Biological samples	LC-UV	[157]
	Biological samples	LC–MS/MS	[158]
Clofarabine	Biological samples	LC–MS/MS	[158]
Cytarabine	Pharmaceutical formulation	FIA	[8]
	Pharmaceutical formulation	LC-UV	[63,90–93]
	Pharmaceutical formulation	LC–MS/MS	[51]
	Biological samples	GC–MS or GC with a nitrogen-sensitive detector	[94]
	Biological samples	LC–MS	[467]
	Biological samples	LC-UV	[63,95–99]
	Biological samples	LC with solid-phase scintillation detection	[100]
	Biological samples	LC–MS/MS	[101–105]
	Biological samples	Supercritical fluid chromatography	[106]
	Biological samples	CE-UV, MEKC-UV	[107–109]
	Wipe samples (surface contamination)	LC–MS/MS	[51,52,89]
	Wastewater	LC–MS/MS	[57]
Fludarabine	Pharmaceutical formulation	FIA	[8]
	Biological samples	LC–MS/MS	[159]
5-Fluorouracil	Pharmaceutical formulation	LC-UV	[8,60,61,63,113]
	Pharmaceutical formulation	CE-UV	[65]
	Biological samples	Review	[24]
	Biological samples	LC-UV	[71]
	Biological samples	LC–MS	[71,88,468,469]
	Biological samples	CE-UV	[73–75,77–79]
	Fundamental study	CE-UV	[76]
	Wipe samples (surface contamination)	GC–MS	[41]
	Wipe samples (surface contamination)	LC-UV	[45,274,279]
	Wipe samples (surface contamination)	LC–MS/MS	[49,89]
	Waste water	LC–MS/MS	[57]
	Hospital effluents	CE-UV	[55]
Gemcitabine	Pharmaceutical formulation	CE-UV	[115]
	Pharmaceutical formulation	LC-UV	[113] [8]
	Pharmaceutical formulation	HPTLC	[114]
	Pharmaceutical formulation	LC–MS/MS	[51]
	Biological samples	Zero-and second order derivative spectrophotometry	[135]
	Biological samples	LC-UV	[63,116–125,135]
	Biological samples	LC–MS	[126]
	Biological samples	LC–MS/MS	[53,127–132,134]
	Fundamental study	LC–MS/MS	[133]
	Wastewater	LC–MS/MS	[57]
	Wipe samples (surface contamination)	LC–MS/MS	[51–53,136]
Hydroxycarbamide	Pharmaceutical formulation	Potentiometry, fluorimetry	[192]
	Pharmaceutical formulation	LC	[193]
	Biological samples (plasma, peritoneal fluid)	LC-ECD	[195]
	Biological samples	LC-UV	[194]
	Biological samples	GC–MS	[196,197]
	Air samples	LC-UV	[198]
Mercaptopurine	Pharmaceutical formulation	CZE-UV	[145]
	Biological samples	Review	[23]
	Biological samples	LC-UV	[149,151]
	Biological samples	LC–MS/MS	[148]

Table 1 (*Continued*)

Compound	Matrix	Analytical technique	References
	Wipe samples (surface contamination)	LC-UV	[156]
Methotrexate	Pharmaceutical formulation	CE-UV	[175]
	Pharmaceutical formulation	FIA	[8,177]
	Pharmaceutical formulation	CD-MEKC	[176]
	Biological samples	Review	[160]
	Biological samples	LC-UV combined with pseudo template molecularly imprinted polymer	[161]
	Biological samples	LC-UV-fluorescence	[162]
	Biological samples	LC–MS/MS	[163]
	Biological samples	CE-UV	[75,164–166,168–171]
	Biological samples	MEKC-UV	[167]
	Biological samples	CE-LIF	[172,174]
	Biological samples	MEKC-LIF	[173]
	Biomonitoring	LC–MS/MS	[27]
	Wipe samples (surface contamination)	LC–MS/MS	[47,51,52,89]
	Wastewater	LC–MS/MS	[58,179,180]
	Fundamental study	PACE (pressure assisted CE)	[178]
Pemetrexed	Pharmaceutical formulation	LC-UV	[181–184]
	Pharmaceutical formulation	LC-ELSD	[184]
	Biological samples	LC-UV	[185,186]
	Biological samples	LC–MS	[187]
Pentostatin	Biological samples	LC–MS	[191]
Raltirexed	Pharmaceutical formulation	CD-MEKC	[189]
	Biological samples	LC–MS	[190]
Tegafur	Pharmaceutical formulation	CE-UV	[65]
	Pharmaceutical formulation	Zero-crossing first derivative spectrometry	[64]
	Biological samples	LC-UV	[470–472]
	Biological samples	GC–MS	[472]
	Biological samples	LC–MS/MS	[473]
Thioguanine	Biological samples	CE-UV	[75]
	Fundamental study	LC–MS/MS	[153]

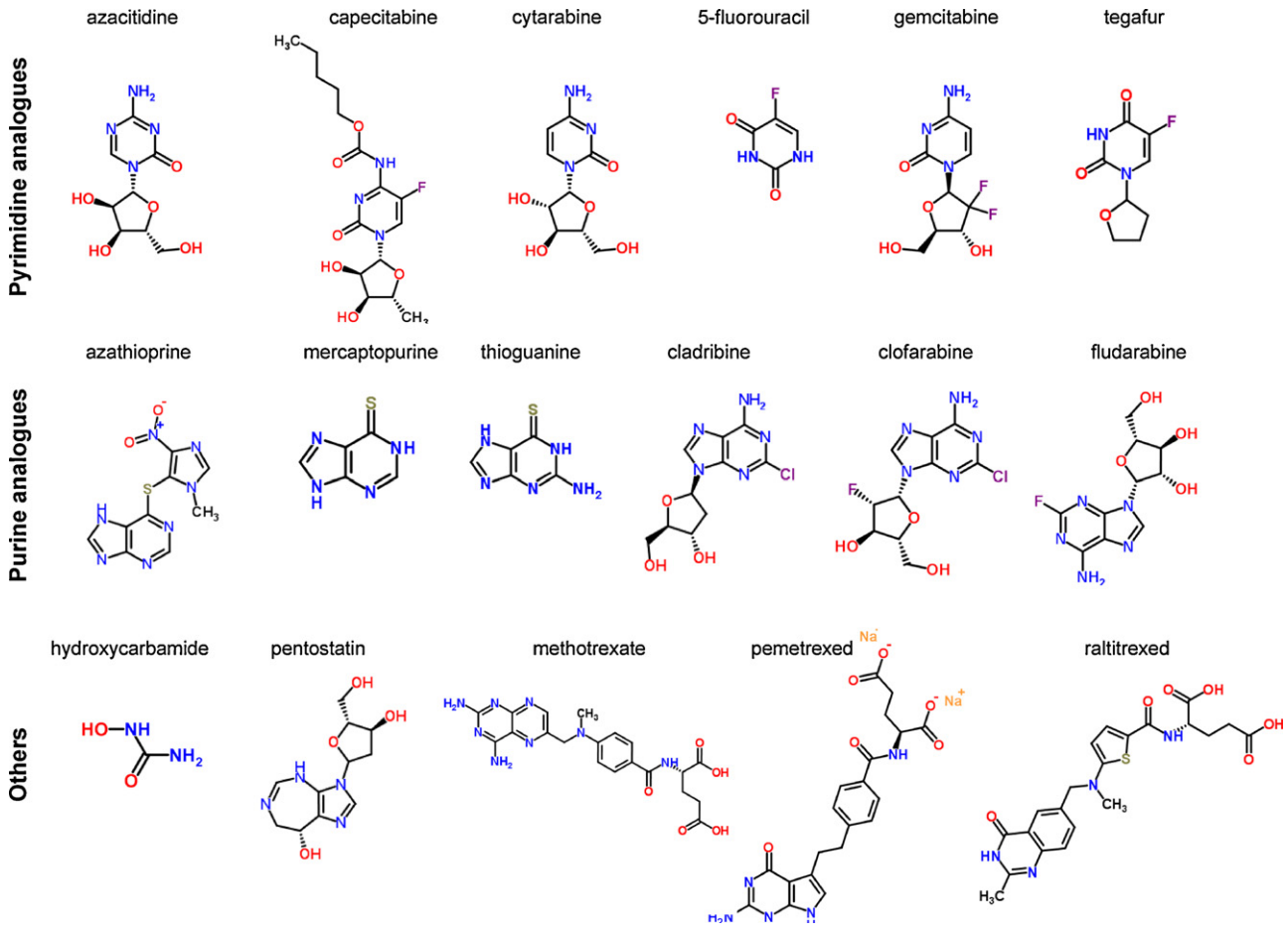

Fig. 1. Chemical structures of antimetabolites.

A large number of analytical methods for the determination of 5-FU, related prodrugs and their metabolites in biological matrices have been developed in the last 30 years. These methods include cell-based culture assays, LC-UV, LC-fluorescence, GC–MS and LC–MS/MS. Advantages and disadvantages of such methods have already been discussed by Breda and Barattè in 2010 [70], including biological sample analysis of tegafur. According to this review, 5-FU monitoring has not yet been widely used, and recent developments with LC–MS/MS and nanoparticle antibody-based immunoassays may facilitate routine monitoring of 5-FU in daily clinical practice. Recently, eight original 5-FU derivatives were synthesized in order to identify new efficient prodrugs of 5-FU and sensitive LC-UV and LC–MS methods were developed to simultaneously quantify 5-FU and its derivatives in human plasma. Sample preparation by centrifugation, filtration and dilution was performed, and MS detection was necessary for characterisation of degradation products [71].

CE methods were not recorded in the review of Breda and Barattè [70], but have also been used for biological samples: CE coupled to amperometric detection for urine and serum samples [72] and CE-UV for plasma [73,74], urine [75], or cell extracts [74,76–79] have been reported. However, the sensitivity was not always sufficient for simultaneous determination of 5-FU and its active metabolites. Indeed, LODs superior to 1 $\mu g\,mL^{-1}$ were achieved for 5-FU and its active metabolite 5-fluoro-29-deoxyuridine-59-monophosphate (FdUMP) and, thus, a preconcentration step (e.g., extraction) and/or the use of more sensitive detection techniques should be investigated [74]. For the determination of capecitabine, LC-UV [80–82] or LC–MS methods [83–88] have been published. With simple protein precipitation followed by LC–MS/MS analysis, very good selectivity and sensitivity values were obtained, with an LOQ of 10 $ng\,mL^{-1}$ for capecitabine in human plasma allowing PK studies [87].

Analysis of 5-FU in environmental samples is particularly interesting because it is one of the most used cytotoxic agents at high doses and therefore an ideal marker compound for other potential contaminants. Surface contamination monitoring using GC–MS [41] or LC [89] was successfully performed. However, due to the high polarity of 5-FU, low retention times were recorded when reversed phase LC columns were used, and separation from different antimetabolites was difficult to obtain. For this reason, the use of hydrophilic interaction liquid chromatography (HILIC) coupled to MS/MS appears to be an attractive approach for the analysis of antimetabolites in wastewater [57]. In the described conditions, baseline separation was obtained for 5-FU, cytarabine, gemcitabine and their metabolites (uracil 1-β-D-arabinofuranoside and 2′,2′-difluorodeoxyuridine) with a resolution superior to 2.4 and an LOQ of 5 $ng\,mL^{-1}$ for 5-FU. In addition, CE-UV allowed the determination of 5-FU in hospital effluents after enrichment by solid-phase extraction (SPE) (concentration factor 500), allowing good quantitative performance with similar quantification limits with an LOQ of 5 $ng\,mL^{-1}$ [55].

3.1.1.2. Cytarabine. Cytarabine is still one of the most effective single agents available for treating acute myeloblastic leukaemia, although myelosuppression is a major side effect [2]. Stability and compatibility data for cytarabine in different containers and admixtures were determined by LC in the 1980s [90–92]. LC methods have also been developed for the analysis of bulk drugs and pharmaceutical formulations containing cytarabine and azacitidine [93]. For biological sample analysis, GC–MS or GC with a nitrogen-sensitive detector was developed for determination of cytarabine in human plasma in 1978 [94]. Different LC-UV methods have also been published for plasma analysis and PK studies within a concentration range in order of $\mu g\,mL^{-1}$ [95–97]. More recently, LC-UV methods were developed and validated for the simultaneous detection of cytarabine and etoposide in pharmaceutical preparations and in spiked human plasma [63]; cytarabine and doxorubicin for TDM [98]; and cytarabine, daunorubicin and etoposide in human plasma for clinical studies [99]. The latter was preceded by SPE with a mixed-mode sorbent and presented LOQs in order of $ng\,mL^{-1}$ [99]. Furthermore, tritium-labelled cytarabine was used to evaluate the intracellular metabolism of cytarabine and was analysed simultaneously with its metabolites by ion-pair LC with solid-phase scintillation detection [100]. Concerning the sample preparation, the incubated cells were lysed by adding a solution containing amphoteric tetrabutylammonium phosphate at pH 3.0, vortexed, centrifuged and filtered before analysis.

Over the last five years, various LC–MS/MS methods for the determination of cytarabine in plasma samples [101–105] or environmental samples [51,52,57] have been reported with good quantitative performance in terms of selectivity and sensitivity. Supercritical fluid chromatography with a simple sample pretreatment procedure showed equivalent accuracy to the analytical results obtained by LC–MS/MS from 50 to 10,000 $ng\,mL^{-1}$ of cytarabine in mouse plasma and have been proven to be reliable for *in vivo* studies [106]. Several CE-UV or micellar electrokinetic chromatography (MEKC)-UV methods also have been found to be suitable for clinical samples and pharmacokinetic studies [107–109]. However, LOQ of cytarabine in human serum was superior by MEKC-UV [109] (3000 $ng\,mL^{-1}$) than by the above mentioned LC–MS/MS methods (i.e.10 $ng\,mL^{-1}$ in rat plasma [104] or 1.0 $ng\,mL^{-1}$ in aqueous solutions [51]).

3.1.1.3. Gemcitabine. Gemcitabine is a more recently introduced compound of the antimetabolites and is used intravenously in association with cisplatin for metastatic non-small cell lung, pancreatic, and bladder cancers. It is generally well tolerated but can cause gastrointestinal disturbances, renal impairment, pulmonary toxicity, and influenza-like symptoms [2].

The first degradation studies were published in 1994 by Lilly Research Laboratories using LC-UV, NMR and MS [110]. Later, physical and chemical stability tests showed good stability for reconstituted solutions up to 35 days at room temperature, but precipitation was observed when stored at 4 °C [111]. Jansen et al. also studied the degradation kinetics of gemcitabine by LC-UV, MS and NMR in acidic solution and identified degradation products [112]. For quality control, preparations of gemcitabine were controlled by LC-UV [113], high performance thin layer chromatography (HPTLC) [114] or LC–MS/MS [51]. A CE-UV method has also been developed for gemcitabine determination in injectable solutions [115]. For biological samples analysis, different LC methods have been published for the determination of gemcitabine and its metabolites in plasma, urine, tissue or cancer cells by LC-UV methods [116–125], LC–MS [126], LC–MS/MS [127–134] and by zero-and second-order derivative spectrophotometric methods [135]. The last method was compared with an LC-UV method for determination of gemcitabine in human plasma and no significant difference was obtained in term of precision with an LOQ of 200 $ng\,mL^{-1}$. Lower LOQs were obtained by LC–MS (i.e. 0.5 $ng\,mL^{-1}$ in human plasma [127]). LC–MS/MS methods were also used for environmental analysis, including surface contamination and wastewater analysis [51–53,57,136] with LOQ values in the order of $ng\,mL^{-1}$ [51,57].

3.1.1.4. Azacitidine. 5-Azacytidine is used for the treatment of myelodysplastic syndromes [137]. LC methods were developed for the determination of cytarabine and azacitidine for bulk drugs and pharmaceutical formulations [93,138,139]. Spectrophotometry and LC-UV were used for degradation studies [140] and for the development of encapsulated drug formulations containing azacitidine [141]. LC-UV [142] and, later, LC–MS/MS [137] were reported for azacitidine determination in plasma. The LC–MS/MS method

was found to be 50 times more sensitive with LOQ of 5 ng mL^{-1} than previously published assays (i.e. LOQ of 250 ng mL^{-1} [142]), and allowed PK and PD studies of azacitidine [137].

3.1.2. Purine analogues

3.1.2.1. Azathioprine, mercaptopurine and thioguanine. Azathioprine, an immunosuppressant agent, is a useful antileukaemic drug and is metabolised to 6-mercaptopurine. Mercaptopurine is also directly used almost exclusively as maintenance therapy for acute leukaemia. Thioguanine is used orally to induce remission in acute myeloid leukaemia [2].

A validated ultra high performance liquid chromatography with UV detection (UHPLC-UV) method was developed for determination of process-related impurities in azathioprine bulk drug. All impurities were well resolved within 5 min and presented LOQs in the range of 490–740 ng mL^{-1} [143]. Quality control for azathioprine in tablets has been performed by ^{1}H NMR spectroscopy [144] and by a stability-indicating CE-UV method, which performed well at separating azathioprine, 6-mercaptopurine and other related substances (including degradation and impurity products) [145]. CE was also useful for determination of 6-thioguanine in urine with an LOQ of 5300 ng mL^{-1} and a simple dilution of urine with water 1:1 [75]. To assess adherence to azathioprine therapy and to identify myelotoxicity and hepatotoxicity, thiopurine metabolite monitoring can be performed by LC-UV [146,147] or LC–MS/MS [147,148]. Additional LC methods for biological samples [149–153], chemical degradation studies [154] or residues after cleaning in production areas [155,156] have been reported. With an LOQ of 290 ng mL^{-1}, the LC-UV method was considered as sensitive enough for routine cleaning validation processes and for quantitative determination of azathioprine in commercial samples [155].

3.1.2.2. Cladribine, clofarabine, fludarabine. Cladribine is given by intravenous infusion for the first-line treatment of hairy cell leukaemia and the second-line treatment of chronic lymphocytic leukaemia in patients who have failed on standard regimens of alkylating agents. Fludarabine is also used for patients with chronic lymphocytic leukaemia after failure of an initial treatment with an alkylating agent. Usefulness is limited by myelosuppression. Clofarabine is approved for treating refractory acute lymphoblastic leukaemia in children after failure of at least two other types of treatment [2].

Yeung et al. developed an LC-UV method preceded by SPE for determination of cladribine in plasma. The described method presented adequate sensitivity and specificity with an LOQ of 50 ng mL^{-1} to study PK of cladribine in rats [157]. Micro-column LC–MS/MS and UHPLC–MS/MS methods were developed for the simultaneous determination of cladribine and clofarabine in mouse plasma samples with a protein precipitation as sample pretreatment [158]. The UHPLC–MS/MS method was sensitive, cost-effective and reliable for high throughput PK screening with a 2 min run time and showed equivalent accuracy (less than 15%) to the analytical results obtained using the micro-column LC–MS/MS method with a one min run time [158]. Simultaneous determination of fludarabine and cyclophosphamide in human plasma has also been successfully performed by a validated LC–MS/MS over a range of 1 to 100 ng mL^{-1} [159].

3.1.3. Other antimetabolites

Methotrexate (MTX) is used as maintenance therapy for childhood acute lymphoblastic leukaemia, in choriocarcinoma, non-Hodgkin's lymphoma, and several solid tumours. It is also administered for the treatment of autoimmune diseases like psoriasis, rheumatoid arthritis, and lupus. Side effects include myelosuppression, mucositis, and gastrointestinal ulceration with potential damage to kidneys and liver that may require careful monitoring. According to the review of Rubino [160], more than 70 papers describing chromatographic assays for MTX and its metabolites have been published in the literature between 1975 and 2000. A wide range of experimental conditions for sample preparation and analyte separation and detection have been employed. Since 2001, LC-UV combined with pseudo template molecularly imprinted polymer [161], LC-UV-fluorimetry [162], and LC–MS/MS [27,163] have been reported for biological samples. LOQ for MTX in human serum was found to be at the level of 10.0 ng mL^{-1} with LC–MS/MS preceded by acetonitrile protein precipitation and filtration [163]. Monitoring of MTX in urine [75,164,165], in whole blood [166,167], plasma [168], serum [169] and tumour samples [170] was also successfully performed by CE-UV. In most of these studies, complete validation for biological samples was achieved. Several sample preparation techniques were used, including simple dilution [75,165], SPE [164,168] and on-line stacking CE [167,168]. CE with high sensitivity cells (Z-cell) showed good precision and accuracy for quantitative analysis of MTX in biological media and led to an approximately 10-fold improvement of the detection limit compared to standard capillaries with LOD in water and urine of 100 ng mL^{-1} [171]. Other improvements to sensitivity were obtained using CE-LIF analysis with detection in the ng mL^{-1} range [172–174].

Another validated CE method allowed chiral separation of racemic MTX in pharmaceutical formulations with precision values below 5% and baseline enantiomers separation within 6 min [175]. Gotti et al. developed and validated (according to ICH guidelines) a cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) method to analyse MTX and its most important impurities [176]. Separation was improved by the addition of methanol in the CD-MEKC system and adequate accuracy between 93 and 106% with RSD values lower than 8% was obtained. Additionally, FIA was successfully used for the determination of methotrexate in pharmaceutical formulations [8,177]. The first method used UV detection and was applied for qualitative and quantitative control of cytotoxic preparations in a hospital preparation unit [8]. The second FIA method was coupled with fluorescence detection preceded by oxidation of MTX into a highly fluorescence product (2,4-diaminopteridine-6-carboxylic acid) with acidic potassium permanganate [177]. Under these conditions, intra and interday precision values (RSD) were inferior to 1%. Finally, fundamental studies on the determination of pK values for MTX and other compounds have been performed by pressure-assisted CE-UV [178].

For environmental analysis, LC–MS/MS was employed for MTX determination in water samples [58,179,180] and on several surfaces [47,51,52]. A wiping procedure coupled to LC–MS/MS allowed determination of surface concentration down to 0.1 ng cm^{-2} of MTX and nine other cytotoxic drugs with completely evaluated quantitative performance in terms of accuracy and precision [52].

Pemetrexed is indicated for the treatment of pleural mesothelioma as well as non-small cell lung cancer. Physical and chemical stabilities were established by LC-UV for different pemetrexed formulations (e.g., in PVC bags or plastic syringes) by Zhang and Trissel [181–183]. Recently, an ion-pairing reversed-phase LC method using a double detection analysis (UV and evaporative light scattering detection (ELSD)) was employed to monitor the stability of pemetrexed preparations [184]. UV detection was used to quantify pemetrexed within a concentration range of 0.45 to 0.60 mg mL^{-1} with a total error inferior to 3%. L-Glutamic acid was identified and quantified as a potential degradation product by ELSD with an LOD of 1800 ng mL^{-1}.

A column-switching LC method for pemetrexed determination in human plasma has been developed to support PK studies with an LOQ of 10 ng mL^{-1} [185]. Other LC-UV [186] and LC–MS [187] methods have also been reported for biological samples analysis. Recently, a new ultrafast and high-throughput MS approach for the

therapeutic drug monitoring of pemetrexed in plasma from lung cancer patients was developed by matrix assisted laser desorption/ionisation (MALDI)–MS/MS with an analysis time of only 10 s and good sensitivity and compliance with FDA regulations (within- and between-run accuracy and precision inferior to 15% RSD) [188].

Raltitrexed, a drug approved in Canada, is given intravenously for palliation of advanced colorectal cancer in cases where 5-FU cannot be used. It is generally well tolerated, but can cause myelosuppression and gastrointestinal toxicity [2]. A rapid and effective method was developed for the chiral separation of raltitrexed enantiomers by CD-MEKC to determine the purity of real synthetic drug samples [189]. The enantiomers of raltitrexed could be separated within 13 min with satisfactory resolution and sensitivity (LOD of 1000 ng mL^{-1} for both enantiomers). Determination of raltitrexed in human plasma was successfully performed by LC–MS and achieved good sensitivity and specificity with an LOQ of 2 ng mL^{-1} [190].

Administered intravenously, pentostatin is highly active in hairy cell leukaemia and is able to induce prolonged remissions [2]. However, only a few analytical methods have been reported for this therapy (e.g., determination of pentostatin in culture broth by LC–MS [191]).

Hydroxycarbamide, also called hydroxyurea, is an antineoplastic drug used in myeloid leukaemia, often in combination with other drugs. It can also be used for the treatment of melanoma and to reduce the rate of painful attacks in sickle-cell disease [2]. For quality control, potentiometry and fluorimetry have been described for the determination of hydroxyurea in capsules [192], as well as LC-UV for pharmaceutical formulations and bulk products [193]. LC-UV [194] and LC-ECD [195] allowed quantification of hydroxyurea in plasma and peritoneal fluids. GC–MS methods have also been developed for the analysis of plasma samples containing hydroxycarbamide [196,197]. Both methods were validated: the LOD was 78 ng mL^{-1} and the LOQ was 313 ng mL^{-1} and intra-day and inter-day variations inferior to 10% [196]. In addition, an LC-UV method has been developed for environmental monitoring to reduce exposure through inhalation of drug dusts or droplets by workers involved in the manufacture of this compound [198]. The reported method successfully detected hydroxyurea in the concentration range of 0.001–0.08 mg m^{-3}.

3.2. DNA interactive agents

Analysis of alkylating agents, cross-linking agents, intercalating agents, topoisomerase inhibitors and DNA-cleaving agents are described in this section. The chemical structures of DNA-interactive agents are shown in Figs. 2–6 and the relevant analytical methods for pharmaceutical formulations, biological and environmental samples are reported in Table 2.

3.2.1. Alkylating agents (dacarbazine, temozolomide, procarbazine, ecteinascidin-743)

Dacarbazine is employed as a single agent to treat metastatic melanoma and in combination with other drugs for soft tissue sarcomas. The predominant side effects are myelosuppression and intense nausea and vomiting [2]. Stability and compatibility assays of pharmaceutical formulations of dacarbazine by LC-UV [13,14,199–201] and LC–MS [202] have been described. LC-UV [203,204] and LC–MS/MS [205] methods have also been used for the quantification of dacarbazine and its degradation products in urine and plasma. Due to the extreme hydrophilic and unstable character of dacarbazine and its terminal metabolite (5-amino-4-imidazole-carboxamide), HILIC–MS/MS method with a two-step extraction process was considered as specially adapted for the analysis of these compounds in human plasma [205]. The method was validated and presented good quantitative performance in terms of accuracy, precision and specificity with an LOQ of 0.5 ng mL^{-1} allowing PK studies. With LC-UV method preceded by simple protein precipitation (methanol), PK studies were also possible, however, LOQ in plasma samples of dacarbazine and its metabolites were superior (about 30 ng mL^{-1} for dacarbazine) with a RSD of 20% [204].

Temozolomide is a more-recently introduced compound for the second-line treatment of brain cancers. Structurally similar to dacarbazine, its main advantage is its good oral bioavailability and distribution properties with penetration into the central nervous system [2]. LC-UV methods were used for the development of new drug formulations containing temozolomide, including a dry powder formulation for inhalation [206], liposomes for nasal administration [207] or intravenous injection with solid lipid nanoparticles [208]. Andrasi et al. developed MEKC-UV methods for stability studies of temozolomide and its degradation products in water and serum with short analysis times (1.2 min) [209]. Short analysis time is very important due to the low stability of temozolomide in solution (half-lives inferior to 10 min in physiological conditions). Furthermore, several publications reported the use of LC-UV methods for the quantification of temozolomide and its metabolites in plasma or urine [210–212] and LC–MS/MS [213] methods for 5-(3-N-methyltriazen-1-yl)-imidazole-4-carboxamide), a bioconversion product of temozolomide. In this study, samples were processed and analysed one at a time with an analysis time of 4.5 min, in order to compensate for the inherent instability of the analyte [213]. In addition, an acidic pH (<5) was recommended throughout the collection, sample preparation and analysis to preserve the integrity of the drug [210,212]. Finally, several temozolomide PK studies have been published [214–217].

Procarbazine has significant activity in lymphomas and carcinomas of the bronchus and in brain tumours. Its toxic effects include nausea, myelosuppression, and a hypersensitivity rash that pre-

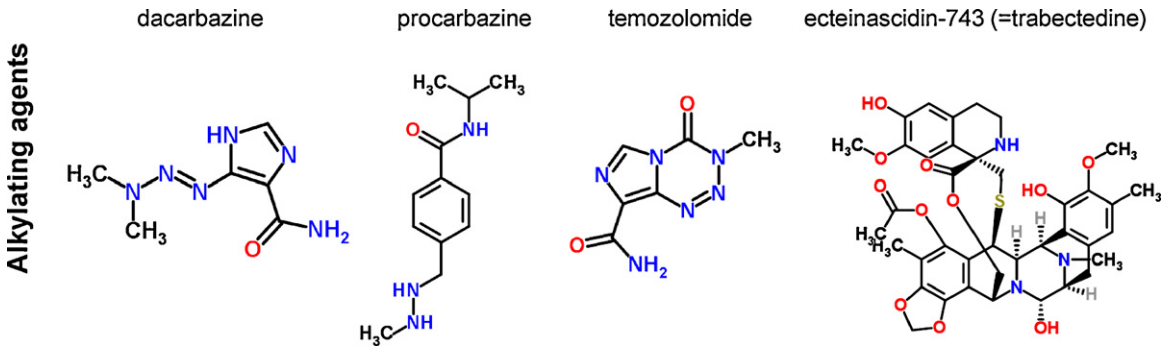

Fig. 2. Chemical structures of DNA-interactive agents: alkylating agents.

Table 2
Analytical methods for DNA interactive agents.

Compound	Matrix	Analytical technique	References
Actinomycin-D	Biological samples	LC–MS/MS	[417–421]
Anthracyclines (aclarubicin, daunorubicin, doxorubicin, idarubicin, epirubicin)	Review	Review	[366–369,423]
	Chemical degradation	LC-UV	[154]
	Pharmaceutical formulation	FIA; LC-UV/Vis	[8,63,370–373]
	Pharmaceutical formulation	LC–MS/MS	[51]
	Biological samples	LC-UV	[63,98,99,393,394]
	Biological samples	LC-chemiluminescence	[385]
	Biological samples	LC-fluorescence	[395–402,413]
	Biomonitoring	LC-fluorescence	[412]
	Biological samples	LC-LIF–MS	[403]
	Biological samples	UHPLC-MS	[409]
	Biological samples	LC–MS/MS	[32,262,263,404–408,411]
	Biological samples	Accelarator mass spectrometry	[410]
	Biological samples	CZE-, MEKC-, MEEKC-UV	[389]
	Biological samples	CE-UV	[374,375]
	Biological samples	CE-LIF	[376–383]
	Biological samples	MEKC-LIF	[386–388]
	Biological samples	CD-MEKC-LIF	[384]
	Biological samples	MALDI-TOF	[383]
	Biological samples	CE-amperometry	[390]
	Fundamental study (pKa)	CE-amperometry	[391]
	Fundamental study	CE-absorption-based wave-mixing detector	[392]
	Wipe samples (surface contamination)	LC-UV	[45,274]
	Hospital effluents	LC-fluorescence	[56]
	Wipe samples (surface contamination)	LC–MS/MS	[49,51,52]
	Wastewater	LC–MS/MS	[58]
Amsacrine	Degradation study	LC-UV	[154]
	Biological sample	Review	[369]
Bleomycin	Pharmaceutical formulation	LC-UV	[439]
	Pharmaceutical formulation	LC–MS	[441]
	Pharmaceutical or biological samples	DNA-based electrochemical strategy	[440]
Busulfan	Review	Review	[22–24]
	Pharmaceutical formulation	HPTLC	[355]
	Pharmaceutical formulation	NIRS	[356]
	Pharmaceutical formulation	LC-UV	[351–353]
	Pharmaceutical formulation	LC-CD	[354]
	Biological samples	LC-UV	[347–349]
	Biological samples	LC-fluorescence	[350]
	Biological samples	LC–MS	[340]
	Biological samples	LC–MS/MS	[341–346]
Camptothecin analogs (irinotecan, topotecan)	Review	Review	[423–427]
	Pharmaceutical formulation	FIA-UV	[8]
	Pharmaceutical formulation	Spectrofluorimetry	[432,474]
	Pharmaceutical formulation	LC-UV	[429–431]
	Pharmaceutical formulation	LC–MS/MS	[51]
	Pharmaceutical formulation	HPTLC	[428]
	Plant extracts	MEKC-UV	[433]
	Biological samples	LC-fluorescence	[475–480]
	Biological samples	Spectrofluorimetry	[474]
	Biological samples	LC–MS/MS	[481]
	Wipe samples (surface contamination)	LC–MS/MS	[51,52]
Chlorambucil	Biological samples	Review	[22,24]
	Biological samples	LC-UV	[296,297]
	Biological samples	LC–MS/MS	[298]
	Biological samples (adducts)	HPLC-MS(n)	[299]
Chlormethine (or nitrogen mustards)	Pharmaceutical formulation, aqueous solution	HPLC-UV	[283–286]
	Biological samples	GC–MS	[288]
	Biological samples	LC-UV	[287]
	Biomonitoring	LC–MS/MS	[292]
	Soil samples	GC–MS	[289,290]
	Aqueous and decontamination solutions	LC–MS	[291]
Cyclophosphamide, ifosfamide	Review	Review	[23,24,255,256]
	Fundamental study (chirality)	Capillary electrochromatography	[282]
	Pharmaceutical formulation	HPTLC	[269]
	Pharmaceutical formulation	LC-UV	[8,268]
	Pharmaceutical formulation	LC–MS/MS	[51]

Table 2 (*Continued*)

Compound	Matrix	Analytical technique	References
	Biological samples	LC–MS	[258–260,467]
	Biological samples	UHPLC-QTOF	[267]
	Biological samples	LC–MS/MS	[159,257,261–265]
	Biomonitoring	LC–MS/MS	[27,32,38,41,53,270–273]
	Wipe samples (surface contamination)	GC–MS	[41,278–280]
	Wipe samples (surface contamination)	LC-UV	[45,274]
	Wipe samples (surface contamination)	LC–MS/MS	[47,49,51–53,136,272,273,275–277]
	Wastewater, surface water	LC–MS/MS	[57,58,179,180,281]
Dacarbazine	Pharmaceutical formulation	FIA-UV	[8]
	Pharmaceutical formulation	LC-UV	[13,14,199–201]
	Pharmaceutical formulation	LC–MS	[202]
	Biological samples	LC-UV	[203,204]
	Biological samples	LC–MS/MS	[205]
Ecteinascidin-743	Biological samples	LC-UV	[224–226]
	Biological samples	LC–MS	[226]
	Biological samples	LC–MS/MS	[226,227]
Estramustine	Biological samples	LC-fluorescence, GC-NPD, GC–MS	[294,295]
	Biological samples	LC–MS/MS	[293]
Etoposide	Review	Review	[369,423]
	Pharmaceutical formulation	LC-UV	[7,8,63]
	Pharmaceutical formulation	LC–MS/MS	[51]
	Biological samples	LC-UV	[63,99]
	Biological samples	LC–MS/MS	[434]
	Biological samples	UHPLC-MS/MS	[435]
	Biological samples	CE-UV	[171]
	Biological samples	CE-LIF	[437]
	Biological samples	MEKC-near-field thermal lens detection	[438]
	Wipe samples (surface contamination)	LC–MS/MS	[51,52]
	Wastewater samples	LC–MS/MS	[58]
Fotemustine	Pharmaceutical formulation	LC-UV	[328]
	Biological samples	LC-UV	[329]
Melphalan	Pharmaceutical formulation	LC-UV	[8,13,316–318]
	Biological samples	Review	[22–24]
	Biological samples	LC-UV	[300–302]
	Biological samples	LC-fluorescence	[304–306]
	Biological samples	LC-ECD	[303]
	Biological samples	LC–MS/MS	[307,308]
	Biological samples (adduct)	LC–MS/MS	[309–314]
	Biological samples (adduct)	LC-ICP-MS	[315]
Mitomycin C	Pharmaceutical formulation	LC-UV	[362,363]
	Biological samples	LC-UV	[358–361]
	Biological samples	LC–MS	[357]
	Biomonitoring	LC-UV	[364,365]
	Ambient air samples	LC-UV	[364,365]
Mitoxantrone	Review	Review	[368,369]
	Biological samples	LC-UV	[414,415]
	Biological samples	LC–MS/MS	[416]
	Aqueous and biological samples	CE-CL	[422]
Nitrosurea (lomustine, carmustine)	Permeability and compatibility studies	LC-UV, LC–MS/MS, spectrophotometry	[13,14,50,321–323]
	Pharmaceutical formulation	LC-UV	[324]
	Biological samples	LC-UV	[319,320,325–327]
Platinum complexes	Review	Review: CE	[228,236–238]
	Pharmaceutical formulation	LC-UV, FIA	[8,229]
	Pharmaceutical formulation	MEKC-UV, MEEKC-UV	[253,254]
	Pharmaceutical formulation	LC–MS/MS	[4]
	Biological samples	Atomic absorption spectra	[482]
	Biological samples	LC-UV	[230]
	Biological samples	LC–MS/MS	[27,231,232]
	Biological samples	LC-ICP-MS	[233,234]
	Biological samples	MEKC, MEEKC	[239–241,243–249]
	Biomonitoring	Voltammetry	[34,35]
	Biomonitoring	ICP-MS	[235]
	Fundamental study	MEEKC-UV	[250,251]
	Fundamental study	MEEKC-ICP-MS	[252]
	Air samples	Voltammetry	[34,35]
	Wipe samples (surface contamination)	Voltammetry	[41]
	Wipe samples (surface contamination)	ICP-MS	[44]
	Wastewater	ICP-MS	[54,233]

Table 2 (*Continued*)

Compound	Matrix	Analytical technique	References
Procarbazine	Pharmaceutical formulation	LC-UV	[199,218,219].
	Pharmaceutical formulation	GC–MS	[199]
	Biological samples	LC-UV	[220]
	Biological samples	LC-amperometry	[221]
	Biological samples	LC–MS	[222,223]
	Sewage water	LC–MS/MS	[58]
Temozolomide	Pharmaceutical formulation	LC-UV	[206–208]
	Pharmaceutical formulation	MEKC-UV	[209]
	Biological samples	LC-UV	[210–212]
	Biological samples	LC–MS/MS	[213]
Teniposide	Review	Review	[369]
	Pharmaceutical formulation	LC-UV	[6]
	Biological samples	UHPLC-MS/MS	[436]
Thiotepa	Review	Review	[23,330]
	Degradation studies	LC-UV	[154,333]
	Pharmaceutical formulation	LC-UV	[334,335]
	Biological samples	GC-NPD	[266,332]
	Biological samples	LC–MS/MS	[264]
	Biological samples	UHPLC-QTOFMS	[331]
Treosulfan	Biological samples	LC-refractometric detection	[336–338]

vents further use of the drug [2]. Procarbazine was determined together with other anticancer drugs in sewage water by selective SPE and UHPLC–MS/MS [58]. In addition, several destruction procedures for toxic compounds including procarbazine were evaluated using LC-UV and GC–MS [199]. Other degradation studies for procarbazine were performed by LC-UV and LC–MS [218,219]. Determination of procarbazine and its metabolites in plasma or urine was achieved by LC-UV [220], LC coupled to amperometric detection [221] and LC–MS [222,223]. With the electrochemical detector, LOD of procarbazine in plasma were obtained in the order of ng mL^{-1}, which was more sensitive than with a typical UV detector [221]. Good sensitivity was also achieved by MS detection with LOQ values of 0.5 ng mL^{-1} for procarbazine in human plasma [223] and 30 ng mL^{-1} for its final metabolite (terephthalic acid isopropylamide) in urine [222].

Ecteinascidin-743 is a novel DNA-binding agent derived from the marine tunicate *Ecteinascidia turbinate*. It has significant activity *in vitro* against melanoma, breast, ovarian, colon, renal, and non-small cell lung and prostate cell lines [2]. For pharmacokinetic or stability studies, LC-UV [224–226], LC–MS [226] and LC–MS/MS [226,227] methods have all been published. Ecteinascidin-743 is administered in $\mu g\,m^{-2}$ dosages, which demands high sensitive analytical method supporting clinical PK studies. Using conventional LC-UV with SPE, an LOQ of 1.0 ng mL^{-1} in plasma was achieved [224], but with SPE followed by LC–MS/MS, an LOQ of 0.01 ng mL^{-1} was obtained [227]. LC–MS/MS was also especially useful in the search for metabolites of ecteinasidin-743 [226].

3.2.2. Cross-linking agents

3.2.2.1. Platinum complexes (cisplatin, carboplatin and oxaliplatin). Platinum complexes belong to the most widely used class of drugs in cancer treatment and possess a pronounced activity in different cancer types. Cisplatin was the first platinum complex used with a pronounced activity in testicular and ovarian cancers. The related analogues carboplatin and oxaliplatin were developed later to reduce the problematic side effects of cisplatin (nephrotoxicity, ototoxicity, and peripheral neuropathy, among others). Carboplatin is used in the treatment of advanced ovarian cancer and lung cancer, while oxaliplatin is licensed for the treatment of metastatic colorectal cancer in combination with fluorouracil and folinic acid [2].

As reported by Espinosa Bosch et al. in 2010 [228], various techniques have been developed for the determination of cisplatin, including derivative spectrophotometry, phosphorescence, atomic absorption spectrometry, GC–MS, CE and LC coupled with different detectors (UV, electrochemical, inductively coupled plasma-atomic emission spectrometry, ICP-MS or electrospray ionisation-mass spectrometry (ESI-MS)). The determination of platinum complexes in biological fluids and tissues presents a particularly interesting challenge because the damage produced in the affected organs is probably due to the association of platinum or the parent drug metabolites with important proteins of the impacted organ [228]. Analytical methods already reported by Espinosa Bosch et al. [228] are not discussed here. In addition, for carboplatin and oxaliplatin in pharmaceutical formulations or biological samples, LC-UV [229,230] LC–MS/MS [4,231,232] and LC-ICP-MS [233,234] have been published. In occupational exposure and environmental studies (air, surfaces, and wastewater), voltammetry [34,35,41] and ICP-MS [44,54,233,235] have been successfully applied with LOD in the order of 0.1 ng mL^{-1}.

According to different authors, CE has emerged as the method of choice for the separation of intact platinum metal complexes and their metabolites due to its high efficiency, versatility and gentle separation conditions for metal complexes [236–238]. Because platinum drugs are non-charged coordination complexes, MEKC or microemulsion electrokinetic chromatography (MEEKC) is often used. The main publications dedicated to the analysis of platinum drugs with MEKC or MEEKC were developed for biological studies, such as clinical sample analysis [239], drug–protein [240–244] and drug–DNA (or nucleotides) binding studies [245–249] and chemical studies [250,251]. Most commonly, UV spectrophotometry was used for the detection of platinum drugs with MEKC or MEEKC, despite ICP-MS also being reported to enhance their selectivity and sensitivity [252]. The LOQs for oxaliplatin samples were slightly lower when ICP-MS detection was used than UV/Vis detection (0.3 mg mL^{-1} instead 0.5 mg mL^{-1}). Few methods of MEEKC and MEKC were also developed for the quality control of diluted formulations of cisplatin, carboplatin, and oxaliplatin [253,254], and the latter was completely validated and successfully applied for cytotoxic preparations at a hospital pharmacy [254].

3.2.2.2. Nitrogen mustards (cyclophosphamide, ifosfamide, melphalan, chlorambucil, chlormethine, estramustine). Cyclophosphamide has a broad spectrum of clinical activity in solid tumours (carcinomas of the bronchus, breast, ovary, and various sarcomas), chronic lymphocytic leukaemia, and lymphomas. Ifosfamide is an analogue

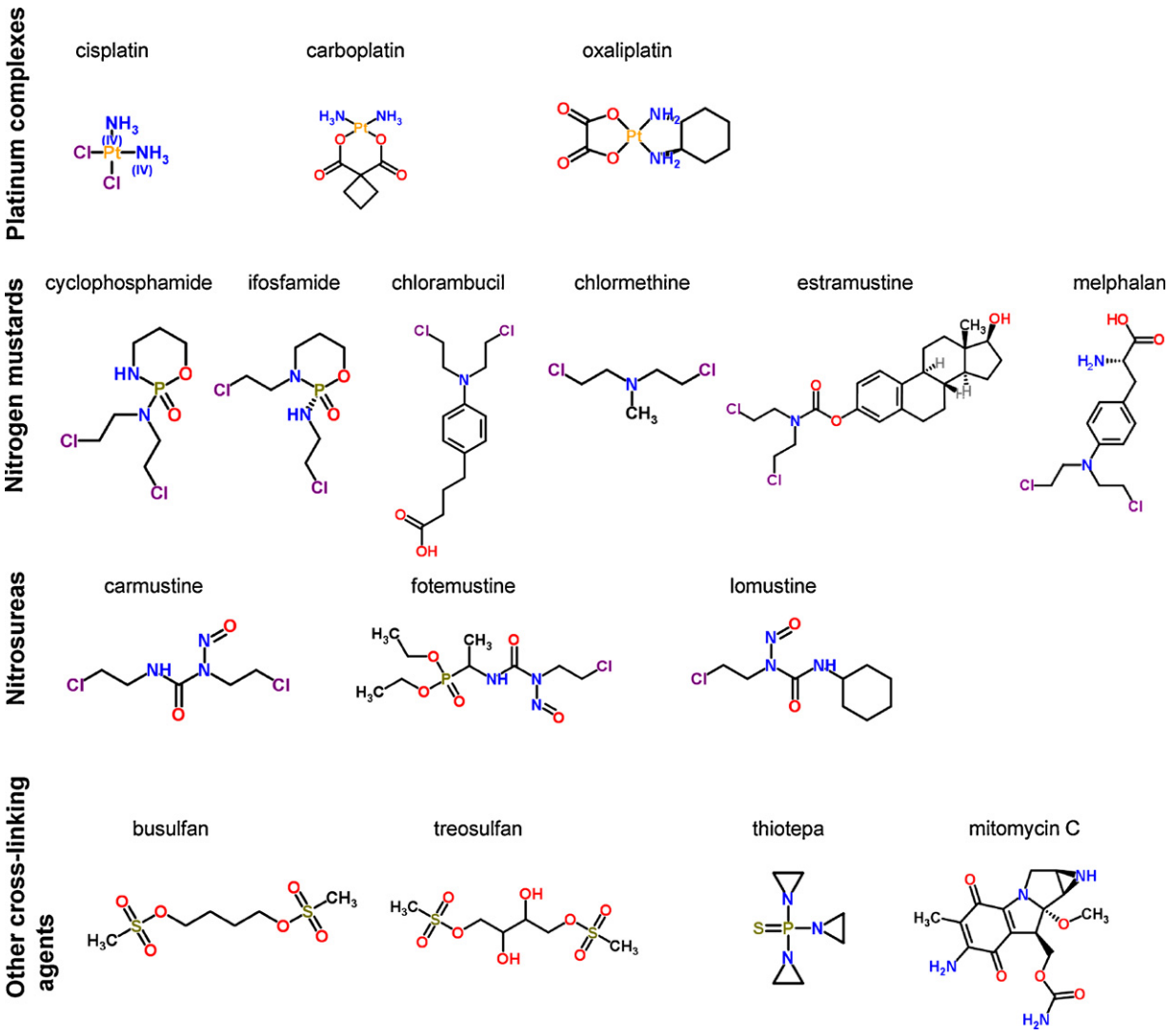

Fig. 3. Chemical structures of DNA-interactive agents: cross-linking agents.

of cyclophosphamide with a similar activity spectrum. Activation of the drugs is obtained after drug metabolism in the liver [2]. Reviews on anticancer drug monitoring, including cyclophosphamide and ifosfamide, using GC–MS [23] and LC–MS [24] were published by Guetens et al. in 2002. Other reviews of the analysis of oxazaphosphorines (cyclophosphamide, ifosfamide, trofosfamide) and their metabolites have given an excellent overview of sensitive and selective analytical methods, but these were published ten years ago [255,256]. GC with nitrogen-phosphorus detection (GC-NPD) was the most used determination technique with and without derivatisation, allowing high selectivity and sensitivity. However, GC–MS, LC-UV and LC–MS for cyclophosphamide and related compounds, and also several analyses of DNA-adducts, were discussed in the review of Baumann and Preiss in 2001 [256]. Moreover, oxazaphosphorines are chiral molecules, administered as a racemic mixture of their two enantiomeric forms, and various assays have been described for studying stereochemical effects [256–258].

Since 2001, LC–MS [258–260] and LC–MS/MS [159,257,261–266] have been characterised by good quantitative performance in terms of sensitivity and selectivity for cyclophosphamide and ifosfamide in biological samples. LOQ in order of ng mL^{-1} were obtained and different sample preparation techniques were used, allowing PK studies. For example, the use of turbulent flow online sample extraction followed by LC–MS/MS analysis decreased sample preparation time and simplified the quantitation of cyclophosphamide and its metabolite carboxyethylphosphoramide mustard (CEPM) in human plasma with sufficient accuracy and precision values (RSD inferior to 3.0%) to allow its application in clinical studies. LOQ of cyclophosphamide and CEPM in human plasma were 500 ng mL^{-1} and 50 ng mL^{-1}, respectively [265]. In another study by LC–MS/MS, sample preparation consisted of dilution of urine with an aqueous solution of the internal standard D4-CP and methanol, and centrifugation. LOD of cyclophosphamide in urine was about 5 ng mL^{-1}, but quantification range was adjusted to the expected concentrations in 24-h urine collections of patients and the urinary concentration of cyclophosphamide was much higher, i.e. in the range of 3000–17,5000 ng mL^{-1} due to the high administrated dosages of this drug [261]. Metabolism profiles of cyclophosphamide and ifosfamide in mice were studied using UHPLC–MS/MS to better understand the selective toxicity of these two compounds [267]. Twenty three urinary metabolites, including five novel drug metabolites, were identified and structurally elucidated. Although cyclophosphamide and ifosfamide went through similar metabolic processes, the amount of metabolites in urine was significantly different between these two drugs.

A stability-indicating LC-UV method allowed the determination of cyclophosphamide in oral suspensions and was used

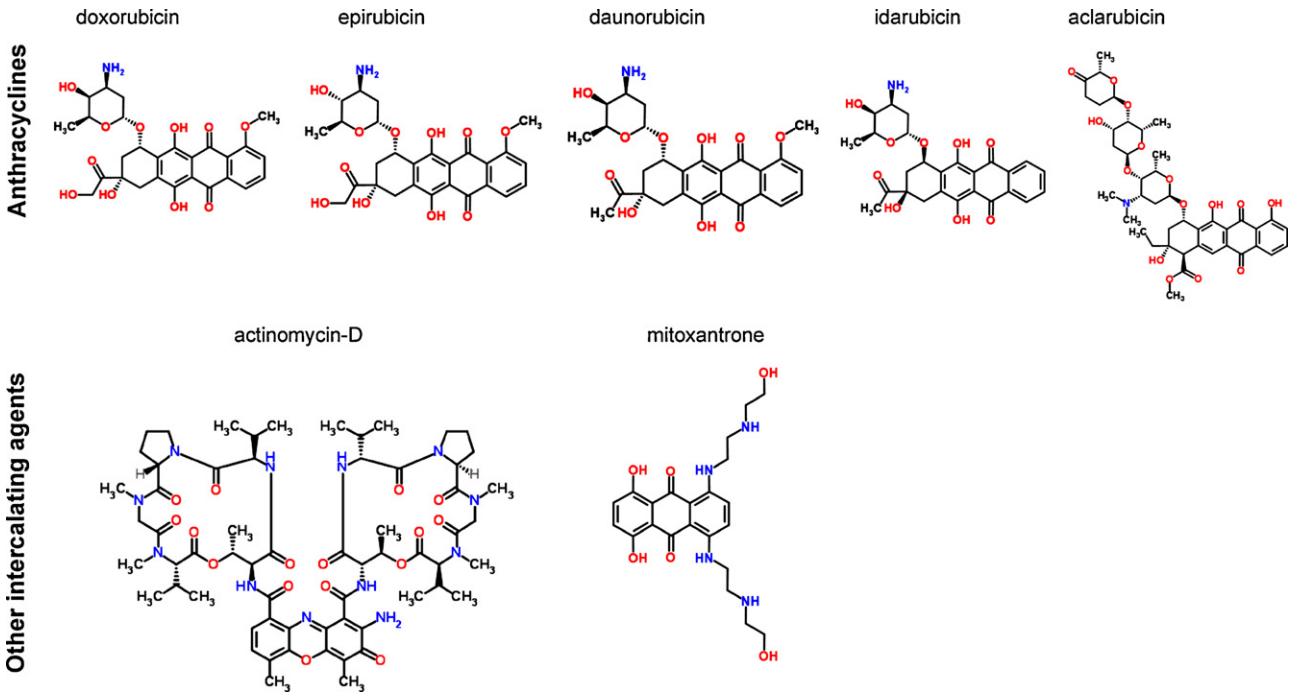

Fig. 4. Chemical structures of DNA-interactive agents: intercalating agents.

to set up storage conditions for simple syrup or suspension [268]. HPTLC [269], LC-UV [8] and LC–MS/MS [51] have been reported for the quality control of hospital formulations. Cyclophosphamide and ifosfamide have also often been analysed in urine samples of healthcare operators for biomonitoring [27,32,38,41,53,270–273], in wipe samples from cytotoxic preparation facilities [41,45,47,49,51–53,57,136,272–280] and in wastewater samples [58,179,180,281]. Cyclophosphamide and

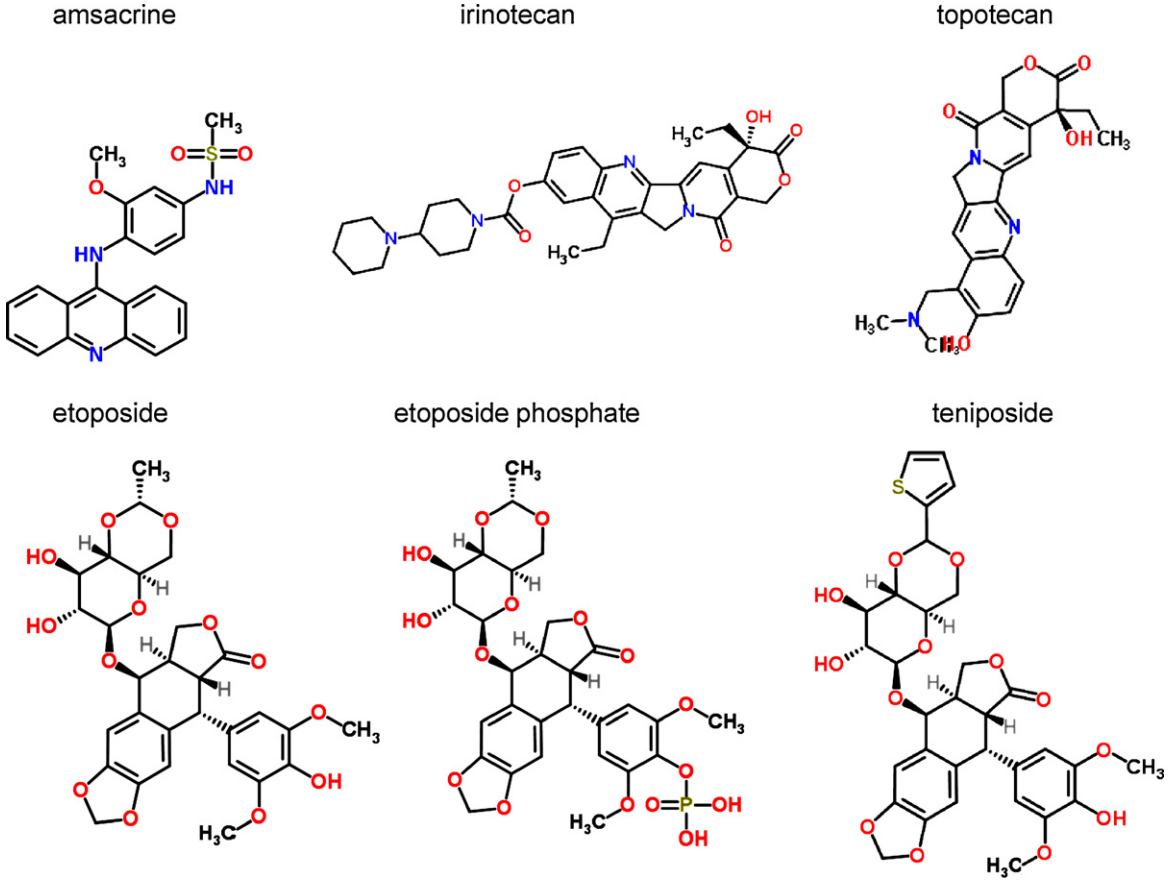

Fig. 5. Chemical structures of DNA-interactive agents: topoisomerase inhibitors.

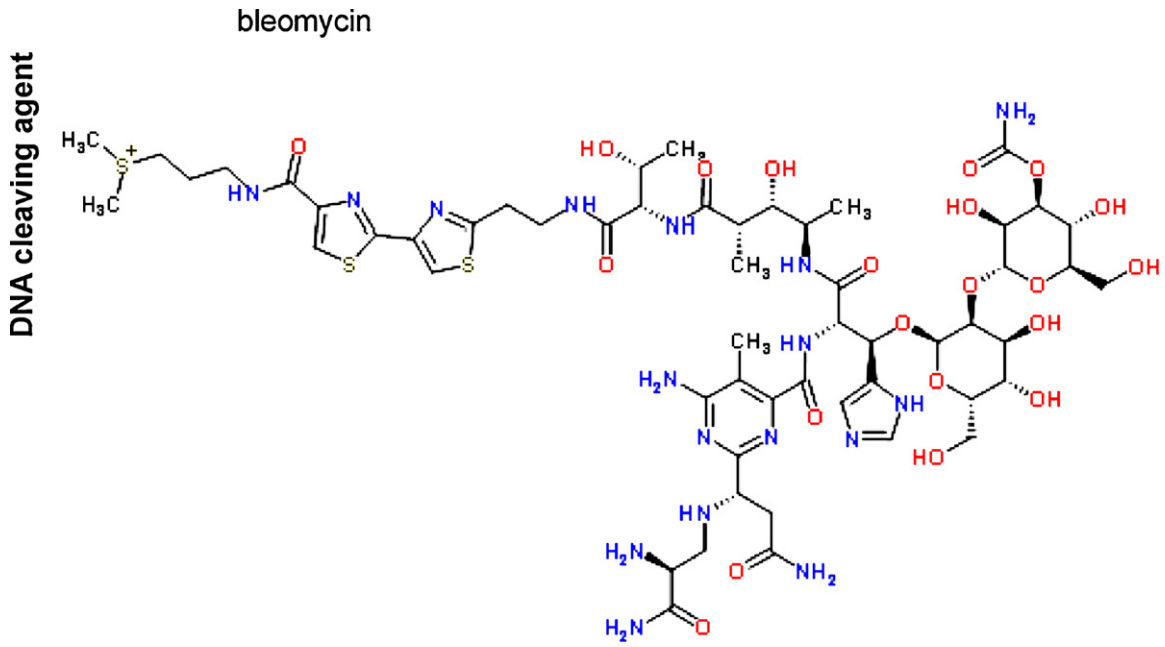

Fig. 6. Chemical structures of DNA-interactive agents: DNA-cleaving agent.

ifosfamide were often used to investigate environmental contamination and LOQs in order of pg to ng mL^{-1} were obtained by LC–MS/MS analysis [51,58,179,180]. Furthermore, Li and Lloyd developed a CE method using capillaries packed with a α1-acid glycoprotein chiral stationary phase for the analysis of enantiomers of cyclophosphamide and ifosfamide [282].

Chlormethine (or mechlorethamine) is used for the treatment of Hodgkin's disease. Due to its chemical reactivity, it must be freshly prepared prior to administration and then delivered via a fast-running intravenous infusion [2]. LC-UV methods, including a pre-derivatisation of mechlorethamine, have been published for the determination of mechlorethamine in aqueous solutions, formulations [283–286] and in plasma [287]. GC–MS methods (with pre-derivatisation) were developed for hydrolysis products of nitrogen mustards in biological samples [288] and for precursors of nitrogen mustards in environmental samples [289,290].

Soil samples were prepared using an on-matrix derivatisation–extraction technique and the method has shown satisfying precision values inferior to 5% within a linearity range from 1 to 12 ng mL^{-1} [289]. Additionally, Chua et al. developed a fast and efficient method of LC–MS for qualitative screening of nitrogen mustards and their degradation products in water and decontamination solutions [291]. Quantification of ultratrace levels (inferior to 1 ng mL^{-1}) of hydrolysis products of nitrogen mustards in human urine was achieved by LC–MS/MS for exposure assessment [292].

Estramustine phosphate is a conjugate consisting of chlormethine chemically linked to an oestrogen moiety. It is usually orally administered to patients with metastatic prostate cancer [2]. A sensitive and selective LC–MS/MS method was developed and validated for the simultaneous determination of estramustine phosphate and its four metabolites (estramustine, estromustine, estrone and estradiol) in human plasma [293]. The assay presented accuracy and precision values inferior to 15% with an LOQ of 10 ng mL^{-1}, and was successfully used for routine analysis of human plasma samples collected in cancer patients with estramustine phosphate treatment. Other studies have used LC with fluorescence detection for estramustine phosphate determination and GC coupled to NPD or MS for metabolite analysis [294,295].

Chlorambucil is useful in the treatment of ovarian cancer, Hodgkin's disease, non-Hodgkin's lymphomas, and chronic lymphocytic leukaemia. Its lower chemical reactivity allows oral dosing [2]. Methods for monitoring anticancer drugs including chlorambucil were published in 1985 by Eksborg and Ehrsson [22] and in 2002 by Guetens et al. [24]. In the last 10 years, LC–UV [296,297] and LC–MS/MS [298] have been used to determine chlorambucil and its metabolite in human serum and plasma. The latter has exhibited specific and sensitive performance for both parent drug and phenyl acetic acid mustard metabolite contained in human serum and plasma with accuracy and precision values inferior to 15%. Moreover, the applied automated SPE procedure was significantly faster than manual sample pre-treatment methods. With LC-UV analysis preceded by acetonitrile protein precipitation, LOQ of chlorambucil in plasma was about 100 ng mL^{-1} [296,297]. In addition, Mohamed et al. reported an LC–MS method for the determination of chlorambucil–DNA adducts [299].

Melphalan is indicated for the treatment of myeloma, solid tumours (e.g., breast and ovarian) and lymphomas [2]. Guetens et al. published a review of hyphenated techniques for anticancer drug monitoring, including GC–MS and LC–MS methods, for melphalan in 2002 [23,24]. LC-UV [300–302], LC-ECD [303] and LC with fluorescence detection [304–306] were also used for the determination of melphalan in biological samples. More recently, LC–MS/MS methods were developed for TDM and pharmacokinetic studies on melphalan [307,308]. Mirkou et al. developed and validated two methods for quantification of melphalan by LC–MS/MS [307]. The first method was adequate for routine use and allowed an accurate determination over a wide range of concentrations (1–500 ng mL^{-1}) with a simple and rapid sample preparation (protein precipitation). The second method using a more selective extraction (i.e. SPE) and HILIC approach allowed quantification of melphalan and its hydrolysis products without matrix effects present with the first one. The hydrolysis products appear rapidly at room temperature and are important to assess a failure during the storage of samples. Several studies on melphalan DNA adducts were published by Van den Driessche et al. [309–313] and Mohamed and Linscheid [314]. Additionally, LC–ICP-MS [315] was also useful for adduct analysis. Furthermore, LC-UV methods were described for the simultaneous determination of melphalan and impurities in

melphalan drug substance [316], for the analysis of pharmaceutical formulations [13,317] and for chemical degradation studies [318]. Chromatographic conditions were able to separate and quantify all impurities found in routine production batches of melphalan at above 0.1% area/area and simple sample preparation by dilution in methanol was used [316].

3.2.2.3. Nitrosurea (lomustine, carmustine, fotemustine). Lomustine is a nitrosurea analogue with a high degree of lipophilicity. Administered orally, it is mainly prescribed for the treatment of certain solid tumours and Hodgkin's disease. Carmustine has a similar activity and toxicity profile to lomustine [2]. Since publications of Hochberg et al. [319] and Yeager et al. [320] reporting LC-UV methods for the analysis of carmustine in biological samples in the 1980s, no further significant developments for this compound have been reported. However, a few papers have been published for the determination of carmustine or lomustine in association with other anticancer drugs. For example, permeability studies on anticancer drugs with different glove materials [50,321,322] and compatibility studies with container materials [13,14,323] were achieved using spectrophotometry, LC-UV and LC–MS/MS techniques. For lomustine, a stability-indicating LC-UV method was recently validated for degradation studies and presented adequate accuracy and precision values with a resolution between impurities and analyte superior to 2.0 [324]. In the case of biological sample analysis and pharmacokinetic studies, few LC-UV methods for lomustine have been developed since 1982 [325–327]. For example, an LC-UV method with a one-step liquid–liquid extraction procedure was used to detect and quantify lomustine and its two monohydroxylated metabolites (trans- and cis-4′-hydroxylomustine) in canine plasma with an LOD of 100 ng mL^{-1} for lomustine [325]. For fotemustine, a chlorethylnitrosourea, LC-UV has been used for both stability [328] and PK studies [329]. In these studies, quantification was performed in the μg mL^{-1} concentration range.

3.2.2.4. Other cross-linking agents. Thiotepa, used as an effective anticancer drug since the 1950s, appears to be one of the most effective anticancer drugs when used in high dose regimens. Its main indications are the treatment of bladder or ovarian cancers, breast cancer and malignant effusions [2]. A review of the chemistry, pharmacology, clinical use, toxicity, pharmacokinetics of thiotepa and analytical methods for its determination was published by Maanen et al. in 2000 [330]. Given that its metabolism is not clearly defined, several studies using UHPLC–MS/MS [331], GC-NPD [266,332] and LC–MS/MS [264] were conducted in the past few years. With the UHPLC–MS/MS method, nine metabolites in urine and five metabolites in serum, including two novel drug metabolites, were elucidated [331]. The LC–MS/MS method was validated for the simultaneous quantification of cyclophosphamide, thiotepa and their respective metabolites in human plasma with an LOQ of 5 ng mL^{-1} and was useful in routine TDM of cancer patients [264]. LC-UV methods were also developed to quantify thiotepa in aqueous solutions [333] and formulations [334,335].

Treosulfan, which is mainly used to treat ovarian cancer, has similar major side effects to nitrogen mustards. LC with refractometric detection methods was developed for pharmacokinetic studies of this compound [336–338]. Centrifugation and microfiltration preceded LC analysis. With this technique, LOQs were 10.0 μg mL^{-1} and 50.0 μg mL^{-1} in plasma and urine, respectively. Since the concentration of treosulfan in plasma and urine after infusion was high, the method was suitable for PK studies of the drug in biological fluids [337,339].

Busulfan is used for the treatment of chronic myeloid leukaemia and as part of conditioning regimens for patients undergoing bone marrow transplantation. Unfortunately, it can cause excessive myelosuppression, resulting in irreversible bone marrow aplasia, and requires careful monitoring [2]. Analytical methods have already been reported in reviews on anticancer drug monitoring in 1985 [22] and 2002 [23,24] and are not discussed in this paper. More recently, the determination of busulfan in serum or plasma was achieved by LC–MS [340] and LC–MS/MS [341–344]. To reduce manual sample preparation, an LC–MS/MS method coupled with turbulent flow on-line sample cleaning technology offered reliable busulfan quantification in serum or plasma and was fully validated for clinical use with an LOQ of 36 ng mL^{-1} [345]. Because of practical limitations in obtaining blood from children, saliva was evaluated as an alternative matrix for therapeutic drug monitoring of busulfan, with subsequent analyses by LC–MS/MS [346]. An online extraction cartridge with column-switching technique was used for sample preparation and LOQs in saliva and plasma were about 10 ng mL^{-1}. In addition, LC-UV [347–349] and LC with fluorescence detection [350] were also used for the determination of busulfan in biological samples. In these studies, precolumn derivatisation was needed for sample preparation and LOQs in plasma about 100 ng mL^{-1} were obtained. Stability studies of several busulfan formulations were performed by LC-UV [351–353] and a method of stability-indicating ion chromatography with conductivity detection was published by Chow et al. [354]. For hospital formulations, an HPTLC method [355] was compared with near infrared spectroscopy [356] for the determination of busulfan in capsules. Similar quantitative performance in terms of accuracy and precision was obtained, but near infrared spectroscopy had the advantage of being a non-invasive technique.

Mitomycin-C is a member of a group of naturally occurring antitumor antibiotics produced by *Streptomyces caespitosus* (*griseovinaceseus*) and was first isolated in 1958. Intravenous mitomycin is used to treat upper gastrointestinal and breast cancers, and administration by bladder instillation allows treating superficial bladder tumours. Adverse events include delayed bone marrow toxicity. It can also be administered in ophthalmology as an adjunctive therapy in trabeculectomy. A simple, fast and reliable LC–MS method was developed for the determination of traces of mitomycin-C in aqueous tumour samples and an LOQ inferior to 0.1 ng mL^{-1} was obtained [357]. LC-UV methods were also reported for the determination of mitomycin C in human ocular tissues [358], in plasma [359–361] and for stability tests of freshly prepared ophthalmic formulation [362] and intravesical instillation solutions [363]. Exposure to mitomycin-C in the operating room during hyperthermic intraperitoneal chemotherapy was monitored in ambient air and in plasma samples from the surgeon by LC-UV [364]. The permeability of the gloves was also investigated using *in vitro* techniques [365].

3.2.3. Intercalating agents

3.2.3.1. Anthracyclines (doxorubicin, epirubicin, daunorubicin, aclarubicin, idarubicin). Anthracyclines are a group of antitumor antibiotics consisting of a planar anthraquinone nucleus attached to an amino-containing sugar. Doxorubicin, daunorubicin, and aclarubicin are natural products extracted from *Streptomyces peucetiusor* or *Streptomyces galilaeus*, while epirubicin and idarubicin are semisynthetic analogues. Doxorubicin is widely used as an anticancer drug because of its broad spectrum of activity (acute leukaemia, lymphomas, and a variety of solid tumours). Adverse events include nausea, vomiting, myelosuppression, mucositis, alopecia and cardiotoxicity by dose accumulation. Daunorubicin is an important agent in the treatment of acute lymphocytic and myelocytic leukaemia, while aclarubicin is used as a second-line treatment for acute nonlymphocytic leukaemia. Epirubicin, a semisynthetic analogue of doxorubicin differing only by its stereochemistry, is similar in terms of efficacy for the treatment of breast cancer. Idarubicin is used in advanced breast cancer after failure of

first-line chemotherapy and in acute nonlymphocytic leukaemia [2].

A review of the physicochemical and analytical properties of anthracycline antitumour agents focused on protolytic equilibria, partition coefficients, self-association, adsorptive properties, metal complexation, spectroscopy and chromatography was published in 1986 [366]. In 2001, various reviews reported analytical methods for anthracyclines and their metabolites [367] or related compounds [368,369]. Generally, separations of these anticancer agents were achieved by LC coupled with various detection techniques including electrochemical or MS. Due to their colour and native fluorescence, UV–Vis or fluorescence detection are particularly adapted.

Quality control of hospital formulations was performed by FIA and LC-UV [8]. Given the similar structure of anthracyclines, FIA-DAD was not able to distinguish all compounds, and a separation by LC was necessary. In addition, pharmaceutical preparations containing a drug mixture of doxorubicin and vincristine [370], doxorubicin and 5-FU [63], or several anthracyclines [371] were successfully analysed by LC-UV. Jelińska and co-workers reported stability studies in the solid state of doxorubicin and daunorubicin [372] and epidoxorubicin [373] by LC-UV.

Due to the cardiotoxicity of the accumulation of anthracyclines, monitoring of plasma or tissue concentrations is of utmost importance. Several studies have reported anthracycline determination in biological samples (plasma, serum, cell extracts) by CE-UV [374,375] and CE-LIF [376–383]. Sweeping preconcentration and electrokinetic injection coupled to CE-UV analysis provided LODs of 1×10^{-9} mol L^{-1} ($\sim$0.5 ng mL^{-1}) for doxorubicin and daunorubicin in plasma samples allowing determination of therapeutic concentrations [374]. LIF detection provided also an extremely sensitive and selective technique for biological samples with LODs in the range of ng mL^{-1}. For example Perez-Ruiz et al. published a CE-LIF method with simple acetonitrile protein precipitation exhibiting LODs inferior to 1.0 ng mL^{-1} for doxorubicin, daunorubicin and idarubicin in serum samples [376]. However, electrophoretic separation between doxorubicin and its metabolite doxorubicinol, which is responsible for the cardiotoxicity, is difficult due to their similar structure and charge. The presence of doxorubicinol was determined separately by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry [383]. Another approach to overcome this problem was the use of a chiral method (i.e., CD-MEKC-LIF) with a resolution of 2.81 [384] or LC with a photosensitisation reaction followed by chemiluminescence detection with complete baseline separation [385].

Other methods for measurement of intracellular accumulation of anthracyclines in cancer cells were reported, including MEKC-LIF [386–388] with LOD values in order of ng mL^{-1}. MEEKC-UV has also shown good potential for the analysis of anthracyclines in biological samples [389]. However, LOD and LOQ for doxorubicin in plasma were 9.7 μg mL^{-1} and 32.5 μg mL^{-1}, respectivley; which was not sufficient for the application of the method to real clinical samples. Additionally, CE with amperometric detection was used for the analysis of idarubicin in human urine with an LOD of 8.0×10^{-8} mol L^{-1} ($\sim$40 ng mL^{-1}) [390] and for the determination of the dissociation constants of anthracyclines [391]. CE with an absorption-based wave-mixing detector method exhibited high selectivity and sensitivity for anthracycline drugs similar to LIF detection with an LOD of 9.9×10^{-10} mol L^{-1} for daunorubicin (i.e. inferior to 1 ng mL^{-1}) [392].

Since 2001, several methods of LC-UV [63,98,393,394] and LC with fluorimetric detection have been reported for the determination of anthracyclines in biological samples [395–402]. For example, Katzenmeyer et al. reported an LC–LIF-MS method to determine *in vitro* metabolism of doxorubicin [403]. LC-LIF detection allowed quantification of the metabolic compounds while MS detection contributed to the metabolites identification. However, the best selectivities were obtained with LC–MS/MS methods [262,263,404–408] with LOQs inferior or close to 1.0 ng mL^{-1}. Wang et al. used UHPLC–MS to profile urinary metabolites for toxicity-related processes and pathogenesis induced by doxorubicin [409]. An accelerator mass spectrometry method allowed cellular quantification of doxorubicin at femtomolar concentrations with the best sensitivity but without discrimination between parent drug and metabolites [410].

Methods of LC–MS/MS [32,411] and LC-fluorescence [412,413] were used for monitoring anthracyclines in urine samples of healthcare workers or employees of drug manufacturers. Environmental monitoring of anthracyclines together with other anticancer drugs has been achieved in wipe and air samples [49,51,52] and in sewage water [58] using LC–MS/MS or LC with fluorescence detection [56]. Before LC-fluorescence analysis, wastewater samples were pre-concentrated by SPE (concentration factor of 100). The method was reproducible and accurate within a range of 0.1–5 ng mL^{-1} for doxorubicin, epirubicin and daunorubicin (recoveries >80%) and successfully applied for determination of these drugs in hospital effluents. Moreover, an LC-UV method was also developed for surface contamination of 5-FU, ifosfamide, cyclophosphamide, doxorubicin, and paclitaxel with LODs of 500 ng mL^{-1} [45,274] while LODs of 1.0 ng mL^{-1} were obtained by MS detection [51].

3.2.3.2. Mitoxantrone and actinomycin-D. The indications of mitoxantrone are the treatment of metastatic breast cancer, adult nonlymphocytic leukaemia and non-Hodgkin's lymphoma. Actinomycin-D is mainly used to treat paediatric cancers, some testicular sarcomas and AIDS-related Kaposi's sarcoma. The side effects of mitoxantrone and actinomycin-D are similar to those of doxorubicin except that the cardiac toxicity is less prominent. However, cardiac examinations and monitoring are still recommended when a certain cumulative dose has been reached [2]. Chen et al. [369] and Loadman and Calabrese [368] published reviews reporting several LC methods for the determination of mitoxantrone in 2001. Thanks to the presence of chromophores, UV detection is frequently used for the analysis of mitoxantrone, with LOD between 1 and 75 ng mL^{-1}. The sensitivity was improved with ECD with LOD of 0.1 ng mL^{-1} [369]. Recently, other LC-UV [414,415] and LC–MS/MS methods for mitoxantrone [416] and actinomycin-D [417–421] were developed for clinical samples with good quantitative performance in terms of sensitivity and selectivity. LOQs of mitoxantrone in plasma and tissues were in the same concentration order than the above mentioned studies. With simple protein precipitation followed by LC–MS/MS analysis, LOQ of actinomycin-D in plasma was about 0.5 ng mL^{-1} [421]. Finally, CE with chemiluminescence detection was reported for mitoxantrone determination in commercial drugs and in spiked biological samples [422].

3.2.4. Topoisomerase inhibitors

3.2.4.1. Topoisomerase I inhibitors (irinotecan, topotecan). Their lead structure is the natural product camptothecin, a cytotoxic quinoline-based alkaloid with a unique five-ring system extracted from the bark of the Chinese *Camptotheca* and the Asian *Nothapodytes* trees. Clinical use of camptothecin is limited due to poor water solubility and a number of serious side effects. However, several derivatives of camptothecin with improved solubility are now used. Topotecan is administered intravenously for the treatment of metastatic ovarian cancer when first-line or subsequent therapy fails. Irinotecan is licensed for metastatic colorectal cancer in combination with 5-FU and folinic acid or as a monotherapy when 5-FU containing treatments have failed. In addition to dose-limiting myelosuppression, side effects include

gastrointestinal disturbances such as delayed diarrhoea, asthenia, alopecia, and anorexia. The drug is hydrolysed *in vivo* to 7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite approximately 200–2000-fold more cytotoxic than irinotecan. However, despite its intrinsic potential as an anticancer agent, its poor solubility in most pharmaceutically acceptable solvents limits its clinical use [2].

In 2001, a paper on traditional Chinese medicines and antineoplastic compounds reviewed LC methods for camptothecin, irinotecan, topotecan, and 9-aminocamptothecin in biological samples [423]. LC with fluorescence detection was the most commonly used technique for determination of these compounds in biological samples. Other reviews reporting methods for camptothecin and related compound determination discussed separation efficiency and detection sensitivity and specificity [424–426]. The chemistry, structure–activity relationships and stability of camptothecin analogues were reported with particular attention on the chemical stability. Because the active lactone structure can undergo ring opening under conditions of extraction, pre-treatment and analysis should be studied carefully.

In 2010, a review of bioanalytical methods for irinotecan and its active metabolite SN-38 provided an exhaustive compilation of published assays, with details on validation parameters and applicability [427]. Pharmacokinetic profiling of irinotecan and its metabolites was studied in various species, including cancer patients, by means of LC-UV, LC with fluorescence detection, LC–MS and LC–MS/MS. Concentrations of irinotecan and SN-38 in biological samples in order of ng mL^{-1} were achieved by LC–MS/MS and LC coupled to fluorescence detection analysis [427]. The developed methods continue to find use in the optimisation of newly designed delivery systems with regard to pharmacokinetics for the safe and effective use of irinotecan or SN-38. Studies already reported in these reviews will not be further discussed in this paper and only some references with analytical techniques other than LC or developed for special application areas will be discussed.

HPTLC [428] and LC–MS/MS [51] methods for camptothecin derivatives were developed for quality control of hospital formulations. LC-UV methods were validated for quantitative determination of irinotecan [429] and topotecan [430] in bulk drug samples and formulations. In addition, an LC-UV method was reported for the simultaneous determination of the carboxylate and lactone forms of SN-38 in nanoparticles [431]. Laser-induced fluorescence and photochemical derivatisation was also suitable for irinotecan and topotecan trace analysis [432]. Another example is the determination of camptothecins in extracts of *Nothapodytes foetida* by MEKC-UV [433]. This method was found to be very suitable for monitoring camptothecin concentrations during the cultivation of the medicinal plant. For surface contamination in cytotoxic preparation units, LC–MS/MS analysis allowed the determination of irinotecan and other cytotoxics with well studied quantitative performance in terms of accuracy and precision. LOQ of irinotecan in aqueous solutions was at 1.0 ng mL^{-1} corresponding to a surface contamination of 0.1 ng cm^{-2} [51,52].

3.2.4.2. Topoisomerase II inhibitors (etoposide, teniposide, amsacrine). The lead structure of drugs that inhibit topoisomerase II is podophyllotoxin, a plant alkaloid isolated from the American mandrake rhizome. Etoposide is a semisynthetic glucoside of epipodophyllotoxin and is one of the most effective agents for treating small-cell bronchial carcinoma. It can also be used for testicular cancer and some lymphomas. The toxic effects of this drug include nausea and vomiting, myelosuppression, and alopecia. Teniposide is an etoposide analogue with a similarly broad clinical activity. Amsacrine, another topoisomerase II inhibitor, has an acridine-based structure. Clinically, amsacrine has an activity and toxicity profile similar to doxorubicin. It is administered intravenously for the treatment of advanced ovarian carcinomas, myelogenous leukaemia, and lymphomas. Its side effects include myelosuppression and mucositis [2].

A review of LC methods for the determination of topoisomerase II inhibitors was published by Chen et al. in 2001, including a compilation of LC methods for the analysis of etoposide, teniposide, and amsacrine, as well as anthracyclines, mitoxantrone and others [369]. Methods based on LC coupled to various detectors, such as UV, fluorescence, ECD, MS and ELISA, were reported for etoposide determination in physiological fluids [369,423,434]. In 2010, Sachin et al. developed an UHPLC–MS/MS method with SPE sample pretreatment for the simultaneous determination of etoposide and a piperine analogue in plasma samples with a total run time of 6 min [435]. LOQs for etoposide and the piperine analogue were 2.0 and 1.0 ng mL^{-1}, respectively. Teniposide has been analysed by LC-UV and LC-ECD [369], but recently an UHPLC–MS/MS method was developed for the determination of teniposide in plasma samples with a simple liquid–liquid extraction procedure and using etoposide as internal standard [436]. LOQ of 10 ng mL^{-1} in rat plasma and short analysis time (3.0 min) were obtained and were particularly adequate for a high sample throughout. The intraday and interday precision values (RSD) were less than 15% and the method was considered as suitable for preclinical pharmacokinetic studies of teniposide in rats. Additionally, the chemical stability of teniposide [6] and etoposide [7] in lipid emulsion was monitored by LC-UV. Separation of etoposide phosphate and methotrexate was also achieved by CE-UV with a high-sensitivity cell in a concentration range between 0.1 and 100.0 μg mL^{-1} [171]. CE-LIF [437] and MEKC with near-field thermal lens detection [438] allowed the simultaneous quantification of etoposide and etoposide phosphate in human plasma with similar LODs in order of 100 ng mL^{-1} for etoposide phosphate and 170 ng mL^{-1} for etoposide. For environmental monitoring, sensitive LC–MS/MS methods were reported for etoposide determination in sewage water with LOD in order of ng L^{-1} [58] and for etoposide phosphate quantification on different surfaces [51,52].

3.2.5. DNA cleaving agents (bleomycin)

Bleomycin accumulates in squamous cells and is therefore suitable for the treatment of tumours of the head and neck, Hodgkin's disease and testicular carcinomas. Pharmaceutical preparations containing bleomycin sulphate consist of a mixture of glycopeptide bases obtained from *Streptomyces verticillus* with individual molecular weights in the region of 1300 Da. The analytical and biological inequivalence of two commercial bleomycin formulations was demonstrated using LC-UV [439]. Recently, Yin et al. demonstrated that a sensitive DNA-based electrochemical strategy appeared to be a promising alternative for the determination of trace amounts of bleomycin in pharmaceutical and clinical samples with LOD in the order of picomolar (~0.1 ng mL^{-1}) [440]. Furthermore, an LC–MS method was developed for pharmacokinetic studies of a new formulation of bleomycin in dog plasma after intramuscular injection [441].

3.3. Antitubulin agents

Analysis of taxanes, vinca alkaloids and ixabepilone are described in this section. Chemical structures of antitubulin agents are shown in Fig. 7, and relevant analytical methods for pharmaceutical, biological and environmental samples are reported in Table 3.

3.3.1. Taxanes (paclitaxel, docetaxel)

Paclitaxel is a highly complex tetracyclic diterpene found in the needles and bark of *Taxus brevifolia*, the Pacific yew tree. Pure paclitaxel was isolated in 1966 and its structure published in 1971. However, it did not appear in clinical practice until the 1990s.

Table 3
Analytical methods for antitubulin agents.

Compound	Matrix	Analytical technique	References
Docetaxel	Review	Review	[423]
	Pharmaceutical formulation	LC-UV	[5,442–445]
	Pharmaceutical formulation	FIA	[8]
	Biological samples	CZE, MEKC, MEEKC	[389]
	Biological samples	LC-UV	[393]
	Biological samples	LC–MS	[451]
	Biological samples	LC–MS/MS	[19,452–454]
Paclitaxel	Review	Review	[423]
	Pharmaceutical formulation	FIA	[8]
	Pharmaceutical formulation	LC-UV	[446–448]
	Pharmaceutical formulation	LC–MS	[449]
	Biological samples	MEKC-UV	[455]
	Biological samples	CZE, MEKC, MEEKC	[389]
	Biological samples	LC-UV	[229,393,450]
	Biological samples	LC–MS	[451,483]
	Wipe samples (surface contamination)	LC-UV	[45,274]
	Wipe samples (surface contamination)	LC–MS/MS	[49]
Vinca alkaloides (vincristine, vinblastine, vindesine, vinorelbine)	Review	Review	[423]
	Pharmaceutical formulation	NACE-DAD	[456]
	Pharmaceutical formulation	HPTLC	[457]
	Pharmaceutical formulation	LC-UV	[370]
	Pharmaceutical formulation	LC–MS/MS	[51]
	Plant extracts	LC-UV	[458]
	Plant extracts	CE-MS	[459]
	Biological samples	LC–MS/MS	[417,418,460,461]
	Wipe samples (surface contamination)	LC–MS/MS	[51,52]
	Sewage water	LC–MS/MS	[58]
Ixabepilone	Biological samples	LC–MS/MS	[462–464]

Docetaxel is a more recently introduced semisynthetic analogue with similar therapeutic and toxicological properties. Paclitaxel has relatively poor water solubility and lack of activity in some cancers with resistance, which has prompted ongoing research into new analogues. Given by intravenous infusion, paclitaxel in combination with cisplatin or carboplatin constitutes the treatment of choice for ovarian cancer. Docetaxel is licensed for initial treatment of advanced breast cancer in combination with doxorubicin or alone when adjuvant cytotoxic chemotherapy has failed. The two taxanes are also used for advanced or metastatic non-small-cell lung cancer or for metastatic breast cancer in cases where first-line therapy has failed [2].

For stability testing or quality control of pharmaceutical formulations of docetaxel [5,442–445] and paclitaxel [446–448], LC-UV methods have been developed. Musteata and Pavliszyn used LC–MS for the determination of free concentration of paclitaxel in a

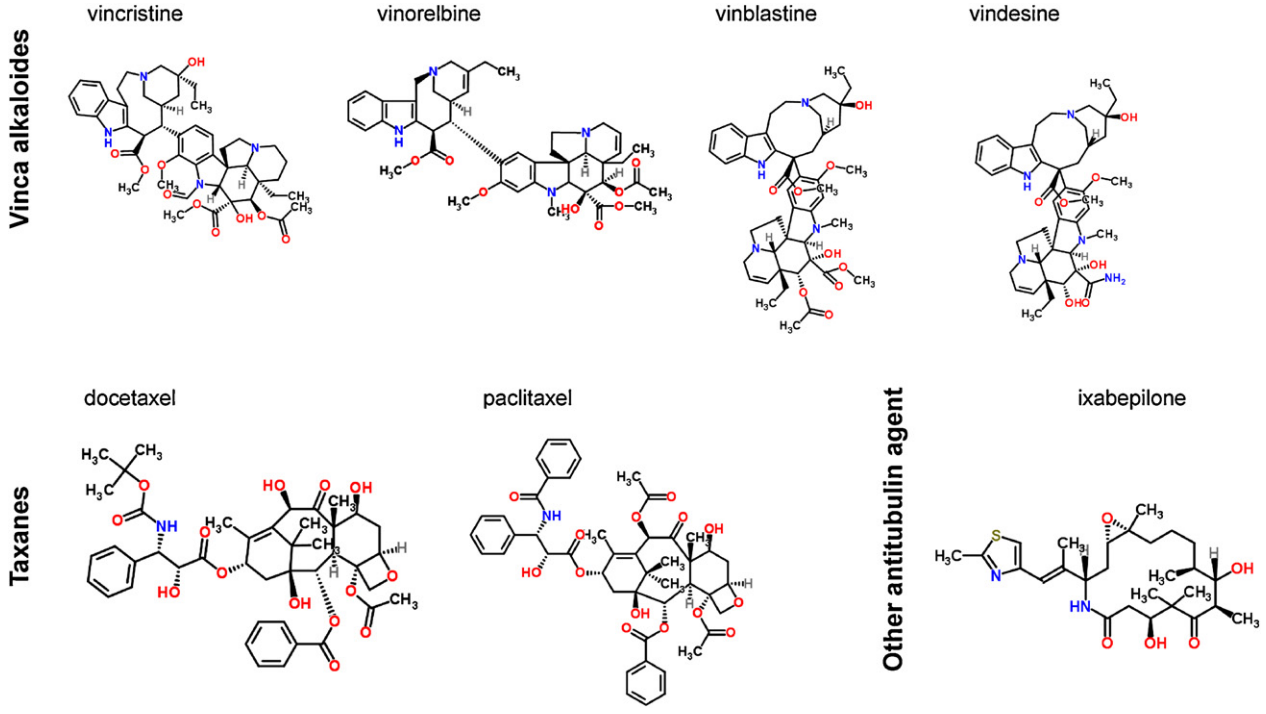

Fig. 7. Chemical structures of antitubulin agents.

liposome formulation [449]. Additionally, control of chemotherapy during preparation was performed by FIA-UV for docetaxel and paclitaxel [8]. In 2001, several methods for the determination of paclitaxel in biological matrices using LC-UV, LC–MS and immunoassays were reported [423].

Since 2001, several LC-UV [229,393,450], LC–MS [451] and LC–MS/MS methods have been developed for taxanes determination in biological samples [19,452–454]. For example, Corona et al. used on-line extraction procedure with LC–MS/MS for high-throughput quantification of docetaxel in plasma. The method was validated and presented LOQ of 0.15 ng mL^{-1} with good accuracy and precision performance and was successfully applied for pharmacokinetics of docetaxel in cancer patients [19]. On-line column-switching was also applied by Bermingham et al. for determination of taxanes and anthracyclines by LC-UV, however the method was not sensitive enough for TDM at low serum concentration because the LOQ was evaluated at 500 ng mL^{-1} [393]. Electrophoretic separation techniques (e.g., CE, MEKC, MEEKC) showed also good potential for taxanes analysis in biological samples [389,455]. For example, a MEEKC-UV method was characterised by a very short separation time and high efficiency and was proven to be flexible for the separation of different combinations of anthracyclines and taxanes [389]. This separation approach could be highly beneficial for biological sample analysis if applied with a sensitive detection system. With UV detection, LOQs were in the order of 84,500 ng mL^{-1} for docetaxel [389].

Contamination and exposure assessment of paclitaxel and other cytotoxic drugs was performed by LC-UV [45,274] and LC–MS/MS [49]. The LC–MS/MS method provided adequate sensitivity for measuring five antineoplastic drugs in air and wipe samples in healthcare environment with LOD of 0.7 ng mL^{-1} for paclitaxel [49].

3.3.2. Vinca alkaloids (vincristine, vinblastine, vinorelbine, vindesine)

The two alkaloids vinblastine and vincristine are constituents of the Madagascar periwinkle (*Vinca rosea*). Isolation and structural identification were reported in the 1960s. Vinblastine synthesis starting from catharanthine and vindoline units was reported in 1979. Because these alkaloids have proven efficacy in therapy to treat certain solid tumours (mainly lung and breast), lymphomas, and acute leukaemia, efforts have been made to design new analogues with reduced toxicity, which resulted in the semisynthetic analogues vindesine and vinorelbine. These agents are given by intravenous administration, and their side effects include neurotoxicity, myelosuppression, and alopecia [2].

A non-aqueous CE-UV method allowed the successful determination of vinorelbine in a commercial pharmaceutical formulation [456]. For quality control of pharmaceutical formulations in hospitals, HPTLC [457], LC-UV [370] and LC–MS/MS [51] have all been used. In 2005, Gupta et al. developed an LC-UV method for the determination of vinca alkaloids in leaf extracts of *Catharanthus roseus* [458]. CE-MS was also successfully used for determination of vinblastine and its precursors vindoline and catharantine in plant samples [459]. As reported in the review on traditional Chinese medicines, analyses of vinblastine, vincristine and vinorelbine in biological samples were achieved by LC-UV, LC with fluorescence detection and LC-ECD [423]. LOQ of these vinca alkaloids in plasma or urine were in order of ng mL^{-1} with LC-fluorescence and LC-ECD. LC–MS/MS methods for vinca alkaloids determination in human plasma [417,418,460,461] and for drug residues in dog urine [262] were also published. For example, Dennison et al. developed a very sensitive LC–MS/MS method with an LOQ of 0.012 ng mL^{-1} for vincristine and its major metabolite in human plasma [460]. For environmental monitoring, an LC–MS/MS method was useful for sewage water analysis [58] and for surface contamination [51,52].

3.3.3. Other antitubulin agents (ixabepilone)

Ixabepilone is a semi-synthetic, microtubule stabilising, epothilone B analogue that displayed activity in taxane-resistant breast cancer patients. A human mass balance study of the novel anticancer agent ixabepilone was performed using accelerator mass spectrometry to investigate elimination pathways [462]. In addition, pharmacokinetics after intravenous and oral administration was established by sensitive and validated LC–MS/MS methods [463,464]. Plasma samples were extracted by acetonitrile protein precipitation and an LOQ of 2 ng mL^{-1} of ixabepilone in human plasma was obtained [464].

4. Conclusion

Over the last thirty years, numerous analytical methods for cytotoxic drug determination in pharmaceutical formulations, biological samples, and environmental samples have been reported in the literature. The first analytical methods, mainly using LC-UV, allowed for the foundations of the use of cytotoxic drugs in treating human cancers to be laid in terms of understanding drug interactions with the organism, developing pharmaceutical formulations and determining the toxicity of these compounds. As with all pharmaceutical substances, more elaborate methods to support pharmacokinetics, pharmacodynamics and therapeutic drug monitoring of cytotoxic drugs have been published thanks to the implementation of detection systems with higher selectivity and sensitivity, such as mass spectrometry. During the last five years, however, particular attention has been focused on the safe handling of cytotoxic drugs and the protection of the environment. Indeed, several papers reporting the analysis of cytotoxic drugs in wastewater, in working environments and in biological samples of healthcare professionals have been published.

Today, with the emergence of new chemotherapy treatments (including biological agents, hormones and molecular targeting agents), the development of useful methods is required for preclinical and clinical studies, but also for the development of formulations containing these compounds, and constitutes the next challenge in the analysis of anticancer drugs.

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Article I*b*

« Antineoplastic drugs and their analysis : a state-of-the-art review »

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Antineoplastic drugs and their analysis: a state of the art review

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The number of patients suffering from cancer is constantly increasing and, consequently, the number of different chemotherapy treatments administered is increasing. Given the high reactivity and toxicity of antineoplastic drugs, analytical methods are required in all pharmaceutical fields, from drug development to their elimination in wastewater; including formulation quality control, environment and human exposure and therapeutic drug monitoring. The aim of this paper is to provide an overview of the analytical methods available for the determination of antineoplastic drugs in different matrices such as pharmaceutical formulations, biological and environmental samples. The applicability and performance of the reported methods will be critically discussed, with focus on the most commonly used antineoplastic drugs. Only conventional compounds and small molecules for targeted therapy will be considered in the present review.

1 Introduction

The World Health Organisation stated that the incidence of cancer increased from 12.7 million in 2008 to 14.1 million in 2012.[1] This trend is expected to continue with the number of new cancer cases increasing by 70% over the next two decades. This bleak prognosis leads to a growing prescription for antineoplastic drugs, which constitute, with surgery and/or radiotherapy, the main treatment in oncology. Two classes of antineoplastic drugs from different generations can be distinguished, namely conventional molecules and drugs from targeted therapy. Conventional chemotherapy appeared at the beginning of the 20th century with the development of chemical weapons[2]. One of the first families of antineoplastic drugs still administered today constitutes molecules with a structure related to mustard gas used on the battlefields of World War I. A side effect observed with this gas (that has a myelosuppressive action) initiated the development of the first antineoplas-

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tic drugs against leukemia.[3] Driven by this success, the USA decided to launch several national programs with the main objective of developing new molecules for cancer treatment. Then, an unbridled race towards the discovery of new anticancer drugs, such as antifolate compounds, purine and pyrimidine analogues and also antibiotics, started for a period of 20 years. Most of the conventional antineoplastic drugs administered today were developed during this period. The war against cancer knew a second offensive with the apparition of targeted therapies in the '90s. However, in spite of the promising discovery of these new molecules which act with precision, thus reducing side effects, heavy weapons represented by the conventional antineoplastic drugs remain the first-line molecules for the chemical treatment of a large number of cancers.

The high chemical reactivity of conventional antineoplastic drugs is responsible for their anticancer activity, but also represents their main drawback. Indeed, this high chemical reactivity makes such molecules extremely unstable and strongly toxic. Particular attention has therefore been paid to these compounds in terms of safety and quality. In this context, numerous methods were reported for the analysis of antineoplastic drugs in different fields, from the development of stable pharmaceutical products to wastewater treatment, including quality control of the formulation and therapeutic drug monitoring.

It can be noted that products containing antineoplastic drugs are almost exclusively intermediate pharmaceutical forms available as a lyophilisate or a concentrated solution of the active drug. Under these conditions, the highest compound stability can be obtained, and thus the desired therapeutic effect can be guaranteed. A reconstitution step, usually performed extemporaneously by the hospital staff (nurse or pharmacy operators), converting the intermediate form to a formulation ready to be administered to the patient, has to be performed. The analysis of reconstituted formulations, which are considered as high-risk products, prior to patient administration, is an unavoidable step from a quality control point of view. Given the toxicity of these molecules, in the last ten years focus has been on the determination of antineoplastic drug traces in the environment (hospital pharmacies, care units or effluents) and in biological fluids from the person handling these compounds, to control exposure.

The aim of this paper is to complete a review already published in 2011 by our group.[4] A critical overview of the reported analytical methods for antineoplastic drugs is provided. A significant number of new references are added and the methods used for the analysis of small molecules from targeted therapy are also discussed. Biological agents such as monoclonal antibodies are not considered in the present review.

2 Conventional antineoplastic drugs

Three different families of conventional antineoplastic drugs can be distinguished according to their action on deoxyribonucleic acid (DNA):

- Molecules acting on DNA synthesis (antimetabolites);
- Molecules with a direct action on DNA, called DNA-interactive agents (alkylating agents, intercalating agents and topoisomerase inhibitors);
- Molecules with an action on mitosis (antitubulin agents).

2.1. Antimetabolites

Antimetabolites belong to one of the oldest families of antineoplastic drugs. They include molecules such as methotrexate, and more recent compounds such as gemcitabine (GemC). In all cases, the mechanism of action of these compounds is to prevent DNA replication. Antimetabolites inhibit the synthesis of DNA components by acting as lures. They are structural analogues of purine and pyrimidine bases (similar to nucleobases or nucleosides), and folic analogues (inhibition of nucleic acid synthesis). Table 1 reports analytical methods published for the determination of antimetabolites.

2.1.1. Folic analogues. These compounds target folic coenzymes involved in the synthesis of nitrogenous bases. The first folic acid antagonist, namely aminopterin, was discovered in the late '40s for the treatment of childhood leukemia.[5] It was rapidly replaced by a less active but also less toxic agent,

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Table 1 Analytical methods for the detection of antimetabolite drugs

Antimetabolites	Matrices	Techniques	Ref.
Methotrexate	Molecule/ formulations	LC-UV	13 and 47
		CE-UV	43, 48 and 49
	Biological matrices	LC-UV	11, 12, 45 and 46
		LC-MS	17, 21, 24, 26, 30–34
		CE-UV	35–40, 42, 45 and 133
		CE-LIF	44
	Environment	LC-MS	15, 16, 19, 20, 22, 23, 25, 27–29, 134 and 135
Pemetrexed	Molecule/ formulations	LC-UV	51, 53–56 and 60
	Biological matrices	LC-UV	50 and 52
		LC-MS	57 and 136
Raltitrexed	Molecule/ formulations	CE-UV	61
	Biological matrices	LC-MS	59
5-fluorouracil	Molecule/ formulations	LC-UV	66, 67, 72, 74 and 137
		CE-UV	108
	Biological matrices	LC-UV	67, 85, 89 and 93
		LC-MS	34, 80, 95, 100–102
		LC-FD	97
		CE-UV	39, 105 and 109
	Environment	LC-UV	138
		LC-MS	28, 29, 78 and 103
		CE-UV	106 and 107
		GC-MS	27, 113 and 114
Azacitidine	Biological matrices	LC-MS	82 and 83
Gemcitabine	Molecule/ formulations	LC-UV	66, 73, 74 and 137
	Biological matrices	LC-UV	68–70, 87, 88, 91, 92, 96, 98 and 99
		LC-MS	34, 75–77, 81, 94, 104 and 136
	Environment	LC-UV	114
		LC-MS	19, 29, 103 and 135
Cytarabine	Molecule/ formulations	LC-UV	13, 67, 71 and 74
		CE-UV	111
	Biological matrices	LC-UV	67
		LC-MS	34, 79, 86, 90 and 139
		CE-UV	110–112
	Environment	LC-MS	18, 19, 29 and 103
6-Mercaptopurine	Biological matrices	LC-UV	123
		LC-MS	33
		LC-FD	130
6-Thioguanine	Biological matrices	LC-UV	120
		LC-MS	33, 121 and 131
		LC-AD	129
		CE-UV	39
Azathioprine	Molecule/ formulations	LC-UV	119
		CE-UV	132
	Biological matrices	LC-UV	118 and 125
	Environment	LC-UV	122 and 140
		LC-MS	134
Fludarabine	Biological matrices	LC-MS	127
Cladribine	Biological matrices	LC-UV	128
		LC-MS	126
Clofarabine	Biological matrices	LC-MS	126

amethopterin, also known as methotrexate, which is still extensively used 70 years after its discovery (for the treatment of osteosarcomas, acute lymphoblastic leukemias, Hodgkin's disease, breast, bladder and lung cancers). In the '90s, new folic analogues emerged, including raltitrexed[6] and pemetrexed,[7] with the aim of preventing methotrexate resistance and toxicity. Fig. 1 shows the structure of folic acid and its antagonists.

Molecules with a structure close to that of folic acid are characterised by the presence of two carboxylic acid groups. Their solubility depends on the pH of the solution. Indeed, with pK_a values of the carboxylic acid groups between 3.3 (pemetrexed) and 4.7 (methotrexate), neutral or basic solutions are required for their solubilisation. The presence of an asymmetric carbon is another property of folic analogues. *S*-Methotrexate, *S*-pemetrexed and *S*-raltitrexed are active forms, while *R*-forms are considered as impurities.[8]

Since 1975, more than 100 articles have described different analytical methods for the extraction, separation and detection of methotrexate.[4,9,10] Most of the reported methods are based on the use of liquid chromatography (LC) coupled with UV spectrophotometry (UV)[11,12,13,14] or mass spectrometry (MS), when there was a need for high selectivity and/or sensitivity.[15–22,23,24,25,26,27,28,29,30,31,32,33,34] Limits of detection

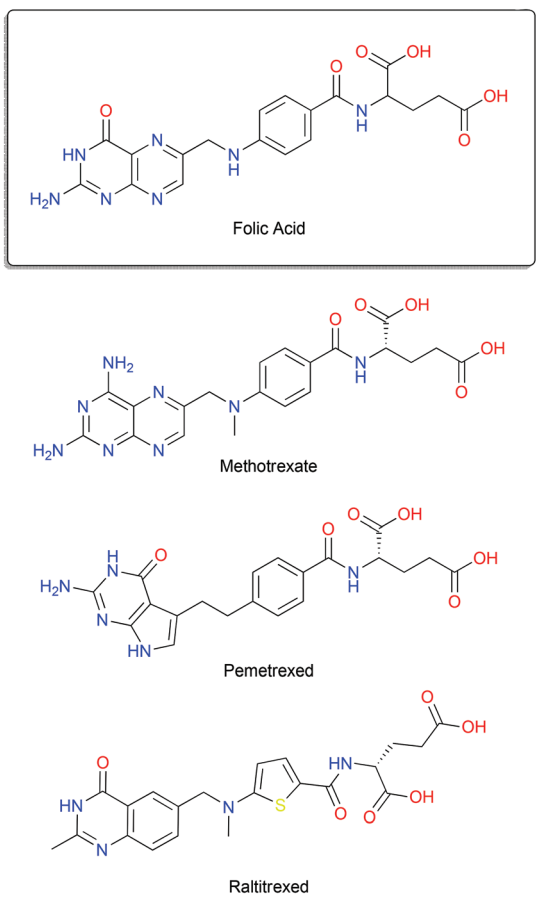


Fig. 1 Structure of folic acid and its analogues.

(LOD) inferior to 1 ng mL^{-1} were usually reached with LC-MS.[24,32] Capillary electrophoresis (CE) coupled with UV detection[35–43] or laser induced fluorescence detection (LIF)[44] was also proposed as a suitable approach for the analysis of methotrexate, since the molecule is ionisable. To improve the sensitivity of CE methods, zeta cell[38] or conventional sample preparation steps were used (LOD in the order of μg mL^{-1}). Solid phase extraction (SPE),[15,16,20,22,35,39,41,32,33,34] liquid–liquid extraction (LLE)[40] and protein precipitation (PP)[24,30,31,11,14,17,36] were among the most widely used sample preparation techniques when analysing methotrexate in complex matrices. Column-switching methods with SPE coupled with the analytical system (column and detection) were also developed for methotrexate analysis.[26] Methotrexate enantiomers separation was carried out by LC-UV[45–47] and CE-UV.[48,49] Contrary to methotrexate, only a few analytical methods have been reported in the literature for the most recent compounds (*i.e.*, raltitrexed and pemetrexed). The analytical methods for pemetrexed were based on LC coupled to UV,[50–55,56] MS[57,58] or evaporative light scattering detector (ELSD).[51] In our opinion, ELSD is not recommended for analysing pemetrexed since this molecule is UV-active and ELSD is often less sensitive and selective than the commonly used UV detectors. A methodology including a PP step followed by LC-MS analysis was also developed for the determination of raltitrexed in human plasma with a limit of quantification (LOQ) of 2 ng mL^{-1}.[59] For impurity profiling of active ingredients, separation of pemetrexed and raltitrexed enantiomers were obtained by LC-UV[60] and CE-UV,[61] respectively. In LC-UV, a polysaccharide chiral stationary phase was employed, while the chiral selector (cyclodextrins) was directly added to the BGE in CE-UV.

2.1.2. Pyrimidine analogues. As the name suggests, these substances have structures close to the endogeneous pyrimidine bases (thymine, cytosine and uracil). Biochemical lures disrupt the synthesis of nucleic acids. The first compound, developed in the '50s,[62] was a uracil molecule with a fluorine atom in the 5-position, named 5-fluorouracil (5FU). This old substance remains the most prescribed molecule in oncology and its main application is the treatment of colorectal cancer. The structures of the most familiar compounds of this subfamily of antineoplastic agents are shown in Fig. 2.

Discovered in the late '50s/early '60s, following research on marine sponges, cytarabine paved the way for a series of cytidine analogues (cytosine nucleoside).[63] In 1964, azacitidine, whose structure differed by the presence of an additional nitrogen atom in the 5-position of the cytosine nucleus, was produced.[64] It was only in the '90s that GemC appeared in the arsenal of antineoplastic drugs, synthesized a decade earlier by Eli Lilly Laboratories.[65]

A wide range of analytical methods have been developed for pyrimidine analogues. Historically, the most commonly used technique was reversed phase LC (RPLC) coupled with UV[66–72,73,74,13] or MS.[18,19,75–82,83,84,29,34] LOD inferior to 1 ng mL^{-1} can be obtained for the analysis of pyrimidine analogues by LC-MS. For example, Marangon *et al.* developed an LC-MS method including a PP step, for the determination of GemC and its metabolite in plasma, achieving a LOD of 0.1 ng mL^{-1}.[77] The main limitation of these methods is the poor retention of pyrimidine analogues on a C18 stationary phase under RPLC conditions. Indeed, pyrimidine analogues are small hydrophilic molecules having log *P* values between −0.9 and −3.5. To have a sufficient retention for these relatively polar molecules, different strategies were applied in LC including the use of ion pairing reagents,[85–88] ion exchange chromatography,[89–92] porous graphitic carbon support,[93] the derivatisation of pyrimidine analogues prior to their analysis by RPLC,[94,95] or the use of normal phase chromatographic supports.[96–99] However, all of these approaches suffer from obvious limitations, including long column equilibration, poor kinetic performance, tedious sample preparation procedures, or incompatibility with MS.

More recently, hydrophilic interaction chromatography (HILIC) has been proposed as an alternative strategy for the analysis of 5FU[100,101,102] and cytidine analogues[103,104] as shown in Fig. 3. In brief, a polar stationary phase is used in HILIC, together with a mobile phase composed of a large proportion of acetonitrile and aqueous buffer. The limitations previously described for ion pairing chromatography, ion exchange chromatography or normal phase LC were all tackled with HILIC, and this is why this analytical technique was successfully applied for the analysis of pyrimidine analogues.

Various CE-UV methods have also been suggested for the analysis of 5FU[39,105–108,109] and cytarabine,[110–112] since CE

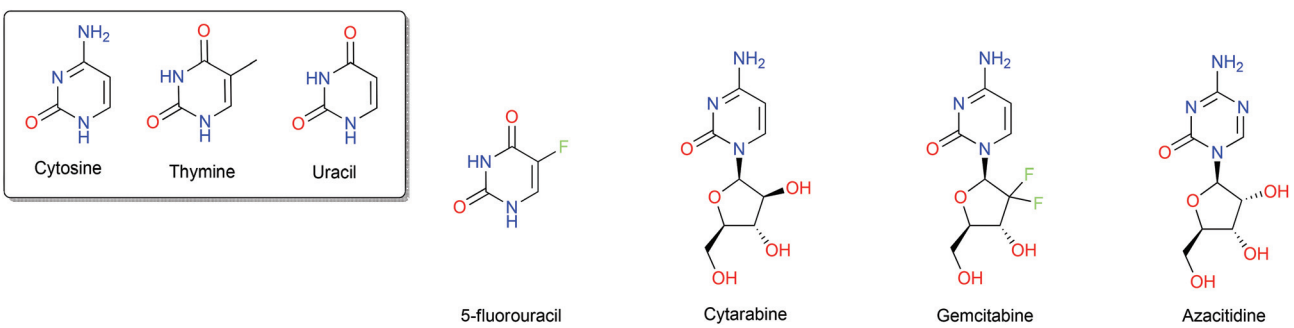


Fig. 2 Structures of pyrimidine bases and their analogues.

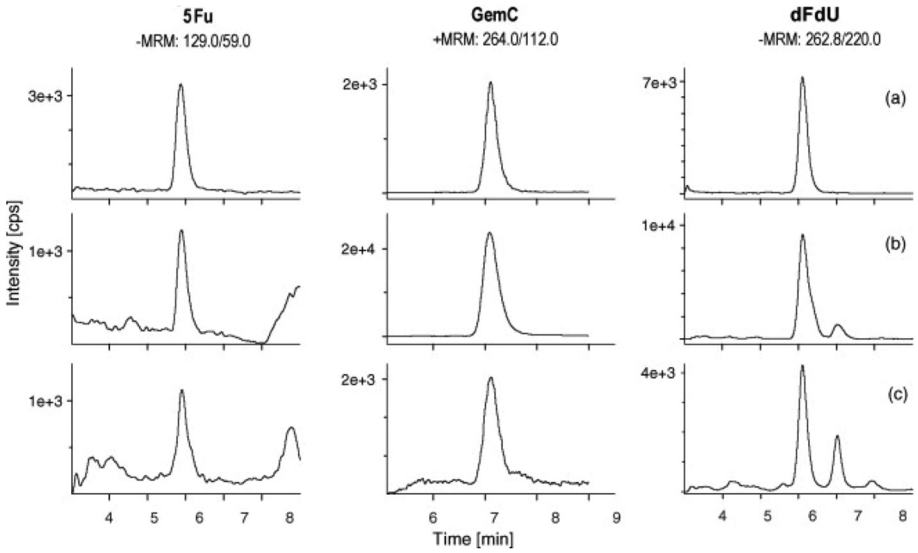


Fig. 3 Multiple reaction monitoring chromatograms of 5FU, GemC and its metabolite (dFdU) obtained for the analysis of calibration standards in (a) spiked hospital wastewater, and (b) hospital wastewater, by hydrophilic interaction chromatography coupled to MS. Adapted from ref. 103, 2009, with permission from Elsevier.

works with any type of ionisable substance, whatever the polarity. LOD of 1.7 ng mL^{-1} was reached for the analysis of 5FU in effluents using an SPE-CE-UV method.[106] Gas chromatography (GC) coupled to MS was also used for the analysis of 5FU,[113,114,27] but the time consuming and tedious derivatisation step means low popularity of GC for the analysis of these non-volatile compounds.

2.1.3. Purine analogues. Like pyrimidine analogues, purine analogues are incorporated into cell components to disrupt the synthesis of nucleic acids. Their structures are inspired by the endogenous purine bases (Fig. 4). Two generations of compounds can be discerned: nitrogenous base analogues (6-mercaptopurine, 6-thioguanine and azathioprine) and nucleoside analogues (fludarabine, cladribine and clofarabine). The first generation appeared in the '50s after a study of more than 100 purine analogues revealed their inhibitory activity on DNA synthesis of guanine and hypoxanthine compounds with a sulfur in the 6-position instead of an oxygen atom.[115,116] 6-Mercaptopurine and 6-thioguanine are mainly used for the treatment of leukemia, while azathioprine (6-mercaptopurine prodrug) is used for its myelosuppressive activity. Despite their significant adverse effects (myelosuppression and digestive disorders) and anticancer activity that is subject to a large inter-individual variation (metabolic pathway by a polymorphic enzyme: thiopurine methyltransferase (TPMT)), these three substances are still present in the chemotherapy arsenal. In the '80s, two additional molecules were discovered (fludarabine and cladribine) and were used ten years later as models for the synthesis of clofarabine, a more stable and active molecule.[117] This second generation of purine analogues are today used against leukemia.

Purine analogues are less hydrophilic than pyrimidine analogues. Thus, RPLC was the main technique employed for the analysis of both the first[118–125] and second generation[126–128] purine analogues. It should be noted that several studies were based on the analysis of the products resulting from the

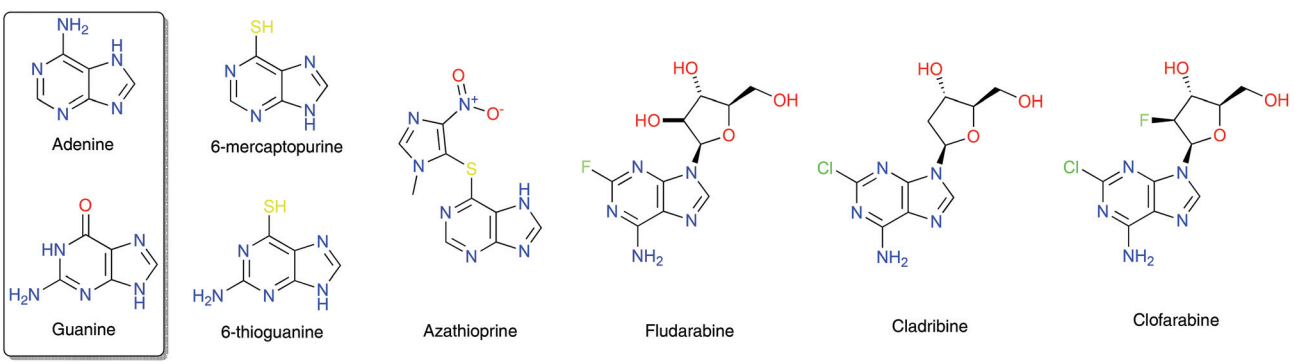


Fig. 4 Structures of purine bases and their analogues.

metabolism of the first generation purine analogues, given their potential toxicity.[120,121,125] 6-Methylmercaptopurine produced by TPMT was frequently sought in globules of patients undergoing treatment to define their phenotyping, since TPMT deficiency led to severe toxicity.[121] In addition to UV detection (which was the most widely used), amperometric detection (AD),[129] fluorescence detection (FD)[130] and MS detection[121,126,127,131] were also coupled to LC for the determination of purine analogues. LOQ of 1 ng mL^{-1} were usually obtained for purine analogues determination by LC-MS.[127,126] Concentrations of 6-mercaptopurine at 0.0615 ng mL^{-1} in urine samples were detected by a LC-FD method using metal palladium to form coordination complexes and enhance the detection signal.[130] A CE-UV method based on the use of borate buffer as background electrolyte (BGE) were also successfully used for the analysis of azathiopurine and its impurities in formulations,[132] or 6-thioguanine in urine[39] with a LOD in the order of 1 μg mL^{-1}.

2.2. DNA interactive agents

2.2.1. Alkylating agents. Alkylating agents represent the oldest family of antineoplastic drugs with the introduction of chlormethine in the late '40s. This family originated from mustard gas, which was used as a chemical weapon during World War I. The observation of an aplastic anemia appearing with severe burns, a few days after exposure, was the starting point for the search for anticancer drugs with reduced side effects. Alkylating agents are organic compounds with one or more electrophilic groups that react with the nucleophilic groups of DNA nucleobases in double helix or proteins, by covalently incorporating alkyl groups (Fig. 5) thereby altering replication and transcription processes. Analytical methods for detecting alkylating agents are reported in Table 2. Today, seven classes of alkylating agents can be distinguished:

- Nitrogen mustard analogues;
- Oxazophosphorines;
- Ethylene imines;
- Nitrosoureas;
- Alkylsulfonates;
- Triazenes and hydrazines;
- Platinum derivatives.

2.2.1.1. Nitrogen mustard analogues. Nitrogen mustard analogues are characterised by the presence of bis(2-chloroethyl)amino groups generating an azyridium ion which binds preferentially to the nitrogen atom at the 7-position of guanine. Given the high reactivity and, consequently, instability in solution, nitrogen mustards are generally orally administered. Only chlormethine and mephalan are administered by injection. To overcome their instability in water, injectable formulations are dry forms that need to be solubilised extemporaneously (patient administration must be carried out within 1 h after solubilisation). This limits the hydrolysis of the molecule in aqueous media (chloride groups are substituted by hydroxy groups in water) which leads to an inactive compound.

The analysis of nitrogen mustard analogues is very difficult because of their instability. Chlormethine was usually analysed by LC-UV after a derivatisation step leading to a stable and UV detectable compound.[141–143] Products resulting from chlor-

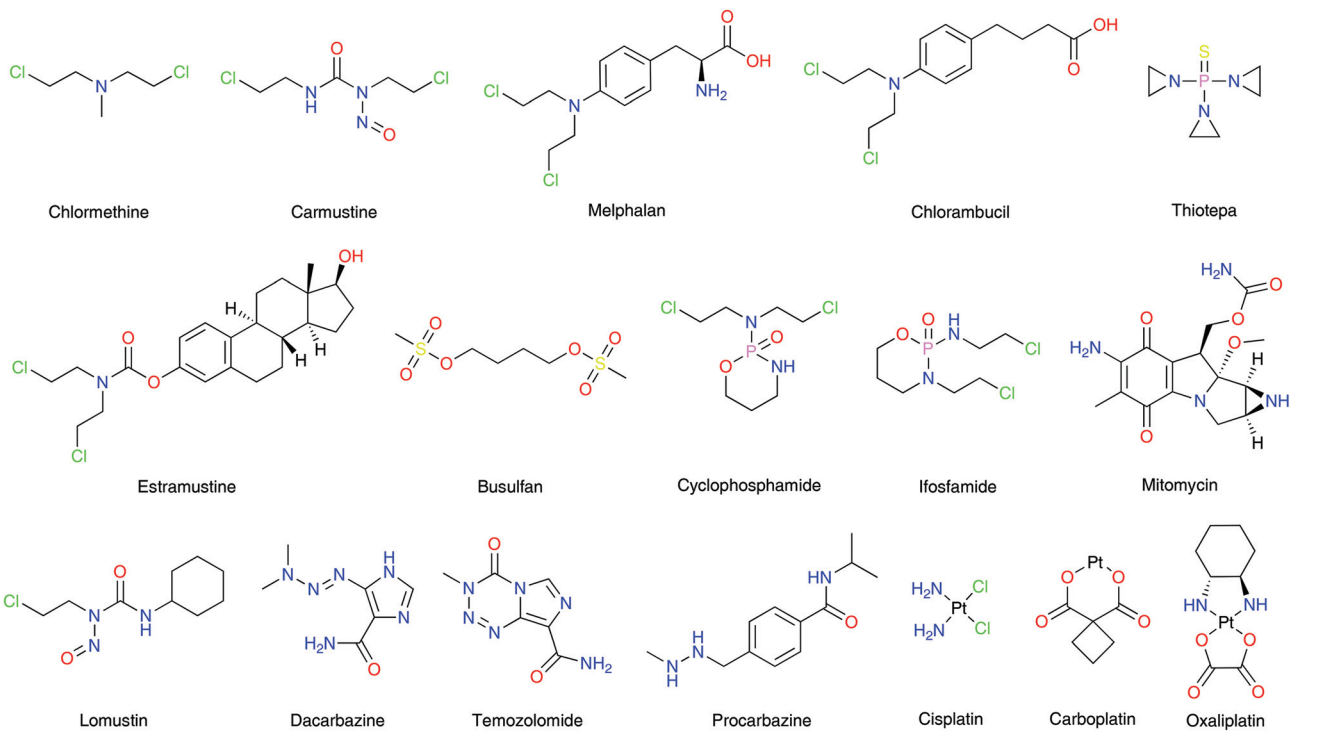


Fig. 5 Structures of alkylating agents.

Table 2 Analytical methods for the detection of alkylating agents

Alkylating agents	Matrices	Techniques	Ref.
Chlorambucil	Biological matrices	LC-UV	45
		LC-MS	162
		CE-UV	45
Chloromethine	Molecule/ formulations	LC-UV	141, 142, 143
Estramustine	Biological matrices	LC-FD	163
		LC-MS	164
		GC-NPD	163
		GC-MS	163
Mephalan	Molecule/ formulations	LC-UV	146, 137
	Biological matrices	LC-UV	147, 148, 149
		LC-MS	153, 154, 155, 156–160 and 161
		LC-FD	150 and 151
		LC-EC	152
Cyclophosphamide	Molecule/ formulations	LC-UV	137 and 186
	Biological matrices	LC-UV	189
		LC-MS	21, 34, 78, 127, 136, 168–180, 193, 194, 305 and 306
	Environment	LC-UV	138
		LC-MS	15, 16, 19, 20, 22, 23, 25, 28, 29, 78, 134, 135 and 307
		GC-MS	27
Ifosfamide	Molecule/ formulations	LC-UV	137
	Biological matrices	LC-UV	188
		LC-MS	21, 34, 136, 170, 176, 179–181, 184, 185, 187 and 191
		GC-NPD	190
		GC-MS	195
	Environment	LC-UV	138
		LC-MS	19, 20, 22, 23, 25, 29, 78, 134, 135, 192 and 307
		GC-MS	27
Mitomycine	Molecule/ formulations	LC-UV	201, 203 and 205
		LC-MS	205
	Biological matrices	LC-UV	208, 209, 211, 212, 214 and 215
		LC-MS	210
	Environment	LC-UV	214 and 215
		LC-MS	29 and 213
Thiotepa	Molecule/ formulations	LC-UV	200, 202 and 204
	Biological matrices	LC-MS	171, 206 and 207
		GC-NPD	207
	Environment	LC-UV	140
Carmustine	Molecule/ formulations	LC-UV	220, 217 and 221
		LC-MS	219
Lomustine	Biological matrices	LC-UV	222 and 223
Busulfan	Molecule/ formulations	LC-UV	238
	Biological matrices	LC-UV	28, 233–237
		LC-FD	239
		LC-MS	178, 183, 240–249
		GC-MS	226–230
		GC-ECD	231 and 232
Procarbazine	Biological matrices	LC-MS	262
	Environment	LC-MS	308
Dacarbazine	Molecule/ formulations	LC-UV	250–252
	Biological matrices	LC-UV	260 and 45
		LC-MS	34 and 266
		CE-UV	45
Temozolomide	Molecule/ formulations	LC-UV	254 and 255
	Biological matrices	LC-UV	253, 256–259 and 261
		LC-MS	263 and 264
		CE-UV	267
	Environment	LC-MS	135
Procarbazine	Biological matrices	LC-MS	265
	Environment	LC-MS	22
Cisplatin	Molecule/ formulations	LC-UV	137
		CE-UV	4, 297, 303 and 304
		CE-MS	302
	Biological matrices	LC-UV	273
		LC-MS	274 and 278
		LC-ICP-MS	280, 283 and 284
		ICP-MS	287 and 21
		CE-UV	295 and 300
		Absorptive voltammetry	288, 289 and 290
	Environment	ICP-MS	286
		LC-ICP-MS	280, 282 and 285
		Absorptive voltammetry	288, 290 and 113
Carboplatin	Molecule/ formulations	LC-UV	269, 271 and 137
		CE-UV	4, 297 and 301
	Biological matrices	LC-UV	270
		LC-MS	275, 136 and 309
		LC-ICP-MS	280 and 283
		ICP-MS	287 and 21
		Absorptive voltammetry	289 and 290
	Environment	LC-MS	29
		ICP-MS	286
		LC-ICP-MS	280 and 282
		Absorptive voltammetry	290 and 113
Oxaliplatin	Molecule/ formulations	LC-UV	291
		CE-UV	4, 298 and 299
		CE-ICP-MS	279
	Biological matrices	LC-MS	276 and 277
		LC-ICP-MS	280, 281 and 283
		ICP-MS	287 and 21
		CE-UV	300
		Absorptive voltammetry	289 and 290
	Environment	LC-MS	310
		ICP-MS	286
		LC-ICP-MS	280
		Absorptive voltammetry	290 and 113

methine hydrolysis were determined either by LC-MS[144] or GC-MS[145] in biological matrices.

LC coupled to a wide range of detectors such as UV,[137,146–149] FD,[150,151] electrochemistry (EC)[152] and MS[153–160] were successfully used for melphalan assay. Based on the chemical structure of the molecule, there is no reason to use fluorescence since the molecule does not possess an extended π conjugation system in its structure. Indeed, similar LOD values (between 5 and 10 ng mL^{-1}) were obtained for LC-UV and LC-FD methods for the analysis of melphalan in plasma. With MS detection, LOQ of 1 ng mL^{-1} was reached for melphalan in biological samples.[154] Chlorambucil was determined by CE-UV and LC-UV in biological matrices with an inferior LOQ of 1 μg mL^{-1}.[45] Studies based on the analysis of DNA/nucleotide adducts with melphalan[155–160,161] and chlorambucil[162] in biological samples by LC-MS have also been published.

Finally, methods based on LC-FD,[163] LC-MS,[164] GC-MS and GC coupled to a nitrogen–phosphorus detector (NPD)[163] were developed for the analysis of estramustine and its main metabolites in biological matrices. With a simple PP step prior to LC-MS analysis, LOQ of 3 ng mL^{-1} was obtained for the estramustine assay in plasma.[164]

2.2.1.2. Oxazophosphorines. Oxazophosphorine compounds appeared in the '50s and are clearly less reactive than nitrogen mustards. They were derived from a concept designed to chemically mask the high reactivity of the active compound up to its target (tumor cell), where specific enzymes were responsible for the conversion to the active form.[165] Indeed, the nitrogen-phosphorus bond does not allow a direct ionisation of the bis(2-chloroethyl) group. The activation, including heterocycle opening resulting from the oxidation of the carbon in the 4-position and formation of chlorethylazirine responsible for the alkylation of DNA, is carried out by P450 cytochromes. The two spearheads of the oxazophosphorines are cyclophosphamide and ifosfamide which have multiple applications (blood cancers, sarcomas, breast cancers...).

Although many methods based on the use of thin layer chromatography (TLC) or GC have been developed for the analysis of oxazophosphorine compounds,[166,167] LC-MS[21,58,127,168–182,23,25,29,183,184,185] and LC-UV[186] remain the techniques of choice. The analysis of the active metabolite of cyclophosphamide (4-hydroxycyclophosphamide) requires an additional derivatisation step immediately after biological sampling, because of its very low stability (half-life of approximately 4 minutes). Various stabilizing agents, such as phenylhydrazine,[174] ansyldrazine,[177] methylhydroxylamine[169] and semicarbazide,[184,171] have been suggested.

Since oxazophosphorines, and more particularly cyclophosphamide, are the most prescribed antineoplastic agents and are administered at high concentrations (mg mL^{-1}), these compounds are often used as markers during studies of exposure to cytotoxic agents. Several LC-MS methods have been developed for the determination of oxazophosphorine traces in environmental and biological matrices, with LOD's lower than 1 ng mL^{-1}. For example, concentrations of 50 pg mL^{-1} oxazophosphorines in urine have been detected by LC-MS including LLE, during biological monitoring of hospital personnel exposed to antineoplastic drugs.[21]

Oxazophosphorine compounds are chiral molecules (asymmetric phosphorus) administered as a racemate mixture to the patient (the *S*-enantiomer has more potent anticancer activity). Protein-based columns[187–192] or polysaccharide-based columns[193,194] allow resolution of oxazophosphorine enantiomers. Ifosfamide enantiomers and their metabolites in urine and serum can be discriminated by a β-cyclodextrine capillary GC column.[195]

2.2.1.3. Ethylene imines. Ethylene imines contain one or more azyridine rings which lead to the formation of aziridinium ions responsible for the alkylating action of the compounds (such as nitrogen mustards). The absence of charge on the aziridine ring makes these molecules less reactive than nitrogen mustards. The first compound of this subfamily, called thiotepa, was discovered in the '50s.[196] Its main indications are ovary, breast and bladder cancers, but it remains scarcely used. The other important molecule belonging to this subfamily is mytomycin C, an antibiotic produced by a bacterium (*Streptomyces caespitosus*) also discovered in the late '50s.[197] It is inactive and requires an enzymatic reduction to induce the opening of the aziridine ring to obtain the alkylating molecule. Mitomycin C is mainly used for the treatment of cancers of the digestive system.

Ethylene imines are unstable in aqueous solution. Indeed, thiotepa degrades rapidly by opening cycles (P–N cleavage) to give the aziridinium ion, polymerising to an insoluble product.[198] In the case of mitomycin C, substitution of an amine group in the 7-position by a hydroxyl group (basic conditions), or loss of a methoxy group and opening of the aziridine ring (acid conditions), can occur.[199] Therefore, pharmaceutical formulations containing ethylene imine molecules are dry forms to be reconstituted and diluted before patient administration.

Due to their low stability in solution, different methods for both thiotepa and mitomycin C were used in degradation studies[140,200] or in stability studies of pharmaceutical formulations.[201–204,205] These methods were mainly based on LC-UV. Since thiotepa metabolism is still uncertain, LC-MS methods were also developed more recently for the identification of potential metabolites.[171,206,207] de Jonge *et al.* have developed an LC-MS method for the determination of thiotepa, cyclophosphosphamide and their main metabolites in plasma, and a LOQ of 5 ng mL^{-1} was achieved for thiotepa.[171] An LOQ of 25 ng mL^{-1} was obtained for the determination of thiotepa in urine by GC-NPD following LLE as the sample preparation step.[207] For mitomycin C, analyses are based on the use of LC-UV and LC-MS and allow the detection of the molecule in biological[208–212] and environmental matrices.[29,213–215] Similar sensitivities are usually obtained for mitomycin (LOD in the order of ng mL^{-1}) with each technique. However, given the high sensitivity and selectivity of MS, it is possible to make the sample preparation step quicker prior to LC analysis, for example a simple dilution of the sample can be included.[210]

2.2.1.4. Nitrosoureas. Nitrosourea compounds have the characteristic of giving, under basic conditions, the diazohydroxide moiety, which generates a highly reactive cation, responsible for the alkylating activity of this subfamily of antineoplastic drugs. The first compound (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine or MNNG) was developed in the '50s.[216] Studies carried out on MNNG allowed the development of new molecules such as:

- MNU (*N*-methyl-*N*-nitrosourea), in the early '60s, which demonstrated good permeability of the blood–brain barrier (treatment of brain tumors);
- BCNU (carmustine), in the late '60s;
- CCNU (lomustine) in the '70s.[216]

The main applications of these molecules are for the treatment of cerebral tumors and melanomas.

Given their relative lipophilic character (log *P* values between 1.5 and 2.5), nitrosourea compounds, in particular carmustine, are interesting for permeability studies on protective gloves[217–219] and for studies dealing with the interaction of substances with the usual containers of pharmaceutical formulations.[220,221] Reported methods are based on the use of spectrophotometry[218] and LC-UV.[217,219–221] Wallemacq *et al.* obtained an LOD of 59 ng mL^{-1} for carmustine in water using LC-UV.[219] Similar sensitivities were obtained with LC-UV methods for the determination of lomustine[222,223] in plasma.

2.2.1.5 Alkylsulfonates. Discovered in the '50s, busulfan is the only compound in this subfamily of alkylating agents.[224] Originally orally administered for the treatment of chronic myeloid leukemia, busulfan was also injected as part of the conditioning regimens for patients undergoing bone marrow transplants at the end of the 20th century. Busulfan is characterized by very low solubility and stability in water, pharmaceutical formulations contain solubilising agents (dimethylacetamide and polyethylene glycol 400) to limit rapid busulfan hydrolysis into tetrahydrofuran and methanesulfonic acid.[225]

Numerous methods have been developed for the analysis of busulfan in blood matrices. Indeed, due to wide inter-patient variation in the pharmacokinetics and the narrow therapeutic range of busulfan, it was essential to introduce pharmacological therapeutic monitoring during treatment with this antineoplastic drug. For this purpose, different analytical techniques were applied. GC coupled to MS,[226–230] or to an electron capture detector (ECD)[231,232] were published for the analysis of busulfan in blood, with an LOQ between 10 and 150 ng mL^{-1}. However, these methods require an additional derivatisation step prior to separation because busulfan is a non volatile and thermolabile compound. LC-UV is not an interesting approach for the determination of busulfan because it does not contain any chromophoric groups. This explains why all reported LC-UV methods also included a derivatisation step, often inspired by those used in GC with similar sensitivities.[28,233–237] An LC-UV method with a derivation step using diethylthiocarbamate provided a successful study of busulfan stability in injectable solutions contained in different medical devices in the concentration range 0.05–0.5 mg mL^{-1}.[238] A similar strategy was applied to the analysis of this compound in plasma by LC-FD with a LOD and LOQ of 9 ng mL^{-1} and 20 ng mL^{-1}, respectively.[239] The derivatisation step became redundant with LC-MS.[178,240–247,248,183] Busulfan concentrations of 0.2 ng mL^{-1} were quantified by a LC-MS, including PP sample preparation and only 100 μL plasma sample.[248]

To analyse busulfan in complex biological matrices, the use of several sample preparation techniques have been reported: LLE,[243,246] SPE on 96-well plates format[178] and in-line[241], as well as PP.[242,244,245,248,183] Recently, busulfan determination was also achieved by LC-MS in whole blood, using dried blood spots followed by methanol desorption[240] with an LOQ of 50 ng mL^{-1}. Danso *et al.* have developed a very fast SPE method coupled to MS for the analysis of busulfan in plasma with an LOQ of 25 ng mL^{-1}.[249] Selectivity was provided by the sample preparation (PP step prior to the SPE) and by the use of a triple quadrupole mass spectrometer.

2.2.1.6 Triazenes and hydrazines. Triazene compounds are characterised by the presence of 3 adjacent nitrogen atoms and are activated by hepatic cytochromes *via* the formation of a methyldiazonium ion (responsible for alkylating action). The two representative compounds of this subfamily are dacarbazine and temozolomide. Discovered in the '70s, dacarbazine is mainly used in the treatment of melanoma and Hodgkin's lymphoma in an injectable form. The development of temozolomide was more recent (late '80s, early '90s). Given its good stability under acidic conditions, possible oral administration, large distribution in the central nervous system and antitumor activity, temozolomide constitutes the treatment of choice for brain tumors (multiform glioblastomas).

Procarbazine is a hydrazine compound (two adjacent nitrogen atoms) developed first as a monoamine oxydase inhibitor in the '60s. In oncology, it is used for the treatment of Hodgkin's lymphoma. Its activation is also achieved by cytochrome enzymes, leading to the formation of diazonium ions.

LC-UV was mainly used for the determination of dacarbazine[250–252] and temozolomide[253–255] in stability/degradation studies. Few methods based on LC-UV[256–261] and LC-MS[262–266] were reported for the analysis of triazene compounds and procardazine in biological matrices. An LOQ of 0.5 ng mL^{-1} was achieved for the analysis of dacarbazine[266] and procarbazine[265] in plasma samples by LC-MS. Lower sensitivity (LOQ of 50 ng mL^{-1}) was reached with LC-MS for the temozolomide assay in plasma.[264]

A micellar electrokinetic chromatography (MEKC) method has also been reported for the determination of temozolomide and its degradation products in water and serum.[267] The choice of the MEKC method was based on: (i) the absence of charge on temozolomide in neutral and acidic conditions; and (ii) its low stability in solution.

2.2.1.7. Platine derivatives. All the compounds belonging to this subfamily of antineoplastic drugs contain a platinum atom in the oxidation state II, the nature of ligands reflects the history of their development.

Even if the first compound of this subfamily, cisplatin, was synthesised initially in 1844, its anticancer activity was accidentally discovered only in the '60s.[268] Its high toxicity (and

more particularly its nephrotoxicity) and various resistance phenomena led to the development of new molecules. The applied strategy focused on a reduction of toxicity by increasing water solubility and stability. In this perspective, the chlorine atoms were substituted by carboxylate chelating groups. Among all the molecules synthesised and evaluated, carboplatin (developed at the end of the '80s) was particularly interesting, due to its wider therapeutic index and reduced toxicity. The bidentate cyclobutanedicarboxylate ligand gives carboplatin greater stability than the chloride ligands. However, this second generation of compounds suffers, under chlorine-rich conditions, from a substitution of the carboxylate group by chlorine, leading to the formation of cisplatin. Therefore, manipulations of carboplatin should be avoided with solutions containing chlorine, to limit toxicity. In addition, because carboplatin has a close structure to cisplatin, it is also inactive against cisplatin-resistant tumors. Consequently, there was a need to develop a new molecule without these problems of resistance. Thus, the third generation of platinum derivatives appeared in the late '90s, with a 1,2-diaminocyclohexane group, whose anticancer activity against tumors resistant to cisplatin and carboplatin, has been demonstrated. Due to the presence of two bidentate ligands, oxaliplatin was the most stable platinum derivative. However, it can be noted that chloride ions are not recommended in the presence of oxaliplatin to avoid any substitution of the ligands by chlorine atoms, leading to highly reactive products (formation of mono or dichloro platinum complexes). Today, cisplatin is still prescribed in the treatment of solid tumors such as neuroblastomas, while carboplatin is mainly used in the treatment of ovarian cancers. Oxaliplatin is mainly used in the treatment of colorectal cancers.

Different analytical techniques were used for the determination of derivative platinum compounds. Given their low UV-absorbance and high instability, the development of methods for the platinum derivatives assay can be considered as difficult. Few LC-UV methods were reported but all of them suffered from poor sensitivity (limit of quantification (LOQ) in the order of a few μg mL^{-1}).[269–271] The introduction of an additional derivatisation step improved sensitivity (up to a factor of 100) with LC-UV methods.[272,273] Better LOQ's, between 2 and 25 ng mL^{-1}, were reached with LC-MS.[274–278] However, the technique of choice for the detection of platinum derivatives was undoubtedly inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS coupled to a separation technique,[279–284,285] or not,[21,286,287] is characterised by an excellent LOQ, in the range 0.1 to 1 μg mL^{-1}. A chromatogram obtained for the analysis of platinum compounds by LC-ICP-MS is reported in Fig. 6.[280] Without separation prior to ICP-MS, the quantification of total platinum was performed. On the other hand, absorptive voltammetry was also successfully applied for the quantification of total platinum with an LOD in the order of pg mL^{-1} in biological matrices. However, similar to oxazophosphorine compounds, platin derivative agents belong to the most administered antineoplastic drugs (often in high concentrations too) and are often used as markers in exposure studies. Hence, all of these techniques are used even if absorptive voltammetry methods offer better sensitivity. This is because this analytical technique requires a sample degradation step *via* photolysis digestion.[113,288–290]

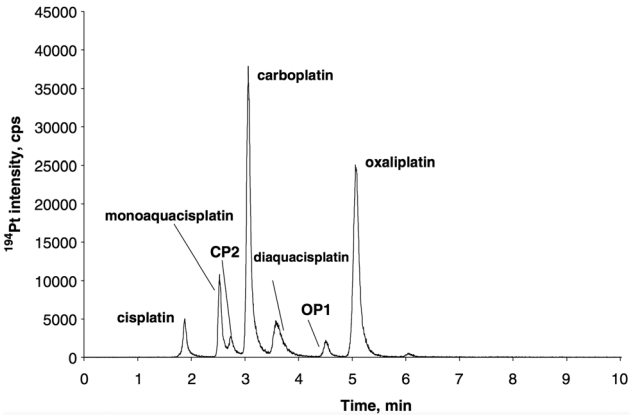


Fig. 6 Chromatogram obtained for the analysis of platinum compounds and degradation products of cisplatin (monoaquacisplatin, diaquacisplatin), carboplatin (CP2) and oxaliplatin (OP1) in water by LC-ICP-MS. Adapted from ref. 280 by permission of Springer, 2005.

For the separation step, different chromatographic supports were used, including reversed phase columns,[269,270,277,281,278] ion exchange columns,[275] HILIC support[285] or cellulose based columns for the enantiomer resolution of oxaliplatin.[291] CE was also reported as a promising technique for the separation of platinum derivatives.[292–294] MEKC methods with UV detection allowed a baseline resolution between cisplatin, carboplatin and oxaliplatin.[295–297] Detection limits in the order of 0.6 μg mL^{-1} were reported for cisplatin in serum after a sample preparation step based on ultrafiltration.[295] Microemulsion electrokinetic chromatography (MEEKC) methods coupled to UV detection were also developed in the context of fundamental studies.[298,299] An MEEKC method coupled to ICP-MS detection was reported for the characterisation of oxaliplatin and other platinum derivatives in drug development.[279] Due to the signal suppression effects of surfactants employed in MEEKC, the sensitivity was only improved by a factor of 1.5 compared to UV detection, to the detriment of the separation (peak broadening due to the interfacing and detector carry-over). More conventional CE methods were also developed in interaction studies (between platinum compounds and blood proteins)[300] and for the analysis of platinum derivatives and nucleoside adducts.[301–304] Under these conditions, the molecule to be analysed was a charged macromolecule.

2.2.2. Intercalating agents. Intercalating agents are planar polycyclic molecules that can be incorporated between contiguous base pairs of DNA. DNA replication and transcription are then inhibited. Direct action on topoisomerase II and I, or formation of free radicals are the two proposed modes of action of these intercalating agents. The structures of the main

intercalating agents and the analytical methods used for their analysis are reported in Fig. 7 and Table 3, respectively.

2.2.2.1 Anthracyclines. Anthracyclines are natural antibiotic molecules derived from pigments produced by *Streptomyces peucetius*.[311,312] They have a common structure composed of an anthracyclinone entity (responsible for their red coloration) and aminoglucoside. The first molecules of this subfamily were discovered in the early '60s and were called daunorubicin and doxorubicin. A slight difference in structure (C9 chain terminated with a primary alcohol for doxorubicin and methyl for daunorubin) resulted in significant changes in the activity spectra of these two molecules. Doxorubicin is used for the treatment of lymphomas, breast, stomach, ovarian and bladder cancers, while daunorubicin is indicated for the treatment of chronic lymphocytic leukemia. Both molecules exhibit high cardiotoxicity. Therefore, the following developments of new anthracyclines focused on molecules with lower side effects. Epirubicin, a semi-synthetic derivative of doxorubicin (an epimer in the hydroxyl group at the carbon in 4-position of aminoglycoside) appeared in the '80s. Although epirubicin has approximately the same indications as doxorubicin, differences in metabolism and pharmacokinetics of the molecule are observed, including an increase in distribution volume, 4-*O*-glucuronidation, an increase of clearance and a decrease of half-life. Thus, high doses of epirubicin can be administered without an increase in cardiotoxicity. At the same time, a semi-synthetic analog of daunorubicin, namely idarubicin, also appeared on the market. Compared to daunorubicin, idarubicin lost the C4 methoxy group, conferring greater lipophilicity. Idarubicin exhibits a wider spectrum of activity and lower cardiotoxicity. Treatment can be orally administered and its main indications are breast cancer and some types of leukemia. Anthracyclines are characterized by stability in solution within a very narrow pH range (between pH 5–7). Indeed under acid pH conditions, the molecules precipitate whereas at basic pH, they degrade rapidly. Liposomal injection solutions of doxorubicin and daunorubicin have been developed to reduce their cardiotoxicity by limiting their distribution to the heart.

Numerous LC and CE methods have been published for the analysis of anthracyclines in the last three decades. For most LC methods, reversed phase chromatographic supports were used. LC-UV methods were applied for the determination of anthracyclines in solution[67,313–317,74] or in biological matrices.[67,318,319] Given the high cardiotoxicity of anthracyclines (due to the accumulation of the drug in myocardium), drug monitoring of patients is generally required. The sensitivity of the developed method was of the utmost importance. For example, an LOD of 5 ng mL^{-1} and LOQ of 30 ng mL^{-1} were obtained for the determination of doxorubicin in different tissues by SPE-LC-UV.[319] Fluorescence spectrophotometry was found to be particularly well suited to the detection of anthracyclines, due to their anthracyclinone ring. Thus, method sensitivity was drastically improved.[320–330] LOD of 0.3–0.75 ng ml^{-1} and LOQ of 1–2.5 ng mL^{-1} were reached for the analysis of doxorubicin, epirubicin, daunorubicin and idarubicin in plasma and saliva (LLE was employed as a sample preparation step prior to LC-FD analysis).[323] The use of MS as a detection system offered even better sensitivity.[172,173,179,331–336,337,338] Indeed, an LOD and LOQ

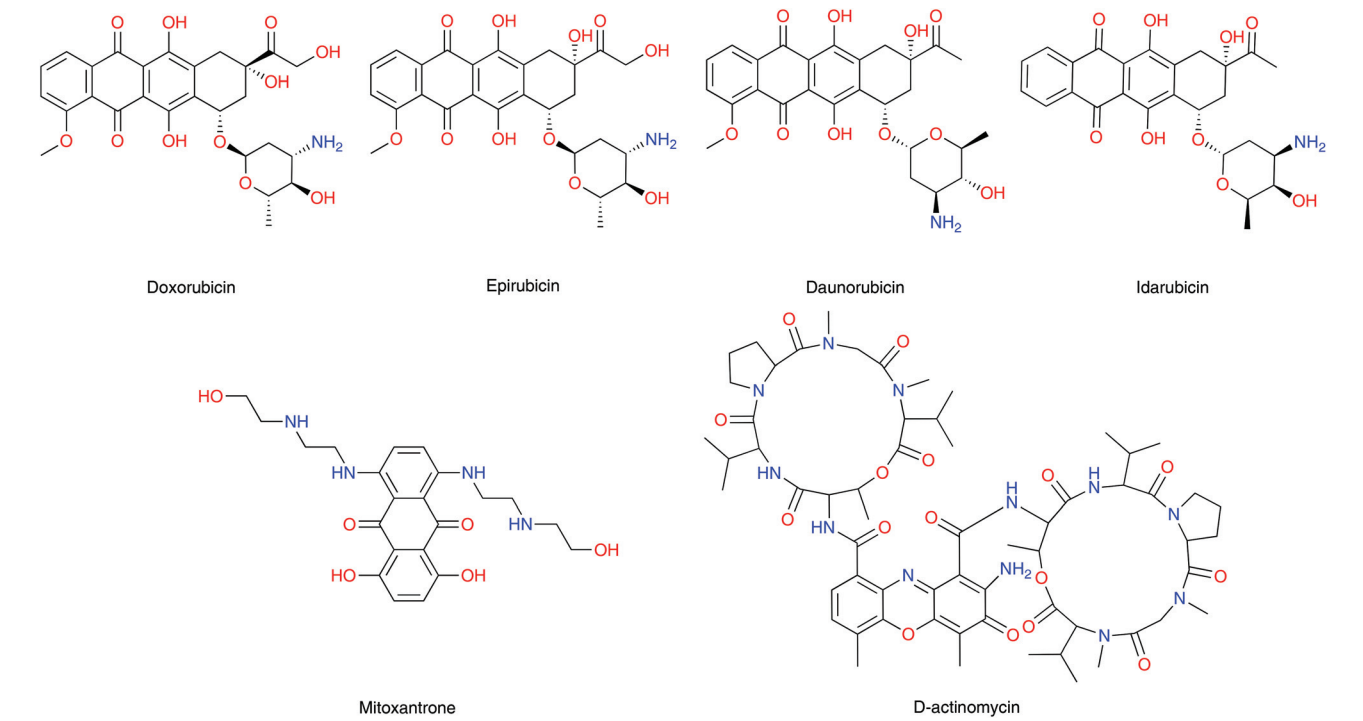


Fig. 7 Structures of intercalating agents.

Table 3 Analytical methods for the detection of intercalating agents

Intercalating agents	Matrices	Techniques	Ref.
Doxorubicin	Molecule/ formulations	LC-UV	313, 314, 67 315 316 and 74
	Biological matrices	LC-UV	67, 318 and 319
		LC-FD	320, 322, 323, 324 325, 326, 327, 328, 329 and 330
		LC-MS	331, 172, 173, 333, 179, 335, 337, 338 and 34
		LC-LIF	339
		CE-LIF	341, 342, 343, 344, 345, 346, 347 and 349
		CE-UV	350 and 363
	Environment	LC-UV	138
		LC-MS	29, 78, 19, 22, 135 and 307
Epirubicin	Molecule/ formulations	LC-UV	313, 317 and 74
	Biological matrices	LC-UV	318
		LC-FD	321, 323, 324 and 325
		LC-MS	334, 179, 335 and 34
		CE-LIF	344, 345, 347 and 349
	Environment	LC-MS	19 and 307
Daunorubicin	Molecule/ formulations	LC-UV	313, 314 and 74
		LC-FD	323 and 324
		LC-ELSD	340
	Biological matrices	LC-UV	318
		LC-FD	364
		LC-MS	179, 335 and 336
		CE-LIF	345, 346, 347, 348 and 349
		CE-UV	350 and 363
Idarubicin	Molecule/ formulations	LC-UV	313 and 137
	Biological matrices	LC-FD	323 and 324
		LC-MS	335
		CE-LIF	345 and 346
Mitoxanthrone	Biological matrices	LC-UV	354 and 355
		LC-MS	361
Actinomycin D	Biological matrices	LC-MS	356–358 and 360

lower than 0.01 ng mL^{-1} and 0.1 ng mL^{-1} were obtained, respectively, for the LC-MS analysis of anthracyclines in urine treated by SPE.[335] LC-LIF-MS was also developed for studying *in vitro* metabolism of doxorubicin: quantification was performed on the results obtained with LIF detection (LOD ~ 1 μg mL^{-1}) and metabolites identification was performed with MS detection.[339] LC-ELSD was also applied for the analysis of daunorubicin and its degradation products for a stability study of injectable solution of anthracycline (0.25 mg mL^{-1} was the lowest concentration of standard solutions tested), but the interest of ELSD for anthracyclines is not obvious since these molecules can be easily detected by UV, FD and MS.[340]

As already mentioned, CE was another separation technique widely used for the determination of anthracyclines. LIF detection[341–349] is usually preferred to UV detection[350] to improve sensitivity. However, the poor UV sensitivity observed in CE (due to the narrow optical path of the capillaries) can be compensated for by a sweeping preconcentration step and an electrokinetic injection. Under such conditions, an LOD of 0.5 μg mL^{-1} was reached for anthracyclines in plasma.[350] LIF detection coupled to conventional CE[343,346,347,349] or MEKC methods[342,344,345] offered better sensitivities (LOD inferior to 1 ng mL^{-1}) without the need for a preconcentration step and using the hydrodynamic injection. The addition of a chiral modifier (hydroxy-propyl-γ-cyclodextrine) to the BGE used in CE provided a resolution baseline between doxorubicin and its metabolite doxorubicinol (slight structural difference: OH group instead of a carbonyl group in 13-carbon), responsible for the cardiotoxicity.[342] Finally, it can be noted that in the majority of the developed CE methods, an organic solvent was added in the BGE to improve the solubility and stability of the anthracyclines and reduce their adsorption on the capillary walls.[351] An electropherogram obtained for the analysis of three anthracycline compounds by CE-LIF and demonstrating the influence of organic solvent in the BGE on the separation efficiency is illustrated in Fig. 8.[349]

2.2.2.2 Mitoxantrone and actinomycin D. Mitoxanthrone and actinomycin D are molecules with a planar tricyclic structure (like anthracyclines). Discovered in the late '80s, mitoxantrone originated from an American program on the development of intercalating molecules.[352] Actinomycin D (or dactinomycin) is a peptide antibiotic isolated from strains of *Streptomyces parvullus* in the early '50s.[353] Mitoxantrone is available as a concentrated acid-buffered solution (because of hydrolysis in a basic medium) and is mainly used for the treatment of prostate cancers. Formulations containing actinomycin D are indicated for the treatment of nephroblastomas, neuroblastomas in children and testicular cancers.

LC was generally used for the determination of mitoxanthrone and actinomycin D. Developed methods were based on C18 stationary phase, coupled to UV[354,355] and MS detection.[356–361] An LOQ of 5 ng mL^{-1} was obtained for the LC-UV analysis of mitoxantrone in plasma.[354,355] For the same compound, a ten-fold increase in sensitivity was observed in LC-MS.[361] Due to the concomitant administration of vincristine and actinomycin D in the treatment of various pediatric cancers, several LC-MS methods have been developed for the simultaneous determination of these two anticancer drugs in biological matrices.[356–359] An LOD of 0.007 ng mL^{-1} and LOQ of 0.05 ng mL^{-1} were reached for the analysis of actinomycin D in plasma using a SPE-LC-MS procedure.[358] A CE method coupled to chemiluminescence detection was also reported for the determination of mitoxantrone in injectable solutions and biological samples.[362]

2.2.3. Topoisomerase inhibitors. Toposiomerases are enzymes responsible for the cleavage, annealing and topological state of DNA. Two categories of topoisomerases can be distinguished: topoisomerase I and topoisomerase II. Topoisomerase I acts on one strand of the DNA, while topoisomerase II acts on both strands of the DNA. Inhibitors of these enzymes are used primarily as anticancer agents (but also as antibacterial and antiparasitic agents). The structures

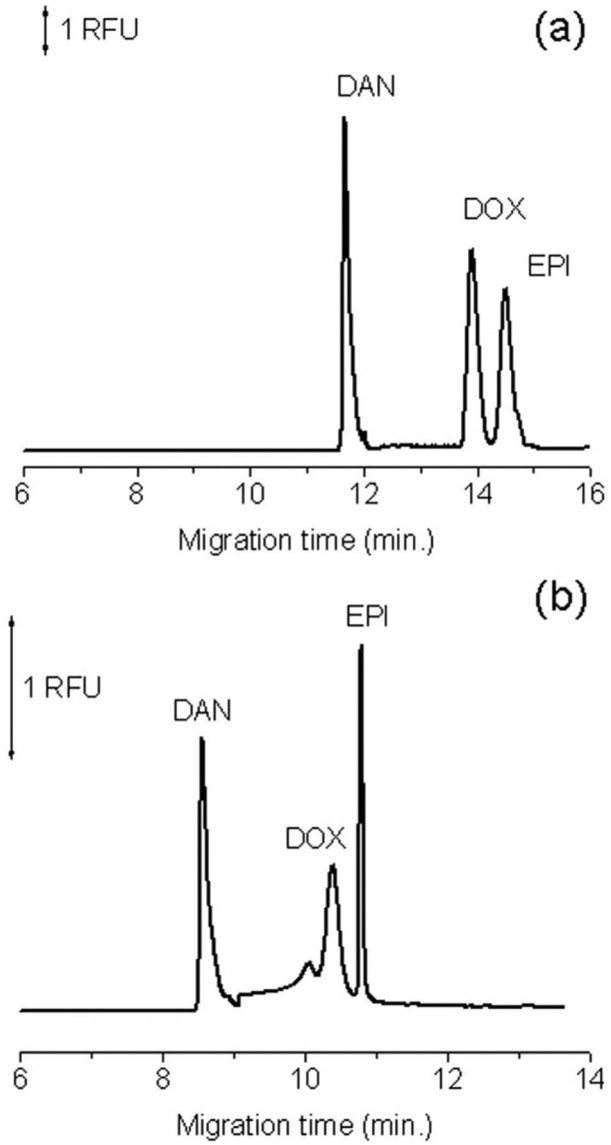


Fig. 8 Electropherograms of daunorubicine (DAN), doxorubicine (DOX) and epirubicine (EPI) obtained by CE-LIF analysis with BGE (105 mM borate pH 9.0) containing 30% methanol (a), and 10% methanol (b). Adapted from ref. 349, 2008, with permission from Wiley.

of the main topoisomerase inhibitors are depicted in Fig. 9. Analytical methods for the analysis of topoisomerase inhibitors are reported in Table 4.

2.2.3.1. Topoisomerase I inhibitors. Topoisomerase I inhibitors are derived from camptothecin first isolated in the '60s from the bark of an Asian tree named *Camptotheca acuminata*. Despite its anticancer activity, camptothecin was rapidly abandoned due to the high toxicity (hemorrhagic cystitis) of the soluble sodium salt. It appeared that the lactone ring of camptothecin is responsible for its anticancer activity. The fact that this cycle opened up in the preparation of sodium salts and could be reformed in the acid environment of the bladder, explained the toxicity of this molecule.[365] The interest in this molecule reappeared in the '80s with the discovery of camptothecin action on topoisomerase I. Thus, camptothecin served

Table 4 Analytical methods for the detection of topoisomerase inhibitors

Topoisomerase inhibitors	Matrices	Techniques	Ref.
Irinotecan	Biological matrices	LC-FD	371, 374, 372, 376, 377, 378 and 380
		LC-MS	370, 375, 373, 381, 375, 385, 387, 388, 389, 390 and 136
		CE-UV-LIF	391
	Environment	LC-MS	19, 135 and 307
Topotecan	Molecule/ formulations	LC-UV	382
	Biological matrices	LC-FD	379, 383 and 384
		LC-MS	386
Etoposide	Molecule/ formulations	LC-UV	67, 393, 394 and 137
		LC-FD	364
	Biological matrices	LC-UV	67
		LC-MS	395, 396, 136 and 34
		CE-LIF	397
		CE-UV	38
	Environment	LC-MS	19, 22, 134 and 135

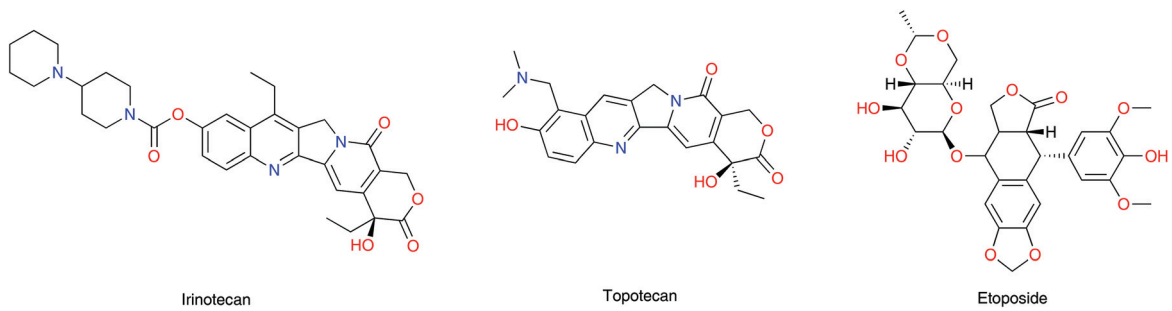


Fig. 9 Structures of topoisomerase inhibitors.

as a model for the development of various water-soluble semi-synthetic compounds, by retaining a lactone ring in which the two main representative compounds were irinotecan and topotecan. Discovered in the '90s, irinotecan is indicated in colorectal cancers and topotecan in certain cancers of the ovary, cervix and lung. Irinotecan is a prodrug that is activated by liver carboxylesterases (active form SN-38: loss of the bipiperidylcarbonyl chain). Both molecules are stable at acid pH (if pH > 4: opening of the lactone ring leads to an inactive carboxylate form).

The methods developed for the analysis of camptothecins are essentially based on LC.[366–369] When developing an analytical method for the determination of camptothecins in biological matrices, several features have to be taken into account. First of all, it should be noted that the lactone and carboxylate forms of camptothecins coexist under biological conditions (equilibrium depending on pH and temperature). In other words, the method must allow the analysis of the desired form (s) (active lactone, inactive or total carboxylate). Then, the lactone (lipophilic) forms are able to diffuse through the cell membranes (and more particularly through the red blood cells) even within the sample. Moreover, the carboxylesterases present in the biological matrices contribute to the conversion of irinotecan into SN-38 within the sample as well. Thus, inactivation of carboxylesterases (in the case of irinotecan) and rapid extraction of the camptothecins contained in biological matrices (and in particular blood fluids) must be carried out rapidly after collection.

LC methods reported the analysis of the lactone form only,[344–346] lactone and carboxylate forms separately[347–351] and all the forms together with an equilibrium shift to the lactone form in acidic conditions. Usually, separations were performed on reversed phase supports. Several studies have succeeded in resolving the lactone forms and carboxylate forms by ion-pair chromatography to increase retention of the carboxylate forms.[377–380] In the case of irinotecan analysis, carboxylesterase inactivation was accomplished by the immediate addition of sodium dodecyl sulfate (SDS)[372] or zinc sulfate[373] to the sample.

Detection using, UV,[382] FD[371,372,377,379,380,383,384] and MS[370,373,375,381,385–390] were the most widespread. LC-UV methods were developed for the analysis of topotecan in pharmaceutical formulations and in blood samples with an LOQ of 0.070 μg mL^{-1}.[382] More sensitive detectors, such as FD or MS, allowed an LOQ in the order of 1 ng mL^{-1} to be reached for camptothecines in biological matrices. Finally, a quantification of SN-38 in plasma concentrations of 50 pg mL^{-1} was achieved thanks to a microfluidic chip-based nano-LC-MS method.[385]

A CE-UV-LIF method including solid-supported liquid extraction (SLE) was developed to quantify irinotecan and SN-38 in urine samples with an LOQ in the order of 30 ng mL^{-1} for both analytes.[391]

2.2.3.2. Topoisomerase II inhibitors. Two antineoplastic drugs act on topoisomerase II: anthracyclines (considered in this paper for their intercalating action) and podophyllotoxins.

Podophyllotoxins extracted from the plant roots of the *podophyllum* family are considered to be highly toxic molecules, even though they were used as medicinal remedies centuries ago thanks to their antimitotic action. In the '50s, a series of podophyllotoxin derivatives were synthesized and studied in the hope of finding a molecule that retained its anticancer action, but with less toxicity.[392] These studies led to etoposide in 1966. Its low solubility in water required the presence of numerous excipients in injectable formulations such as sorbate 80, polyethylene glycol 300 solubilizing agents and buffering agents such as citric acid (to avoid the *cis*-lactone epimerization of molecules occurring in basic conditions). However, precipitation of the molecule can be observed during dilution of the formulation and rapid administration of etoposide is hampered by the high volumes injected to cover the prescribed dose. The etoposide phosphate appeared in the '90s to overcome the solubility problem of the original molecule. This prodrug is rapidly converted in the blood to etoposide, by alkaline phosphatases.

Etoposide was mainly analysed by LC-UV,[67,393,394] LC-FD[364] and LC-MS.[58,395,396,34] UV sensitivity was sufficient to achieve etoposide analysis in pharmaceutical formulations.[393,394] However, more sensitive detectors such as FD (LOQ of 52.5 ng mL^{-1}) and MS (LOQ between 2 and 10 ng mL^{-1}) were required for etoposide assays in plasma samples. CE allowed the determination of etoposide in plasma with an LOQ of 0.1–0.2 μg mL^{-1}, using either a UV zeta-cell[38] or LIF detector.[397]

2.3. Antitubulin agents

Antitubulin agents interfere with microtubule dynamics (*i.e.*, spindle formation or disassembly), block division of the nucleus and lead to cell death. The main members of this family include vinca alkaloids and taxanes.

2.3.1. Vinca alkaloids. The antineoplastic properties of Madagascar periwinkle alkaloids (*Catharanthus roseus*) were discovered during research on its use as an antidiabetic by Malagasy people. The first anticancer alkaloids extracted from the leaves of the plant were vinblastine in 1958 and vincristine three years later. These two molecules consist of a catharanthine nucleus and a vindoline nucleus. The low natural abundance of these compounds (a few ppm in the leaves), the multitude of different alkaloids present in the leaves and their toxicity (neurotoxicity for vincristine and myelosuppression for vinblastine), have encouraged studies on the synthesis of structural analogues. The latter gave rise to two semi-synthetic molecules: vindesine and vinorelbine in the '80s. Recently, a fluorinated derivative also appeared, namely vinflunine, which is a molecule close to vinorelbine, but with two fluorine atoms in 20′-position and a single 3′–4′ bond. The structures of vinca alkaloids are reported in Fig. 10. Today, vinca alkaloids are used in the treatment of leukemias, lymphomas and some solid tumors. Table 5 reports the analytical methods for vinca alcaloids.

The analysis of vinca alkaloids has been carried out on plant extracts,[398–403] pharmaceutical formulations,[316,404] biological samples[358,359,405–420,421] and environmental

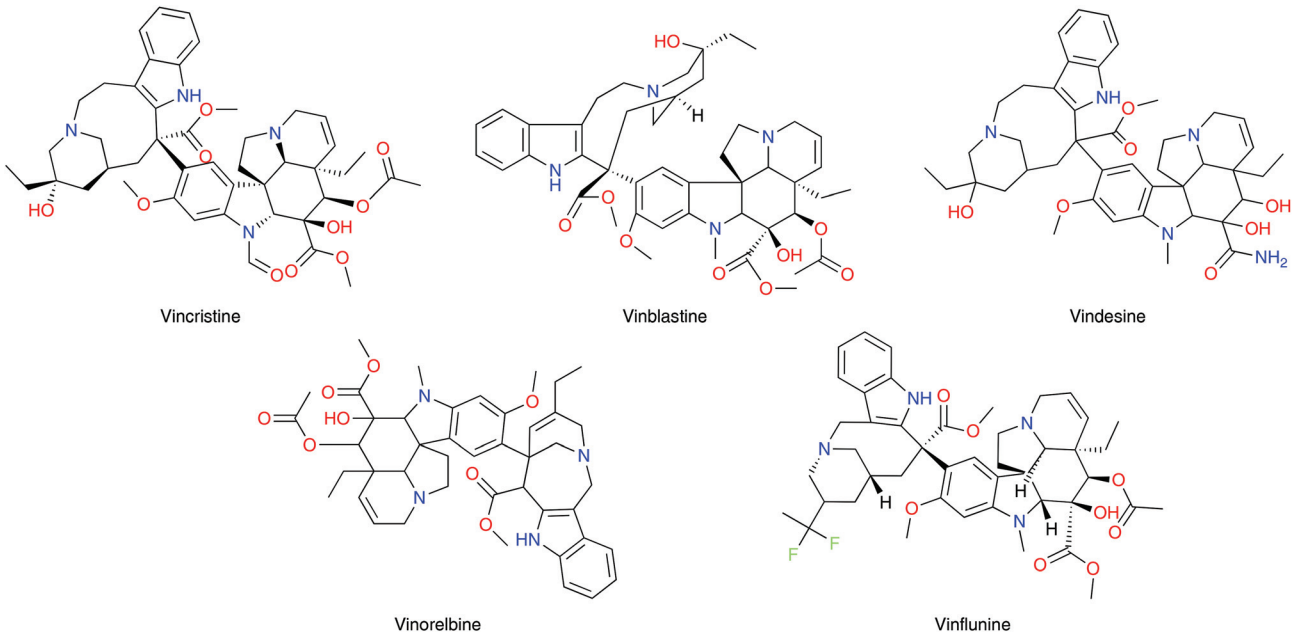


Fig. 10 Structures of vinca alkaloids.

Table 5 Analytical methods for the detection of vinca alcaloids

Vinca alcaloids	Matrices	Techniques	Ref.
Vincristine	Molecule/ formulations	LC-UV	316
		CE-UV	404
	Plant extracts	LC-UV	400, 402 and 403
		LC-MS	401
	Biological matrices	LC-UV	402
		LC-MS	34, 173, 356, 358, 359, 407, 409, 413 and 421
	Environment	LC-MS	19, 22, 28, 134 and 307
Vindesine	Molecule/ formulations	CE-UV	404
	Biological matrices	LC-MS	418
	Environment	LC-MS	307
Vinblastine	Molecule/ formulations	CE-UV	404
	Plant extracts	CE-MS	398
		LC-MS	399 and 401
		LC-UV	400, 402 and 403
	Biological matrices	LC-UV	402
		LC-MS	34, 173, 405, 410 and 411
	Environment	LC-MS	307
Vinorelbine	Molecule/ formulations	CE-UV	404
	Biological matrices	LC-MS	406, 408, 410, 414 and 416
		CE-ECL	423
Vinflunine	Biological matrices	LC-UV	419
		LC-MS	415, 417 and 420

samples.[18,19,134,307,422] Vinca alcaloid extraction from plants was generally performed by ultrasound in acid media followed by LLE.[45,398–401] For the sample preparation of biological samples, SPE,[358,359,405,407,408,411,414,418] LLE[409,410,413,415–417,419,421] and PP[420] were used. Although some methods based on CE have been published, LC was the most widely used separation technique for the analysis of vinca alkaloids.

Most of the LC methods are based on reversed phase C18 supports. Nevertheless, cyano,[419,420] pentafluorophenylpropyl[405] or HILIC[418] columns also allowed vinca alkaloids separation and quantification, with good analytical performance in terms of efficiency and reproducibility.

Detection of the molecules separated by LC was achieved by UV[316,400,402,419,403] or MS.[18,19,134,307,358,359,399,401,405,407–411,413–418,420,422] Acidic conditions were usually applied due to the good stability of vinca alkaloids at these pH values. Thus, MS detection was performed on the $[M + H]^+$ molecular ion or the doubly-charged $[M + 2H]^{2+}$ ion. The LOQ ranged from 1 ng mL^{-1} (or slightly lower) by MS to a few μg mL^{-1} by UV.

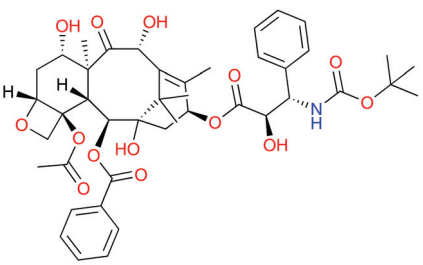
CE methods are scarcely reported for the determination of vinca alkaloids. A CE-UV method in non-aqueous conditions (NACE) allowed the separation of ten different vinca alkaloids in less than 10 min.[404] Extracts from *Catharanthus roseus* containing vinblastine were also successfully analysed by CE-MS.[398] Finally, the analysis of vinorelbine in urine samples was achieved by CE coupled to an electrochemiluminescence detection (ECL) with LOD in the order of 7 ng mL^{-1}.[423]

2.3.2. Taxanes. A compound extracted from the bark of *Taxus Brevifolia* demonstrated anticancer activity in the '60s, namely taxol or paclitaxel.[424] However, it appeared that the first clinical trials conducted on taxol raised toxicity problems linked to the formulation and not to the active molecule. Since taxol is a molecule poorly soluble in water and therapeutically

active at high concentrations, the presence of solubilizing agents is essential in the pharmaceutical formulation. The developed formulation contained a mixture of polyoxyethylated triglycerides (which are toxic and allergenic). In addition, more than 2500 trees needed to be felled to harvest 1 kg of taxol. A solution was introduced in the '80s by Pierre Potier (also author of the discovery of vinorelbine) with the hemi-synthesis of paclitaxel from a precursor (non-cytotoxic) available from a renewable source (*Taxus baccata* needles).[425] This route also gave rise to another taxane with anticancer properties, docetaxel. Slightly soluble in water, docetaxel injectable formulations contain ethanol and polysorbate 80 (a less toxic excipient). The main indication of docetaxel is the treatment of breast and lung cancer. Paclitaxel is also used in ovarian cancer. The structures of the taxanes and their analytical methods are shown in Fig. 11 and Table 6, respectively.

Most methods for taxanes analysis are based on RPLC coupled to MS[305,309,426–442] and UV detection.[138,269,443–453] LODs lower than 0.05 ng mL^{-1} were reached for the analysis of docetaxel in blood using a column-switching method involving a preconcentration step on a trapping column, prior to LC-MS analysis.[435] Paclitaxel in plasma was quantified down to a concentration of 45 ng mL^{-1} using an LLE-LC-UV procedure.[447] Several column-switching methods were proposed to reduce sample handling and/or concentrate paclitaxel[438] or docetaxel[429,434,435,442] as shown in Fig. 12. Given their strong binding to plasma proteins, free or bound fractions of taxanes were evaluated in biological samples. Thus, an ultrafiltration step was included at the start of the whole analytical procedure.[305,436]

CE was also employed for taxanes determination.[363,454] Different CE, MEEKC and MEKC methods coupled to UV



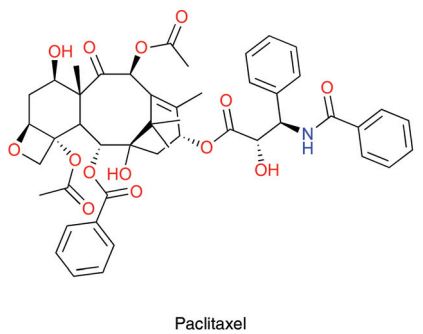


Fig. 11 Structures of taxanes.

Table 6 Analytical methods for the detection of taxanes

Taxanes	Matrices	Techniques	Ref.
Paclitaxel	Molecule/ formulations	LC-UV	269, 443–445, 447–449, 452 and 453
	Biological matrices	LC-UV	446, 447, 451 and 452
		LC-MS	34, 309, 410, 426, 428, 430, 431, 438 and 441
		CE-UV	454
	Environment	LC-UV	138
		LC-MS	28, 134, 135 and 307
Docetaxel	Molecule/ formulations	LC-UV	450
	Biological matrices	LC-MS	305, 410, 427 and 429, 431–437, 439, 440 and 442
		CE-UV	363
	Environment	LC-MS	134 and 307

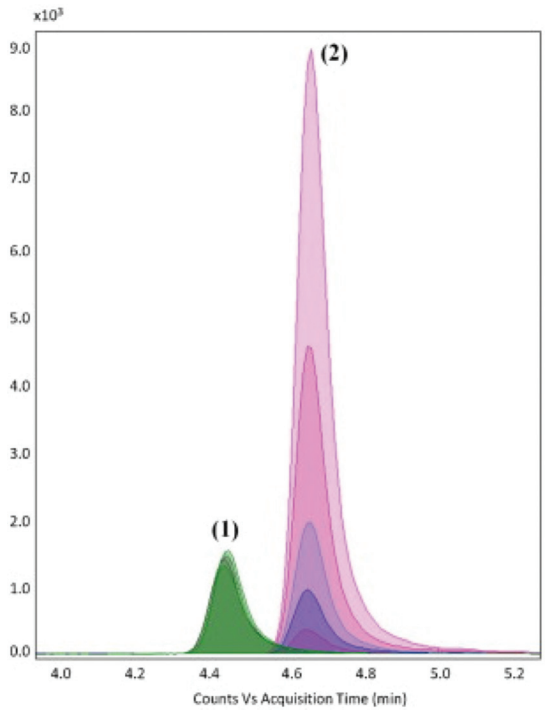


Fig. 12 On-line SPE-LC-MS chromatograms of docetaxel[2] over the concentration range 10–200 μg L^{-1} in plasma with paclitaxel[1] as the internal standard. Adapted from ref. 435, 2013, with permission from Elsevier.

allowed the separation of docetaxel and several anthracyclines in plasma samples.[363] An LOD of 20 ng mL^{-1} was observed for the analysis of placlitaxel in plasma by LLE-MEKC-UV.[454]

3. Small molecules for targeted therapy

Conventional anticancer molecules act without discrimination between normal and tumor cells. This lack of specificity is

responsible for almost all the adverse effects of these anticancer treatments described so far. The apparition of molecules whose action is targeted specifically on tumor cells gave new breath to chemotherapy and new hope in the treatment of certain cancers. These "search-head missiles" are characterized by wider therapeutic indexes and reduced toxicity. Two main classes of molecules constitute targeted therapies: small molecules and monoclonal antibodies. Only small molecules are discussed in this paper.

3.1. Tyrosine kinase inhibitors

Tyrosine kinase inhibitors are low molecular weight compounds whose target is the inhibition of tyrosine kinase enzymes (TK). TK enzymes catalyse the transfer of phosphate to proteins from adenosine triphosphate (ATP) and play a major role in cell regulation such as proliferation, survival, migration and differentiation. TK inhibitors react with membranes (intracellular) or cytoplasmic enzymes, which induce molecule diffusion through the cell membrane. TK inhibitors are used in oncology when targeted TK's are activated by mutations and are responsible for tumor progression.[455]

Imatinib was the first TK inhibitor used as an anticancer agent. Discovered in the '90s, imatinib was derived from drug development efforts to target the bcr-abl protein. It corresponds to 2-phenylaminopyrimidine to which methyl and benzamide groups have been added to improve the interaction with the protein. Imatinib is indicated for the treatment of chronic myeloid leukemia. Two analogues appeared later on the market to tackle the phenomena of resistance and intolerance: dasatinib and nilotinib. Lapatinib and erlotinib, which act on epidermal growth factor receptors (EGFR), were commercialized in the 2000s for the treatment of breast cancer and some lung cancers, respectively. Sunitinib, used in the treatment of gastrointestinal and renal cancers, completes the family of TK inhibitors by targeting vascular endothelial growth factor (VEGFR) receptors. Fig. 13 shows the structures of TK inhibitors.

Different techniques have been used for the determination of TK inhibitors in pharmaceutical formulations, biological and environmental samples (Table 7). LC is the technique of choice as shown in the literature.[456] Indeed, 90% of the published methods are based on LC, while the remaining 10% involve another analytical technique (CE, GC or UV). LC was carried out essentially in the reversed phase mode, although the use of a HILIC column allows the simultaneous determination of imatinib, dasatinib and nilotinib in plasma.[457] An ion pairing LC method also contributed to the improvement of the imatinib peak shape compared to RPLC.[458] It may be noted that sunitinib can exist as two isomers (*cis* and *trans*). Available in the *cis* form in pharmaceutical formulations, sunitinib is converted into a *trans* form in solution under light. Several methods are able to resolve the two isomers using a conventional reversed phase column.[459,460]

Different sample preparations were used to extract TK inhibitors from biological matrices such as PP, LLE and SPE (off-line and on-line). Several methods involving column-switching systems were reported for the purification and concentration of the analytes before analytical separation. For example, Couchman *et al.* developed a method including a large particles support for the extraction of nine TK inhibitors and their metabolites in blood samples before separation on a C18 chromatographic support and MS detection.[461] With a sample volume of 50 μL, an LOQ of 1 ng mL^{-1} was obtained for dasatinib. Using a larger sample volume (100 μL after a PP step), an LOQ of 0.03 ng mL^{-1} was reached for the analysis of imatinib in biological matrices with a column-switching

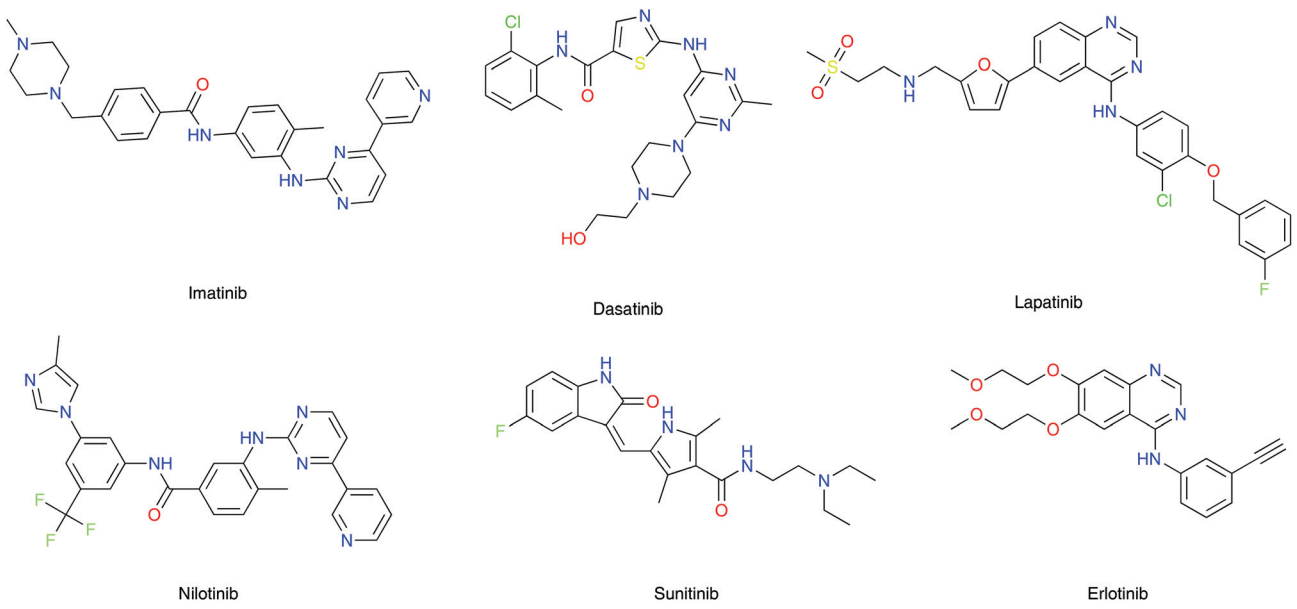


Fig. 13 Structures of tyrosine kinase inhibitors.

Table 7 Analytical methods for the detection of TK inhibitors

TK inhibitors	Matrices	Techniques	Ref.
Imatinib	Molecule/ formulations	LC-UV	466–470
		CE-UV	58 and 471
	Biological matrices	LC-UV	458, 464, 472–485
		LC-MS	457, 461, 462, 472, 486–512
		CE-UV	39, 41 and 513
		CE-MS	514
	Environment	LC-MS	135
Dasatinib	Molecule/ formulations	LC-UV	515
		CE-UV	515
	Biological matrices	LC-UV	479, 474, 441, 516, 517 and 518
		LC-Fluo	465
		LC-MS	457, 461, 486, 491, 493, 494, 497, 500, 507, 510, 519 and 520
Nilotinib	Molecule/ formulations	LC-UV	521
	Biological matrices	LC-UV	463, 475, 479, 522, 523
		LC-MS	457, 461, 486, 491, 493–495, 497, 500, 507, 510, 524 and 525
Lapatinib	Molecule/ formulations	LC-UV	526 and 527
	Biological matrices	LC-UV	464, 528, 529
		LC-MS	461, 486, 491, 493, 495, 497, 500, 510, 520, 530 and 531
Erlotinib	Molecule/ formulations	LC-UV	532
	Biological matrices	LC-UV	464, 516, 533–536 and 529
		LC-MS	461, 486, 491, 492, 495, 500, 510, 537–544, 545, 546 and 547
		CE-UV	548
	Environment	LC-MS	135
Sunitinib	Molecule/ formulations	LC-MS	549
	Biological matrices	LC-UV	464, 550 and 551
		LC-MS	459–461, 486, 491, 495, 497, 500, 507, 510, 537, 552–559
		CE-MS	560

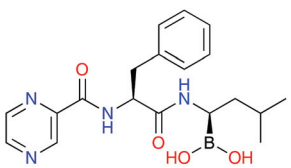
system and LC-MS.[462] A similar approach involving a sample preconcentration step and on-line extraction was applied for the analysis of nilotinib in plasma using UV detection.[463] In this case, an LOQ of 5 ng mL^{-1} was achieved. Garrido-Cano *et al.* developed an LC-UV method using a micellar mobile phase allowing direct injection of filtered plasma for the determination of 4 TK inhibitors.[464] A total analysis time of about 20 min and LOQ of 50 ng mL^{-1} were obtained. Finally, even if most of the TK inhibitors determination was performed with LC-UV and LC-MS, a LC-FD method was also published for the analysis of dasanitib in plasma with sensitivities close to those obtained in LC-UV (*i.e.*, 50 ng mL^{-1}).[465]

CE coupled to UV or MS was also used for the analysis of TK inhibitors. Conventional CE or NACE methods allow the determination of imatinib, erlotinib and sunitinib for drug purity testing or biological samples analysis (Table 1). A CE-UV method involving β-cyclodextrins in the BGE has demonstrated real potential for the quality control of imatinib (drug and impurities).[58] Concentration values of 5–10 ng mL^{-1} were quantified thanks to a stacking injection of large volume sample. Sunitinib and its main metabolites in urine were finally analysed by NACE-MS after a simple one-tenth dilution of the sample at concentrations between 0.5 and 50 μg mL^{-1}.[560]

3.2. Proteasome inhibitor

Synthesized in the '90s, the only representative compound of this anticancer family is bortezomib (Fig. 14). Bortezomib affects the capacity of cancer cells (myeloma cells) to interact with the microenvironment of the bone marrow and thus promotes cell death by its inhibitory action on the proteasome (a proteinaceous complex that degrades proteins). Bortezomib is produced as a trimer of boronic anhydride. The formulations are in the form of lyophilisates (also containing mannitol). Mannitol reacts with boroxine to form a stable monomeric diester which is hydrolysed in the reconstitution step (adding 0.9% NaCl) to bortezomib (active boric acid).[561] Bortezomib is used primarily in the treatment of multiple myelomas. Analytical methods for the analysis of bortezomib are reported in Table 8.

LC remains the main separation technique employed for the analysis of bortezomib in pharmaceutical formulations[562–564] or in biological samples.[486,565,566] A fast LC-MS (less than 2 min) method was developed for the determination of bortezomib within cultured myeloma cells and media. This ultra-fast analysis limits the degradation of bortezomib, which is known to be instable in solution.[565] MS detection was carried out on the dehydrated protonated molecular ion ($[M - H_2O + H]^+$) in positive ESI mode. Shu *et al.* developed an LC-MS method including an off-line SPE step for the quantification of bortezomib and 5 other drugs commonly used in multiple myeloma chemotherapy in biological



Bortezomib

Fig. 14 Bortezomib structure.

Table 8 Analytical methods for the detection proteasome inhibitor

Proteasome inhibitor	Matrices	Techniques	Ref.
Bortezomib	Molecule/ formulations	LC-UV	562, 563 and 564
	Biological matrices	LC-MS	486, 565, 566 and 306

matrices with an LOQ of 2 ng mL^{-1}.[306] Byrn *et al.* propose different methods to compare the contents and impurity profile of two pharmaceutical formulations available on the US market.[563] The presence of an additional impurity as well as a different inactive/active form ratio between the two formulations demonstrated that they were not strictly equivalent.

3.3. mTOR inhibitors

Discovered more than 30 years ago, sirolimus (or rapamycin) is a macrolide produced by *Streptomyces hygroscopicus* and was initially used as an immunosuppressant. It would be necessary to wait until the end of the '90s to witness its use in oncology. Its action is based on an intracellular serine/threonine kinase (mTOR for mammalian target of rapamycin), which is an enzyme involved in several cellular processes such as angiogenesis, metabolic modulation, cell cycle and apoptosis. This protein complex is involved in the tumor progression of certain cancers. In the mid-2000s, a water-soluble sirolimus ester was developed, namely temsirolimus, which is a prodrug whose active form is sirolimus (rapid hydrolysis). A few years later, a second analogue of sirolimus, everolimus, enlarged the family of mTOR inhibitors. Although widely used as immunosuppressants after organ transplantation, mTOR inhibitors can also be used for the treatment of kidney and lymphoma cancers. Fig. 15 shows the structures of the major inhibitors of mTOR and Table 9 lists the reported analytical methods.

Inhibitors of mTOR were mainly analysed in biological matrices even if some methods were published for their determination in solution or in pharmaceutical formulations, as in the case of stability studies or for quality control purposes.[567–570] Their narrow therapeutic window and highly variable blood levels (for the same administered dose) make mTOR inhibitors analysis in whole blood essential (mTOR inhibitors are concentrated in the red blood cells). In recent years, many analytical methods have been reported for the analysis of mTOR inhibitors in biological samples.[571–574,84]

Table 9 Analytical methods for the detection of mTOR inhibitors

mTOR inhibitors	Matrices	Techniques	Ref.
Sirolimus	Molecule/ formulations	LC-UV	567 and 570
	Biological matrices	LC-UV	575, 576, 578, 581, 586, 592, 593, 594, 595, 596 and 597
		LC-MS	577, 580, 582, 584, 585, 598, 599, 602, 603, 605, 606, 607, 609, 617, 618, 619, 600, 601, 604, 608, 610, 620, 621, 624 and 626
		LC-EC	627
		CE-UV	628
		CE-MS	630
Temsirolimus	Molecule/ formulations	LC-UV	569
Everolimus	Molecule/ formulations	LC-UV	568
	Biological matrices	LC-UV	589, 590 and 591
		LC-MS	577, 579, 583, 587, 588, 598, 599, 602, 612, 603, 605, 613, 614, 606, 607, 609, 615, 622, 616, 617, 618, 619, 625 and 626

Two analytical techniques can be discerned: immunoassays and LC. The comparison of these two approaches shows their comparable performances.[575–586,587,588] The main limitation of immunoassays is the cross-reactivity with metabolites. Only separative techniques are considered in this review.

LC-UV is an interesting technique for the analysis of mTOR inhibitors in blood.[575,576,578,581,589–597] Nevertheless, the achieved sensitivity was too limited and an additional extrac-

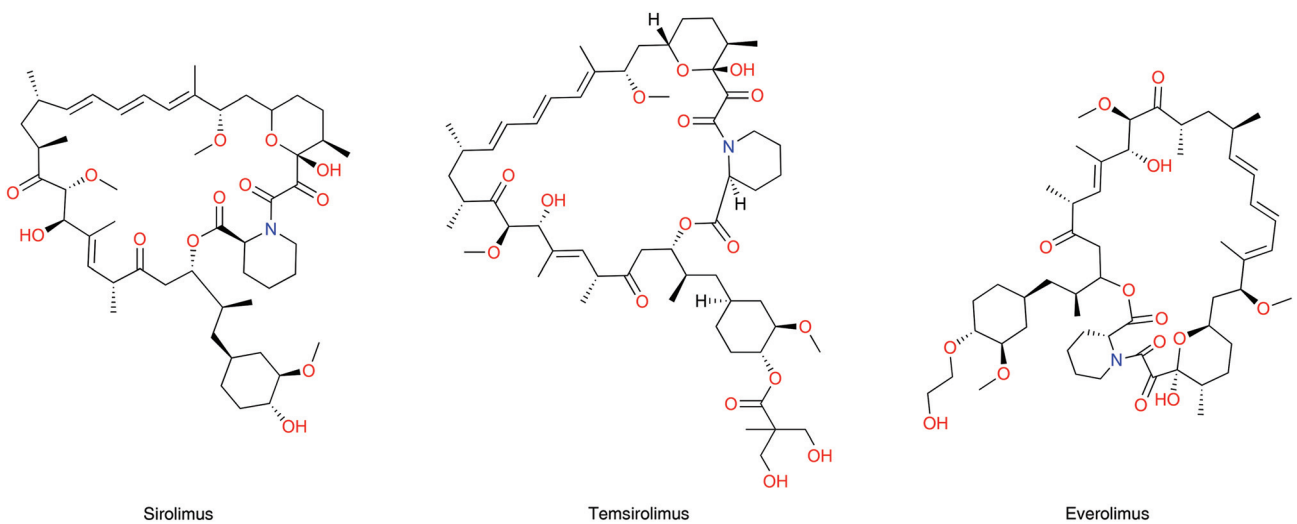


Fig. 15 Structures of mTOR inhibitors.

tion step allowing purification and preconcentration of the analyte was required, such as LLE[575,581,591,592,595–597] or SPE including a prior PP.[580,589,590,593,594,596] As an example, an LLE-LC-UV method allowed the determination of everolimus in whole blood at concentrations between 1 and 200 ng mL^{-1}.[589] The sensitivity and selectivity provided by MS helped to ease sample preparation in clinical chemistry laboratories. Column-switching systems coupling the extraction support to the chromatographic column before MS detection were also used for the analysis of sirolimus[598–611] and everolimus[598,599,602,603,605–607,609,612–614] in whole blood. LLE[615] or SPE[616] on 96-well plates constitutes another approach for sample preparation automatisation. The automatisation of a PP step with a liquid handling platform was also applied for the LC-MS analysis of mTOR inhibitors in blood.[617] Blood sample analysis on blotting paper was particularly useful in the case of mTOR inhibitors by facilitating the collection and processing of the sample prior to LC-MS analysis.[618–622]

Under such conditions, concentrations in the order of ng mL^{-1} were detected. It can be noted that MS detection of mTOR inhibitors was mainly performed *via* adducts formation including sodium adducts[601,602] or ammonia adducts[623,624,625] because of the neutral character of these molecules. An LOQ often inferior to 1 ng mL^{-1} was obtained with MS detection.[626] Electrochemistry detection was also successfully used for sirolimus analysis in blood at concentrations of 1 to 50 ng mL^{-1}.[627]

CE was also used for the determination of mTOR inhibitors. A CE-UV method with a BGE containing SDS and acetonitrile allowed the analysis of sirolimus in blood with an LOQ of 0.2 ng mL^{-1} thanks to a preconcentration factor of 10 obtained with SPE and a large injection volume (focusing technique).[628] Screening for mTOR inhibitors in extracts from natural products was finally performed by CE-LIF.[629]

4 Conclusion

The analytical procedures developed for the analysis of anticancer agents chronologically follow progress in the field of analytical sciences. Two major periods can be distinguished. The first one corresponds more or less to the second half of the 20th century. During this period, the majority of analytical methods developed for the determination of antineoplastic drugs were based on chromatographic techniques such as LC or GC using poorly selective and sensitive detectors. These analytical tools were mostly used in the quality control framework of the anticancer molecule (impurity profiling) and in therapeutic drug monitoring (dose adjustment). Then, there has been increasing interest on the impact of these highly toxic molecules on humans and the environment. To deal with these more complex matrices and attain sufficient limits of detection, the introduction of highly sensitive and selective techniques such as LC-MS opened the era of trace analysis of antineoplastic drugs. Indeed, most analytical methods published since the early 2000s were based on LC-MS. Even if these methods are mainly applied for therapeutic drug monitoring of antineoplastic agents, an increasing numbers of publications concerned exposure studies. The trace detection in humans handling these molecules (other than patients) and in the environment, is becoming more and more powerful thanks to more efficient analytical techniques, which raise discussion on their real impact on health at very low concentrations.

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Chapitre 3 : Formulations pharmaceutiques

Caractérisés par une marge thérapeutique étroite et une haute toxicité, les chimiothérapies sont considérées comme des formulations à haut risque pour lesquelles toutes erreurs de préparation peuvent engendrer des conséquences dramatiques. Aux côtés des contrôles et précautions appliqués en cours de préparation, l'analyse du produit fini figure parmi les stratégies les plus courantes pour réduire au minimum le risque d'erreur. Le Laboratoire de Contrôle Qualité de la Pharmacie des HUG a développé une méthode MEKC-UV pour l'analyse des dérivés du platine (cisplatine, carboplatine, oxaliplatine) qui a été appliquée avec succès pour le contrôle qualité des chimiothérapies produites à la Pharmacie des HUG (article II). Cette méthode permettant une séparation entre les trois composés et leurs produits de dégradation peut également être utilisée dans le cadre d'une étude de stabilité.

Du fait de leur haute instabilité, le flux prescription-préparation et administration des agents anticancéreux peut être compromis. Même si le temps entre la préparation et l'administration se réduit à une ou deux heures dans la plupart du temps, il se peut que pour des raisons pratiques ou logistiques ce temps dépasse plusieurs heures avec un impact sur la qualité de la chimiothérapie administrée. Les données disponibles ne permettent pas généralement au pharmacien de répondre de manière claire à ses propres problématiques. Avec l'objectif de disposer des données de stabilité chimique des chimiothérapies de busulfan produites à la pharmacie des HUG, le Laboratoire de Contrôle Qualité a mis au point une méthode LC-ESI-MS/MS indicatrice de stabilité (article III). Au cours de ce travail, des durées ainsi que des conditions de conservation ont été définies pour les poches et seringues de busulfan produites à la Pharmacie des HUG ; ce qui a permis d'optimiser la logistique entourant la reconstitution de cet anticancéreux.

Article II

« Quality control of pharmacetical formulations containing cisplatin, carboplatin and oxaliplatin by micellar and microemulsion electrokinetic chromatography (MEKC, MEEKC).»

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Quality control of pharmaceutical formulations containing cisplatin, carboplatin, and oxaliplatin by micellar and microemulsion electrokinetic chromatography (MEKC, MEEKC)

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ABSTRACT

A micellar electrokinetic chromatography (MEKC) method was developed for the determination of cisplatin, carboplatin, and oxaliplatin in pharmaceutical formulations. The background electrolyte consisted of a phosphate buffer (pH 7.0; 25 mM) with sodium dodecyl sulfate (80 mM). The applied voltage was 30 kV and the sample injection was performed in the hydrodynamic mode. All analyses were carried out in a fused silica capillary with an internal diameter of 50 μm and a total length of 64.5 cm. The detection of target compounds was performed at 200 nm. Under these conditions, a complete separation of cisplatin, carboplatin and oxaliplatin was achieved in less than 10 min. The MEKC-UV method was validated and trueness values between 99.7% and 100.8% were obtained with repeatability and intermediate precision values of 0.7–1.4% and 1.1–1.7%, respectively for the three drugs. This method was found appropriate for controlling pharmaceutical formulations containing platinum complexes and successfully applied in quality control at the Geneva University Hospitals.

1. Introduction

Platinum complexes belong to the most widely used drugs in cancer chemotherapy treatment and possess a pronounced activity in different cancer types by binding to the DNA and modifying its structure. Cisplatin, carboplatin, and oxaliplatin are the most important worldwide clinically approved platinum compounds (Fig. 1). Cisplatin was the first used platinum complex with a pronounced activity in testicular and ovarian cancers. The related analogs, carboplatin and oxaliplatin, were developed later to reduce the problematic side effects of cisplatin (nephrotoxicity, ototoxicity, peripheral neuropathy, etc.). Carboplatin is used in the treatment of advanced ovarian cancer and lung cancer, while oxaliplatin is licensed for the treatment of metastatic colorectal cancer in combination with fluorouracil and folinic acid [1].

Despite the use of platinum compounds for several decades, there are only few published analytical methods. As reported in the review by Espinosa Bosch et al., different techniques were developed for the determination of cisplatin, such as derivative spectrophotometry, phosphorescence, atomic absorption spectrometry, gas chromatography, capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) coupled with different detectors (UV–vis, electrochemical, inductively coupled plasma–atomic emission spectrometry, inductively coupled plasma–mass spectrometry (ICP–MS) or electrospray ionization–mass spectrometry) [2]. Regarding carboplatin and oxaliplatin, no specific reviews about analytical methods have been published to the author's knowledge. Most common techniques for these compounds are HPLC or CE coupled to UV–vis or MS detection. During the last years, ICP–MS has become very popular for the determination of the three platinum compounds in environmental, biological, and clinical samples [2].

According to Hartinger et al., CE has emerged as the method of choice for the separation of intact platinum metal complexes and their metabolites due to its high efficiency, versatility and gentle separation conditions for metal complexes [3–5]. Analysis of anticancer drugs by CE appears to be very interesting due to the toxicity of these compounds, because the separation is performed in a closed system and the waste volume is on the μL range.

The three tested platinum drugs are non-charged coordination complexes. Therefore, simple CZE is not adapted for resolving these compounds and other separation techniques are necessary, such as micellar electrokinetic chromatography (MEKC) or microemulsion electrokinetic chromatography (MEEKC). In MEKC, an ionic surfac-

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tant, generally sodium dodecyl sulfate (SDS), is added to the BGE at a higher concentration than its critical micelle concentration and micelles act as pseudo-stationary phase allowing solute partition simultaneously to electrophoretic process [6]. MEEKC has a similar operating principle except that a microemulsion (ME) is used. As reported in several studies, MEEKC may present advantages over MEKC such as enhanced solubilization power and an enlarged migration window [7,8].

The main publications dedicated to the analysis of platinum drugs with MEKC or MEEKC were developed for biological studies, such as clinical sample analysis [9], drug–protein [10–14] and drug–DNA (or nucleotides) binding studies [15–19] or for chemical studies [20,21]. Usually, UV spectrophotometry was used for the detection of platinum drugs with MEKC or MEEKC even if ICP–MS was also reported to enhance selectivity and sensitivity [22].

For quality control of pharmaceutical formulations, UV detection was found sufficient in terms of sensitivity because the limit of quantification of platinum compounds was inferior to their concentration in drug products. To our knowledge, only one MEKC method has been reported in the literature for quality control of platinum formulations and no complete validation was performed [23].

At the pharmacy of the Geneva University Hospitals (HUG), more than 20% of prepared chemotherapies are platinum formulations (including cisplatin, carboplatin, and oxaliplatin). The role of the hospital pharmacy is to dilute or dissolve commercially available pharmaceutical formulations in appropriate conditions to ensure the protection of nurses and the sterility of the injectable solution. For the quality control of such reconstituted formulations, a method for the determination of these compounds is necessary.

The objective of this study was to develop and validate a simple MEEKC or MEKC–UV method to determine cisplatin, carboplatin, and oxaliplatin in pharmaceutical formulations and to apply it in quality control.

2. Experimental

2.1. Chemicals

The study was performed with the following commercially available cytotoxic drugs (see Fig. 1): Cisplatin Ebewe® 1 mg mL^{-1} was purchased from Sandoz Pharmaceuticals SA (Steinhausen, Switzerland) and Carboplatin Teva® 10 mg mL^{-1} from Teva Pharma AG (Aesch, Switzerland). Eloxatin® (containing oxaliplatin, 50 mg) was obtained from Sanofi-Aventis (Meyrin, Switzerland) and reconstituted with glucose 5% from Sintetica-Bioren SA (Couvet, Switzerland) to obtain a final concentration of 5 mg mL^{-1}.

Caffeine citrate used as internal standard (IS) was purchased from Fagron GmbH (Barsbüttel, Germany).

Concentrated phosphoric acid and NaOH 1 M were obtained from Merck (Darmstadt, Germany), sodium dodecyl sulfate (SDS) from Fluka (Buchs, Switzerland) and ultra-pure water was supplied by a Milli-Q Plus unit from Millipore (Bedford, MA, USA). n-heptane was purchased from Merck (Darmstadt, Germany), n-octane and n-butanol from Fluka (Buchs, Switzerland). Ceofix® kit was from Analis (Suarlée, Belgium).

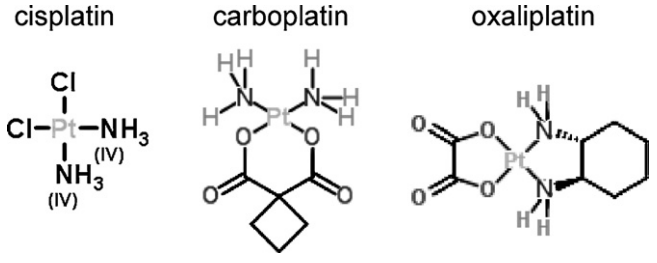

Fig. 1. Structures of cisplatin, carboplatin and oxaliplatin.

Water for injection and NaCl 0.9% used in the preparation of pharmaceutical formulations were obtained by Bichsel laboratories (Interlaken, Switzerland) and glucose 5% was from Sintetica-Bioren SA (Couvet, Switzerland).

2.2. BGE preparation

For MEEKC, different microemulsions (ME) were prepared from a 20 mM phosphate buffer set at pH 2.0, 7.0 and 10 mM borate buffer set at pH 9.0. Different ratios of SDS, n-butanol and n-octane or n-heptane were tested: 6.6% (w/v) n-butanol, 3.3% (w/v) SDS, and 0.78% (w/v) n-heptane; 7.3% (w/v) n-butanol, 2.3% (w/v) SDS, and 0.82% (w/v) n-octane; 6.6% (w/v); n-butanol, 3.3% (w/v) SDS, and 0.78% (w/v) n-octane. SDS was partially dissolved in approximately 80% of the buffer before adding n-butanol and n-heptane. The mixture was then carefully shaken until SDS was completely dissolved, and the remaining buffer added. The solution was left to stand for 1 h at room temperature. Before use, the ME was filtered through a 0.45 μm microfilter (BGB Analytik, Böckten, Switzerland). The ME was stored at room temperature and remained stable for at least one week.

For MEKC, different BGEs were tested: borate (pH 9.2; 50 mM) with SDS (80 mM); acetate (pH 4.75; 50 mM) with SDS (80 mM), phosphate (pH 7.0) with different buffer concentration (10, 25 and 50 mM) and SDS concentration (30, 60 and 80 mM). The final composition consisted of 25 mM phosphate at pH 7.0 with 80 mM SDS. The aqueous BGE was prepared by an adequate dilution of the concentrated acidic solution, and a volume of NaOH 1 M was added to adjust the solution at pH 7.0. The solution was then diluted to the final volume with water and SDS dissolved to obtain a final concentration of 80 mM. The BGE was degassed in an ultrasonic bath for 10 min before use.

2.3. Instrumentation

CE experiments were carried out with an HP^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. A CE ChemStation (Agilent) was used for CE control, data acquisition and data handling.

Analyses were performed in uncoated fused silica (FS) capillaries from BGB Analytik AG (Böckten, Switzerland) with an internal diameter (i.d.) of 50 μm, an outside diameter (o.d.) of 375 μm and a total length of 64.5 cm (effective length of 56.5 cm).

The capillary was thermostated at 25 °C in a high velocity air stream and a voltage of 30 kV was applied in the positive mode. The generated current was between 20 and 70 μA depending on the BGE. Samples were kept at ambient temperature in the autosampler and injected in the hydrodynamic mode to fill approximately 1% of the effective capillary length (40 mbar for 10 s). The detection was achieved at 200 nm with a band width of 10 nm and a response time of 0.1 s.

Before first use, FS capillaries were sequentially rinsed with methanol, 0.1 M NaOH, water, methanol, 0.1 M HCl, water and BGE for 5 min. Prior to each sample injection, the capillary was rinsed by pressure (940 mbar) for 3 min with fresh BGE ensuring good repeatability of migration times. When not in use, the capillary was washed with water and methanol. As the electrophoresis process altered the running buffer pH by electrolysis, the separation buffer was refreshed every six runs at the inlet and outlet vials.

For MEEKC performed at pH 2, the capillary was coated with Ceofix® according to the publication of Henchoz et al. [24] to ensure a high EOF at low pH. Before an analytical series, several wash-

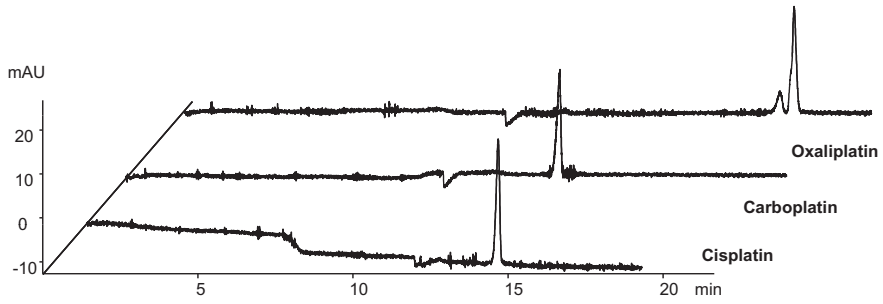

Fig. 2. Electropherogram obtained for the MEEKC–UV analysis of standard samples containing cisplatin, carboplatin, and oxaliplatin at 0.5 mg mL^{-1} in an aqueous solution. BGE: 20 mM phosphate at pH 7.0 with 6.6% (w/v) n-butanol, 3.3% (w/v) SDS, and 0.78% (w/v) n-heptane. Voltage: 20 kV. All other experimental conditions are described in Section 2.3.

ing steps (1 bar) were carried out: water (1 min), Ceofix® initiator (1 min), Ceofix® accelerator (1 min), BGE (5 min), and then the separation voltage (20 kV) was applied for 5 min. Prior to each sample injection (preconditioning step), the capillary was rinsed (1 bar) with BGE for 3 min. No postconditioning was performed.

2.4. Method validation

A validation was performed to estimate quantitative parameters of the method for the analysis of cisplatin, carboplatin, and oxaliplatin in pharmaceutical formulations. The validation was based on ICH guidelines following the recommendations of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [25]. Quantitative performance was estimated in three separate series ($j = 3$) with the V2 protocol. This protocol involves three concentration levels ($k = 3$) with two repetitions ($n = 2$) for calibration standards (CS) and three concentration levels ($k = 3$) with four repetitions ($n = 4$) for validation standards (VS). Each series involved the injection of a freshly prepared BGE, complete washing of the capillary with water and methanol, and instrument shut-off. Caffeine citrate was used as internal standard (IS). Calculations were performed using normalized area (area/migration time) ratios of the three platinum drugs on the internal standard.

2.5. Sample preparation

All solutions were prepared in appropriate conditions for handling hazardous compounds as cytotoxic agents. Moreover, the development of the method was performed with drug specialities to avoid direct contact of the operator to cytotoxic powder and to minimize contamination risk by preparing working solutions. For the validation, standard solutions of cisplatin and oxaliplatin were compared with pharmacopeia reference standards and no difference between the electropherograms was observed (data not shown). Therefore, the validation was also performed with drug specialities.

CS and VS were independently prepared for each platinum compound. For stability reasons and to avoid drug interactions, the three platinum complexes were separately analysed. Cisplatin was prepared in NaCl 0.9% to avoid hydrolysis, while carboplatin is modified to cisplatin in presence of chloride. The IS stock solution was prepared by dissolving caffeine citrate in ultra-pure water at a concentration of 1.0 mg mL^{-1}. CS and VS were stable for at least three days at 25 °C and no degradation was observed during the analysis.

2.5.1. Calibration standard

For CS, three concentration levels at 0.05, 0.5, and 1 mg mL^{-1} of cisplatin, carboplatin, and oxaliplatin were prepared by diluting the appropriate volume of drug specialities in water. 50 μL of caffeine citrate at 1 mg mL^{-1} (IS) was added to 500 μL of the prepared solutions.

2.5.2. Validation standard

For VS, three concentration levels at 0.05, 0.5, and 1 mg mL^{-1} of cisplatin, carboplatin, and oxaliplatin were prepared by diluting the appropriate volume of drug specialities in NaCl 0.9% for cisplatin and glucose 5% for carboplatin and oxaliplatin. 50 μL of caffeine citrate at 1 mg mL^{-1} (IS) was added to 500 μL of the prepared sample.

2.6. Application to pharmaceutical formulations

Cisplatin, carboplatin, and oxaliplatin were determined in pharmaceutical formulations prepared by the HUG pharmacy. The formulations were diluted in distilled water to obtain a final concentration between 0.05 and 1 mg mL^{-1} of the platinum compound. Quantitative analyses were repeated in duplicate for each formulation.

3. Results and discussion

3.1. Method development

3.1.1. Microemulsion electrokinetic chromatography (MEEKC)

To ensure the identity of the platinum compound in formulations, separation of the three drugs was mandatory. Different MEs were tested: 20 mM phosphate at pH 2.0 and pH 7.0, 20 mM borate at pH 9.0, with different ratios of SDS, n-butanol, n-octane and n-heptane, respectively. Among the tested experimental conditions, best separation of the three platinum compounds was obtained with a phosphate ME at 20 mM and pH 7.0, 6.6% (w/v) n-butanol, 3.3% (w/v) SDS, and 0.78% (w/v) n-heptane and an applied voltage of 20 kV (Fig. 2). Analysis time was long (20 min), but high resolution between the three compounds (>7) and good efficiency was obtained ($N > 70{,}000$). With the phosphate ME at pH 2.0, resolution between cisplatin and carboplatin was lower ($Rs \sim 2$) and analysis time was inferior to 10 min for all compounds due to Ceofix® coating. Similar efficiency was obtained for all compounds ($N > 70{,}000$). With the borate ME at pH 9.0, also good separation was obtained, but a better stability of platinum complexes was observed at lower pH [4]. The tested ratios of SDS, n-heptane, n-octane and n-butanol did not influence the separation significantly (data not shown).

For oxaliplatin, two peaks were observed in all selected conditions. The experiments were performed with the commercially available Eloxatin and the second peak was supposed to be an additive or impurity present in the formulation. Therefore, the analysis was repeated with a Pharmacopeia Reference Standard of oxaliplatin and with the Pharmacopeia Impurities A, B, C and D. The same electropherogram was obtained with the Reference Standard as with Eloxatin. Impurity A was not detected in the separation

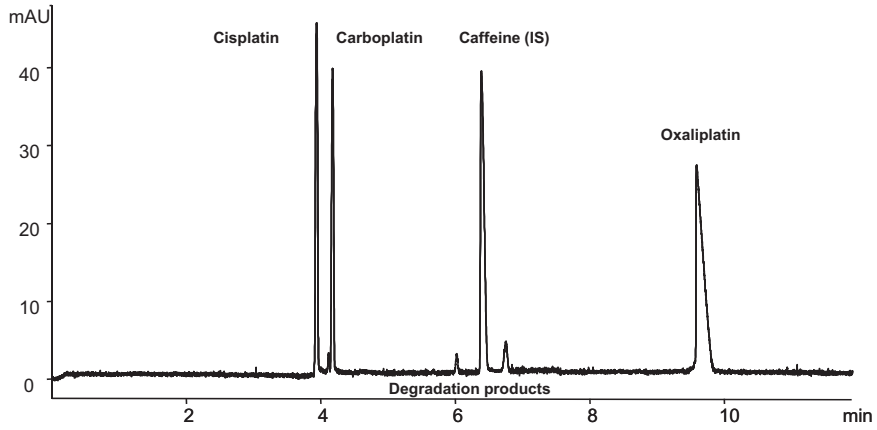

Fig. 3. Electropherograms obtained for the MEKC–UV analysis of a standard sample containing cisplatin, carboplatin, and oxaliplatin at 0.5 mg mL^{-1} with caffeine (IS) at 0.1 mg mL^{-1} in an aqueous solution. BGE: 25 mM phosphate at pH 7.0 with SDS 80 mM. All other experimental conditions are described in Section 2.3.

window. Impurities B and C were completely separated from oxaliplatin and did not migrate with the unknown peak. No resolution between oxaliplatin and the Impurity D, corresponding to the S–S enantiomer, was observed (data not shown). Thus, these experiments demonstrated that the second peak observed for oxaliplatin was not due to impurities or additives present in the formulation.

To exclude a degradation of oxaliplatin due to the separation conditions (20 kV, 25 °C), instrument parameters were modified and different conditions of voltage (15 kV and 30 kV) and temperature (15 °C and 35 °C) were applied. However, no difference was obtained for the second peak of oxaliplatin (data not shown). MEs were also prepared with solvents from different origins, to exclude a reaction between oxaliplatin and an impurity in the BGE system. With all tested MEs, both peaks for oxaliplatin were observed.

Some hypotheses found in the literature could explain this behavior. Oxaliplatin possesses a 1,2-diaminocyclohexane (DACH) carrier ligand and according to Tyagi et al. [26], several conformers coexist at room temperature, which might explain the presence of a second peak for oxaliplatin with MEEKC. Another study reported intramolecular transformations of platinum complexes with aminoalcohol ligand and the possibility of separating singly ring-opened and doubly ring-opened species. This apparently takes place due to the shifting of the equilibrium toward the ring-opened species induced by adduct formation between SDS and the platinum complex [27]. But to our knowledge, this behavior has never been reported for oxaliplatin. Moreover, in the following experiments with MEKC, only one peak was observed for oxaliplatin.

Another problem of the MEEKC method might be the quantification of platinum drugs in very low concentrated formulations, especially for preparations containing cisplatin, because of insufficient sensitivity. Given the presence of two peaks for the analysis of oxaliplatin and the limited sensitivity, an alternative strategy based on MEKC was investigated to perform the quality control of platinum drugs in hospital formulations.

3.1.2. Micellar electrokinetic chromatography (MEKC)

Different BGEs were tested including borate, phosphate and acetate buffer at different concentrations, pH and SDS concentrations. At increased pH value, platinum complexes can be hydrolyzed [4] and therefore, the borate BGE (pH 9.2) was excluded. However, with acetate BGE (pH 4.5) cisplatin was co-migrating with the EOF. Finally, a phosphate buffer (pH 7.0) was chosen as compromise and no degradation was observed during the analysis.

The first analyses were performed with a buffer concentration of 10 mM. Nevertheless, to improve the resolution between cisplatin, carboplatin, oxaliplatin and EOF, buffer solutions with different molarities (25, 50 mM) and different SDS concentrations (30, 60, 80 mM) were studied. Among the tested BGEs, best separation was obtained with 25 mM phosphate and 80 mM SDS. Analysis time was inferior to 10 min and acceptable resolution (Rs > 4) and efficiency ($N \sim 70{,}000$) was obtained for all compounds. Under these conditions the generated current was still acceptable (~50 μA). With higher SDS amount, the generated current was too high and capillary breakdown was observed.

The separation between the three platinum drugs was also studied in presence of 5, 10 and 20% of acetonitrile. As reported, solvent modified MEKC could sometimes achieve better separation conditions [28,29]. For oxaliplatin, the migration time decreased with increased ACN concentration. But the resolution between cisplatin and carboplatin was also lowered with ACN (data not shown). Therefore, a purely aqueous phosphate BGE (pH 7.0; 25 mM) containing 80 mM of SDS was selected (Fig. 3).

Comparing to MEEKC, the selected MEKC method presented similar efficiency and shorter analysis time. Moreover, better sensitivity allowed the analysis of low concentrated formulations. Therefore, the MEKC method was selected for quality control of pharmaceutical formulation and a complete validation was performed.

3.2. Method validation

The concentrations of the prescribed platinum drugs at HUG were considered for the determination of the concentration range used in the validation. For cisplatin, concentrations between 0.05 and 0.4 mg mL^{-1} (median: 0.16 mg mL^{-1}), for carboplatin 0.1 and 2.5 mg mL^{-1} (median: 1.4 mg mL^{-1}) and for oxaliplatin 0.1 and 1.0 mg mL^{-1} (median: 0.4 mg mL^{-1}) were prescribed in 2009. In order to decrease the number of manipulations with toxic compounds, formulations were injected with simple or without dilution. Therefore, the concentration range was fixed from 0.05 to 1 mg mL^{-1} for all three compounds. Caffeine citrate chosen as IS was detected between carboplatin and oxaliplatin.

The calibration curve was obtained for each series with conventional least-squared linear regression using the three concentration levels (0.05 mg mL^{-1}, 0.5 mg mL^{-1} and 1.0 mg mL^{-1}). After establishing the calibration curves for each series, concentrations of VS were computed from the analytical response to obtain trueness, repeatability and intermediate precision. Trueness was expressed (in percentage) as the ratio between theoretical and average measured values at each concentration level. Repeatability and intermediate precision were expressed as the relative standard deviation (RSD%), i.e., the ratio of the intra-day standard deviation (s_r) and between-day standard deviation (s_R), respectively,

Table 1
Validation results: trueness, repeatability and intermediate precision of the developed MEKC–UV method for the analysis of cisplatin, carboplatin and oxaliplatin in pharmaceutical formulations.

Theoretical concentration [mg mL^{-1}]	Trueness	Repeatability (RSD)	Intermediate precision (RSD)
Cisplatin			
0.05	100.6%	1.0%	1.7%
0.5	100.1%	0.7%	1.4%
1	100.8%	1.1%	1.1%
Carboplatin			
0.05	100.7%	0.8%	1.6%
0.5	100.0%	0.7%	1.4%
1	99.7%	0.7%	1.3%
Oxaliplatin			
0.05	100.2%	1.4%	1.4%
0.5	99.9%	0.9%	1.3%
1	100.3%	1.3%	1.4%

on the theoretical concentrations [30]. The s_r and s_R values were obtained using ANOVA analysis. As reported in Table 1, trueness and precision values were in accordance with recommendations for the analysis of pharmaceutical formulations over the tested concentration range. The RDS (repeatability and intermediate precision) was lower than 2%, with trueness values between 99.7 and 100.8%. To visualize the overall method variability, the accuracy profile was built combining trueness and intermediate precision as the confidence interval [31]. As presented in Fig. 4, the total error did not exceed acceptance limits (±5%) for all concentration levels. Consequently, the developed MEKC–UV method could be considered accurate for the three platinum drugs over the tested range.

3.3. Application to pharmaceutical formulations

In order to demonstrate the applicability of the MEKC–UV method to real samples, determination of the three platinum drugs was achieved in pharmaceutical formulations for quality control. The concentrations of the cytotoxic agents were calculated with reference to a calibration curve constructed the same day. CS at three concentration levels were replicated twice, and conventional least-squared linear regression was applied. Since two independent analyses ($N = 2$) were performed on each pharmaceutical formulation, the result of the analysis could be expressed as:

$$\mathrm{cnf}(x) = \bar{x} \pm t_{\mathrm{df},\alpha}\sqrt{\frac{s_r^2}{N} + s_g^2} \quad (1)$$

where N is the number of analyses performed and $\bar{x}$ is the mean result. The $t_{\mathrm{df},\alpha}$ (Student's constant depending on df and α set at 5%), s_r^2 and s_g^2 variance values were determined during the validation step with the regular ANOVA-based variance decomposition [31]. The analysis repetition was useful to obtain a smaller confidence interval, since most of the variability came from repeatability (s_r^2). As shown in Table 2, prescribed concentrations of pharmaceutical formulations were confirmed to be in the range of ±10% of the target value by the MEKC–UV method, which corresponds to the acceptance limits for these formulations at the HUG pharmacy.

Table 2
Analysis of the three cytotoxic drugs by MEKC–UV in pharmaceutical formulations prepared at the HUG pharmacy.

Batch number	Concentration
CYT/10-123162 104 mg cisplatin in 604 mL NaCl 0.9%	107.0 ± 2.8%
CYT/10-122999 140 mg cisplatin in 640 mL NaCl 0.9%	100.8 ± 2.8%
CYT/10-121694 40 mg cisplatin in 540 mL NaCl 0.9%	106.9 ± 2.8%
CYT/10-122599 529 mg carboplatin in 303 mL glucose 5%	96.8 ± 2.8%
CYT/10-122482 260 mg oxaliplatin in 302 mL glucose 5%	95.0 ± 2.0%
CYT/10-122846 114 mg oxaliplatin in 273 mL glucose 5%	94.3 ± 2.0%
CYT/10-123120 120 mg oxaliplatin in 274 mL glucose 5%	97.1 ± 2.0%

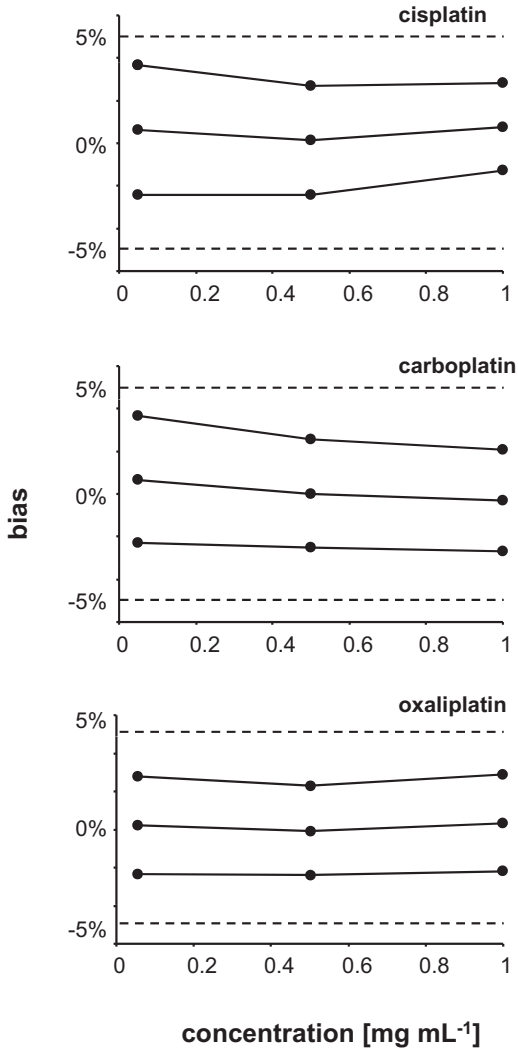

Fig. 4. Accuracy profiles of the developed MEKC–UV method for the determination of cisplatin, carboplatin, and oxaliplatin in a pharmaceutical formulation using a linear regression model. The dashed lines represent the acceptance limits of ±5%.

4. Conclusions

Different methods based on MEKC and MEEKC were developed for the quantitative determination of cisplatin, carboplatin, and oxaliplatin in pharmaceutical formulations. The MEKC method exhibited very good quantitative performance in terms of accuracy and precision with an analysis time of less than 10 min for the three platinum compounds. The manipulation steps, including the handling of cytotoxic agents, are reduced to dilution and addition of the IS to the pharmaceutical formulation. Therefore, the presented MEKC–UV method can be used as a very simple technique in quality control and was successfully applied in routine analysis at HUG pharmacy.

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Article III

« Stability determination of busulfan diluted solutions in polypropylene containers by UHPLC-MS.»

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Stability of busulfan solutions in polypropylene syringes and infusion bags as determined with an original assay

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Purpose. The stability of busulfan solution in 0.9% sodium chloride and stored in polypropylene syringes or infusion bags was evaluated.

Methods. Busulfan solutions (0.54 mg/mL) were prepared and transferred to 50-mL polypropylene syringes and 100- and 500-mL polypropylene infusion bags and stored at 2–8 and 23–27 °C. Chemical stability was measured using a stability-indicating, ultrahigh performance liquid chromatography coupled to mass spectrometry method. The stability of busulfan was assessed by measuring the percentage of the initial concentration remaining at the end of each time point of analysis. The initial busulfan concentration was defined as 100%. Stability was defined as retention of at least 90% of the initial busulfan concentration. A visual inspection of the samples for particulate matter, clarity, and color without instrumentation of magnification was conducted at each time point of analysis.

Results. The visual inspection demonstrated no influence of the storage container when busulfan infusions diluted in 0.9% sodium chloride injection were stored at 23–27 °C. No color change or precipitate was observed at this temperature; however, a rapid decrease of the busulfan content in all containers stored at room temperature was observed. Busulfan in syringes was chemically stable for 12 hours, while busulfan in infusion bags (100 and 500 mL) was stable only for 3 hours at 23–27 °C.

Conclusion. Busulfan 0.54-mg/mL solution in 0.9% sodium chloride injection was physically and chemically stable for 30 hours when stored in 50-mL polypropylene syringes at 2–8 °C and protected from light.

Keywords: antineoplastic agents; busulfan; chemical precipitation; chromatography, liquid; drug stability; mass spectrometry

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Busulfan, a bifunctional alkylating agent in the class of alkyl sulfonate antineoplastic drugs, is extensively used in myeloablative regimens before hematopoietic stem cell transplantation.[1] This molecule was discovered in the early 1950s and orally administered in clinical trials for the palliative treatment of chronic myeloid leukemia.[2] However, this treatment demonstrated highly variable pharmacokinetics, mainly induced by poor absorption after oral administration or presence of vomiting and variations in dietary intake, which can influence the outcome of the bone marrow transplantation.[1,3-5] In order to increase the bioavailability of busulfan, an i.v. form was developed. Since busulfan has an extremely low solubility in water, the use of cosolvents was required to allow its solubilization. The i.v. form was initially commercially available as Busulfex (Otsuka) in the U.S. market as a clear, colorless concentrated solution of 6 mg/mL dissolved in *N,N*-dimethylacetamide (DMA, 33% by volume) and poly-

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ethylene glycol 400 (PEG400, 67% by weight).[6] The same formulation was approved in Europe under the brand name Busilvex (Pierre Fabre).

Currently, busulfan is indicated in conjunction with other antineoplastic agents such as cyclophosphamide (for adults) or melphalan (for children) for conditioning before hematopoietic stem cell transplantation. Other antineoplastic agents, such as fludarabine or thiotepa, are also used with busulfan.[7,8]

According to the summary of product characteristics for parenteral busulfan, the concentrate must be diluted before administration in 0.9% sodium chloride injection or 5% dextrose injection to a final concentration of 0.54 mg/mL.[9] The solution is stated to be stable for 8 hours at room temperature when the product is diluted in 5% dextrose injection. When the dilution is performed with 0.9% sodium chloride injection, the solution is stable for 12 hours under refrigeration (2–8 °C) or 3 hours at room temperature.

Busulfan is usually administered over 2 hours every 6 hours for 4 consecutive days, for a total of 16 doses. The limited stability of this product induces organizational problems for cytotoxic preparation units in hospital pharmacies.

A few studies have been conducted to investigate how to increase the stability of diluted busulfan solutions. Busulfan is known to undergo rapid hydrolysis degradation at room temperature, yielding tetrahydrofuran and methanesulfonic acid.[10,11] Nevertheless, a U.S. study conducted in 1996 found that busulfan was chemically and physically stable at room temperature when reconstituted in 0.9% sodium chloride injection or 5% dextrose injection and stored in polyvinyl chloride (PVC) or polyolefin bags for 8 hours.[12] A more recent German study highlighted the physical instability of busulfan stored at low temperatures (2–8 °C) due to precipitation and concluded that busulfan diluted in 0.9% sodium chloride injection and stored at 10–15 °C is stable for up to 48 hours.[13] That intermediate temperature was chosen to strike a balance between busulfan hydrolysis and the occurrence of precipitation. In 2013, a French group reported on the stability of busulfan in polypropylene syringes, PVC bags, and glass bottles at 3 different temperatures (5, 14, and 20 °C) and confirmed the precipitation observed by the German group when busulfan in 0.9% sodium chloride injection was stored at a low temperature.[14] However, stability was the longest (24 hours) when diluted bisulfan was stored in polypropylene syringes at 5 °C.

Most of the busulfan stability studies were based on quantitation with high-performance liquid chromatography (HPLC) with ultraviolet (UV)-light detection. However, busulfan has no chromophore functional group, and a derivatization step before analysis is required in order to make the compound visible under UV light. Derivatization can be tedious, as it often requires a long reaction time (more than 1 hour) or heat to be achieved. Given the unstable nature of busulfan, a method with a short overall analysis time without a derivatization step seems to be more suitable for determining the stability of pharmaceutical formulations containing this molecule.

Ultra-HPLC (UHPLC) coupled to mass spectrometry (UHPLC-MS) is a sensitive, highly specific, and fast analytic technique. Moreover, no prior derivatization step is required for busulfan analysis. This technique is widely used for monitoring plasma busulfan levels and making dosage adjustments, particularly for pediatric patients.[15-19] Nevertheless, to our knowledge no UHPLC-MS method has been developed for evaluating the stability of busulfan.

This study investigated the stability of busulfan diluted in 0.9% sodium chloride injection and stored in polypropylene syringes and infusion bags at 2–8 and 23–27 °C using UHPLC-MS.

KEY POINTS

- The stability-indicating method used was more sensitive, more specific, and faster than conventional high-performance liquid chromatography with ultraviolet light, with prior derivatization step methods used for already published stability studies.
- The physical stability of diluted busulfan solutions was very labile and strongly dependent on container type.
- Busulfan 0.54-mg/mL solution in 0.9% sodium chloride injection was physically and chemically stable for 30 hours when stored in 50-mL polypropylene syringes at 2–8 °C and protected from light.

Methods

Materials. Busulfan,[a] [$^2H_8$]-busulfan[b] (internal standard), Busilvex[c] from 3 different lots (designated as lots A, B, and C), syringes,[d] syringe caps,[e] and infusion bags containing 0.9% sodium chloride injection[f] were obtained commercially. Ultrapure water,[g] liquid chromatography–MS grade acetonitrile,[h] formic acid,[i] and ammonium formate[j] were used for mobile-phase preparation. WFI-grade water[k] was used for sample dilution. Forced degradation studies were performed with sodium hydroxide,[l] hydrochloric acid,[m] 30% hydrogen peroxide,[n] and heat (80 °C). DMA[o] and PEG400 (Macrogol 400) [p] were used for signal enhancement/suppression effect investigations.

Preparation of reference solutions. Busulfan reference solution for UHPLC-MS assay was prepared by dissolving 54 mg of busulfan powder in acetonitrile using a 20-mL volumetric flask. Because the solution was demonstrated to be stable for at least 5 days at –20 °C, aliquots were placed in 500-µL plastic vials and stored at –20

°C for the whole duration of the study.

The internal standard was prepared by dissolving 10 mg of [$^2H_8$]-busulfan in acetonitrile using a 20-mL volumetric flask. Because the solution was demonstrated to be stable for 2 months, aliquots were placed in 500-µL plastic vials and stored at –20 °C until use. A working solution of 10 µg/mL was obtained by dilution with WFI-grade water and a 10-mL volumetric flask. The working solution was freshly prepared for each time point of analysis.

UHPLC-MS assay method. The optimized method to assay busulfan by UHPLC-MS used an ultrafast liquid chromatograph system coupled to a mass spectrometer consisting of a high-vacuum pump;[q] a thermostated autosampler;[r] a quaternary-flow solvent-delivery system;[s] a 2.1 × 50 mm, 1.7-µm C_{18} column[t] fitted with a guard column;[u] a thermostatic column compartment;[v] and a triple-quadrupole mass spectrometer detector.[w] The column compartment was maintained at 35 °C, and the sample-manager temperature was maintained at 10 °C to limit sample degradation during the run time. The injection volume was set to 2 µL.

The mass spectrometer was operated in positive-ion electrospray ionization mode. Electrospray settings were as follows: capillary voltage, 3.00 kV; cone voltage, 18 V; and source and desolvation temperatures, 150 and 450 °C, respectively. Desolvation and cone gas flow were set to 800 and 30 L/hr, respectively. Positive ionization with selected-ion recording mode was used, and the multiplier gain was set on 1. Busulfan and [$^2H_8$]-busulfan were detected as ammonium adducts at m/z 264.1 and 272.1, respectively. Peak areas of busulfan and [$^2H_8$]-busulfan were analyzed, and subsequent calculation of calibration curves and quantification of busulfan were performed with MassLynx software, version 4.1 (Waters, Milford, MA).

Two mobile phases, designated A and B, consisted of 5 mmol/L ammonium formate and 0.1% formic acid in water (A) and pure acetonitrile (B). Gradients were programmed as follows: 20% mobile phase B for 0.4 minutes, increased to 80% B for 0.5 minutes, and then equilibrated with 20% B for 0.8 minutes. A flow rate of 0.6 mL/min was applied. A column-switching valve was used to switch the liquid flow into the detector between 0.4 and 1.0 minutes after injection. Each analysis was confirmed by a second injection of the sample, with an acceptance specification lower than 1% of the difference in response. The results were reported as the average of both analyses.

Antineoplastic substances handling. All manipulations of toxic substances were executed inside a class II biological-safety cabinet with appropriate and certified personal protective equipment, including safety goggles, mask, gloves, hand sleeves, and gown.[20]

Method validation. The developed method was validated according to the International Conference on Harmonisation (ICH) guidelines following the recommendations of the Commission of the French Society of Pharmaceutical Science and Technology (SFSTP).[21-23] The calibration curves ($r^2 > 0.999$) were obtained for each series with conventional least-square linear regression using 3 concentrations (378, 540, and 648 ng/mL) by plotting the area ratio m/z (264.1 and 272.1 for busulfan and [$^2H_8$]-busulfan, respectively) against busulfan concentrations. No weighing function was applied. Calibration samples were prepared by diluting an adequate volume of busulfan reference solution to reach concentrations of 378, 540, and 648 ng/mL using 10-mL volumetric flasks and WFI-grade water. Next, 500 µL of [$^2H_8$]-busulfan working solution was added as internal standard to obtain a final concentration of 500 ng/mL in the sample. Validation samples were prepared by diluting 900 µL of Busilvex in 0.9% sodium chloride injection using a 10-mL volumetric flask, yielding a final busulfan concentration of 0.54 mg/mL. The solution was then diluted in 10-mL volumetric flasks with WFI-grade water to final busulfan concentrations of 378, 540, and 648 ng/mL. Next, 500 µL of [$^2H_8$]-busulfan working solution was added to each calibration sample to obtain a final concentration of 500 ng/mL in the sample. Calibration and validation samples were analyzed twice, and the average result was used to determine calculations and method validation.

The quantitative performance of the developed method was estimated using 3 separate series (n = 3). For each series, 6 independent calibration samples (2 for each concentration) and 12 independent validation samples (4 for each concentration) were analyzed. After establishing the calibration curves for each series, concentrations of validation samples were computed from the analytic response to obtain trueness, repeatability, and intermediate precision. Trueness, expressed as a percentage, was the ratio between theoretical and average measured values at each concentration. Repeatability and intermediate precision were expressed as the relative standard deviation (RSD) (i.e., the ratio of the intraday standard deviation [sr] and interday standard deviation [sR], respectively, to the theoretical concentrations).

As reported in Table 1, trueness and precision values were in accordance with recommendations for the analysis of pharmaceutical formulations over the tested concentration range. The RSD values (repeatability and intermediate precision) were lower than 1.3%, with trueness values between 100% and 101%. The accuracy profile was then obtained according to the SFSTP 2006 recommendations (β = 95%). The upper and lower tolerance limits represented the total error of the method, based on the tolerance interval (Table 1).

Forced degradation studies. Forced degradation studies were performed to provide an indication of the stability-indicating properties and specificity of the method. Intentional degradation of busulfan was at-

tempted using hydrochloric acid (0.1, 0.5, and 1 mol/L), sodium hydroxide (0.1, 0.5, and 1 mol/L), 30% hydrogen peroxide, and heat (80 °C), following the ICH recommendations and methodological guidelines for stability studies of hospital pharmaceutical preparations.[24] After the degradation treatments were completed, all samples were allowed to cool to room temperature, neutralized with acid or base (if needed), and prepared according to the assay procedures. Because of the high specificity of MS and the low molecular mass of already identified degradation products (tetrahydrofuran and methanesulfonic acid), there was no ambiguity on main peak product identification (m/z, 264.1), and no interference between busulfan and its degradation products was observed (data not shown).

Physical assessment. A visual inspection of the samples for particulate matter, clarity, and color without instrumentation of magnification was conducted at each time point of analysis.

Data analysis. The stability of busulfan was assessed by measuring the percentage of the initial concentration remaining at the end of each time point of analysis. The initial busulfan concentration was defined as 100%. Stability was defined as retention of at least 90% of the initial busulfan concentration.

Sample preparation. *Syringes.* Three bulk solutions from 3 Busilvex lot numbers were prepared by diluting 19.73 g of Busilvex in 180.8 g of 0.9% sodium chloride injection. These solutions were homogenized with a magnetic stirrer for 5 minutes prior to dispensing in 12 50-mL syringes (4 for each lot number). A 300-µL sample was withdrawn in each syringe for initial busulfan quantification. For each batch, 3 syringes were stored at 2–8 °C in a regulated climatic chamber and 1 syringe was stored at room temperature (23–27 °C). All syringes were protected from light.

Infusion bags. Four 100-mL infusion bags were prepared for each different Busilvex lot number. From each container, 9 mL of 0.9% sodium chloride injection was withdrawn with a 10-mL syringe[x] and a 20G needle[y] followed by an injection of the same volume of Busilvex. The containers were then vigorously shaken prior to individual busulfan concentration determination by UHPLC-MS. For each batch, 3 containers were stored at 2–8 °C in a regulated climatic chamber and 1 container was stored at room temperature (23–27 °C). All containers were protected from light.

Four 500-mL infusion bags were prepared from 3 different Busilvex lot numbers (2 from lot A and 1 each from lots B and C). From each container, 45 mL of 0.9% sodium chloride injection was withdrawn with a 50-mL syringe[y] and a 18G needle[d] followed by an injection of 45 mL of Busilvex. The containers were then vigorously shaken prior to individual quantification of busulfan by UHPLC-MS. All bags were stored at 2–8 °C in a regulated climatic chamber except 1 bag from lot A that was stored at room temperature (23–27 °C). All containers were protected from light.

Table 1. Validation Results for Busulfan Assay[a]

Theoretical Busulfan Conc. (ng/mL)	%			
	Trueness	Repeatability, RSD	Intermediate Precision, RSD	Tolerance Interval
378	100.0	0.8	1.3	97.0–103.0
540	100.8	1.1	1.3	97.9–103.8
648	101.0	1.2	1.2	98.2–103.7

[a]RSD = relative standard deviation.

Sample analysis. At each time point of analysis, all containers were vigorously shaken and observed for physical assessment prior to withdrawal of the sample with 1-mL syringes[z] and 22G needles.[aa] The number of containers, sampling times, and volumes are summarized in Table 2.

Results and discussion

Ion suppression or enhancement effect. A modification of the MS signal can be observed when several compounds are coeluted into the mass spectrometer. Such a phenomenon might induce an error in the quantification of the target compound. In the case of pharmaceutical formulations, particular attention should be paid to the possible coelution of excipients and target compound. For example, PEG (widely used as a solubilization agent) is known to cause strong signal suppression.[25] An assay was conducted to investigate this effect for both the excipients of Busilvex—PEG400 and DMA. Three solutions were prepared: 1 with PEG400 only, 1 with DMA only, and 1 with a mixture (2:1 ratio, weight/weight) of these 2 products. These solutions were diluted following the standard assay procedure and injected in the UHPLC-MS system with the parameters described above. A busulfan solution (100 ng/mL in acetonitrile) at a 50-µL/min flow rate was continuously injected into the mass spectrometer with a postcolumn-injection procedure. The busulfan signal recording was examined to visualize the effects of excipients on the signal. DMA did not have any influence; however, PEG400 showed numerous ion suppression zones at different retention times. Optimization of the gradient and column temperature was performed to remove overlap between ion suppression zones and busulfan retention time (data not shown).

Stability study results. The visual inspection demonstrated no influence of the storage container when busulfan infusions diluted in 0.9% sodium chloride injection were

Table 2. Experimental Conditions in Determining Stability of Busulfan 0.54 mg/mL in 0.9% Sodium Chloride Injections

Containers	Storage Temperature, °C	No. Containers	Sample Volume, µL	Sampling Times, hr
50-mL polypropylene syringes	2–8	9	300	0, 3, 6, 12, 17, 24, 30, 33, 36, 50
	23–27	3	300	0, 3, 6, 12, 17, 24, 30, 36, 50
100-mL polypropylene infusion bags	2–8	9	300	0, 3, 6, 9, 12, 15, 19, 23, 28, 33, 100
	23–27	3	300	0, 3, 6, 9, 12, 15, 19, 23, 28, 31
500-mL polypropylene infusion bags	2–8	3	500	0, 3, 5, 9, 13, 16, 19, 22
	23–27	1	500	0, 3, 5, 9, 13, 16, 19, 22

stored at 23–27 °C. No color change or precipitate was observed at this temperature; however, a rapid decrease of the busulfan content in all containers stored at room temperature was observed. As shown in Table 3, busulfan in syringes was chemically stable for 12 hours; busulfan in infusion bags (100 and 500 mL) was stable only for 3 hours at 23–27 °C if a 10% threshold limit was considered (Tables 4 and 5). These results were consistent with those obtained by Houot et al.,[14] who demonstrated the stability of busulfan for 15 hours when stored in polypropylene syringes and for 6 hours when stored in PVC infusion bags at room temperature. Moreover, Houot et al.[14] found that the stability of busulfan in infusion bags stored at room temperature was shorter than expected compared with that found in the summary of product characteristics.[9]

Table 3. Stability of Busulfan 0.54 mg/mL in 0.9% Sodium Chloride Injection Stored in 50-mL Polypropylene Syringes

	Mean ± S.D. % Initial Busulfan Conc. Remaining at Indicated Storage Temperature	
Study Time, hr	2–8 °C[a]	23–27 °C[b]
3	97.14 ± 0.79	93.60 ± 0.42
6	96.41 ± 0.47	93.26 ± 0.45
12	99.25 ± 0.52	98.34 ± 3.97
17	96.93 ± 0.67	85.42 ± 0.87
24	97.99 ± 1.29	80.18 ± 1.53
30	93.93 ± 1.52	75.57 ± 1.00
33	88.89 ± 1.93	NA[c]
36	88.93 ± 3.35	68.11 ± 1.30
50	78.91 ± 11.39	68.04 ± 0.17

[a]Actual mean ± S.D. initial busulfan concentration = 0.56 ± 0.005 mg/mL (n = 9). The sample volume was 300 µL.
[b]Actual mean ± S.D. initial busulfan concentration = 0.56 ± 0.005 mg/mL (n = 3). The sample volume was 300 µL.
[c]Not applicable; not a predetermined study time for this temperature.

At a lower temperature (2–8 °C) the stability of diluted busulfan solution was mainly driven by the physical assessment. Indeed, white crystals were detected on the syringes' surface after 33 hours of storage. This observation could be correlated with the quantification results obtained for the syringes stored at 2–8 °C (Table 3). Indeed, all the tested solutions remained above the 90% threshold limit for 30 hours (n = 9, RSD <2%). The variability in crystallization kinetics resulted in the relatively large standard deviations observed for time points after 30 hours.

Busulfan crystallization was also detected in the 100-mL infusion bags, but the precipitate took the form of a granular film on the bags' surface. Interestingly, some bags showed precipitate formation after only 15 hours, while others showed no precipitate after 100 hours. For each bag, the appearance of precipitate was correlated with a strong diminution of busulfan concentration found by UHPLC-MS (Figure 1). In the worst case, the 100-mL infusion bags remained stable at 2–8 °C for 12 hours (Table 4), consistent with the manufacturer's storage recommendations. The same variability in precipitate formation was observed for the 500-mL infusion bags (Figure 2). However, the stability time was shorter than expected (9 hours instead of 12 hours) (Table 5).

The results of this study confirmed that polypropylene syringes offered the best stability times, regardless of the storage temperature. However, the stability of busulfan at 2–8 °C in these syringes was considerably increased compared with the manufacturer's

Table 4. Stability of Busulfan 0.54 mg/mL in 0.9% Sodium Chloride Injection Stored in 100-mL Polypropylene Infusion Bags

	Mean ± S.D. % Initial Busulfan Conc. Remaining at Indicated Storage Temperature			
	2–8 °C[a]			
Study Time (hr)	Lot A	Lot B	Lot C	23–27 °C[b]
3	99.59 ± 1.38	99.79 ± 1.70	98.58 ± 1.08	96.03 ± 0.23
6	95.28 ± 1.75	96.04 ± 1.03	95.89 ± 1.30	90.78 ± 1.05
9	97.04 ± 1.56	96.99 ± 0.09	97.49 ± 1.35	90.01 ± 0.53
12	96.75 ± 1.89	98.22 ± 1.19	98.10 ± 2.73	88.55 ± 1.32
15	99.43 ± 2.15	99.42 ± 0.93	95.90 ± 6.19	87.59 ± 0.38
19	96.61 ± 1.48	97.30 ± 0.62	75.02 ± 44.1	84.20 ± 0.61
23	97.07 ± 1.18	97.23 ± 0.59	56.96 ± 67.5	79.36 ± 0.57
28	94.04 ± 0.63	87.19 ± 12.1	43.22 ± 104	73.79 ± 1.57
31	95.40 ± 2.23	79.83 ± 33.5	44.49 ± 84.7	74.12 ± 4.55
33	98.71 ± 2.26	72.26 ± 51.9	37.93 ± 124	NA[c]
100	86.94 ± 1.70	61.47 ± 37.4	34.06 ± 66.4	NA

[a]Mean ± S.D. initial busulfan concentration = 0.53 ± 0.004 mg/mL (n = 3 for each batch). The sample volume was 300 µL.
[b]Mean ± S.D. initial busulfan concentration = 0.53 ± 0.004 mg/mL (n = 3). The sample volume was 300 µL.
[c]Not applicable; not a predetermined study time for this temperature.

Table 5. Stability of Busulfan 0.54 mg/mL in 0.9% Sodium Chloride Injection Stored in 500-mL Polypropylene Infusion Bags

	% Initial Busulfan Conc. Remaining at Indicated Storage Temperature	
Study Time, hr	2–8 °C[a]	23–27 °C[b]
3	NA[c]	98.41
5	96.12 ± 0.36	91.25[d]
9	92.63 ± 1.08	85.03
13	87.89 ± 10.8	86.42
16	80.27 ± 15.3	81.68
19	71.19 ± 26.0	77.36
22	53.02 ± 30.5	71.87

[a]Results expressed as mean ± S.D. (n = 3). Mean ± S.D. initial busulfan concentration = 0.53 ± 0.004 mg/mL (n = 3). The sample volume was 500 µL.
[b]Initial busulfan concentration = 0.53 mg/mL (n = 1). The sample volume was 500 µL.
[c]Not applicable; not a predetermined study time for this temperature.
[d]A systematic error (1.3%) should be considered when examining results.

recommendations. As discussed in other studies, there are 2 independent phenomena that explain busulfan degradation. At a high temperature, the main cause is hydrolysis; at a low temperature, the precipitation is the main source of product instability. Crystallization of a metastable solution is initiated by a nucleation process followed by crystal growth resulting from consecutive additions of molecules onto the nucleus. Both steps are influenced by numerous factors and are difficult to predict. Indeed, temperature, agitation, and interactions between product and container are known to influence the precipitation kinetics.[26] Other potential variables that could affect busulfan degradation include the roughness of the container, surface area:volume ratio, and adsorption affinity of the drug molecule to the surface. These factors might explain the variability observed between the container types. They could also explain the variability observed within the same container type, such as with the 100-mL bags.

Karstens and Kramer[13] tried to extrapolate the influence of temperature on busulfan crystallization. They found that an intermediate storage temperature of 14 °C could extend the shelf life of diluted busulfan solutions. This result was not confirmed by Houot et al.[14] An intermediate temperature was excluded from our study because it would have created additional storage problems, as climatic chambers would have been necessary in all care units that use busulfan.

Published data and the results of the current study suggest that the physical stability of diluted busulfan

Figure 1. Stability of busulfan at 0.54 mg/mL in 0.9% sodium chloride injection at 2–8 °C and stored in 3 100-mL infusion bags for lot C. Each line represents 1 of the 3 bags. The red dashed lines correspond to the higher and lower acceptance limits.

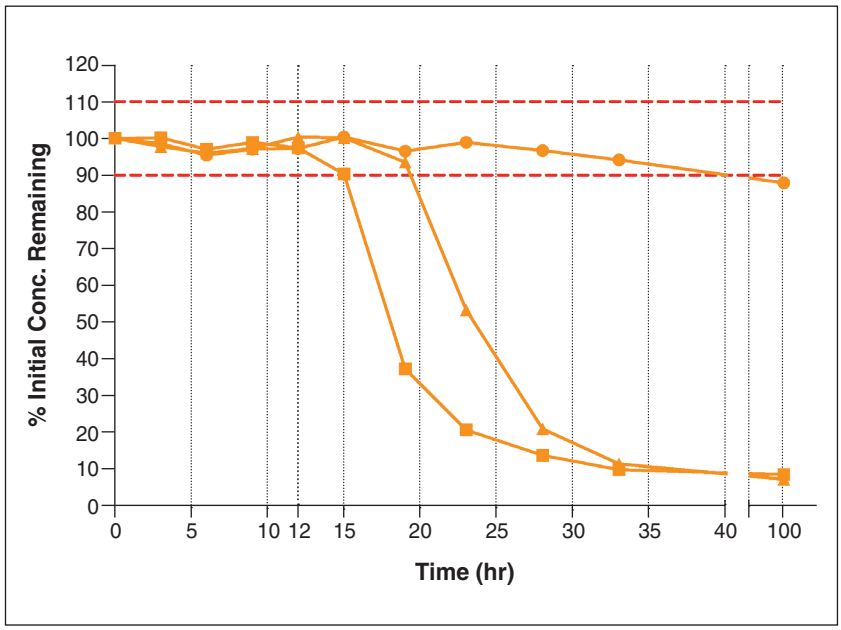
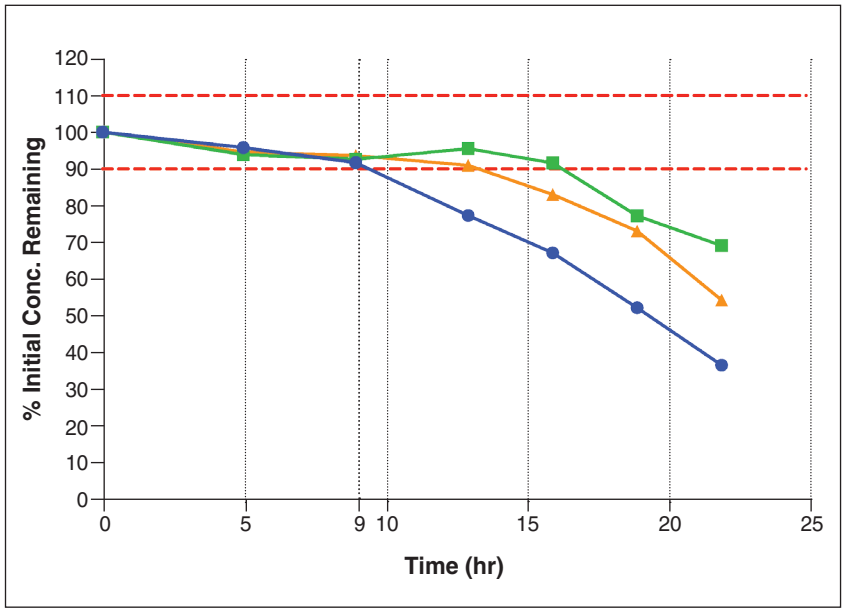


Figure 2. Stability of busulfan at 0.54 mg/mL in 0.9% sodium chloride injection at 2–8 °C and stored in 500-mL infusion bags for lot A (blue dots), B (orange triangles), and C (green squares). The red dashed lines correspond to the higher and lower acceptance limits.



solutions is very labile and strongly dependent on container type. Indeed, of the containers tested in this study, syringes offered the best stability regardless of the storage temperature. Moreover, the solutions remained stable for a longer period of time when they were stored in refrigerated conditions. Because high variability was observed in the precipitation time and rate of busulfan, inspection of busulfan containers before transport to care units and before administration to patients is strongly recommended.

The extended shelf life of busulfan infusion stored in syringes allowed for better organization of the chemotherapy preparation unit by reducing busulfan production to once daily. This allowed the 4 doses of busulfan required for a patient each day to be prepared at the same time and sent to care units in advance.

Conclusion

Busulfan 0.54-mg/mL solution in 0.9% sodium chloride injection was physically and chemically stable for 30 hours when stored in 50-mL polypropylene syringes at 2–8 °C and protected from light.

Disclosures

The authors have declared no potential conflicts of interest.

[a]Busulfan, analytic standard for drug analysis, Sigma-Aldrich, St. Louis, MO, lot BCBN8120V.

[b][$^2H_8$]-busulfan, Alsachim, Strasbourg, France, batch CM-ALS-11-167-B1.

[c]Busilvex, busulfan 6 mg/mL (10 mL), Pierre Fabre Pharma SA, Allschwill, Switzerland, lots AD5362B, AD9058B_1, and AD7703D_1 (called lots A, B, and C, respectively, in this study).

[d]BD Luer-Lok Plastipak syringes, 50 mL, Becton Dickinson, Franklin Lakes, NJ, ref. no. 300865, lot 1505235.

[e]Tamper-evident cap, B. Braun Medical, Bethlehem, PA, ref. no. 418004, lot 61458781.

[f]Sodium chloride 0.9% Bioren 500- and 100-mL bags, Sintetica, Mendrisio, Switzerland, ref. no. 420028, lot 15112269B (for 500-mL bags) and ref. no. 420025, lot 15092230A (for 100-mL bags).

[g]Type 1 ultrapure water, Milli-Q purification system, Merck-Millipore, Darmstadt, Germany.

[h]Acetonitrile hypergrade for LC-MS, Merck, Darmstadt, Germany, lot I784629529.

[i]Formic acid for mass spectrometry, 98%, Sigma-Aldrich, lot BCBP4740V.

[j]Ammonium formate for mass spectrometry, >99.0%, Sigma-Aldrich, lot BCBP6806V.

[k]Aqua ad iniect. Ph. Eur. steril, Laboratorium Dr. G Bichsel AG, Unterseen, Switzerland, lot 1000247.

[l]Sodium hydroxide solution, 1 mol/L, Merck, lot HC681199637.

[m]Hydrochloric acid, 1 mol/L, Merck, lot HC253654.

[n]Hydrogen peroxide 30% for analysis, Merck, lot K46362809512.

[o]*N,N*-dimethylacetamide anhydrous, 99.8%, Sigma-Aldrich, lot STBF4798V.

[p]Polyethylene glycol, Macrogol 400 Ph. Eur., Hanseler, Herisau, Switzerland, lot 2015.07.0183.

[q]Sogevac SV 40 BI, Leybold, Cologne, Germany.

[r]Waters Aquity UPLC H-Class Sample manager-FTN, Waters, Milford, MA.

[s]Waters Acquity UPLC H-Class QSM, Waters.

[t]Waters Acquity UPLC BEH Shield RP18, 2.1 × 50 mm, 1.7-µm particle size, Waters, lot 01503326015908.

[u]Waters Acquity UPLC BEH Shield RP18 2.1 × 5 mm, 1.7-µm VanGuard Pre-Column, Waters, lot 0161352101.

[v]Waters Acquity CH-A column heater, Waters.

[w]Waters TQD, Waters.

[x]Omnifix 10 mL, B. Braun, Melsungen, Germany, lot 15L19C8.

[y]BD Microbalance 3 18G needle, Becton Dickinson, ref. no. 304622, lot 150805.

[z]BD 1-mL Luer-Lok Tip syringe, Becton Dickinson, ref. no. 309628, lot 5201845CAV08.

[aa]Neolus 22G hypodermic needle, Terumo, Tokyo, Japan, ref. no. NN-2232S, lot 1401005.

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Chapitre 4 : Echantillons de surface

Pour évaluer une contamination de surface par des agents anticancéreux, il faut disposer d'une méthode analytique sélective (différenciation entre les différents composés) et sensible (recherche de traces). La LC-MS est indubitablement une technique de choix qui répond à ces deux critères. C'est pourquoi dans un premier temps, le Laboratoire de Contrôle Qualité de la Pharmacie des HUG s'est consacré au développement d'une méthode de prélèvements d'échantillons de surface couplée à une analyse LC-ESI-MS/MS pour la détermination d'un cocktail d'agents anticancéreux. Ce développement s'est déroulé en deux étapes successives. La première étape reposait sur le développement d'une méthode LC-ESI-MS/MS pour l'analyse de plusieurs agents anticancéreux. Au terme de ce travail (article IV), 10 agents anticancéreux (cytarabine, gemcitabine, méthothrexate, étoposide phosphate, cyclophosphamide, ifosfamide, irinotécan, doxorubicine, épirubicine et vincristine), dont la sélection est issue d'un compromis entre le taux de prescription au sein des HUG et des aspects analytiques, sont analysés par LC-MS en 21 minutes. Les performances analytiques de la méthode ont été évaluées : des limites de quantification comprises entre 0.25 et 2 ng/mL pour les 10 composés ont été obtenues et des recouvrements entre 85 et 110% ont été observés avec des déviations standards relatives (répétabilités et précisions intermédiaires) inférieures à 15%. Dans un deuxième temps, une méthode de prélèvement d'échantillons sur différentes surfaces, préalable à l'analyse LC-MS (précitée), a été mise au point pour les 10 agents anticancéreux choisis (article V). Elle consiste à essuyer à l'aide d'un papier filtre imbibé d'une solution hydro-organique la surface d'intérêt et puis à désorber les éventuels agents anticancéreux prélevés du papier dans le même solvant (après passage aux ultrasons et centrifugation). Au cours de ce travail, les performances quantitatives de la procédure complète (prélèvement et analyse LC-MS) ont été évaluées pour les 10 agents anticancéreux. Des limites de quantification de l'ordre de 10 ng par prélèvement (soit 0.1 ng/cm^2) ont pu être atteintes. Des taux de recouvrement dépendants du type de surface et du composé ont été définis avec des déviations standards relatives (précision intermédiaire) inférieures à 20%.

La méthode de prélèvements de surface couplée à l'analyse LC-ESI-MS/MS développée a été utilisée dans le cadre d'un projet d'ampleur national qui consistait à évaluer les contaminations de surface par des agents anticancéreux au sein de différentes pharmacies hospitalières suisses (article VI). Soutenu par l'Association Suisse des Pharmaciens de l'Administration et des Hôpitaux (GSASA), ce projet a

démontré que la plupart des sites testés volontaires présentaient des contaminations de surface par des agents anticancéreux. Aucune contamination n'a été décelée dans deux sites par contre pour les 22 autres sites volontaires, des contaminations totales (sommes des contaminations pour les 10 agents anticancéreux) comprises entre 8 ng et 41 000 ng par échantillon ont été détectées. La majeure partie des contaminations provenaient des surfaces situées dans les postes de sécurité microbiologique. Toutefois il n'en demeurait pas moins que des traces d'agents anticancéreux ont été prélevées dans les salles à atmosphère contrôlée et dans les locaux dédiés aux tâches logistiques. Des corrélations, basées sur une analyse statistique, entre les taux de contamination et les lieux de prélèvements, le nombre de chimiothérapies préparées par an et le type de solution de nettoyage ont été établies. Les résultats de cette étude ont été transmis anonymement à tous les sites ayant participé à ce projet qui ont pu alors sur la base de leurs résultats entamer des réflexions sur les précautions employées et sur d'éventuelles mesures à mettre en place afin de réduire au minimum les contaminations de surface au sein de leur site.
La manipulation des agents anticancéreux lors des étapes de reconstitution est considérée comme la source principale de contamination de surface au sein des pharmacies hospitalières. Toutefois, la présence d'agents anticancéreux sur les parois extérieures des flacons commercialisés contribue à cette contamination de surface. Une étude visant à évaluer la contamination de surface de plus de 100 flacons d'agents anticancéreux disponibles sur le marché suisse a été conduite par le Laboratoire de Contrôle Qualité de la Pharmacie des HUG (article VII). Les prélèvements effectués au cours de ce travail ont été analysés par la méthode LC-ESI-MS/MS développée. Des contaminations externes ont été détectées sur plus de 60% des flacons testés avec des teneurs pouvant atteindre plus de 1 mg par flacon. Des contaminations par des agents anticancéreux autres que la molécule active ont été observées pour 35% des flacons testés.

Les résultats issus des travaux précités (articles VI et VII) démontrent l'importance de la décontamination chimique lors de la manipulation d'agents anticancéreux. Or si les techniques de décontamination microbiologique sont parfaitement définies, les solutions de décontaminations chimiques des agents anticancéreux sont méconnues. La décontamination chimique peut s'effectuer de deux manières différentes : par action mécanique ou par action chimique. Si cette dernière est la

plus efficace, elle soulève certaines interrogations en regard de son innocuité vis-à-vis du matériel (action corrosive) et des opérateurs (action corrosive et possibilité d'obtenir des produits de dégradation toxiques). Dans le but d'évaluer différents agents de décontamination chimique sur les anticancéreux, deux études ont été menées au sein de la Pharmacie des HUG. La première a permis de définir une solution efficace et sans danger pour la décontamination chimique de surface par des agents anticancéreux (article VIII). Au cours de ce travail, plusieurs agents de décontaminations chimiques ont été testés sur de l'inox et du verre et la méthode de prélèvements couplé à l'analyse LC-ESI-MS/MS a également été utilisée comme outil analytique. Il a été démontré qu'une solution de SDS à 10^{-2} M en présence de 20% d'isopropanol permettait d'ôter plus de 90% des 10 agents anticancéreux testés. Cette solution de décontamination chimique a également été testée dans des conditions réelles (article IX). Pendant une année, cette solution a servi hebdomadairement d'agent de décontamination chimique au sein d'un PSM utilisé en routine pour la reconstitution de chimiothérapie à la pharmacie des HUG. Les prélèvements de surface effectués avant et après application de la solution de décontamination ont été analysés par la méthode LC-ESI-MS/MS. Les résultats obtenus ont été comparés à ceux obtenus dans les mêmes conditions au sein d'un second PSM identique utilisé également pour la reconstitution des chimiothérapies à la pharmacie des HUG mais ne bénéficiant pas d'une étape de décontamination chimique spécifique (uniquement décontamination microbiologique par l'isopropanol). Il est démontré que la solution de SDS est plus efficace (20% supérieure) pour ôter 8 des 10 agents anticancéreux testés.

Article IV

« Simultaneous quantification of ten cytotoxic drugs by a validated LC-ESI-MS/MS method »

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ORIGINAL PAPER

Simultaneous quantification of ten cytotoxic drugs by a validated LC–ESI–MS/MS method

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Abstract A liquid chromatography separation with electrospray ionisation and tandem mass spectrometry detection method was developed for the simultaneous quantification of ten commonly handled cytotoxic drugs in a hospital pharmacy. These cytotoxic drugs are cytarabine, gemcitabine, methotrexate, etoposide phosphate, cyclophosphamide, ifosfamide, irinotecan, doxorubicin, epirubicin and vincristine. The chromatographic separation was carried out by RPLC in less than 21 min, applying a gradient elution of water and acetonitrile in the presence of 0.1% formic acid. MS/MS was performed on a triple quadrupole in selected reaction monitoring mode. The analytical method was validated to determine the limit of quantification (LOQ) and quantitative performance: lowest LOQs were between 0.25 and 2 ng mL^{-1} for the ten investigated cytotoxic drugs; trueness values (i.e. recovery) were between 85% and 110%, and relative standard deviations for both repeatability and intermediate precision were always inferior to 15%. The multi-compound method was successfully applied for the quality control of pharmaceutical formulations and for analyses of spiked samples on potentially contaminated surfaces.

Keywords Cytotoxic · Antineoplastic drugs · LC–MS/MS · Pharmaceutical formulation · SRM · Validation

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Introduction

During the last decades, the number of patients receiving anticancer chemotherapy treatments based on cytotoxic drugs has steadily increased. Simple analytical methods are thus required in different pharmaceutical fields, such as quality control or environmental monitoring. Different methods have already been published such as simple flow injection analysis and high-performance liquid chromatography (HPLC)-UV/Vis assays for cytotoxic drugs by Delmas et al. for quality control of cytotoxic preparations in a centralised parenteral preparation unit [1] or simultaneous determination of three anthracycline drugs (doxorubicin, daunorubicin and idarubicin) in serum samples by capillary electrophoresis (CE) with laser-induced fluorescence by Pérez-Ruiz et al. for therapeutic drug monitoring [2]. Due to their toxicity, the analysis of cytotoxic drugs is also useful for environmental monitoring and control of cytotoxic traces in wastewater. As an example, a CE-DAD method for the quantification of 5-fluorouracil in wastewater of hospital effluents was published by Mahnik et al. [3].

Despite safety standards for handling cytotoxic agents, it has been shown that health care professionals are still exposed to these toxic compounds. For instance, several studies reported low-level contamination of these compounds on workbenches, floors, vials, gloves and isolators [4–12].

Moreover, cytotoxic traces have been found in urine of health care professionals [12–15]. Rapid, reliable and validated analytical methods are thus needed for the safety of the operator handling these hazardous drugs and to reduce the exposure at the lowest possible level [16]. A review about analytical methods used for biological and environmental monitoring of hospital personnel exposed to antineoplastic agents was published by Turci et al. [17]. Different instrumental techniques were used depending on the studied analyte. Most of the presented studies used a specific method for the determination of a single cytotoxic drug. For example, a very sensitive voltammetry for platinum drugs, as well as GC–MS methods for the quantification of cyclophosphamide, ifosfamide and fluorouracil, was developed by Schmaus et al. for environmental and biological monitoring [18]. These methods presented very good quantitative performance and detection limits (i.e. 0.1 to 1 pg per sample) and are thus ideal for establishing target guideline values for cytotoxic contamination or for selecting a single compound as a model marker for potential contaminations. On the other hand, such methods are time consuming and not very cost-effective to get an overview of several cytotoxic contaminations. For the latter, multi-compound methods are required. Different approaches have

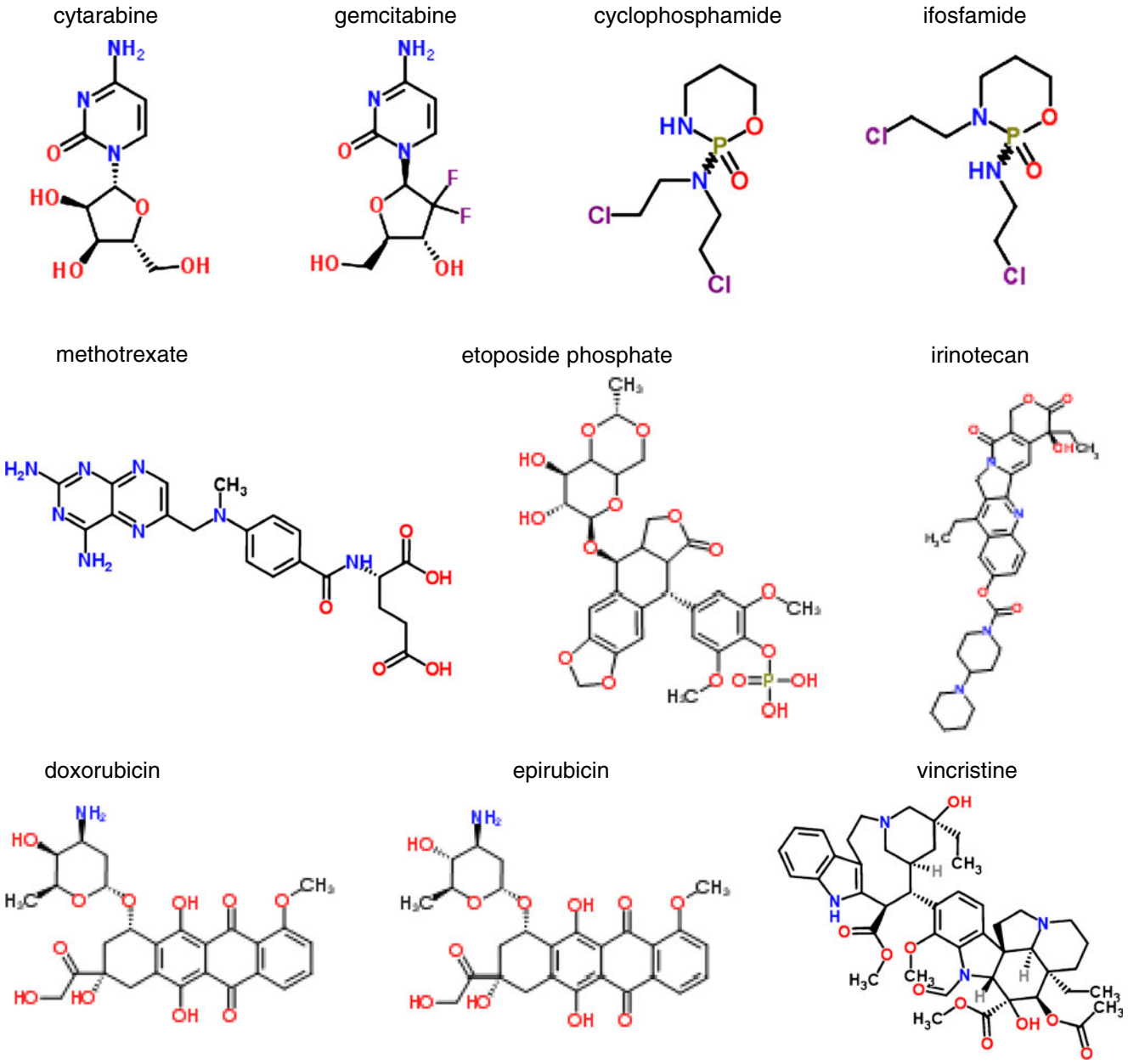

Fig. 1 Chemical structure of the ten studied cytotoxic drugs

Table 1 Preparation of cytotoxic stock solutions

Brand name	Manufacturer	Drug	Drug concentration (mg mL^{-1})	S1 (20 μg mL^{-1}) (in 100 mL H_2O)
Vincristine Teva®	Teva (Aesch, Switzerland)	Vincristine	1	2.000 mL
Doxorubicin Ebewe®	Ebewe Pharma Schweiz (Cham, Switzerland)	Doxorubicin	2	1.000 mL
Epirubicin Actavis Solution®	Actavis (Regensdorf, Switzerland)	Epirubicin	2	1.000 mL
Methotrexate Farmos®	Orion Pharma (Zug, Switzerland)	Methotrexate	2.5	0.800 mL
Endoxan®	Baxter AG (Volketswil, Switzerland)	Cyclophosphamide	20	0.100 mL
Cytosar®	Pfizer AG (Zürich, Switzerland)	Cytarabine	20	0.100 mL
Etopophos®	Bristol-Myers Squibb SA (Baar, Switzerland)	Etoposide phosphate	20	0.100 mL
Gemcitabine Teva®	Teva (Aesch, Switzerland)	Gemcitabine	20	0.100 mL
Campto®	Pfizer AG (Zürich, Switzerland)	Irinotecan	20	0.100 mL
Holoxan®	Baxter AG (Volketswil, Switzerland)	Ifosfamide	40	0.050 mL

been developed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the simultaneous determination of gemcitabine, taxol, cyclophosphamide and ifosfamide by Sottani et al. [19] and for cyclophosphamide, methotrexate and 5-fluorouracil for surface contamination by Sabatini et al. [11].

However, to our knowledge, there is still a lack of simple validated methods for the simultaneous determination of different cytotoxic agents. For drug treatment monitoring or quality control of pharmaceutical formulations, single-compound strategies are often sufficient, but generic multi-compound methods allow simplifying the control. In addition, multi-compound methods are mandatory for environmental analyses. The aim of this work was to develop and validate a simple and sensitive LC–MS/MS method for the simultaneous quantitative determination of ten cytotoxic drugs contained in aqueous samples.

Table 2 Gradient elution programme

Time [min]	Solvent A (%)	Solvent B (%)	Solvent C [%]
0	88	2	10
2.0	88	2	10
2.5	69	21	10
10	69	21	10
13	60	30	10
13.5	40	50	10
15.5	40	50	10
16	88	2	10
21	88	2	10

Mobile phase: *A* water, *B* acetonitrile, *C* formic acid 1%
Flow rate of 200 μL min^{-1}

Experimental

Chemicals and reagents

The study was performed with the following commercially available cytotoxic drugs (see Fig. 1 for their structure and Table 1): Campto® (irinotecan, 20 mg mL^{-1}) and Cytosar® (cytarabine, 20 mg mL^{-1}) were purchased from Pfizer AG (Zürich, Switzerland), gemcitabine Teva® (gemcitabine reconstituted in water at 20 mg mL^{-1}) and vincristine Teva® (vincristine 1 mg mL^{-1}) from Teva Pharm AG (Aesch, Switzerland), Holoxan® (ifosfamide reconstituted in water at 40 mg mL^{-1}) and Endoxan® (cyclophosphamide reconstituted in glucose 5% at 20 mg mL^{-1}) from Baxter AG (Volketswil, Switzerland), methotrexate Farmos® (methotrexate 2.5 mg mL^{-1}) from Orion Pharma (Zug, Switzerland), Etopophos® (etoposide phosphate reconstituted in water at 20 mg mL^{-1}) from Bristol-Myers Squibb SA (Baar, Switzerland), Doxorubine Ebewe® (doxorubicin 2 mg mL^{-1}) from Ebewe Pharma (Cham, Switzerland) and Epirubicin Actavis Solution® (epirubicin 2 mg mL^{-1}) from Actavis (Regensdorf, Switzerland).

The reconstitution of Etopophos, Gemcitabine Teva and Holoxan was done with water for injectables, obtained from Bichsel Laboratories (Interlaken, Switzerland); glucose 5% for the reconstitution of Endoxan was from Sintetica-Bioren SA (Couvet, Switzerland). The internal standard (IS) [^{13}C, $^{2}H_3$]-methotrexate was purchased from Alsachim (Illkirch, France).

Equipment

Analyses were carried out with a high-performance liquid chromatography system Accela from Thermo Fisher Scien-

Table 3 Instrument method for the LC–MS/MS analysis for ten cytotoxic drugs with [^{13}C, $^{2}H_3$]-methotrexate as internal standard

Time segment (min)	Scan event	Drug	Parent (*m*/*z*)	Product (*m*/*z*)	CE (eV)	Mean RT (min)
0–2.2	1	Cytarabine	244.0	112.3	15	1.9
2.2–4	2	Gemcitabine	264.7	112.3	20	2.6
4–7	3	Methotrexate	455.2	308.0	20	6.0
	4	[^{13}C, $^{2}H_3$] methotrexate	459.2	312.2	20	6.0
7–10	5	Etoposide phosphate	691.0	691.0	15	7.5
10–13	6	Ifosfamide	261.1	92.3, 140.2 154.1, 232.9	20	11.4
		Cyclophosphamide	261.1	92.3; 140.2 154.1, 232.9	20	12.2
13–14	7	Irinotecan	587.9	587.3	20	13.7
14–21	8	Doxorubicin	544.6	379.2, 397.1	15	14.8
		Epirubicin	544.6	379.2, 397.1	15	15.3
	9	Vincristine	413.3	353.2	30	15.2

tific Inc. (Waltham, MA) consisting of a quaternary pump equipped with an online degasser, an auto-sampler and a solvent platform. The chromatographic system was coupled to a triple quadrupole Quantum Discovery MS from Thermo Fisher Scientific equipped with an ion max electrospray ionisation (ESI) interface and operated with Xcalibur software (Thermo Fisher Scientific).

Separations were done on a ZORBAX SB-C18 RR 2.1× 100 mm 3.5-μm column from Agilent Technologies (Waldbronn, Germany).

Solutions

Mobile-phase solutions

Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (ACN) and ultrapure water from Merck (Darmstadt, Germany) and formic acid (FA) 99% from Biosolve (Valkenswaard, the Netherlands). The mobile phase constituted of three solutions: ultrapure water (solution A), ACN (solution B) and FA 1% (solution C).

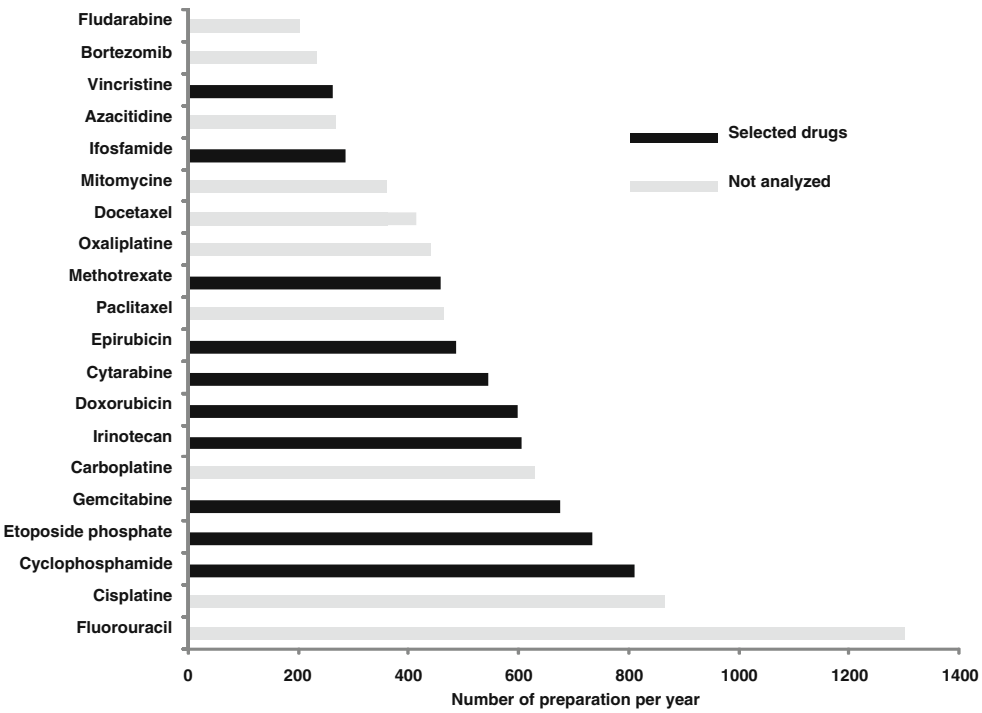

Fig. 2 Overview of the 20 most prepared cytotoxic drugs at the pharmacy of Geneva University Hospitals in 2009. *Black backgrounds* represent the selected compounds

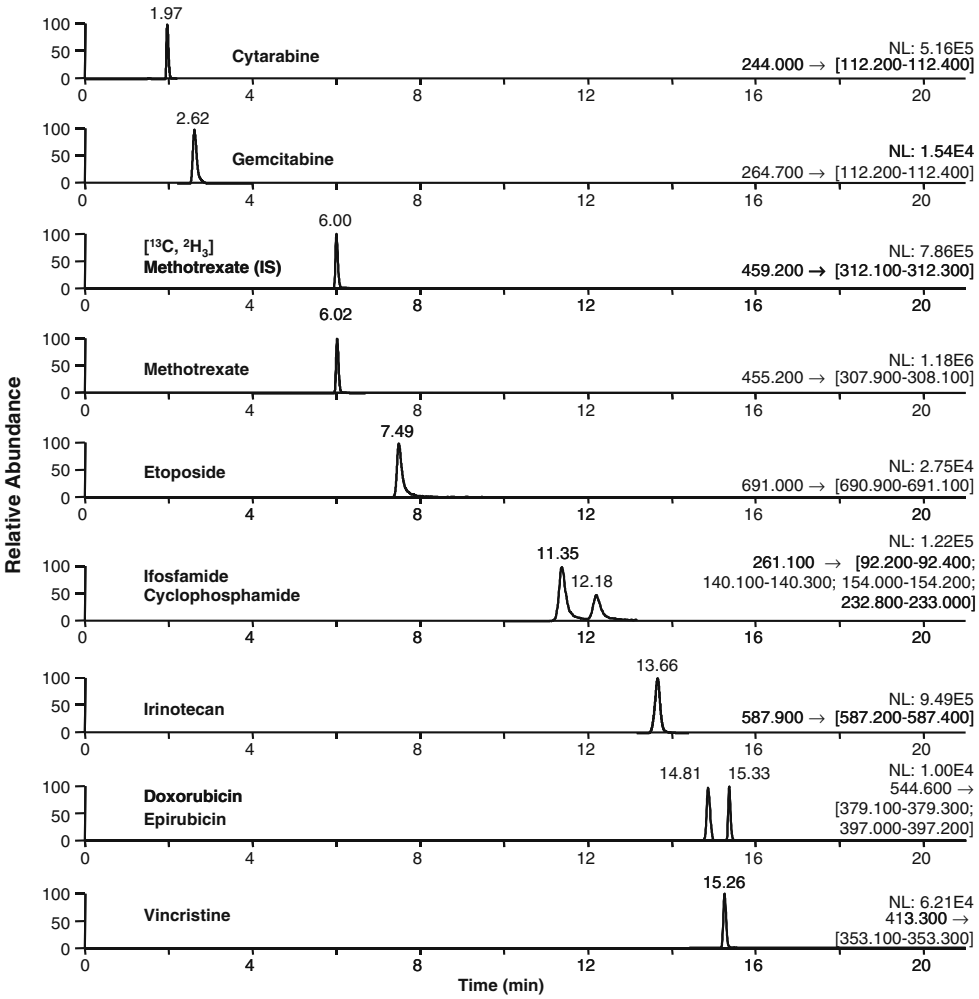

Fig. 3 LC–MS/MS chromatogram of a sample containing ten cytotoxic drugs and the internal standard at 50 ng mL^{-1}. Column: ZORBAX SB-C18 RR 2.1×100 mm 3.5 μm; flow rate 200 μL min^{-1}; gradient conditions and scan events are reported in Tables 2 and 3, respectively

New solvents were prepared for each series of analysis. Washing of the needle and the injection loop was performed with 5% ACN in water after each injection.

Cytotoxic stock solutions, calibration standards, validation standards and internal standard

The operator prepared all solutions (i.e. drug reconstitutions and sample dilution) in appropriate conditions for handling hazardous compounds as cytotoxic agents. Moreover, the development of the method was performed with drug specialities to avoid direct contact of the operator to cytotoxic powder and to minimise contamination risk by preparing working solutions.

A main stock solution (S1) containing the ten cytotoxic drugs was prepared by diluting each compound in water at a concentration of 20 μg mL^{-1} (see Table 1). This solution was further diluted to obtain two independent intermediate stock solutions: S2 at 2 μg mL^{-1} and S3 at 200 ng mL^{-1} in FA 0.1%. The calibration standards and validation standards were prepared by diluting S2 and S3 to 12 concentration levels in FA 0.1% (0.25–200 ng mL^{-1}). All samples were immediately stored at 15 °C in the LC auto-sampler and analysed within the day.

Aliquots of the IS, [^{13}C, $^{2}H_3$]-methotrexate, were prepared with a mixture of ACN and water (75:25 *v*/*v*) at 250 μg mL^{-1} and stored at −22°C for 6 months. No sample degradation could be observed. Stock solutions of IS were regularly diluted at 1 μg mL^{-1} in water, and they were stable for at least 2 weeks at 2–8 °C.

LC–MS/MS conditions

The mobile-phase flow rate was set at 200 μL min^{-1} using the gradient elution programme described in Table 2. The thermostated auto-sampler was maintained at 15 °C, and the injection volume was 25 μL.

Positive ESI conditions were capillary temperature set at 325 °C, spray voltage at 4 kV and sheath and auxiliary gas (nitrogen) flow rate at 45 and 2 psi, respectively. MS/MS was acquired in selected reaction monitoring (SRM) mode in Q1 and Q3. The Q2 collision gas (argon) pressure was set at 1.5 mTorr. Determination of Q2 potential settings and MS/MS transitions (Q1 and Q3) was carried out by direct

Table 4 Validation results for the simultaneous analysis of ten cytotoxic drugs by LC–MS/MS

	Cytarabine	Gemcitabine	Methotrexate	Etoposide phosphate	Ifosfamide	Cyclophosphamide	Irinotecan	Doxorubicin	Epirubicin	Vincristine
Trueness (%)	99–102	96–109	97–104	99–106	98–101	99–104	90–106	86–103	89–103	91–100
Repeatability (%)	1–3	1–5	1–2	1–3	1–8	1–4	1–4	2–10	2–9	1–7
Intermediate precision (%)	1–4	2–9	1–2	1–7	2–8	2–5	3–10	3–10	2–13	1–10
Limit of detection (ng mL^{-1})	0.025	0.25	0.01	0.5	0.25	0.25	0.025	0.5	0.5	0.25
Limit of quantification (ng mL^{-1})	0.25	0.5	0.25	1	1	0.5	1	2	2	1
Linearity range (ng mL^{-1})	1–200	1–200	1–200	1–200	1–200	1–200	1–200	2–200	2–200	1–200
Determination coefficient (r^2)	0.9996	0.9994	0.9997	0.9994	0.9993	0.9990	0.9992	0.9962	0.9976	0.9988

Trueness, repeatability and intermediate precision established at 12 concentration levels ranging from lowest to upper LOQ

infusion of each cytotoxic drug solution at a concentration of 1 μg mL^{-1} diluted in 50:50 of water/methanol with 0.1% FA. Selected *m*/*z* transitions and collision energy for each analyte are reported in Table 3. Seven segments with nine scan events of data acquisition were programmed in the positive mode during the entire analytical run (Table 3).

Chromatographic data acquisition, peak integration and quantification were performed using the Xcalibur software (ThermoQuest, San Jose, CA, USA).

Method validation

Method validation was performed to estimate quantitative performance of the analytical method. The validation was carried out over three series: each series involved (1) freshly prepared calibration, validation samples and solvents, (2) washing of the column and LC system, (3) LC shutoff, (4) cleaning the capillary and cone of the MS with water and methanol and (5) tuning/calibration of the MS system. Calculations were performed using area ratios of the ten cytotoxic drugs on the IS ($[^{13}C, {}^{2}H_3]$-methotrexate). There were two independent sample preparations (calibration and validation samples) at 12 concentration levels (0.25–200 ng mL^{-1}) with injections in triplicate.

Application to cytotoxic formulations and environmental samples

For quality control, cytotoxic drugs were determined in formulations prepared at the Geneva University Hospitals pharmacy. Therefore, formulations were diluted in 0.1% FA to obtain a final concentration of 200 ng mL^{-1} with 50 ng mL^{-1} of the IS.

For environmental applications, a standard solution of the ten cytotoxic drugs was spiked over a polypropylene infusion bag (NaCl 0.9% 50 mL) and over a stainless steel surface (100 cm^2) to obtain a concentration of 2.5 ng cm^{-2}. The spiked area was wiped with a blotting paper (Whatman 903®) and an aqueous solution of IS was added. Then, drugs were extracted from the paper in 5 mL of FA 0.1% in glass vials by ultrasonication for 15 min. The procedure was repeated in triplicate (N=3) for each surface.

Results and discussion

Method development

Selection of the cytotoxic agents and internal standard

The developed LC–MS/MS method allows the determination of ten cytotoxic drugs, namely cytarabine, gemcitabine,

methotrexate, etoposide phosphate, cyclophosphamide, ifosfamide, irinotecan, doxorubicin, epirubicin and vincristine. These ten compounds were selected among the 20 most prepared cytotoxic drugs at the pharmacy of the Geneva University Hospitals (Fig. 2). The selection was a compromise between the most prescribed drugs and their toxicity and analytical considerations. For example, 5-fluorouracil, which is the most prepared chemotherapy drug in 2008, was not included because it is not classified as a carcinogen for humans [20], and a contamination of this compound is less hazardous for exposed personal. Furthermore, 5-fluorouracil is a very polar compound poorly retained in our analytical conditions. Three platinum compounds (i.e., cisplatin, carboplatin and oxaliplatin) presented a high percentage of prescribed chemotherapies and belong to the most toxic compounds, but they need other detection techniques such as ICP–MS or voltammetry [17, 18, 21, 22] and were thus not included in this study. Finally, the ten selected drugs belong to different cytotoxic families with different toxicities, giving an excellent overview of possible contamination.

In order to exclude a cross contamination of the IS, a deuterated compound was chosen: [^{13}C, ^{2}H$_3$]-methotrexate was used as IS as it exhibited high ESI–MS/MS response and was eluted in the middle retention time window of the ten investigated cytotoxic drugs.

Optimisation of LC–ESI–MS/MS conditions

The described method enables the quantification of ten cytotoxic compounds with a mobile-phase composition of water, acetonitrile and 0.1% formic acid. With the selected gradient (Table 2), vincristine and epirubicin were the only unresolved cytotoxic drugs but presented different scan events in SRM mode. All other compounds were separated in less than 16 min (21 min including the column reconditioning). A typical chromatogram, obtained from the analysis of a calibration sample with 50 ng mL^{-1} of each analyte and 50 ng mL^{-1} of IS in FA 0.1%, is shown in Fig. 3.

ESI–MS/MS conditions were optimised for each analyte, and the best compromise for all compounds was chosen. All drugs were analysed in positive mode. Different values for capillary temperature, spray voltage, sheath and auxiliary gas were tested (data not shown). Response signals of compounds increased with capillary temperature, but

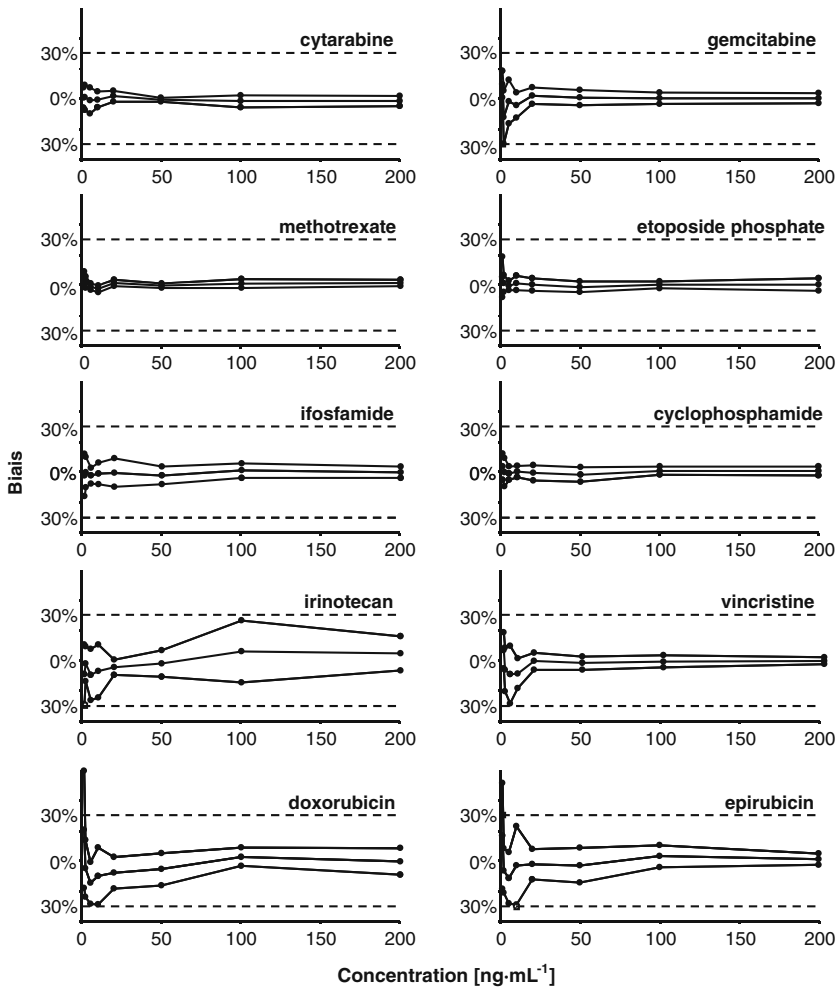

Fig. 4 Accuracy profile for the quantification of ten cytotoxic drugs by LC–MS/MS in the concentration range from 1 to 200 ng mL^{-1}, calculated from the trueness and intermediate precision reported in Table 4; *dashed lines* represent the acceptance limits of ±30%

Table 5 Analysis of the ten cytotoxic drugs by LC–MS/MS in quality control samples and on a spiked surface

	Cytarabine	Gemcitabine	Methotrexate	Etoposide phosphate	Ifosfamide	Cyclophosphamide	Irinotecan	Doxorubicin	Epirubicin	Vincristine
Quality control of drug formulations										
CYT/10-115058 gemcitabine 1,800 mg in 340 mL NaCl 0.9%	–	97±2%	–	–	–	–	–	–	–	–
CYT/10-114026 methotrexate 30 mg in 62 mL glucose 5%	–	–	97±2%	–	–	–	–	–	–	–
CYT/10-113521 ifosfamide 2,316 mg in 1,080 mL glucose 5%	–	–	–	–	97±2%	–	–	–	–	–
CYT/10-116360 cyclophosphamide 860 mg in 293 mL glucose 5%	–	–	–	–	–	100±2%	–	–	–	–
CYT/10-116778 doxorubicin 44 mg in 72 mL NaCl 0.9%	–	–	–	–	–	–	–	106±4%	–	–
CYT/10-115322 epirubicin 190 mg in 145 mL NaCl 0.9%	–	–	–	–	–	–	–	–	98±4%	–
CYT/10-116578 vincristine 1 mg in 51 mL in glucose 5%	–	–	–	–	–	–	–	–	–	93±2%
Environmental applications										
Wiping sample 1:250 ng of the 10 drugs spiked on a polypropylene infusion bag (NaCl 0.9% 50 mL)	94±10%	91±10%	82±2%	85±2%	84±4%	84±8%	49±12%	33±10%	29±10%	18±12%
Wiping sample 2:250 ng of the 10 drugs spiked on a stainless steel surface (100 cm^2)	89±10%	89±10%	67±8%	74±12%	86±12%	85±10%	34±18%	25±8%	23±12%	20±14%

doxorubicin and epirubicin were degraded at temperatures above 325 °C. Final optimised conditions were obtained with a spray voltage of 4 kV, a capillary temperature at 325°C, sheath gas at 45 psi and auxiliary gas at 2 psi. The *m*/*z* transitions and collision energy for each analyte were successfully determined and reported in Table 3.

Triple quadrupole instruments provide excellent sensitivity and selectivity in SRM. Additionally, the MS/MS method can be segmented into various time windows containing different SRM events to increase the signal-to-noise ratio. Thus, seven time segments with nine scan events of data acquisition were used during the entire analytical run. This approach is particularly useful in environmental monitoring because of the relatively low concentration of each individual analyte. With the presented method, cytarabine, gemcitabine, etoposide phosphate and irinotecan were detected in their own time segment with a specific scan event. Doxorubicin and epirubicin, as cyclophosphamide and ifosfamide, were analysed with the same scan event in the same segment, but they were well separated by LC. Vincristine was detected in the same segment as doxorubicin and epirubicin, but with another scan event. Therefore, all cytotoxic drugs could be quantified in satisfied analytical conditions.

Method validation

The method was validated with calibration and validation samples in 0.1% formic acid. Quantitative performance was estimated in three separate series at 12 concentration levels, with three repetitions for calibration standard and three repetitions for validation standard. As a result, 72 injections were carried out per series, for a total of 216 analyses.

Calibration model and concentration range

From all calibration standards, different regression models were tested to determine the best response function for the ten cytotoxic drugs: a weighted linear regression model with a weight equal to $1/x$ gave the best quantitative performance in the studied concentration range with a determination coefficient (r^2) of about 0.999 and superior to 0.996 for all tested compounds (Table 4).

The limit of detection was set at a signal-to-noise ratio of 3:1. LODs were significantly below 1 ng mL^{-1} for all cytotoxic drugs (Table 4). The lowest limit of quantification (LOQ) was determined to ensure relative standard deviation (RSD) inferior to 15%. Lowest LOQs were determined between 0.25 and 2 ng mL^{-1} for all cytotoxic drugs (Table 4). The upper LOQ was set at 200 ng mL^{-1} for all cytotoxic drugs. Higher LOQ values could theoretically be used, but in order to avoid unnecessary exposure of the operator to the cytotoxic agents, concentrations above 200 ng mL^{-1} were not analysed.

Due to practical considerations, the concentration range for all cytotoxic drugs was limited from 1 to 200 ng mL^{-1}.

Accuracy, trueness and precision

Concentrations of validation standards were calculated from the calibration model. Trueness was expressed in percent as the ratio between theoretical and average measured values at each concentration level. Trueness values were between 85% and 110%, as reported in Table 4.

Repeatability and intermediate precision were expressed as RSD of the ratio of the intra-day standard deviation (sr) and between-day standard deviation (sR), respectively. The sr and sR values were obtained using ANOVA analyses. RSD values below 15% were obtained for each compound (Table 4).

To visualise the overall method variability, the accuracy profile of each cytotoxic drug was built from 1 to 200 ng mL^{-1}, combining trueness and intermediate precision as the confidence interval [23]. As presented in Fig. 4, the total error did not exceed ±30% for all compounds in their quantification concentration range. Only doxorubicin and epirubicin presented a superior limit at 1 ng mL^{-1} outside the tolerance of 30%, further justifying the selected LOQ of 2 ng mL^{-1} for these two compounds ("Calibration model and concentration range").

Consequently, the developed LC–ESI–MS/MS method presents quantitative performance fully compatible with environmental monitoring of cytotoxic drugs on surfaces or in combination with a sample preparation for biological sample analyses. Alternatively, the method can be used for quality control or stability studies of pharmaceutical formulations due to the very satisfactory performance at 200 ng mL^{-1} with a total error inferior to 5%.

Applications

In order to demonstrate the applicability of the LC–ESI–MS/MS method to real samples, determination of the ten cytotoxic agents was achieved in pharmaceutical formulations for quality control and in wiping samples for environmental monitoring. Concentrations of the cytotoxic agents were calculated with reference to a calibration curve constructed the same day with five levels of calibration standard (1, 2, 10, 50 and 200 ng mL^{-1}) containing the ten drugs and weighted linear regression with a weight equal to $1/x$ for each compound.

For quality control, pharmaceutical formulations were analysed by diluting the samples to a target value of 200 ng mL^{-1}. As shown in Table 5, concentrations of the tested pharmaceutical formulations were found to be ±10% (including both trueness and precision) of the prescribed concentration which corresponds to the acceptance limit for preparations of the HUG pharmacy.

The method was also successfully applied to environmental samples. The recovery rate was determined for each cytotoxic compound according to the surface type (see Table 5).

Conclusions

A simple LC–ESI–MS/MS method was successfully developed for the simultaneous quantification of ten cytotoxic drugs (cytarabine, gemcitabine, methotrexate, etoposide phosphate, cyclophosphamide, ifosfamide, irinotecan, doxorubicin, epirubicin and vincristine) in 21 min, gradient equilibration time included. This method was validated and exhibited satisfactory quantitative performance in terms of limit of quantification, domain range, trueness and precision: the accuracy profile showed total errors inferior to ±30% for all compounds in their quantification domain range, from 1 or 2 ng mL^{-1} up to 200 ng mL^{-1}, and total errors inferior to ±5% at 200 ng mL^{-1}. Therefore, the method can be used for different applications, as shown by its successful utilisation for quality control of pharmaceutical formulations and environmental monitoring.

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Article V

« Wipe sampling procedure coupled to LC-MS/MS analysis for the simultaneous determination of 10 cytotoxic drugs on different surfaces»

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ORIGINAL PAPER

Wipe sampling procedure coupled to LC–MS/MS analysis for the simultaneous determination of 10 cytotoxic drugs on different surfaces

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Abstract A simple wipe sampling procedure was developed for the surface contamination determination of ten cytotoxic drugs: cytarabine, gemcitabine, methotrexate, etoposide phosphate, cyclophosphamide, ifosfamide, irinotecan, doxorubicin, epirubicin and vincristine. Wiping was performed using Whatman filter paper on different surfaces such as stainless steel, polypropylene, polystyrol, glass, latex gloves, computer mouse and coated paperboard. Wiping and desorption procedures were investigated: The same solution containing 20% acetonitrile and 0.1% formic acid in water gave the best results. After ultrasonic desorption and then centrifugation, samples were analysed by a validated liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) in selected reaction monitoring mode. The whole analytical strategy from wipe sampling to LC–MS/MS analysis was evaluated to determine quantitative performance. The lowest limit of quantification of 10 ng per wiping sample (i.e. 0.1 ng cm^{-2}) was determined for the ten investigated cytotoxic drugs. Relative standard deviation for intermediate precision was always inferior to 20%. As recovery was dependent on the tested surface for each drug, a correction factor was determined and applied for real samples. The method was then successfully applied at the cytotoxic production unit of the Geneva University Hospitals pharmacy.

Keywords Cytotoxic · Antineoplastic drugs · Surface contamination · Environmental monitoring · LC–MS/MS · Wipe sampling

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Introduction

Cytotoxic drugs are widely used in cancer therapy, and an increasing number of patients receiving chemotherapy have been observed during the last decades. These drugs have been recognized as hazardous for healthcare professionals such as oncology nurses or pharmacists and technicians [1]. Despite the existence of safety standards for handling cytotoxic drugs, several studies report low-level contamination of these compounds on workbenches, floors, vials, gloves and storage shelves [2–10]. Traces of cytotoxic agents have also been found in urine [11–17] and blood of healthcare professionals [18], indicating that this working population is at risk. Nevertheless, a direct relationship between exposure to cytotoxic contamination and harmful effects has not been established yet, and no maximal acceptable amount of surface contamination for these drugs has been set up by regulatory offices until now. According to precautionary principles, exposure should nevertheless be kept at the lowest possible level [19]. Monitoring of contamination is essential to ascertain the contamination risk in order to improve working conditions by effective cleaning procedures or other measures.

In Germany, threshold guidance values for platinum and 5-fluorouracil have been set on the basis of data from a large multicenter study including 102 pharmacies [20, 21]. Wiping samples have been obtained by compound-specific wiping procedures and analysed by voltammetry for platinum drugs and by GC-MS for fluorouracil. This approach presented very good quantitative performance and detection limits (i.e., 0.1–1 pg per sample) and estimated potential surface contamination using platinum drugs or 5-FU as model markers. However, a wide range of chemotherapy formulations with different drugs and with different preparation procedures are produced daily in hospital cytotoxic units. To get an overview of several contaminations, multi-compound methods are required. To the author's knowledge, only few generic wiping procedures have been developed, for example a wipe sampling procedure coupled to liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the simultaneous determination of gemcitabine, paclitaxel, cyclophosphamide and ifosfamide [22] or for methotrexate, 5-fluorouracil and cyclophosphamide [9]. Other toxic compounds, such as irinotecan, anthracyclines or vincalcaloïdes, which are also largely used in chemotherapies, have rarely been included in surface contamination studies.

Recently, we developed and validated a LC–MS/MS method for the simultaneous quantification of ten cytotoxic drugs [23]. In the continuity of this method, we present the development of a wiping and extraction procedure of the same ten cytotoxic drugs on different surfaces and their quantitative analysis by the LC–MS/MS method. This wipe sampling procedure was then applied to evaluate the surface contamination in the cytotoxic production unit at the pharmacy of Geneva University Hospital (HUG).

Experimental

Chemicals and reagents

The study was performed with the following commercially available cytotoxic drugs: Campto® (irinotecan, 20 mg mL^{-1}) and Cytosar® (cytarabine, 20 mg mL^{-1}) were purchased from Pfizer AG (Zürich, Switzerland); Gemcitabin Teva® (gemcitabine reconstituted in water at 20 mg mL^{-1}) and Vincristin Teva® (vincristine, 1 mg mL^{-1}) from Teva Pharma AG (Aesch, Switzerland); Holoxan (ifosfamid reconstituted in water at 40 mg mL^{-1}) and Endoxan® (cyclophosphamide reconstituted in glucose 5% at 20 mg mL^{-1}) from Baxter AG (Volketswil, Switzerland); Methotrexat Farmos® (methotrexate, 2.5 mg mL^{-1}) from Orion Pharma (Zug, Switzerland); Etopophos® (etoposide phosphate reconstituted in water at 20 mg mL^{-1}) from Bristol-Myers Squibb SA (Baar, Switzerland); Doxorubine Ebewe® (doxorubicin, 2 mg mL^{-1}) from Ebewe Pharma (Cham, Switzerland); and Epirubicin Actavis Solution® (epirubicin, 2 mg mL^{-1}) from Actavis (Regensdorf, Switzerland).

The reconstitution of Etopophos, Gemcitabine Teva and Holoxan was performed with water for injection, obtained from Bichsel Laboratories (Interlaken, Switzerland). Glucose 5% for the reconstitution of Endoxan was from Sintetica-Bioren SA (Couvet, Switzerland). The internal standard (IS) [^{13}C, $^{2}H_3$]-methotrexate was purchased from Alsachim (Illkirch, France).

Wiping and desorption material

The evaluated materials for wipe sampling were filter paper (Protein Saver™ 903 Card) from Whatman (Dassel, Germany), filter card DMPK-300 from Ahlstrom (Helsinki, Finland) and blend wipers TX 612 from Texwipe (Cabuyao, Philippines).

Desorption was performed in 1.5-mL polyethylene (PE) safe-lock tubes from Eppendorf AG (Hamburg, Germany).

Equipment

Analyses were carried out with the liquid chromatography system Accela from Thermo Fisher Scientific Inc. (Waltham, MA, USA) consisting of a quaternary pump equipped with an online degasser, an autosampler and a solvent platform. The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Discovery MS from Thermo Fisher Scientific equipped with an Ion Max electrospray ionization (ESI) interface and operated with Xcalibur software (Thermo Fisher Scientific). Separations were done on a ZORBAX SB-C18 RR 2.1 × 100-mm, 3.5-μm column from Agilent Technologies (Waldbronn, Germany).

Solutions

Mobile phase solutions

Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (ACN) and ultrapure water from Merck (Darmstadt, Germany) and formic acid (FA) from Biosolve (Valkenswaard, the Netherlands). The mobile phase used for chromatography was constituted of three solutions: ultrapure water (solution A), ACN (solution B) and FA 1% (solution C). Washing of the needle and the injection loop was performed with 5% ACN in water after each injection.

Cytotoxic stock solutions, calibration standard, internal standard, desorption and wiping samples

All solutions (i.e. drug reconstitutions and sample dilution) were prepared in appropriate conditions for handling

hazardous compounds as cytotoxic agents. Moreover, the development of the wiping procedure was performed with brand drugs to avoid direct contact of the operator to the cytotoxic powder and to minimize contamination risk when preparing the working solutions.

Aliquots of the IS were prepared with a mixture of ACN and water (75:25, *v*/*v*) at 250 μg mL^{-1} and stored at −22°°C for 12 months. No sample degradation was observed. Stock solutions of IS were regularly diluted at 50 ng mL^{-1} in ACN 20% with FA 0.1%, and they were stable for at least 2 weeks at 2–8°°C.

A main stock solution containing the ten cytotoxic drugs was prepared by diluting each compound in water at a concentration of 20 μg mL^{-1}. This solution was further diluted to obtain five independent stock solutions at 20, 40, 200, 1,000 and 4,000 ng mL^{-1} in ACN 20% with FA 0.1%. For calibration standards (CS), these solutions were diluted by the IS solution at 50 ng mL^{-1} to obtain five CS at 1, 2, 10, 50 and 200 ng mL^{-1}. For desorption samples, the cytotoxic stock solutions were spiked on a wiping paper and desorbed with 1 mL of the IS solution at 50 ng mL^{-1}. For wiping samples, 50 μL of cytotoxic stock solutions was spread over the studied surface (10×10 cm) by an adjustable volume micropipette, followed by wiping and desorption with 1 mL of the IS solution at 50 ng mL^{-1}. The final concentrations of the ten cytotoxic compounds were at 10, 50 and 200 ng mL^{-1} for desorption and wiping samples. All samples were immediately stored at 15°°C in the LC autosampler and analysed within the day.

Analytical conditions

LC–MS/MS method

The LC–MS/MS conditions have been described elsewhere [23]. Briefly, the mobile phase flow rate was set at 200 μL min^{-1} using the following gradient elution programme with a constant concentration of 0.1% FA during the entire run: 2% of ACN at 0–2 min, 21% of ACN at 2.5–10 min, 30% of ACN at 13 min, 50% of ACN from 13.5 to 15.5 min and a re-equilibration step to the initial solvent from 16 up to 21 min. The thermostated autosampler was maintained at 15°C and the injection volume was 25 μL. Positive ESI conditions were: capillary temperature set at 325°°C, spray voltage at 4 kV, and sheath and auxiliary gas (nitrogen) flow rates at 45 and 2 psi, respectively. MS/MS was acquired in selected reaction monitoring mode in Q1 and Q3. The Q2 collision gas (argon) pressure was set at 1.5 mTorr. Chromatographic data acquisition, peak integration and quantification were performed using the Xcalibur software (ThermoQuest, San Jose, CA, USA). Different time segments were used as reported in Table 1.

Desorption step development

Standard solution of the ten cytotoxic drugs at 1,000 ng mL^{-1} and the IS at 1,000 ng mL^{-1} were spotted on wiping papers to obtain a final amount of 50 ng per sample for each compound. Then, desorption was performed with different desorption solutions (FA 0.1%, 20% ACN with 0.1% FA) and desorption procedures (gentle mixture, ultrasonication for 5 and 20 min). Before LC–MS/MS analysis, a centrifugation step at 2,880 RCF for 5 min was performed to obtain samples without particulate matter such as fibres from the filter paper. Each desorption procedure was repeated in triplicate (N=3).

The best desorption procedure was then evaluated with three series at three concentrations (10, 50 and 200 ng mL^{-1}), with four repetitions each. Cytotoxic solutions at 200, 1,000, 4,000 ng mL^{-1} were spiked on the filter paper to obtain 10, 50 and 200 ng per sample, put in a PE tube, and 1 mL of the desorption solution (20% ACN with 0.1% FA)

Table 1 Instrument method for the LC–MS/MS analysis of the ten cytotoxic drugs with [^{13}C, $^{2}H_3$]-methotrexate as internal standard: time segment description

Time segment (min)	Drug	Parent (*m*/*z*)	Product (*m*/*z*)	Collision energy (eV)	Mean RT (min)
0–4	Cytarabine	244.0	112.3	15	1.6
	Gemcitabine	264.7	112.3	20	1.6
4–7	Methotrexate	455.2	308.0	20	6.0
	[^{13}C,$^{2}H_3$] methotrexate	459.2	312.2	20	6.0
7–10	Etoposide phosphate	691.0	691.0	15	7.5
10–13	Ifosfamide	261.1	92.3; 140.2 154.1, 232.9	20	11.4
	Cyclophosphamide	261.1	92.3; 140.2 154.1, 232.9	20	12.2
13–14	Irinotecan	587.9	587.3	20	13.7
14–21	Doxorubicin	544.6	379.2, 397.1	15	14.8
	Epirubicin	544.6	379.2, 397.1	15	15.3
	Vincristine	413.3	353.2	30	15.2

containing the IS at 50 ng mL^{-1} was added. Desorption was performed by ultrasonication for 20 min followed by centrifugation at 2,880 RCF for 5 min. Finally, 0.5 mL of the clear solution was placed in a vial and LC–MS/MS analysis was performed.

Wiping step development

Standard solutions of the ten cytotoxic drugs at 1,000 ng mL^{-1} in water were spread over a stainless steel plate (10×10 cm) with an adjustable volume micropipette to obtain a final concentration of 0.5 ng cm^{-2} for each drug. After solvent evaporation, wiping was performed with three different wiping papers (Whatman, Ahlstrom and Texwipe). Filter papers were previously wetted with different wiping solutions (water, NaOH 0.01 M, FA 0.1%, ACN 20% with 0.1% FA, ACN 50% with 0.1% FA, ACN with 0.1% FA and isopropyl alcohol 70%) and the tested surface wiped using tweezers. Each wiping procedure was followed by the desorption procedure, and LC–MS/MS analysis was repeated in triplicate (N=3).

Quantitative performance of the method applied to different surfaces

Quantitative performance was evaluated with the best wiping and desorption conditions for different surface types, i.e. stainless steel, polypropylene infusion bags,

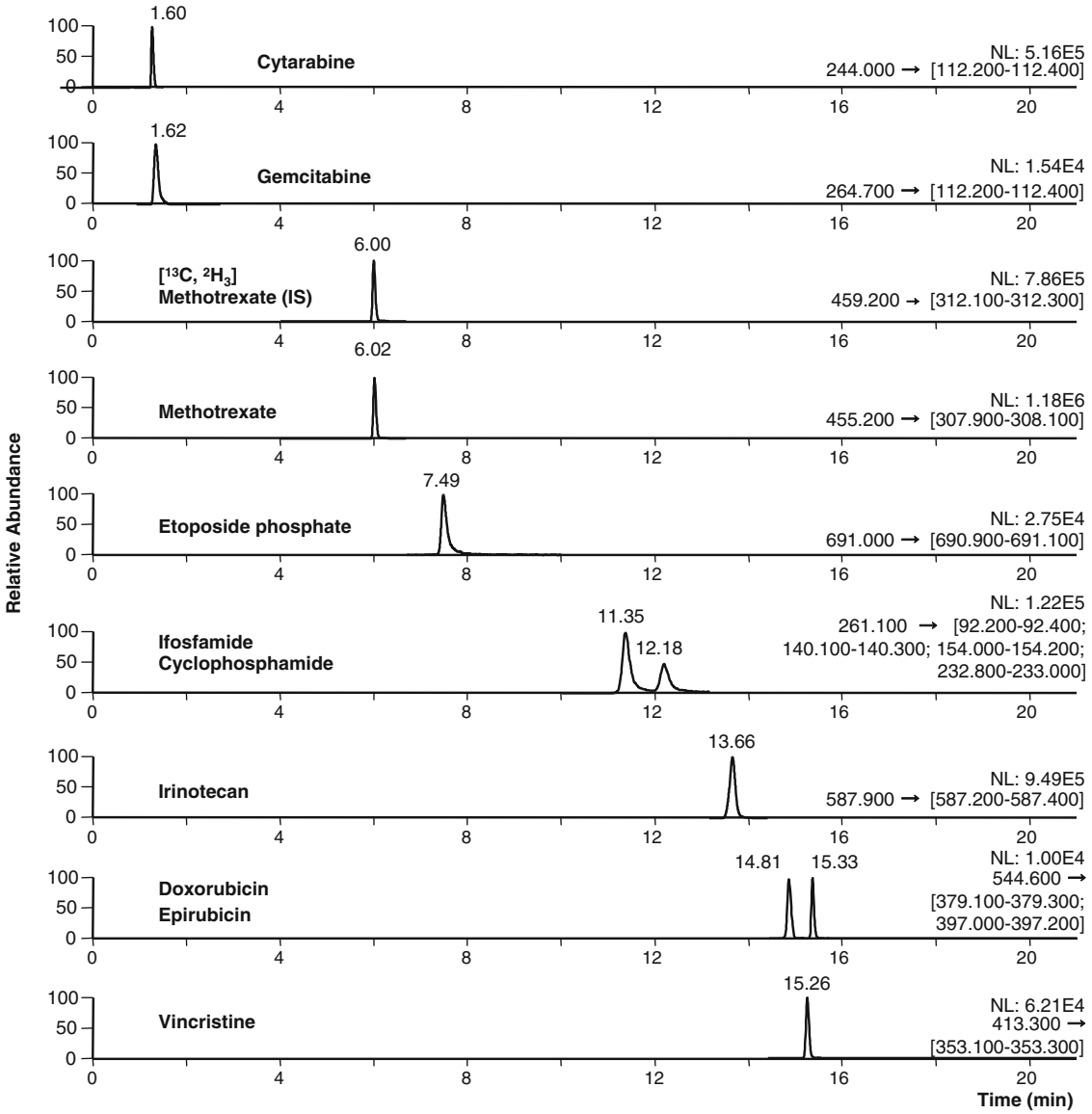

Fig. 1 LC–MS/MS chromatogram of a calibration sample containing the ten cytotoxic drugs and the internal standard at 50 ng mL^{-1} in ACN 20% with 0.1% FA. Column: ZORBAX SB-C18 RR 2.1 × 100 mm, 3.5 μm; flow rate, 200 μL min^{-1}; gradient conditions are reported in "Experimental" and scan events are shown in Table 1

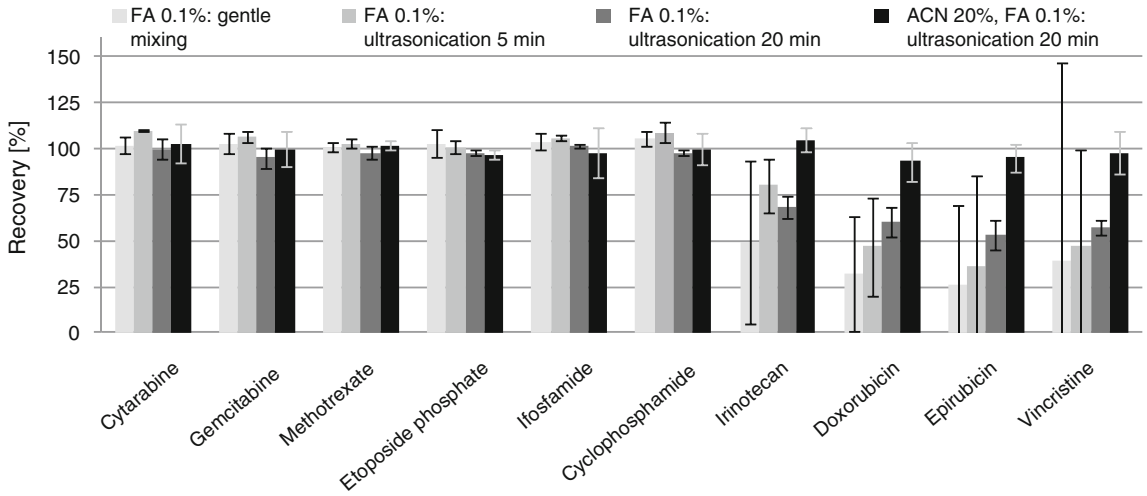

Fig 2 Desorption step development with Whatman papers: recovery of cytotoxic drugs as function of different procedures: FA 0.1% with gentle mixing, FA 0.1% with ultrasonication for 5 min, FA 0.1% with ultrasonication for 20 min, and 20% ACN, FA 0.1% with ultrasonication for 20 min

polystyrol bags, glass, latex gloves, computer mouse and coated paperboard. Solutions containing the ten cytotoxic drugs at 200, 1,000 and 4,000 ng mL^{-1} were spiked on a stainless steel or polypropylene surface to obtain a final surface concentration of 0.1, 0.5 and 2 ng cm^{-2}. For the other surfaces, only one concentration (0.5 ng cm^{-2}) was studied. After solvent evaporation for at least 1 h, wiping with Whatman filter paper wetted with 0.1 mL of ACN 20% with 0.1% FA was performed. Desorption was carried out with 1 mL of ACN 20% with 0.1% FA containing the IS at 50 ng mL^{-1} by ultrasonication for 20 min and then centrifugation at 2,880 RCF for 5 min. Finally, 0.5 mL of the clear solution was placed in a vial, which was followed by LC–MS/MS analysis.

Evaluation of quantification performance was performed over three independent series with three repetitions for each surface and concentration. Each series involved (1) freshly prepared calibration, wiping samples (corresponding to the whole procedure: to spread cytotoxic stock solutions on surfaces, evaporation and dried surface wiping) and solvents (LC, desorption and wiping solvents); (2) washing of the column and LC system; (3) LC shut off; (4) cleaning the capillary and cone of the MS with water and methanol; and (5) tuning/calibration of the MS system.

Table 2 Quantitative performance for the desorption of the ten cytotoxic drugs on Whatman filter paper (50 ng per sample) with 1 mL of ACN 20% with FA 0.1%

	Recovery (%)	Intermediate precision (%)
Cytarabine	102	5.7
Gemcitabine	99	5.2
Methotrexate	99	1.6
Etoposide phosphate	95	1.9
Ifosfamide	102	7.6
Cyclophosphamide	100	4.9
Irinotecan	102	3.7
Doxorubicin	93	3.9
Epirubicin	94	3.6
Vincristine	98	6.4

Concentrations of the cytotoxic agents were calculated with reference to a calibration curve constructed the same day with five levels of CS containing the ten drugs (1, 2, 10, 50 and 200 ng mL^{-1} in ACN 20% with 0.1% FA) and weighted linear regression ($1/x$) for each compound. Calculations were performed using peak area ratios of the ten cytotoxic drugs versus the IS.

Stability of cytotoxic drugs on wiping papers

The stability of the cytotoxic drugs on the filter paper was studied over 3 months. A solution containing the ten compounds was spiked on the Whatman filter paper and the papers kept at 25, at 4 and at −22°C. Desorption and LC–MS/MS analyses were performed in triplicate on day 0, week 1, and months 2 and 3. Concentrations of the cytotoxic agents were calculated with reference to a calibration curve constructed the same day with five levels of CS (1, 2, 10, 50 and 200 ng mL^{-1} in ACN 20% with 0.1% FA containing the ten drugs) and weighted linear regression ($1/x$) for each compound.

Application to surface contamination at the cytotoxic production unit of HUG pharmacy

For the proof of concept, the developed method was applied to the measurement of cytotoxic contamination at HUG pharmacy. Several surfaces of 10×10 cm were tested in the

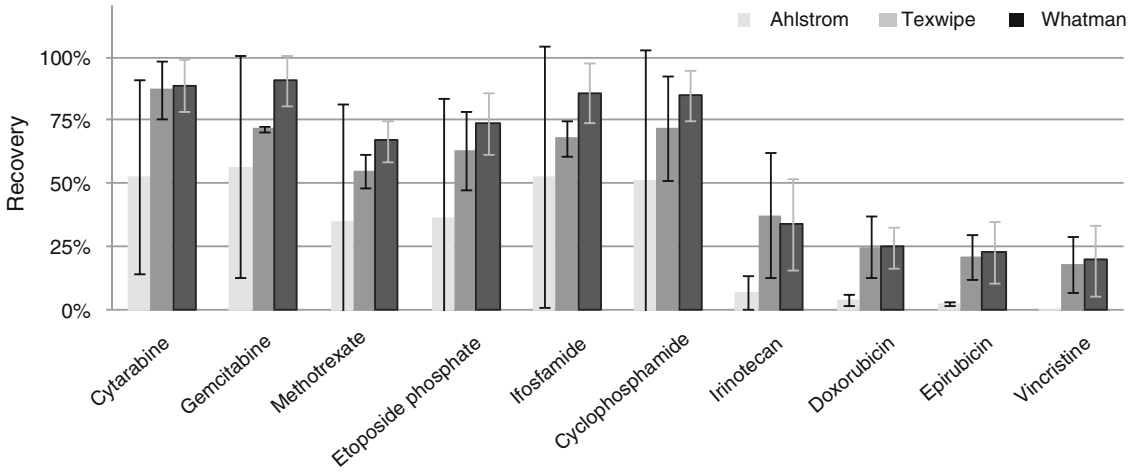

Fig 3 Recovery of ten cytotoxic drugs from a stainless steel surface obtained with different wiping papers (Ahlstrom, Texwipe, Whatman) previously wetted by water during wiping step development

cytotoxic unit, in the preparation and the logistic areas. However, for some special wiping places such as the door handle and phone, the wiping surface was reduced for practical reasons. Concentrations of the cytotoxic drugs were calculated with reference to a calibration curve constructed the same day with five levels of CS (1, 2, 10, 50 and 200 ng mL^{-1} in ACN 20% with 0.1% FA containing the ten drugs) and weighted linear regression ($1/x$) for each compound. A correcting factor for each surface and compound was applied to calculate the surface concentration (see "Quantitative performance of the method applied to different surfaces").

Results and discussion

Three steps can be distinguished during the wipe sampling procedure: (1) wiping of cytotoxic drugs from the investigated surface to the filter paper; (2) desorption of drugs from the filter paper to the solution; and (3) LC–MS/MS analysis. Then, quantitative performance of the whole procedure was determined for different surfaces and the stability of the cytotoxic drugs on the wiping papers was studied. The method was finally applied for surface contamination determination at the cytotoxic unit of HUG pharmacy.

LC–MS/MS method

Development and validation of the LC–MS/MS method for the determination of ten cytotoxic drugs has been reported elsewhere [23]. The compounds have been selected among the 20 most prepared cytotoxic drugs at the HUG pharmacy according to toxicity and analytical considerations. These compounds are cytarabine, gemcitabine, methotrexate, etoposide phosphate, cyclophosphamide, ifosfamide, irino-

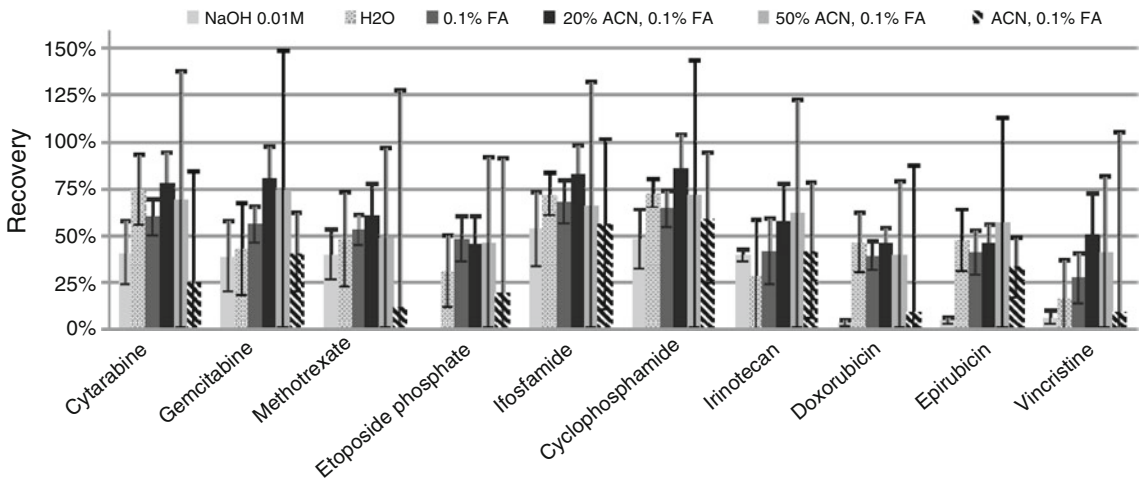

Fig 4 Wiping step development: recovery of cytotoxic drugs as function of different wiping solvents (water, FA 0.1%, NaOH 0.01 M, 20% ACN with 0.1% FA, 50% ACN with 0.1% FA and ACN with 0.1% FA). Wiping was performed with Whatman filter paper on stainless steel surface as described in "Experimental"

tecan, doxorubicin, epirubicin and vincristine. It can be noted that with 20% of ACN in the wiping samples, cytarabine and gemcitabine were not separated by chromatography. The presence of ACN is discussed below, and time segments had to be modified accordingly (Table 1). Calibration samples were also prepared in 20% ACN with FA 0.1%, instead of FA 0.1%. A typical chromatogram, obtained from the analysis of a calibration sample with 50 ng mL^{-1} of each analyte and 50 ng mL^{-1} of the IS in 20% ACN with FA 0.1%, is shown in Fig. 1.

Desorption step development

Desorption of cytotoxic drugs from the filter paper was performed in PE tubes using 1 mL of the desorption solution. This small volume allowed obtaining a small quantity of cytotoxic waste and satisfying quantification limits ("Quantitative performance of the method applied to different surfaces").

Several desorption procedures, including gentle mixing and ultrasonication for 5 and 20 min, were compared using FA 0.1% as the desorption solution. Recovery values and confidence interval were calculated for each assay. As shown in Fig. 2, cytarabine, gemcitabine, methotrexate, etoposide phosphate, ifosfamide and cyclophosphamide were not affected by the desorption procedure and exhibited recoveries close to 100% in all the tested conditions. For the four other compounds, ultrasonication for 20 min improved the desorption rate. In addition, it decreased their confidence interval (inferior to 10%) compared with desorption by gentle mixing and ultrasonication for 5 min.

FA 0.1% was first tested to be fully compatible with the LC–MS/MS method [23], but recoveries of only 50–70% were obtained for irinotecan, doxorubicin, epirubicin and vincristine. In order to increase the recovery of these hydrophobic tested substances, 20% ACN was added. Hence, recoveries were close to 100% for all compounds (Fig. 2).

The desorption procedure with 20% ACN, FA 0.1% and ultrasonication for 20 min was evaluated with three series at three concentrations (10, 50 and 200 ng mL^{-1}), with four repetitions each. Concentrations of the desorption samples were calculated from a calibration model constructed the same day. Recovery was expressed in per cent as the ratio between the theoretical and average measured values at each concentration level. Intermediate precisions were expressed as relative standard deviation (RSD) of the ratio of the between-day standard deviation (sR). The sR values were obtained using ANOVA. Recovery values were between 93% and 102% and intermediate precision inferior to 8% at 10, 50 and 200 ng mL^{-1} for all compounds (Table 2). Therefore, the chosen procedure allowed desorption of the ten cytotoxic drugs from filter paper with satisfying quantitative performance in the studied concentration range.

Table 3 Quantitative performances of the wiping method for the ten cytotoxic drugs on different surfaces at 0.5 ng cm^{-2}

	Surface material	Stainless steel	Polypropylene	Polystyrol	Glass	Latex gloves	Computer mouse	Paperboard
Cytarabine	Recovery (%)	81	79	76	74	58	69	5
	Intermediate precision (%)	8.3	7.8	5.8	7.3	18.0	8.8	2.9
Gemcitabine	Recovery (%)	82	79	76	74	59	81	5
	Intermediate precision (%)	9.5	8.8	6.0	7.7	15.2	6.4	2.5
Methotrexate	Recovery (%)	63	85	75	72	50	64	5
	Intermediate precision (%)	9.8	5.1	5.2	6.5	12.8	9.8	3.2
Etoposide phosphate	Recovery (%)	45	82	73	68	58	81	5
	Intermediate precision (%)	7.8	8.2	10.4	11.7	11.0	22.6	1.9
Ifosfamide	Recovery (%)	82	91	79	85	65	98	7
	Intermediate precision (%)	10.4	8.2	11.2	11.7	17.0	24.8	2.5
Cyclophosphamide	Recovery (%)	86	94	71	80	57	77	5
	Intermediate precision (%)	10.8	4.8	10.0	15.3	11.4	20.4	2.5
Irinotecan	Recovery (%)	57	84	67	65	27	45	15
	Intermediate precision (%)	11.8	11.9	14.5	7.7	11.8	12.0	5.3
Doxorubicin	Recovery (%)	46	54	47	53	20	35	9
	Intermediate precision (%)	5.1	6.1	10.2	10.6	6.8	12.2	3.7
Epirubicin	Recovery (%)	46	58	45	55	16	19	13
	Intermediate precision (%)	5.2	6.2	6.0	7.6	5.0	11.1	10.6
Vincristine	Recovery (%)	50	55	35	56	27	22	13
	Intermediate precision (%)	12.0	11.9	13.3	7.6	13.0	12.3	7.1

Wiping step development

Several wiping procedures were evaluated, including different wiping papers and wiping solvents. Wipe sampling was performed on stainless steel surfaces (10×10 cm) to compare the different procedures in the same conditions. Recovery values and confidence interval were calculated for each wiping procedure.

The tested wiping papers were filter paper from Whatman, which have already been successfully used for the desorption of dried blood spots [24, 25]; filter paper from Ahlstrom; and wipers from Texwipe. The papers were wetted with water before wiping. As shown in Fig. 3, the best recovery values with confidence intervals inferior to 20% were achieved with filter papers from Whatman. Wipers from Texwipe possessed slightly inferior recovery values with similar precision than Whatman papers, but they were not easy to handle for wiping. Filter papers from Ahlstrom gave much lower recovery values with higher variability than the other two papers. Therefore, further experiments were performed with Whatman filter papers.

Filter papers were wetted with 0.1 mL of different wiping solutions, i.e. water, NaOH 0.01 M, FA 0.1%, 20% ACN with 0.1% FA, 50% ACN with 0.1% FA, ACN with 0.1% FA and isopropyl alcohol 70%. As shown in Fig. 4, NaOH 0.01 M was discarded because etoposide phosphate was not detected in wiping samples, probably due to its degradation in basic conditions. Moreover, recovery values inferior to 20% were obtained for doxorubicin, epirubicin and vincristine. Addition of FA 0.1% to water increased the recovery values for gemcitabine, etoposide phosphate, irinotecan and vincristine; better precision values were also obtained for most cytotoxic drugs. Therefore, an acidic wiping solution was selected and different amounts of ACN (20%, 50% and 100%) were tested. The addition of an organic solvent was used to reduce the adsorption of more hydrophobic compounds onto the surface. However, with amounts of ACN superior to 20%, the papers were not easy to handle anymore, resulting in an increased confidence interval. The same phenomenon was also observed with isopropyl alcohol 70% as the wiping solution. The presence of 20% ACN increased the recovery values, and better precision values (confidence interval inferior to 20%) were achieved.

To improve the recovery rate, a second wiping with a new filter paper and a separated desorption procedure was

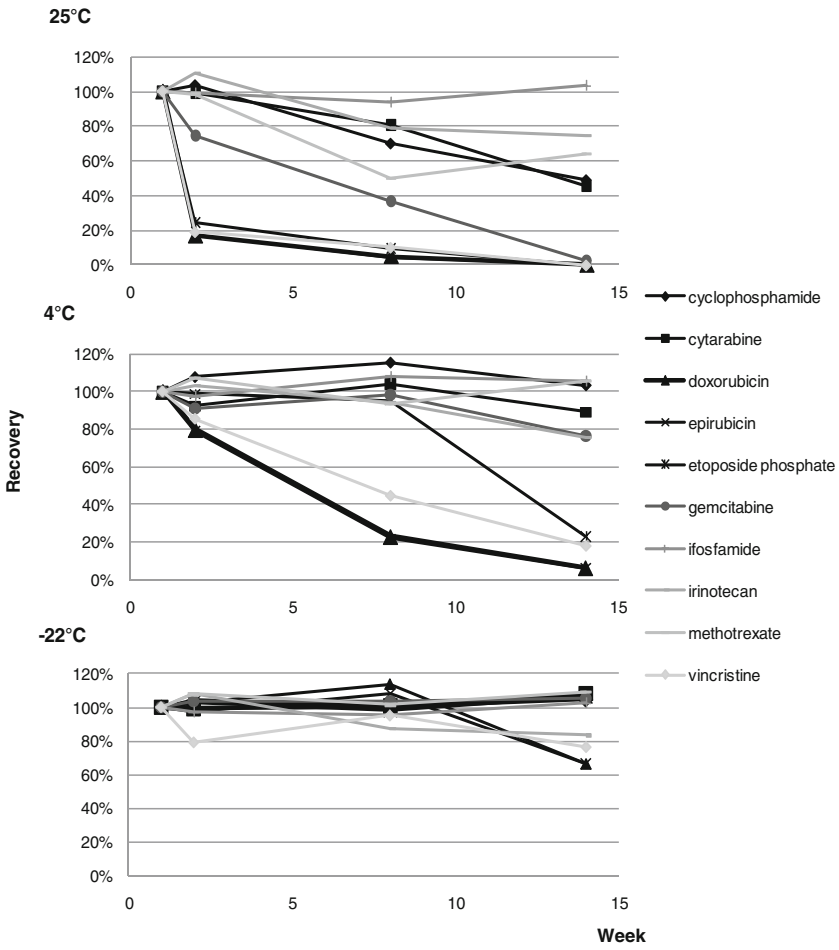

Fig 5 Stability data of the ten cytotoxic drugs on wiping papers stored at −22, 4 and 25°C for 3 months

performed on the same surface. Recovery values between 10% and 15% of the initial spiked amount were found. In addition with the amount found by the first wiping, the total recovery values were still inferior to 75% for methotrexate, etoposide phosphate, irinotecan, doxorubicin, epirubicin and vincristine. This loss of cytotoxicity might be due to adsorption or degradation issues. Only cytarabine, gemcitabine, ifosfamide and cyclophosphamide presented total recovery values between 90% and 100%. Given the low improvement of recovery values with a second wiping, only one wipe was performed in the final procedure and a correcting factor per compound and surface was determined (see below).

Quantitative performance of the method applied to different surfaces

Quantitative performance was studied with three independent series at three concentrations with three repetitions for the stainless steel plate and polypropylene infusion bag. Among the investigated surfaces, these two areas were most commonly in contact with cytotoxic drugs during the daily preparation of drug formulations. Concentrations of wiping samples were calculated from the calibration model constructed the same day. Recovery was expressed in per cent as the ratio between the theoretical and average measured values at each concentration level. Intermediate precision was expressed as the RSD of the ratio of the between-day standard deviation (sR). The sR values were obtained using ANOVA. Surface-dependent correcting factors for each compound were determined according to the recovery value. This factor was used to calculate the surface contamination for real wiping samples.

Constant recovery values were found for 0.1, 0.5 and 2 ng cm^{-2} (corresponding to 10, 50 and 200 ng mL^{-1}) for all compounds (data not shown). Therefore, the recovery value could be considered as independent of the concentration between 0.1 and 2 ng cm^{-2}. The quantitative performance of the other surfaces (polystyrol, glass, latex gloves, computer mouse and coated paperboard) were determined at only one concentration, i.e. 0.5 ng cm^{-2} (50 ng mL^{-1}). A wide range of recoveries (5–98%) were obtained according to the investigated surface and compound (Table 3). In general, wiping of smooth surfaces, such as stainless steel, polypropylene, polystyrol and glass, presented higher recovery and smaller RSD values than irregular surfaces such as latex gloves or computer mouse. Wiping of coated paperboard was not successful (recovery values between 5% and 15%), probably due to the absorption of drugs inside the coated paperboard. For the other surfaces, most recovery values were superior to 50% and RSD for intermediate precision inferior to 20%. As expected, some compounds (irinotecan, doxorubicin, epirubicin and vincristine) presented lower recovery values, probably due to adsorption or degradation issues.

Table 4 Cytotoxic surface contamination at the cytotoxic unit of HUG pharmacy

Sampled surface	Cytotoxic contamination (nanograms per wiping sample)									
	Cytarabine	Gemcitabine	Methotrexate	Etoposide phosphate	Ifosfamide	Cyclophosphamide	Irinotecan	Doxorubicin	Vincristine	Epirubicin
Middle of bench flow	ND	<LOQ	<LOQ	ND	70	38	<LOQ	ND	ND	ND
Sleeves inside isolator	10	24	<LOQ	62	>200	28	94	ND	<LOQ	ND
Balance	ND	<LOQ	<LOQ	ND	85	24	<LOQ	ND	ND	ND
Door handle inside isolator	<LOQ	65	<LOQ	20	>200	98	28	ND	ND	<LOQ
Floor in front of isolator	ND	ND	ND	42	ND	ND	<LOQ	ND	ND	<LOQ
Product storage box at 4°C	<LOQ	<LOQ	ND	10	<LOQ	<LOQ	<LOQ	ND	ND	ND
Product storage box at 25°C	<LOQ	>200	<LOQ	ND	27	<LOQ	ND	ND	ND	ND
Phone	ND	ND	ND	ND	ND	ND	< LOQ	ND	ND	ND

ND not detected

Limits of quantification (LOQs) were based on the validation of the LC–MS/MS [23] method and the determined correction factor for the wiping procedure. LOQs for all cytotoxic drugs were set at 10 ng per wiping sample, or 0.1 ng cm^{-2}.

Stability of cytotoxic drugs on wiping papers

According to the number of wipe samples, the wipe location and the reduced availability of the LC–MS/MS instrumentation, storage of filter papers might be very interesting. Therefore, the stability of cytotoxic drugs on the filter paper was studied over 3 months at three storage temperatures (−22, 4 and 25°C) in a light-protected area (Fig. 5). At 25°C, only ifosfamide was stable for 3 months, whilst all other drugs presented recoveries inferior to 80% after 2 months. Concentrations of etoposide phosphate, doxorubicin, epirubicin and vincristine were already decreased to 20% after 1 week. At 4°C, the wiping samples were stable for 1 week, with at least 80% of the initial concentration found for all drugs. After 2 months, the concentrations of doxorubicin, epirubicin and vincristine were inferior to 50% of the initial amount, and at 3 months, 50% of etoposide phosphate was lost. The other compounds were stable for 3 months at 4°C. However, the best storage conditions for all drugs were obtained at −22°C: All cytotoxic drugs presented acceptable recoveries between 90% and 110% after 2 months. After 3 months, values above 50% were obtained. Therefore, wiping samples could be kept at −22°C for 2 months until desorption procedure and LC–MS/MS analysis.

Application to surface contamination at the cytotoxic production unit of HUG pharmacy

In order to demonstrate the applicability of the developed method to real samples, the contamination rate of the ten cytotoxic agents was measured on different places of the cytotoxic unit at HUG pharmacy. The sample locations included places in the cytotoxic production area such as working surfaces, floor in front of the isolator door, and in the storage and checking room for cytotoxic drugs, including phone, refrigerator, storage shelves and others (Table 4). Gemcitabine, ifosfamide and cyclophosphamide were the most commonly detected drugs. This finding could be explained by the necessity of the operators to reconstitute these drugs before dilution, the high prescribed concentrations or the quantity of preparations during the days before wiping. Most of the contamination was found inside the isolator, and only some traces of cytotoxic drugs were outside the production zone (inferior to LOQ). To reduce contamination inside the isolators, some actions should be applied, such as more efficient cleaning procedure with the use of different cleaning solvents or enhanced cleaning frequency.

Conclusions

A wipe sampling procedure coupled to LC–MS/MS was developed for the determination of ten cytotoxic drugs on different surfaces. To the author's knowledge, this work describes the first wiping method allowing the simultaneous quantification of ten cytotoxic drugs from different therapeutic families, with well-established quantitative performance in terms of recovery and precision. Seven surfaces, the most commonly encountered in hospital production units, were investigated, and the recovery values were clearly defined according to surfaces and compounds. Therefore, this approach is particularly suitable for environmental monitoring and can be used to identify exposure of hospital staff who handles cytotoxic drugs and to validate decontamination procedures. Finally, the method was successfully applied for the determination of surface contamination at the cytotoxic production unit of HUG pharmacy.

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Article VI

« Evaluation of chemical contamination of surfaces during the preparation of chemotherapies in 24 hospital pharmacies»

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Evaluation of chemical contamination of surfaces during the preparation of chemotherapies in 24 hospital pharmacies

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ABSTRACT

Purpose To evaluate the chemical contamination of surfaces by cytotoxic agents during preparation of injectable chemotherapies in hospital pharmacies.

Methods 526 wipe samples collected in 24 Swiss hospital pharmacies were analysed using a validated liquid chromatography–mass spectrometry/mass spectrometry method able to quantify 10 cytotoxic agents: cytarabine, gemcitabine, cyclophosphamide, ifosfamide, methotrexate, etoposide phosphate, irinotecan, doxorubicin, epirubicin and vincristine. Information on chemotherapies produced, equipment and production processes used were collected from all the hospital pharmacies on a voluntary basis in order to investigate their association with contamination rates.

Results In two pharmacies, no trace of the 10 cytotoxic agents was detected. Chemical contamination was found in the other 22 hospital pharmacies, with combined total contamination of the 10 cytotoxic agents ranging from 8 ng to more than 41 000 ng per sample. Most contaminated samples came from inside biosafety cabinets, but some came from other clean room areas and logistics rooms. Statistically significant associations were observed between contamination rates and sampling locations, the number of chemotherapies prepared per year and types of cleaning solutions used.

Conclusions This study demonstrated that most of the hospital pharmacies tested had some contamination of surfaces by different cytotoxic agents. Even if highest levels of contamination were mainly detected inside biosafety cabinets, technicians were also exposed to cytotoxic agents detected in logistical and storage areas. Protective measures should therefore be maintained or even reinforced in these areas in order to limit technicians' risks of exposure when handling cytotoxic products.

INTRODUCTION

Cytotoxic drugs are widely used in cancer therapy, and since the 1970s, they have been recognised as hazardous to healthcare professionals such as oncology nurses, pharmacists and technicians.[1] Several studies have found cytotoxic drugs in the urine or blood of healthcare workers despite recommended safe handling practices for these compounds.[2–10] However, a direct relationship between exposure to cytotoxic contamination and harmful effects remains difficult to establish, and according to precautionary principles, exposure should therefore be kept to the lowest levels possible.[11] To improve safety, most hospitals have set up centralised chemotherapy preparation units, but specific infrastructure and protective measures vary greatly between institutions. Previous studies have detected traces of cytotoxic drugs on gloves, floors and work surfaces inside and/or outside biological safety zones; even on wards and in pharmacies.[12–18] Several analytical methods for the quantification of contamination by one-model or two-model cytotoxic agents have been published, as have generic methods for assessing contamination by several drugs simultaneously.[19] When using marker compounds, wipe samples have been obtained using compound-specific wiping procedures followed by appropriate analytical techniques (eg, voltammetry for platinum drugs[20]). However, hospitals usually produce a wide range of chemotherapy formulations with different drugs and different preparation procedures. Therefore, to get an overview of several contaminants at once, multicompound methods are required involving generic wiping procedures. For sufficient selectivity and sensitivity, liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) have often been chosen as analytical approaches.[13 18 21]

To determine the performance of confinement methods, it is important to benchmark contamination levels between similar structures, and environmental monitoring in areas where cytotoxics are handled is essential. In Germany, a national contamination study called Monitoring-Effect Study of Wipe Sampling in Pharmacies was performed using a multicompound LC-MS/MS method for the simultaneous determination of eight antineoplastic drugs (5-fluorouracil (5-FU), gemcitabine, methotrexate, cyclophosphamide, ifosfamide, etoposide, docetaxel and paclitaxel).[22] Another study evaluated the chemical contamination of surfaces in 102 German pharmacies using cytotoxic platinum drugs (cisplatin, carboplatin and oxaliplatin) and 5-FU as marker compounds.[20 23] Other studies on occupational exposure to antineoplastic drugs among healthcare workers were recently performed in Italy,[6 24] Sweden,[10] the Netherlands,[16] and the USA.[5] In almost all cases, traces of cytotoxic compounds were detected on surfaces that had come into contact with chemotherapies, such as workbench surfaces, floors and storage shelves.

In the present work, the chemical contamination of surfaces by different cytotoxic agents was

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studied in 24 Swiss hospital pharmacies, all participating voluntarily. It tested for traces of 10 cytotoxic agents (cytarabine, gemcitabine, cyclophosphamide, ifosfamide, methotrexate, etoposide phosphate, irinotecan, doxorubicin, epirubicin and vincristine) on multiple surfaces in both preparation and logistics areas by means of a validated wiping procedure coupled to a validated quantitative LC-MS/MS analysis.[25 26] Associations between contamination rates and the pharmacies' institutional structures and organisational characteristics were investigated.

METHODS

Selection of hospital pharmacies and involvement in the study

In 2011, 46 hospital pharmacies, all members of the Swiss Association of Public Health Administration and Hospital Pharmacists, were invited to participate in this study. Twenty-four pharmacies replied positively. Before the sampling campaign began, participating pharmacies filled in a questionnaire to report on the number of different chemotherapies prepared, equipment (biosafety cabinet class II or III, isolator) and production processes (eg, how the cleaning-up step was carried out and the type of drug transfer system). Subsequently, a single investigator (MM) collected all the study samples in the 24 pharmacies. The pharmacies were specifically asked not to clean their installations and rooms more thoroughly than usual before sampling was due to take place. After analyses, each participating hospital pharmacy received a personalised report with their own results and anonymous results from other tested sites. Each hospital pharmacy decides whether or not to deploy corrective measures to limit the operator exposure to cytotoxic agents according to their results.

Wipe sampling of surfaces and storage

Sampling of contamination was performed on various surfaces in the 24 hospital pharmacies. The investigator collected most samples from locations chosen in advance and common to each hospital pharmacy. These included critical locations (workbench surfaces and objects inside biosafety cabinets), but also other locations that could come into contact with technicians' hands, such as other places in clean rooms (surrounding the biosafety cabinet) or logistics rooms (room where all the logistical and administrative tasks are conducted, ie, from prescription validation to preparation of materials and products, and then final reconciliation outside the clean rooms). Due to the huge differences between the sampling sites (eg, type and number of biological safety cabinets, logistic organisation), it was not possible to get an overview of the whole cytotoxic processes (production, logistics) by collecting the same number of samples per site. Therefore, the number of samples was chosen according to the infrastructure of the sampling site to achieve this total overview.

Samples were obtained by wiping a 1 cm^2 Whatman filter paper (Dassel, Germany) over 10×10 cm surfaces made of plastic, stainless steel, glass or latex inside the biosafety cabinets, background environment or logistics rooms. On some surfaces —door handles, computer mouse devices and telephones—the surface wiped was smaller for practical reasons. Before wiping, the filter paper was wet with 100 µL of a solution of acetonitrile (ACN) and water (20:80, v/v) with 0.1% formic acid (FA). After wiping the surface, the filter paper was placed in a 1.5 mL polyethylene safe-lock tube from Eppendorf AG (Hamburg, Germany), stored in a cold chain at 2–8°C for transport to the laboratory in Geneva (1–5 h) and then frozen at −22°C up to 2 months until analysis.

Analysis of samples: from preparation to LC-MS-MS analysis

Chemicals and reagents

Calibration of the analysis method was performed using the following commercially available cytotoxic drugs: Campto 300 mg in a 15 mL vial (irinotecan, 20 mg/mL) and Cytosar 100 mg in a 5 mL vial (cytarabine, 20 mg/mL) were purchased from Pfizer AG (Zürich, Switzerland); Gemcitabine Teva 1 g (gemcitabine reconstituted in water at 20 mg/mL) and Vincristine Teva 2 mg in a 2 mL vial (vincristine 1 mg/mL) from Teva Pharma AG (Aesch, Switzerland); Holoxan 2 g (ifosfamide reconstituted in water at 40 mg/mL) and Endoxan 1 g (cyclophosphamide reconstituted in glucose 5% at 20 mg/mL) from Baxter AG (Volketswil, Switzerland); Methotrexat Farmos 20 mg in an 8 mL vial (methotrexate 2.5 mg/mL) from Orion Pharma (Zug, Switzerland); Etopophos 113.6 mg (etoposide phosphate reconstituted in water at 20 mg/mL) from Bristol-Myers Squibb SA (Baar, Switzerland); Doxorubicin Ebewe 200 mg in a 100 mL vial (doxorubicin 2 mg/mL) from Ebewe Pharma (Cham, Switzerland); and Epirubicin Actavis Solution 200 mg in a 100 mL vial (epirubicin 2 mg/mL) from Actavis (Regensdorf, Switzerland).

The reconstitution of Etopophos, Gemcitabine Teva and Holoxan for injection was performed with water from Bichsel Laboratories (Interlaken, Switzerland). Glucose 5% for the reconstitution of Endoxan was from Sintetica-Bioren SA (Couvet, Switzerland). The internal standard (IS) [13C, 2H3]-methotrexate was purchased from Alsachim (Illkirch, France).

Cytotoxic stock solutions, calibration standards and IS

All solutions (ie, drug reconstitutions and sample dilutions) were prepared in appropriate conditions for handling hazardous compounds such as cytotoxic agents (ie, biosafety cabinets, personal protective equipment). Aliquots of the IS were prepared with a mixture of ACN and water (75:25, v/v) at 250 µg/mL and stored at −22°C for 12 months. No sample degradation was observed. Stock solutions of IS were regularly diluted at 50 ng/mL in ACN 20% with FA 0.1% and remained stable for at least two weeks at 2–8°C.

A main stock solution containing the 10 cytotoxic drugs was prepared by diluting each compound in water at a concentration of 20 µg/mL. This solution was further diluted to obtain five independent stock solutions at 20, 40, 200, 1000 and 4000 ng/mL in ACN 20% with FA 0.1%. For calibration standards (CS), these solutions were diluted with IS solution at 50 ng/mL to obtain 5 CS at 1, 2, 10, 50 and 200 ng/mL. All CS were immediately stored at 15°C in the LC autosampler and analysed within the day.

Sample preparation

After defrosting the samples in their safe-lock tubes, 1 mL of the IS solution at 50 ng/mL in ACN/water (80:20 v/v) with 0.1% FA was added. Samples were ultrasonicated for 20 min before centrifugation at 2880 relative centrifugal force for 5 min. Finally, 25 µL of clear solution was injected into the LC-MS/MS. The validated wiping procedure was followed by validated LC-MS/MS analysis.[25] Concentrations of the cytotoxic drugs detected were calculated with reference to a calibration curve built up on the same day with five levels of CS (1, 2, 10, 50 and 200 ng/mL in ACN 20% with 0.1% FA containing the 10 drugs) and a weighted linear regression (1/x) for each compound.

Equipment and LC-MS/MS conditions

Analyses were carried out using an Accella LC system from Thermo Fisher Scientific (Waltham, Massachusetts, USA); this

consists of a quaternary pump equipped with an online degasser, an autosampler and a solvent platform. The chromatographic system was coupled to a Triple Stage Quadrupole Quantum Discovery mass spectrometer from Thermo Fisher Scientific equipped with an Ion Max electrospray ionisation interface and operated using Xcalibur software (Thermo Fisher Scientific). Separations were done on a ZORBAX SB-C18 RR 2.1×100 mm, 3.5 µm column from Agilent Technologies (Waldbronn, Germany). The LC-MS/MS method has already been described in detail elsewhere.[25] Limits of quantification were 0.25 ng/mL (0.25 ng/sample) for cytarabine and methotrexate, 0.5 ng/mL (0.5 ng/sample) for gemcitabine and cyclophosphamide, 1 ng/mL (1 ng/sample) for etoposide phosphate, ifosfamide, irinotecan and vincristine, and 2 ng/mL (2 ng/sample) for doxorubicin and epirubicin.

Expression of results and statistical analysis

Results were expressed as the sum of the quantities, in ng, of the 10 cytotoxic compounds analysed. The total contamination was calculated as the sum of the quantities of the 10 drugs of all samples per sampling site. Even if it was demonstrated that the recovery mainly depends on cytotoxic compounds and the kind of surface, results published in this paper were not modified by correction factors. Indeed, as the materials implied in this study were multiple, it was difficult to ensure that the suitable correction factor was applied. Moreover, results obtained were not drastically different with or without correction factors and the orders of magnitude for the rate of contamination were kept. For the determination of percentiles, values below the limit of detection (LOD) were set as ½ LOD.

Associations between cytotoxic contamination and its possible explanatory factors were investigated using univariate and multivariate logistic regression models. Unadjusted and adjusted ORs and 95% CIs were estimated using the maximum likelihood method.[27] The absence or presence of contamination (detection of at least 1 of 10 cytotoxic compounds) was the dependent variable. Five predictors were selected: sampling location, number of preparations per year, type of the cleaning carried out, drug transfer system and safety cabinet type (class II and III). For each predictor, the reference category (OR=1) was the one with the lowest percentage of contaminated samples. All statistics were computed using SPSS software, V.21 from IBM (Armonk, New York, USA). The significance level was set at 0.05.

RESULTS

Twenty-four hospital pharmacies agreed to participate in the study (out of 46 contacted, 52%). All pharmacies had a cytotoxic medication preparation unit, which at least partially centralised the preparation of injectable cytotoxic drugs in their hospitals. Most samples were collected from similar locations in each of the 24 pharmacies, but some were collected from locations specific to only one or a few of them.

In total, 526 samples were collected (12–30 per hospital), inside biosafety cabinets (189), in background environment (room where the biosafety cabinets were installed) (138) and in logistics rooms (199). An overview of the results obtained for each sample group (independent of the hospital) is presented in figure 1.

Evidence of at least one contamination was found in 22 of the 24 (92%) participating hospital pharmacies; no trace (superior to LODs of the used analytical method) of the 10 tested cytotoxic compounds was detected in two pharmacies.

Of 526 samples, 238 (45%) were positive. The mean and median contamination levels of positive samples were 231.3 and 6.0 ng, respectively (range 0.2–38.2 µg). Most contaminations were found inside biosafety cabinets (123 positive samples), but a few were also found in background environment (63) and in logistics rooms (52). The total contamination, number of positive samples/total samples and the number of preparations produced in each hospital are summarised in table 1.

Four cytotoxic drugs were detected in >25% of positive samples, namely cytarabine, gemcitabine, ifosfamide and cyclophosphamide. Table 2 reports 50th, 75th and 90th percentiles for cytarabine, gemcitabine, ifosfamide and cyclophosphamide, respectively, depending on the localisation of wiping sample. Table 3 indicates the numbers of positive samples/total samples for the six other compounds.

Biosafety cabinets/isolators

Sixty-five per cent of samples collected from inside biosafety cabinets (class II or III) and isolators were positive; it is thus the most contaminated area in this study.

Twenty pharmacies evaluated in this study were equipped with an electronic double-control system (CATO). This system is based on gravimetric monitoring of the volumes withdrawn from vials by technicians. In order to be used according to the manufacturer's recommendations, this system requires the use of a small, square keyboard and weighing scales within the biosafety cabinet. These two devices were highly contaminated, both in terms of frequency and contamination levels. The two highest total contamination levels across all the biosafety cabinet samples were also collected on these devices: 38 170 ng of cytotoxic drugs was detected on the keyboard in one hospital (on which traces of 'dirt' were even visible), and 5284 ng were detected on the weighing scales in another hospital. In these two hospitals, significant contamination of workbench surfaces was also found, but in smaller amounts.

Four pharmacies were equipped with a class III biosafety cabinet and one was equipped with an isolator (with preliminary sterilisation of all materials entering it). For these closed installations, additional samples were collected on the left glove (finger tips) and on the airlock handles. Of the eight samples collected on gloves, seven were positive; no contamination was found on the eight glove, but no handling had been done between the time the glove was installed and the sample was taken.

In terms of quantities of cytotoxic drugs detected, the inner handles of class III biosafety cabinet airlocks were among the least contaminated points of these cabinets.

In addition to samples collected from common locations, 13 of 19 samples collected from within biosafety cabinets were positive. Two high contamination rates were obtained in the same hospital: in a bowl used with the CATO system (1963 ng) and on an armrest (96 ng).

Background environment

Forty-six per cent of samples collected in background environment—where biosafety cabinets were installed—were positive. The most contaminated background environment location was the floor in front of the biosafety cabinet (just below where materials and vials enter the cabinet).

Twenty-three pharmacies used a material hatch, for the transfer of material and products between background environment and logistics rooms. The 24th hospital had no specialised means of transferring materials and products (a box carried by a technician was used instead). Twenty-three samples were collected from the handles of the hatches on the background environment

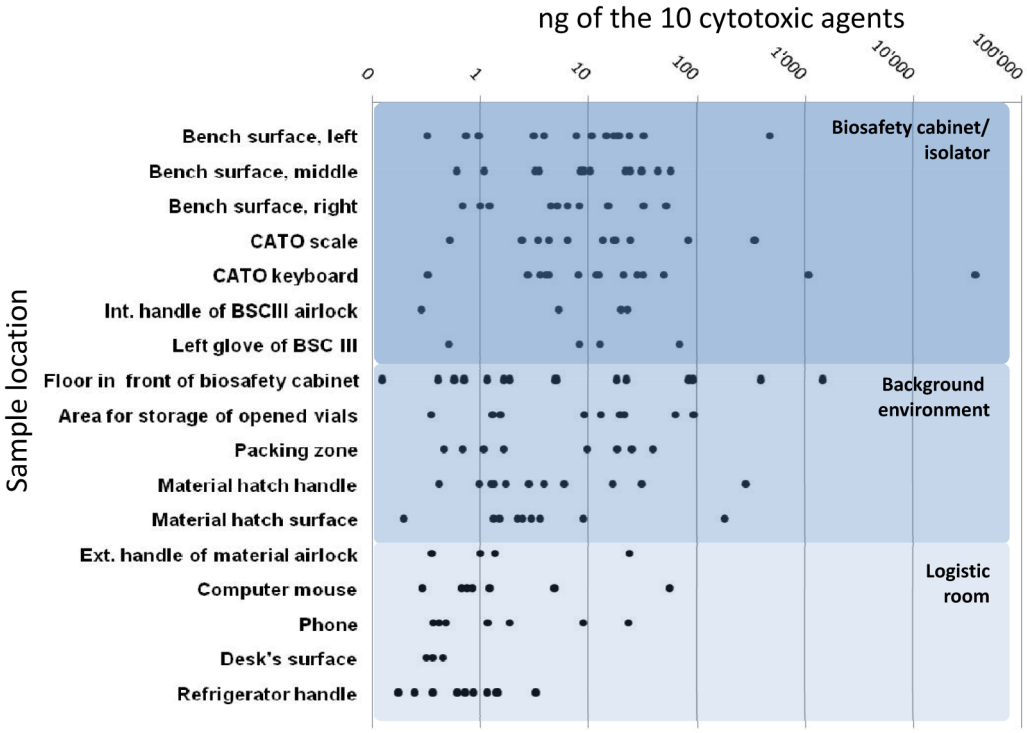

Figure 1 Overview of samples collected in different locations of 24 hospital pharmacies. BSC III, biological safety cabinets class III.

sides. Contamination of the surfaces in the material hatch was also evaluated. For pharmacies with a two-hatch circuit, contamination from the surfaces of both hatches was evaluated.

Table 1 Total contamination, number of preparations per year, number of positive samples/total samples (in ng/sample) for each hospital pharmacy

Ranking	Number of preparations per year	Number of positive samples/total samples per hospital	Total contamination (ng) per hospital
1	2500	0/21	0
2	2000	0/21	0
3	6500	1/21	8
4	6200	13/17	12
5	3900	6/21	22
6	5200	10/26	25
7	3500	3/20	31
8	750	5/12	33
9	4000	12/22	40
10	7000	6/21	47
11	3000	7/21	61
12	4700	4/20	65
13	7000	12/23	72
14	3000	16/21	150
15	6000	7/25	236
16	14 100	8/25	313
17	12 500	21/26	376
18	3500	18/21	518
19	9000	17/25	528
20	11 000	18/30	946
21	5000	7/18	1624
22	4000	7/29	2254
23	13 000	21/29	6261
24	8800	21/21	41 438

Of 33 samples collected in specific locations from each hospital, 10 were positive. The highest contamination rate was observed on a background environment doorknob (127 ng). Contamination of one computer mouse device (37 ng) was also collected.

Logistics room

Twenty-six per cent of samples from the logistic room were positive.

All contaminated samples were found in pharmacies that had a single hatch for both entrance and exit. The pharmacy with the most contaminated handle on the background environment side (286 ng) had no contamination on the logistics room side.

The potential contamination of telephones used in logistics rooms was evaluated. The whole surface of the receiver—those parts in contact with hands or the head—was wiped for sampling. Twenty-five per cent of samples were positive whose two samples had contamination levels above 2 ng. One was a sample from a cellular phone, which had been carried into background environment inside a plastic bag. In this pharmacy, other contaminated samples were found in the logistics and clean rooms, but at lower levels. In other pharmacies, contamination under 2 ng was found on fixed phones or ones that could not have been taken into clean rooms. It is notable that the contamination of three fixed phones located in background environment was also evaluated, but no contamination was found; indeed, they were rarely used as these preparation rooms were equipped with interphones.

Thirty-two per cent of samples collected from computer mouse devices were contaminated, with the highest contamination being 98 ng. Contamination of computer mouse devices in the other pharmacies was low, however (<1 ng except for one pharmacy with 5 ng). Furthermore, contamination of desk surfaces was also evaluated; only 12% of samples were positive and all were lower than 1 ng. Contamination was also assessed on

Table 2 Surface contaminations by cytarabine, gemcitabine, ifosfamide and cyclophosphamide detected in the 24 hospital pharmacies in ng/sample (corresponding approximately to 10 pg/cm^2)

Samples collected	Location	Cytarabine			Gemcitabine			Ifosfamide			Cyclophosphamide		
		50th percentile	75th percentile	90th percentile	50th percentile	75th percentile	90th percentile	50th percentile	75th percentile	90th percentile	50th percentile	75th percentile	90th percentile
Biosafety cabinets													
Common samples	Bench surface, left	0.01	0.38	2.27	0.13	0.50	9.81	0.13	0.13	0.61	0.13	0.50	2.92
	Bench surface, middle	0.01	0.59	5.56	0.13	0.91	9.40	0.13	0.13	2.43	0.13	0.13	10.46
	Bench surface, right	0.01	0.21	5.29	0.13	0.13	1.40	0.13	0.13	0.13	0.13	0.13	4.43
	CATO keyboard	0.01	0.42	5.35	0.13	0.85	2.63	0.13	0.13	1.83	0.13	4.83	15.44
	CATO scale	0.01	1.90	14.38	0.13	1.12	5.49	0.13	0.13	5.28	0.13	0.94	16.44
	Inner hatch handle, class III biosafety cabinet	0.01	2.00	9.09	0.13	1.24	5.81	0.13	0.13	1.62	0.13	0.13	8.07
	Left glove, class III biosafety cabinet	0.16	1.15	27.07	0.32	1.83	3.55	0.13	3.98	15.69	0.13	2.06	12.73
Other samples		0.01	3.00	44.56	0.13	0.13	0.89	0.13	0.13	4.40	0.13	0.13	0.97
Mean of percentile from all biological safety cabinets (BSC) locations		0.03	1.21	14.20	0.15	0.84	4.87	0.13	0.61	4.00	0.13	1.11	8.93
Median of percentile from all BSC locations		0.01	0.87	7.33	0.13	0.88	4.52	0.13	0.13	2.13	0.13	0.32	9.27
Background environment													
Common samples	Floor in front of biosafety cabinet	0.01	0.69	2.24	0.13	0.13	4.59	0.13	0.13	0.13	0.13	1.31	7.68
	Area for storage of opened vials	0.01	0.62	4.09	0.13	0.50	4.48	0.13	0.13	4.45	0.13	0.13	0.13
	Packing zone	0.01	0.35	4.81	0.13	0.13	2.58	0.13	0.13	4.83	0.13	2.77	10.24
	Material hatch handle	0.01	0.01	0.69	0.13	0.13	1.18	0.13	0.13	0.13	0.13	0.13	0.13
	Material hatch surface	0.01	0.01	2.34	0.13	0.13	1.13	0.13	0.13	1.92	0.13	0.63	5.56
Other samples		0.01	0.01	0.72	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	7.72
Mean of percentile from all background environment locations		0.01	0.28	2.48	0.13	0.19	2.35	0.13	0.13	1.93	0.13	0.85	5.20
Median of percentile from all background environment locations		0.01	0.18	2.29	0.13	0.13	1.88	0.13	0.13	1.03	0.13	0.38	6.62
Logistic rooms													
Common samples	Material hatch handle	0.01	0.01	0.01	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
	Computer mouse	0.01	0.22	0.84	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
	Phone	0.01	0.01	0.59	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
	Desk surface	0.01	0.01	0.01	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
	Refrigerator handle	0.01	0.01	0.48	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
	Area for storage of opened vials	0.01	0.01	3.28	0.13	0.13	0.82	0.13	0.13	0.13	0.13	0.13	6.33
	Packing zone	0.01	0.16	1.23	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13
Other samples		0.01	0.01	0.01	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.36
Mean of percentile from all logistic rooms locations		0.01	0.06	0.81	0.13	0.13	0.22	0.13	0.13	0.13	0.13	0.13	0.93
Median of percentile from all logistic rooms locations		0.01	0.01	0.54	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13

Table 3 Positive samples/total samples for the six other cytotoxic compounds: methotrexate, etoposide, irinotecan, doxorubicin, vincristine and epirubicin

Samples collected	Location	Methotrexate	Etoposide	Irinotecan	Doxorubicin	Vincristine	Epirubicin
Biosafety cabinets							
Common samples	Bench surface, left	4/33	1/33	0/33	0/33	0/33	0/33
	Bench surface, middle	4/34	2/34	1/34	0/34	0/34	0/34
	Bench surface, right	3/26	2/26	0/26	0/26	0/26	0/26
	CATO keyboard	3/26	4/26	0/26	0/26	0/26	0/26
	CATO scale	3/30	2/30	1/30	1/30	0/30	0/30
	Inner hatch handle, class III biosafety cabinet	2/13	2/13	0/13	0/13	0/13	0/13
	Left glove, class III biosafety cabinet	1/8	1/8	1/8	0/8	0/8	0/8
Other samples		3/19	0/19	0/19	0/19	0/19	0/19
Background environment							
Common samples	Floor in front of biosafety cabinet	3/32	1/32	1/32	0/32	0/32	4/32
	Area for storage of opened vials	1/7	0/7	0/7	0/7	0/7	0/7
	Packing zone	0/15	0/15	0/15	0/15	0/15	0/15
	Material hatch handle	2/23	2/23	0/23	0/23	0/23	0/23
	Material hatch surface	1/28	0/28	0/28	0/28	2/28	0/28
Other samples		1/33	0/33	1/33	0/33	0/33	0/33
Logistic rooms							
Common samples	Material hatch handle	1/23	0/23	0/23	0/23	0/23	0/23
	Computer mouse	1/22	2/22	0/22	0/22	0/22	0/22
	Phone	1/28	0/28	0/28	0/28	0/28	1/28
	Desk surface	1/26	0/26	0/26	0/26	0/26	0/26
	Refrigerator handle	1/27	0/27	0/27	0/27	0/27	0/27
	Area for storage of opened vials	1/14	0/14	1/14	0/14	0/14	0/14
	Packing zone	0/8	0/8	0/8	0/8	0/8	0/8
Other samples		2/51	0/51	0/51	0/51	0/51	0/51

the handles of 27 refrigerators located in logistics rooms where vials of cytotoxic medicines were stored. Eleven samples were positive (41%), and the highest contamination was 3 ng of cytotoxic drugs. In other pharmacies, the contamination level was under 2 ng.

The highest contaminations (for samples collected in others locations in logistics rooms) were detected in two boxes used for chemotherapy transport in a logistics room. Contamination rates inferior to 4 ng were also detected on other positive samples from locations where chemotherapies or cytotoxic medicine vials were stored or handled.

Factors associated with contamination

Contamination was investigated as a function of five predictors considered contextually relevant to cytotoxic preparation: preparation area (biosafety cabinets, background environment, logistics rooms), as described above; number of preparations, that is, total number of chemotherapy preparations produced each year (≤4000, 4000–7000, ≥7000); type of cleaning agent (alcohol, sodium hydroxide (NaOH), others, that is, formaldehyde and glucoprotamine); class II or III biosafety cabinets; and two categories of drug transfer system, namely 'open system devices' (needle, spike, Codan Cyto-Ad Z and Chemoprotech) and 'closed system devices' (Phaseal and Tevadaptor).

Univariate logistic regression models were first used to estimate the role of each individual predictor with respect to contamination. Table 4 summarises these results. As expected, contamination rates were significantly higher in biosafety cabinets (OR=5.20, p<0.001) and background environment (OR=3.01, p<0.001) than in logistics rooms. The higher the number of preparations per year, the higher the risk of contamination (>7000 vs ≤4000, OR=4.33, p<0.001). An open system device was associated with increased contamination risk compared with a closed system device (OR=3.06, p=0.001). The type of cleaning agent and class of biosafety cabinet were not significantly associated with contamination.

When the five predictors were considered together in a multivariate model, preparation area and number of preparations per year remained significantly associated with contamination, whereas using an open system device did not reach statistical significance (p=0.062). Furthermore, using a cleaning agent other than alcohol or NaOH was associated with increased contamination (OR=3.69, p=0.009), but the number of samples in this category was low (n=17). The correlation matrix of estimated coefficients did not reveal any multicollinearity problem in this model.

DISCUSSION

Chemical contamination was detected in all but two of the hospital pharmacies evaluated. It is noteworthy that results expressed in terms of medians were markedly inferior to those expressed in terms of means. These differences highlighted the fact that the great majority of contamination rates were low, despite the occasional presence of highly contaminated samples. Of the samples analysed as positive, 89% and 60% presented contamination levels inferior to 50 and 10 ng, respectively. To our knowledge, no acceptable or recommended limits exist for total quantities of cytotoxic drugs on hospital pharmacy surfaces, but common sense suggests that they aim for an absence of contamination or the lowest amount possible. Moreover, the applied analytical method used in the present work only tested for 10 cytotoxic compounds, but >50 different ones might be handled by a typical hospital pharmacy. This study's results gave an indication of the level of chemical contamination on surfaces in hospital pharmacies at a given time.

The greater the number of preparations a hospital pharmacy produced per year, the greater the contamination rate observed. Indeed, the two hospital pharmacies with no contamination

Table 4 Factors associated with contamination in 24 hospital pharmacies

				Univariate logistic regression models					Multivariate logistic regression model (Nagelkerke R^2=0.3)			
Predictor	Category	n total	n contaminated	OR	95% CI inf	95% CI sup	p Value	Nagelkerke R^2	OR	95% CI inferior	95% CI superior	p Value
Area	Logistics room	176	40 (23%)	1				0.16	1			
	Biosafety cabinets	172	104 (60%)	5.20	3.26	8.29	<0.001		5.76	3.47	9.58	<0.001
	Background environment	83	39 (47%)	3.01	1.73	5.26	<0.001		3.47	1.91	6.30	<0.001
Number of preparations per year	≤4000	120	42 (35%)	1				0.11	1			
	4000–7000	221	78 (35%)	1.01	0.64	1.61	0.96		2.52	1.11	5.73	0.028
	>7000	90	63 (70%)	4.33	2.41	7.79	<0.001		9.62	4.13	22.41	<0.001
Type of cleaning agent	Alcohol	87	28 (32%)	1				0.002	1			
	Sodium hydroxide	182	74 (41%)	0.91	0.60	1.38	0.66		0.87	0.41	1.84	0.72
	Others	17	14 (82%)	1.14	0.65	2.01	0.65		3.69	1.39	9.82	0.009
Drug transfer system	Closed system device	60	13 (22%)	1				0.04	1			
	Open system device	139	61 (44%)	3.06	1.60	5.84	0.001		2.38	0.96	5.92	0.062
Safety cabinet type	Class II	318	134 (42%)	1				0.000	1			
	Class III	113	49 (43%)	1.05	0.68	1.62	0.82		0.78	0.42	1.46	0.44

belonged to the group of pharmacies that produced the fewest chemotherapies (<4000 preparations per year).

Contamination rates differed significantly depending on the area where samples were collected. The highest contamination rates were observed in biosafety cabinets and decreased in background environment and logistics rooms. Even if it is reassuring to note that chemical contamination was mainly confined to biosafety cabinets or isolators, the contamination levels higher than 1 μg found in these locations could increase the chances of that contamination dispersing inside cabinets and background environment. Of the different elements evaluated inside these confined areas, the workbench surfaces, gloves, square keyboards and weighing scales (for gravimetric control) were the most contaminated. Initially, the contamination of workbench surfaces may appear surprising because they are usually protected by a mat. However, the size of these mats was always smaller than the workbench surface itself, and the technicians used the left and right sides of the workbench surface to store vials or other materials required for the chemotherapy preparation. Materials and vials employed can be considered as potential sources of chemical contamination in addition to the preparation itself.[28–31] The presence of chemical contamination on the middle and sides of a workbench could be explained by dispersion of the contamination over the entire surface during the cleaning procedure. The highest rates of contamination (by an order of several micrograms) were observed on the square keyboards and weighing scales in the biosafety cabinets of two hospital pharmacies. It was difficult to explain the sources of these high contamination levels, but the most probable causes include extraordinary events (eg, a broken vial, leaking infusion bag) happening inside the biosafety cabinet, followed by an inappropriate or ineffective cleaning procedure. It is notable that 67% of the samples collected on square keyboards and weighing scales were positive. Given that these instruments are constantly handled by the technicians during chemotherapy preparation, their gloves were also contaminated and contributed to the dispersion of contamination within the biosafety cabinet and also onto the external surfaces of syringes or infusion bags to be administered to oncology ward patients. The single-use gloves used in most hospital pharmacies were not evaluated because all samples were taken in the absence of drug preparation activity in the biosafety cabinets and these gloves are replaced after each preparation period. Nevertheless, gloves in class III cabinets (eight samples from five hospital pharmacies) were tested, and 88% of them proved positive. Frequently replacing the gloves of class III biosafety cabinets is therefore of the utmost importance. Indeed, it would be appropriate to wear a second pair of surgical gloves that could be thrown away and replaced after every drug preparation session in order to avoid cross-contamination. A similar chemical contamination was observed on the inner handles of hatches for class III biosafety cabinets. Despite these doors generally being opened by pressing a foot pedal, technicians touched the inner handle of the airlock to close it.

In one pharmacy, 100% of samples were positive. This was also the pharmacy that had the highest total contamination level and was perhaps a pertinent example of contamination dispersion along the cytotoxic preparation process. The significant contamination found on the CATO keyboard in its biosafety cabinet may well have been the source of the contamination found on the other locations, including the table for the preparation of materials in the logistics room (0.4 ng). It can be noted that the four most detected substances (cytarabine, gemcitabine, ifosfamide and cyclophosphamide) were logically substances used at high concentrations for therapeutic indication. Less administered but used also at high concentrations, etoposide phosphate, ifosfamide and methotrexate were also detected but in lesser extent in terms of frequency and rate. Doxorubicin, epirubicin, irinotecan and vincristine were rarely detected and in concentration inferior to 30 ng per sample, which is not surprising given their poor consumption and their low concentration administered.

Today, no limit value for occupational exposure to antineoplastic drugs exists. However, several authors suggested guidance values based on the determination of percentile from surface contamination measures performed in several locations depending on cytotoxic agents.[23 27] For example, Schierl *et al* proposed two threshold guidance values (TGVs) assigned by the 50th (TGV1) and the 75th percentile values (TGV2) for platinum compounds and 5-FU in a study including over a thousand wiping samples taken from 102 hospitals. Contamination values inferior to TGV1 were considered as negligible while

contamination values superior to TGV2 implicated an improvement of working procedures. TGVs defined for platinum compounds and 5-FU were between 0.6 and 30 pg/cm^2.[23] In our study, percentile values obtained for the four most detected compounds (cytarabine, gemcitabine, ifosfamide and cyclophosphamide) were slightly superior (0.01–44.56 ng per sample corresponding approximately to 0.1–446 pg/cm^2) (table 2). Even if marked compounds were different in both studies, these drugs were administered at high concentrations, which implicated that working procedures can be improved to reduce surface contaminations. Nevertheless, it can be noted that surface contamination levels by ifosfamide and cyclophosphamide were lower (0.13–8.93 ng per sample corresponding approximately to 1.3–89 pg/cm^2) than those obtained elsewhere.[27] Indeed, Hedmer and Wohlfart[27] defined hygienic guidance values, based on 90th percentile, between 1.2 and 500 pg/cm^2 for ifosfamide and cyclophosphamide.

Two kinds of corrective action can be implemented to reduce chemical contamination in safety cabinets: measures applied during the preparation and measures applied after the preparation (decontamination procedures). The use of a larger mat or several underlayers on which to deposit all the materials required for a specific chemotherapy preparation could be one possible strategy. Moreover, our study confirms previous studies elsewhere on the positive impact of using closed system devices for transferring drugs.[28] Another approach is to carry out an effective and validated chemical decontamination of surfaces in biosafety cabinets after preparation. Indeed, although chemical decontamination should be considered a critical process, the cleaning solutions whose main job is surface disinfection are not always suitable for chemical decontamination.[29–32] It appeared that hospital pharmacies using alcohol or NaOH solutions in their cleaning procedures presented lower contamination rates than those using other solutions such as formaldehyde and glucoprotamin (see table 4). Although the chemical hydrolysis of organic compounds by NaOH easily explains its decontamination effects, the decontamination effects of alcohol-based solutions are questionable. The ability of alcohol solutions to dissolve traces of cytotoxic agents on surfaces better than formaldehyde and glucoprotamin solutions could be one hypothesis. Queruau Lamerie *et al*[33] demonstrated the effectiveness of a decontamination solution containing an anionic surfactant and isopropyl alcohol to eliminate cytotoxic agents on a stainless steel surface. Chemical surface contamination rates were not associated with the type of biosafety cabinet used (see table 4). Even if class III biosafety cabinets are less ergonomic for technicians, no significant difference was observed between type II and type III biosafety cabinets.

Traces of contamination were also detected in background environment where biosafety cabinets were located. Floor surfaces in front of biosafety cabinets were the most contaminated background environment location in terms of frequency and rates. Methods of putting cytotoxic vials inside cabinets could be an explanation. Indeed, spraying ethanol or isopropanol onto vials for microbiological disinfection can dissolve traces of cytotoxic powder on the external surfaces of vials,[34–37] but can contaminate the floor. Despite three pharmacies having replaced spray disinfection of vials by a wiping method in order to avoid this possible contamination route, two of them still had significant rates of floor contamination (mean 207 ng, median 18 ng). Indeed, this was in a similar range to the contamination found in pharmacies using spray disinfection (mean 186 ng, median 2 ng). Finally, the nine pharmacies that did not apply disinfection to vials also had contamination (mean 5 ng, median 4 ng). These results show that multiple sources of contamination must be considered, such as a contamination resulting from direct contact with contaminated material or contamination dispersion due to cleaning of other clean room areas.

Vial storage and packing areas were the least contaminated areas in background environment (of common locations) with rates inferior to 50 ng and a percentage of positive samples between 43 and 53. A similar percentage was detected on samples from the handles and surfaces of material hatches. It seems that technicians on the outside of biosafety cabinets, who could be contaminated by touching the cytotoxic formulations produced, could then disperse that contamination to other areas of the background environment via their gloves. Even if the formulation was packed in a protective bag, the latter remained in contact with the contaminated gloves of the technician who placed it in the hatch.

On the outside of the hatch, in the logistics room, lower contamination levels were detected, with a percentage of positive samples inferior to 45% and mean and median values inferior to 45 and 25 ng, respectively. Contamination in logistics rooms could have two different sources: handling cytotoxic vials before chemotherapy preparation and handling material (syringes, flex and packaging) after chemotherapy preparation. This study demonstrated that when material from biosafety cabinets was contaminated and then handled in other areas of the clean room this contributed to the dispersion of contamination into logistics room, although at lower rates. In addition, the chemical contamination of cytotoxic vials supplied by manufacturers could also contribute to the chemical contamination observed in logistics rooms. Traces of contamination on the handles of refrigerators storing cytotoxic vials confirmed this hypothesis. Even if contamination rates were low in logistics rooms, the chronic exposure to which healthcare professionals are subjected to should not be minimised. It is very important that the handling of cytotoxic vials and the formulations produced should be made with gloves, and that the latter should be removed for desk tasks. This is a basic safety instruction in chemistry laboratories. Moreover, this study's results strongly suggest that the material used in logistics rooms, such as mobile phones, should remain in logistics rooms in order to avoid the dispersion of contamination. Although this study was limited to the evaluation of the surface contamination by cytotoxic compounds in hospital pharmacies, it is clear that this contamination exists in other hospital areas such as patient care units.[38]

CONCLUSION

This work aimed to evaluate the contamination of surfaces by cytotoxic drugs in different hospital pharmacies. Ninety-two per cent of the pharmacies studied presented a chemical contamination, with a large variability in terms of rates. In all cases, the sources of the chemical contamination were inside the biosafety cabinets; however, traces of these contaminations were also detected in different parts of the pharmacy background environment and logistics room.

Protective measures should therefore be maintained or even reinforced in these areas in order to limit technicians' risks of exposure when handling cytotoxic products.

It would be interesting to conduct a new surface sampling campaign hospital pharmacies tested to see the evolution of surface contamination by cytotoxic agents. Thus, it would be possible to determine the impact of any action taken as a result of this campaign to limit contamination detected in some sites. On the other hand, the evaluation of surface contamination by

other cytotoxic agents such as 5-FU or platinum derivatives should be constituted as a complementary approach to this work.

Key messages

What is already known on this subject?

- Handling cytotoxic agents present a major risk for healthcare workers, and surface contamination is the major route of exposure.
- Several studies were reported on the surface contamination in hospital pharmacies in different countries, and usually only a few antineoplastic compounds (maximum 5 and by different analytical methods) were researched by the authors.

What this study adds?

- To our knowledge, no study was published about the surface contamination by cytotoxic drugs in Swiss hospital pharmacies.
- This study used only one validated analytical method able to detect up to 10 different cytotoxic compounds.
- Moreover, a statistical approach was used to explain the potential association between the surface contamination observed and processes used for the chemotherapies produced.

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Article VII

« Determination of the external contamination and cross-contamination by cytotoxic drugs on the surfaces of vials available on the Swiss market.»

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Determination of the external contamination and cross-contamination by cytotoxic drugs on the surfaces of vials available on the Swiss market

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Abstract

Introduction: The external contamination and cross-contamination by cytotoxic drugs on the surface (outside and septum) of 133 vials from various manufacturers and available on the Swiss market were evaluated. All of the tested vials contained one of the following active ingredients: cyclophosphamide, cytarabine, doxorubicin, epirubicin, etoposide phosphate, gemcitabine, ifosfamide, irinotecan, methotrexate or vincristine.

Methods and materials: The validated wiping liquid chromatography-mass spectrometry method used in this study allowed for the simultaneous determination of these 10 cytotoxic drugs in less than 30 min.

Results: External contamination by cytotoxic drugs was detected on 63% of tested vials (outside and septum). The highest contamination level was observed on etoposide phosphate vials with 1896.66 ng of active ingredient on the outside of the vial. Approximately 20% of the contaminated vials had greater than 10 ng of cytotoxic drugs. Chemical contamination on the septum was detected on 38% of the vials. No contamination or very low levels of cytotoxic drugs, less than 1 ng per vial, were detected on the vials protected by plastic shrink-wrap. Traces of cytotoxic drugs different from the active ingredient were detected on 35% of the tested vials.

Conclusion: Handling cytotoxic vials with gloves and having a procedure for the decontamination of vials are of the utmost importance for reducing exposure to cytotoxic drugs. Moreover, manufacturers must improve their procedures to provide products free from any contamination.

Keywords
Cytotoxic drugs, external chemical contamination, vials, packaging, cross-contamination

Introduction

Cytotoxic drugs are known for their high toxicity for tumour cells and healthy cells of patients as well as all people in contact with the drugs.[1] During recent years, a special effort has been focused on the exposure of

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healthcare professionals, including pharmacists, technicians and nurses, to cytotoxic agents during the preparation and administration steps. This was made possible thanks to the use of detection systems with higher selectivity and sensitivity such as the mass spectrometry which allowed developing numerous analysis methods to detect very low amounts of drugs in different matrices.[2] For example, several studies have demonstrated the presence of cytotoxic drugs in the biological samples, including urine[3,4] and blood[5] from operators. To identify the origins of this unwanted exposure, surfaces in hospital pharmacies and oncology units was investigated for potential contamination.[3–9] Despite the use of recommended safe handling practices, traces of cytotoxic drugs were detected on floors and working surfaces, inside and/or outside biological safety cabinets, on gloves, in nurse areas and in pharmacies with a concentration range from 1 $pg \cdot cm^{-2}$ to greater than 100 $ng \cdot cm^{-2}$.

Although the handling by healthcare professionals represents the main source of contamination, another potential reason for the presence of cytotoxic drugs is the external surface contamination of the commercially available drug vials.[7,10–20] The most commonly tested vials contained cyclophosphamide and ifosfamide as active ingredients[7,11–14,16–20] with levels of contamination between a few ng (e.g., 0–5 ng) and 500 ng per vial (a median of 100 ng per vial). However, Connor et al.[12] have detected more than 69,000 ng of cyclophosphamide on a vial containing 2 g of drug from the US market. Commercially available vials containing platinum compounds were also tested.[10–15] Contamination levels between a few ng and 800 ng of platinum drugs per vial were detected on the exterior surface of vials available in Sweden,[10] the United Kingdom,[11] the Netherlands,[12] Germany[14] and Japan.[15] For 5-fluorouracil vials, levels greater than 10,000 ng per vial were detected.[12] Lower contamination levels on the external surface of the 5-fluorouracil vials were observed in Germany, with a median level of 13.3 ng per vial.[14] Favier et al.[16] have also reported external contamination on vials containing doxorubicin and docetaxel with mean amounts of 29 ng and 366 ng per vial, respectively. Trace amounts, at approximately 5 ng per vial, of active ingredient were also found on the external surface of 30 vials containing methotrexate.[11] In response to these studies, some manufacturers have developed a strategy for preventing cytotoxic exposure that consists of coating the vial with a plastic shrink-wrap at the end of the filling process. Under these conditions, contamination was reduced by a factor between 1.5 and 2.[14]

The above-mentioned studies used analytical methods capable of determining only one cytotoxic agent. In these cases, the analysis for cross-contamination by other cytotoxic agents was not possible. However, an LC-MS/MS method that simultaneously detected cyclophosphamide and ifosfamide was used by Hedmer et al.[13] and found contamination by both compounds on vials containing cyclophosphamide as active ingredient from a Swedish distributor. To the best of our knowledge, no other publications have reported the determination of potential cross-contaminations on the surface of vials containing cytotoxic drugs.

In this paper, the external chemical contamination on 133 vials that contained various cytotoxic agents from 12 different manufacturers and available on the Swiss market was studied. Traces of 10 cytotoxic agents (cytarabine, gemcitabine, cyclophosphamide, ifosfamide, methotrexate, etoposide phosphate, irinotecan, doxorubicin, epirubicin and vincristine) were detected on the outside surface and on the septum of the vials using validated wiping and quantitative LC-MS/MS methods.[21,22]

Experimental methods

Chemicals and reagents

The calibration was performed with the commercially available cytotoxic drugs (Table 1). The reconstitutions of Etopophos®, Gemcitabin-Teva® and Holoxan® were performed with water for injection from Bichsel Laboratories (Interlaken, Switzerland). Glucose (5%) from Sintetica-Bioren SA (Couvet, Switzerland) was used for the reconstitution of Endoxan®. The internal standard (IS) [13C, 2H3]-methotrexate was purchased from Alsachim (Illkirch, France).

Stock solutions of the cytotoxic drugs, calibration standard and internal standard

All solutions, including the drug reconstitutions and sample dilutions, were prepared in the appropriate conditions for handling hazardous compounds such as cytotoxic agents. Stock solutions of the IS at 250 $\mu g \cdot mL^{-1}$ and stored at −22°C for 12 months were regularly diluted to 50 $ng \cdot mL^{-1}$ in 20% ACN with 0.1% formic acid (FA) and were stable for at least 2 weeks at 2–8°C.

A main stock solution containing the 10 cytotoxic drugs was prepared by diluting each compound in water at a concentration of 20 $\mu g \cdot mL^{-1}$. This solution was further diluted to obtain 5 independent stock solutions at 20, 40, 200, 1000 and 4000 $ng \cdot mL^{-1}$ in 20% ACN with 0.1% FA. For the calibration standards (CS), these solutions were diluted with the IS solution at 50 $ng \cdot mL^{-1}$, to obtain 5 CS at 1, 2, 10, 50 and 200 $ng \cdot mL^{-1}$. All samples were immediately stored at 15°C in the LC auto-sampler and analysed within 1 day.

Table 1. Tested vials commercially available on the Swiss market.

Brand name	Manufacturer	Drug	Drug concentration ($mg \cdot mL^{-1}$)
Vincristine Teva®	Teva (Aesch, Switzerland)	Vincristine	1
Doxorubicin Ebewe®	Ebewe Pharma Schweiz (Cham, Switzerland)	Doxorubicin	2
Epirubicin Actavis Solution®	Actavis (Regensdorf, Switzerland)	Epirubicin	2
Methotrexat Farmos®	Orion Pharma (Zug, Switzerland)	Methotrexate	2.5
Endoxan®	Baxter AG (Volketswil, Switzerland)	cyclophosphamide	20
Cytosar®	Pfizer AG (Zürich, Switzerland)	cytarabine	20
Etopophos®	Bristol-Myers Squibb SA (Baar, Switzerland)	etoposide phosphate	20
Gemcitabine Teva®	Teva (Aesch, Switzerland)	gemcitabine	20
Campto®	Pfizer AG (Zürich, Switzerland)	irinotecan	20
Holoxan®	Baxter AG (Volketswil, Switzerland)	ifosfamide	40

Wipe-sampling of the drug vials

Two wipe samples were collected with filter paper (1 cm^2) from Whatman (Dassel, Germany) for each tested vial (Table 2). One sample was obtained by wiping the external surface of the vial, and the second was obtained by wiping the septum of the vial after removing the flip-off cap. Before wiping, the filter paper was wet with 100 µL of a solution consisting of ACN and water (20:80, v/v) with 0.1% FA. After wiping the surface, the filter paper was placed in 1.5-mL polyethylene (PE) safe-lock tubes from Eppendorf AG (Hamburg, Germany) with 1 mL of the IS solution at 50 $ng \cdot mL^{-1}$ in ACN/water (80:20 v/v) with 0.1% FA. After ultra-sonication for 20 min, the samples were centrifuged at 2880 RCF for 5 min. Finally, 25 µL of the cleared solution was injected into the LC-MS/MS apparatus. Vials were removed from their primary packaging when performing the two wipes samples. The operator held the vial with one hand by the capsule (aluminum part), what was not tested in this study, with the flip-off for during walls wiping and without the flip-off during the septum wiping. With the other hand, the operator held a clamp (washed after each sampling with a solution of ACN and water (20:80, v/v) with 0.1% FA). Thus, the vial was immediately returned in their packaging. A new pair of gloves was used for vials with a different batch number. The validation of the wiping procedure was previously published.[22]

Equipment and LC-MS/MS conditions

Analyses were performed with the Accela liquid chromatography system from Thermo Fisher Scientific Inc. (Waltham, MA). The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Discovery mass spectrometer (MS) from Thermo Fisher Scientific equipped with the Ion Max electrospray ionisation (ESI) interface and operated with Xcalibur software (Thermo Fisher Scientific). The separations were performed on a ZORBAX SB-C18 RR 2.1 × 100 mm, 3.5-µm column from Agilent Technologies (Waldbronn, Germany). The LC-MS/MS method was previously described in detail.[21] The limits of quantifications (LOQ) were 0.5 $ng \cdot mL^{-1}$ for gemcitabine and cyclophosphamide, 1 $ng \cdot mL^{-1}$ for cytarabine, methotrexate, etoposide phosphate, ifosfamide, irinotecan and vincristine, and 2 $ng \cdot mL^{-1}$ for doxorubicin and epirubicin. To ensure the absence of carryover effect, already described during the validation of the LC-MS/MS method,[21] blank samples were regularly injected in the samples sequence (approximately 1 blank for 10 samples).

Results and discussion

The aim of this work was to evaluate the external contamination on cytotoxic drug vials available on the Swiss market. For this purpose, 133 vials containing one of the 10 following cytotoxic drugs were tested: cytarabine (CYT), gemcitabine (GEM), cyclophosphamide (CP), ifosfamide (IF), methotrexate (MTX), etoposide phosphate (ETO), irinotecan (IRI), doxorubicin (DOX), epirubicin (EPI) and vincristine (VCR). The choice of these compounds was a compromise between the most prescribed drugs in the Geneva University Hospitals, their toxicity and analytical considerations. The choice of only one IS allowed to the limited use of toxic compounds in the analysis samples to reduce operator exposure and preserve the environment. The [13C, 2H3]-methotrexate was selected because it exhibited high ESI–MS/MS response and was eluted in the middle retention time window of the 10 investigated cytotoxic drugs.

As reported in Table 2, a wide range of formulations was selected in terms of concentration/amounts of

Table 2. Vial characteristic and surface contamination results obtained for the evaluation of 133 cytotoxic vials available on a Swiss market.

				Results (O: outside vial; S: septum)	
Manufacturer	Specific drug (amount in vial, form)	Packaging characteristics	Vial ref (batch number)	Amount of active ingredient in ng per vial	Amount of other cytotoxic drugs in ng per vial
Actavis®	Doxorubicin (50 mg, powder)	Glass vial in break-proof plastic container	DOX 1 (BQ08016A)	O: NF	O: NF
				S: <LOQ	S: NF
			DOX 2 (BQ08016A)	O: NF	O: NF
				S: NF	S: NF
			DOX 3 (BQ08016A)	O: NF	O: NF
				S: NF	S: NF
	Epirubicin (200 mg, solution)	Glass vial in break-proof plastic container	EPI 1 (AN09035C)	O: NF	O: IRI<LOQ; GEM: 1.19
				S: NF	S: NF
			EPI 2 (AN09035C)	O: NF	O: IRI<LOQ; GEM: 1.30
				S: NF	S: IRI<LOQ
			EPI 3 (AN09035C)	O: NF	O: GEM: 1.17
				S: NF	S: NF
	Gemcitabine (1 g, powder)	Glass vial in break-proof plastic container	GEM 1 (09K0073)	O: 1.92	O: NF
				S: NF	S: NF
			GEM 2 (09K0073)	O: 1.38	O: NF
				S: 1.03	S: NF
			GEM 3 (09K0073)	O: 3.54	O: NF
				S: 0.84	S: NF
	Irinotecan (100 mg, solution)	Glass vial protected by a plastic shrink vial	IRI 1 (02J0038)	O: NF	O: NF
				S: NF	S: NF
			IRI 2 (02J0038)	O: NF	O: NF
				S: NF	S: NF
			IRI 3 (02J0038)	O: NF	O: NF
				S: NF	S: NF
	Irinotecan (500 mg, solution)	Glass vial in break-proof plastic container	IRI 4 (02K0051)	O: NF	O: NF
				S: NF	S: NF
			IRI 5 (02K0051)	O: NF	O: NF
				S: NF	S: NF
			IRI 6 (02K0051)	O: NF	O: NF
				S: NF	S: NF
			IRI 7 (CW100007B)	O: 2.18	O: GEM: 4.55; MTX: 0.41
				S: NF	S: NF
Baxter®	Cyclophosphamide (1 g, powder)	Glass vial	CP 1 (1A707K)	O: NF	O: NF
				S: NF	S: NF
			CP 2 (1A707K)	O: NF	O: NF
				S: NF	S: NF
			CP 3 (1A707K)	O: NF	O: NF
				S: NF	S: NF
			CP 4 (1D716D)	O: NF	O: IF: 1.37
				S: NF	S: NF
	Ifosfamide (2 g, powder)	Glass vial	IF 1 (OK314M)	O: 14.77	O: NF
				S: 1.72	S: NF
			IF 2 (OK314M)	O: 25.93	O: NF
				S: 6.52	S: NF
			IF 3 (OK314M)	O: 0.57	O: NF
				S: 1.13	S: NF
			IF 4 (1C317F)	O: 79.11	O: NF
				S: 368.50	S: NF

(continued)

Table 2. Continued

Manufacturer	Specific drug (amount in vial, form)	Packaging characteristics	Vial ref (batch number)	Results (O: outside vial; S: septum)	
				Amount of active ingredient in ng per vial	Amount of other cytotoxic drugs in ng per vial
Bristol Myers Squibb ®	Etoposide phosphate (113.6 mg, powder)	Glass vial	ETO 1 (OJ59001)	O: NF	O: NF
				S: NF	S: NF
			ETO 2 (OJ59001)	O: 12.74	O: NF
				S: NF	S: NF
			ETO 3 (OJ59001)	O: NF	O: NF
				S: NF	S: NF
			ETO 4 (1C00530)	O: NF	O: NF
				S: NF	S: NF
	Etoposide phosphate (1.136 g, powder)	Glass vial	ETO 5 (1C0030)	O: 1890.60	O: CP<LOQ
				S: 6.08	S: NF
			ETO 6 (1C0030)	O: 203.63	O: CP: 0.83
				S: 3.19	S: IF <LOQ
			ETO 7 (1C0030)	O: 150.72	O: CP: 0.79
				S: 2.26	S: NF
Ebewe®	Doxorubicin (50 mg, solution)	Glass vial in break-proof plastic container	DOX 4 (96589409)	O: NF	O: NF
				S: NF	S: NF
			DOX 5 (96589409)	O: NF	O: NF
				S: NF	S: NF
			DOX 6 (96589409)	O: NF	O: NF
				S: NF	S: ETO:4.52
	Doxorubicin (100 mg, solution)	Glass vial in break-proof plastic container	DOX 7 (95607110)	O: NF	O: NF
				S: NF	S: NF
			DOX 8 (95607110)	O: NF	O: NF
				S: NF	S: NF
			DOX 9 (95607110)	O: NF	O: NF
				S: NF	S: NF
	Doxorubicin (200 mg, solution)	Glass vial in break-proof plastic container	DOX 10 (96624106)	O: 3.25	O: CYT:9.42; GEM: 5.62; IRI: 2.77
				S: NF	S: NF
			DOX 11 (96624106)	O: 4.05	O: CYT: 1.37; GEM: 2.47
				S: <LOQ	S: NF
			DOX 12 (96624106)	O: 2.62	O: CYT: <LOQ; GEM: 6.48
				S: <LOQ	S: NF
	Epirubicin (200 mg, solution)	Glass vial in break-proof plastic container	EPI 4 (96172306)	O: 2.80	O: GEM: 2.06; MTX: 0.39
				S: NF	S: MTX<LOQ
			EPI 5 (96172306)	O: 2.67	O: GEM: 2.06; MTX: 3.43
				S: NF	S: MTX<LOQ
			EPI 6 (96172306)	O: 17.30	O: GEM: 2.78; MTX: 0.67
				S: NF	S: NF
	Gemcitabine (1 g, powder)	Glass vial in break-proof plastic container	GEM 4 (95883305)	O: NF	O: NF
				S: 20.54	S: NF
			GEM 5 (95883305)	O: NF	O: NF
				S: 24.76	S: NF
			GEM 6 (95883305)	O: NF	O: NF
				S: 7.55	S: NF
	Irinotecan (40 mg, solution)	Glass vial	IRI 8 (95573904)	O: 7.24	O: GEM: 23.44; MTX: 0.35
				S: <LOQ	S: NF
	Irinotecan (100 mg, solution)	Glass vial	IRI 9 (93082705)	O: 5.41	O: MTX<LOQ
				S: <LOQ	S: MTX<LOQ
	Irinotecan (150 mg, solution)	Glass vial	IRI 10 (95536004)	O: 3.10	O: CYT: 101.72; GEM: 13.72; MTX: 52.28
				S: <LOQ	S: GEM: 0.56; MTX: 0.52
	Irinotecan (300 mg, solution)	Glass vial in break-proof plastic container	IRI 11 (96546204)	O: 50.99	O: GEM: 2.42; MTX: 8.65
				S: <LOQ	S: GEM: 1.71, MTX: <LOQ
	Irinotecan (500 mg, solution)	Glass vial in break-proof plastic container	IRI 12 (93083005)	O: NF	O: NF
				S: NF	S: NF

(continued)

Table 2. Continued

Manufacturer	Specific drug (amount in vial, form)	Packaging characteristics	Vial ref (batch number)	Results (O: outside vial; S: septum)	
				Amount of active ingredient in ng per vial	Amount of other cytotoxic drugs in ng per vial
Janssen Cilag ®	Doxorubicin (20 mg, solution)	Glass vial	DOX 13 (101211504)	O: NF	O: NF
				S: NF	S: IRI: <LOQ; MTX: <LOQ
			DOX 14 (101211504)	O: NF	O: NF
				S: NF	S: IRI: <LOQ
			DOX 15 (101211504)	O: NF	O: NF
				S: NF	S: NF
Labatec®	Gemcitabine (1 g, powder)	Glass vial protected by a plastic shrink vial	GEM 7 (AH0004)	O: NF	O: NF
				S: 47.15	S: NF
			GEM 8 (AH0004)	O: NF	O: NF
				S: NF	S: NF
			GEM 9 (AH0004)	O: NF	O: NF
				S: 10.53	S: NF
	Irinotecan (100 mg, solution)	Glass vial protected by a plastic shrink vial	IRI 13 (90607-1)	O: NF	O: NF
				S: NF	S: NF
			IRI 14 (90607-1)	O: NF	O: NF
				S: NF	S: NF
			IRI 15 (90607-1)	O: NF	O: NF
				S: NF	S: NF
Lilly®	Gemcitabine (1 g, powder)	Glass vial protected by a plastic shrink vial	GEM 10 (A749451D)	O: NF	O: IRI<LOQ
				S: 9.33	S: IRI<LOQ
			GEM 11 (A749451D)	O: NF	O: NF
				S: 12.47	S: NF
			GEM 12 (A749451D)	O: NF	O: IRI<LOQ
				S: NF	S: NF
Mepha®	Epirubicin (100 mg, solution)	Glass vial	EPI 7 (L10149/12)	O: NF	O: NF
				S: NF	S: NF
			EPI 8 (L10149/12)	O: NF	O: NF
				S: NF	S: NF
			EPI 9 (L10149/12)	O: NF	O: NF
				S: NF	S: NF
Orion Pharma®	Irinotecan (100 mg, solution)	Glass vial	IRI 16 (280410/2)	O: NF	O: NF
				S: NF	S: NF
			IRI 17 (280410/2)	O: NF	O: NF
				S: NF	S: NF
			IRI 18 (280410/2)	O: <LOQ	O: NF
				S: NF	S: NF
	Methotrexate (20 mg, solution)	Glass vial	MTX 1 (1355741)	O: 0.43	O: NF
				S: NF	S: NF
			MTX 2 (1355741)	O: 0.50	O: NF
				S: NF	S: NF
			MTX 3 (1355741)	O: NF	O: NF
				S: NF	S: NF
			MTX 4 (1367609)	O: NF	O: NF
				S: NF	S: NF
			MTX 5 (CC11050067)	O: <LOQ	O: NF
				S: <LOQ	S: NF
Pfizer®	Cytarabine (500 mg, solution)	Plastic vial	CYT 1 (FA17B)	O: <LOQ	O: NF
				S: NF	S: NF
			CYT 2 (FA17B)	O: <LOQ	O: NF
				S: NF	S: NF
			CYT 3 (FA17B)	O: <LOQ	O: NF
				S: NF	S: NF
			CYT 4 (FE13E)	O: <LOQ	O: NF
				S: NF	S: NF

(continued)

Table 2. Continued

Manufacturer	Specific drug (amount in vial, form)	Packaging characteristics	Vial ref (batch number)	Results (O: outside vial; S: septum)	
				Amount of active ingredient in ng per vial	Amount of other cytotoxic drugs in ng per vial
	Cytarabine (2 g, solution)	Plastic vial	CYT 5 (FE89C)	O: <LOQ	O: NF
				S: NF	S: NF
			CYT 6 (FE89C)	O: <LOQ	O: NF
				S: NF	S: NF
			CYT 7 (FE89C)	O: <LOQ	O: NF
				S: NF	S: NF
	Doxorubicin (50 mg, powder)	Glass vial	DOX 16 (OPL0094)	O: NF	O: CYT: 0.35; EPI: 2.85
				S: NF	S: NF
			DOX 17 (OPL0094)	O: NF	O: CYT: 0.94
				S: NF	S: NF
			DOX 18 (OPL0094)	O: NF	O: CYT: 2.18
				S: NF	S: NF
	Epirubicine (50 mg, solution)	Plastic vial	EPI 10 (FD41H)	O: NF	O: NF
				S: NF	S: NF
			EPI 11 (FD41H)	O: NF	O: NF
				S: NF	S: NF
			EPI 12 (FD41H)	O: NF	O: NF
				S: NF	S: NF
	Irinotecan (300 mg, solution)	Plastic vial	IRI 19 (FD67B)	O: NF	O: CYT: 8.63
				S: NF	S: NF
			IRI 20 (FD67B)	O: NF	O: CYT: 1.35
				S: NF	S: NF
			IRI 21 (FD67B)	O: NF	O: CYT: 0.67
				S: NF	S: NF
	Methotrexate (10 mg, solution)	Glass vial	MTX 6 (V851)	O: 1.10	O: GEM: 1.55
				S: <LOQ	S: NF
			MTX 7 (V851)	O: 1.07	O: GEM: 0.87
				S: <LOQ	S: NF
			MTX 8 (V851)	O: 1.74	O: NF
				S: NF	S: NF
	Vincristine (2 mg, solution)	Plastic vial	VCR 1 (FE14H)	O: NF	O: CYT: <LOQ
				S: NF	S: NF
			VCR 2 (FE14H)	O: NF	O: CYT: <LOQ
				S: NF	S: NF
			VCR 3 (FE14H)	O: NF	O: CYT: <LOQ
				S: NF	S: NF
Sandoz®	Cytarabine (100 mg, solution)	Glass vial	CYT 8 (12190711)	O: 9.87	O: NF
				S: 5.47	S: NF
	Cytarabine (500 mg, solution)	Glass vial	CYT 9 (96519814)	O: 24.50	O: NF
				S: 4.58	S: NF
	Cytarabine (1 g, solution)	Glass vial in break-proof plastic container	CYT 10 (96623310)	O: 32.35	O: NF
				S: <LOQ	S: NF
	Cytarabine (2 g, solution)	Glass vial in break-proof plastic container	CYT 11 (12477906)	O: 2.61	O: NF
				S: 0.22	S: NF
	Irinotecan (100 mg, solution)	Glass vial	IRI 22 (96772512)	O: 3.44	O: CYT: 19.20; GEM: 4.56; MTX: 0.76
				S: <LOQ	S: NF
			IRI 23 (96772512)	O: 3.85	O: CYT: 3.49; GEM: 22.63; MTX: 0.25
				S: <LOQ	S: NF
			IRI 24 (96772512)	O: 3.53	O: CYT: 4.33; GEM: 7.01; MTX: 0.33
				S: <LOQ	S: GEM 4.56

(continued)

Table 2. Continued

Manufacturer	Specific drug (amount in vial, form)	Packaging characteristics	Vial ref (batch number)	Results (O: outside vial; S: septum)	
				Amount of active ingredient in ng per vial	Amount of other cytotoxic drugs in ng per vial
	Methotrexate (5 g, solution)	Glass vial in break-proof plastic container	MTX 9 (12020305)	O: <LOQ	O: GEM 1.74; IRI: <LOQ
				S: NF	S: NF
			MTX 10 (12020305)	O: NF	O: NF
				S: NF	S: NF
			MTX 11 (12020305)	O: NF	O: NF
				S: NF	S: NF
Teva®	Doxorubicin (50 mg, solution)	Glass vial protected by a plastic shrink vial	DOX 19 (10F23LL)	O: NF	O: MTX: 0.70
				S: NF	S: NF
			DOX 20 (10F23LL)	O: NF	O: MTX:0.32
				S: NF	S: NF
			DOX 21 (10F23LL)	O: NF	O: MTX:0.59
				S: NF	S: NF
	Gemcitabine (1 g, powder)	Glass vial protected by a plastic shrink vial	GEM 13 (10J06PB)	O: NF	O: MTX<LOQ
				S: 1.29	S: NF
			GEM 14 (10J06PB)	O: NF	O: MTX<LOQ
				S: 1.52	S: NF
			GEM 15 (10J06PB)	O: NF	O: MTX<LOQ
				S: 4.23	S: NF
	Irinotecan (100 mg, solution)	Glass vial	IRI 25 (CD075C)	O: 46.79	O: CYT: 30.31
				S: 0.93	S: CYT: <LOQ
			IRI 26 (CD075C)	O: 67.58	O: CYT:30.46
				S: 6.13	S: CYT: <LOQ
			IRI 27 (CD075C)	O: 145.14	O: CYT: 34.38
				S: 20.74	S: CYT: <LOQ
	Methotrexate (50 mg, solution)	Glass vial protected by a plastic shrink vial	MTX 12 (10A13KA)	O: <LOQ	O: NF
				S: 0.43	S: NF
			MTX 13 (10A13KA)	O: <LOQ	O: NF
				S: <LOQ	S: NF
			MTX 14 (10A13KA)	O: <LOQ	O: NF
				S: NF	S: NF
	Methotrexate (500 mg, solution)	Glass vial protected by a plastic shrink vial	MTX 15 (10I24NH)	O: NF	O: NF
				S: NF	S: NF
			MTX 16 (10I24NH)	O: NF	O: NF
				S: NF	S: NF
			MTX 17 (10I24NH)	O: NF	O: NF
				S: NF	S: NF
	Methotrexate (5 g, solution)	Glass vial protected by a plastic shrink vial	MTX 18 (10D29MB)	O: 0.70	O: NF
				S: 1.40	S: NF
			MTX 19 (10D29MB)	O: 1.34	O: NF
				S: 1.56	S: NF
			MTX 20 (10D29MB)	O: 0.41	O: NF
				S: <LOQ	S: NF
	Vincristine (2 mg, solution)	Glass vial protected by a plastic shrink vial	VCR 4 (10H11NF)	O: NF	O: NF
				S: NF	S: NF
			VCR 5 (10H11NF)	O: NF	O: NF
				S: NF	S: NF
			VCR 6 (10H11NF)	O: NF	O: NF
				S: NF	S: NF
	Vincristine (1 mg, solution)	Glass vial	VCR 7 (10K08PB)	O: NF	O: NF
				S: NF	S: NF
			VCR 8 (10K08PB)	O: NF	O: NF
				S: NF	S: NF
			VCR 9 (10K08PB)	O: NF	O: NF
				S: NF	S: NF

(continued)

Table 2. Continued

Manufacturer	Specific drug (amount in vial, form)	Packaging characteristics	Vial ref (batch number)	Results (O: outside vial; S: septum)	
				Amount of active ingredient in ng per vial	Amount of other cytotoxic drugs in ng per vial
Teva®	Epirubicine (200 mg, solution)	Glass vial protected by a plastic shrink vial	EPI 13 (11B14SD)	O: NF	O: NF
				S: NF	S: NF
			EPI 14 (11B14SD)	O: NF	O: NF
				S: NF	S: NF
			EPI 15 (11B14SD)	O: NF	O: NF
				S: NF	S: NF

NF: not found; LOQ (limit of quantification) were reported in experimental section for each compound

active ingredients in the vial, drug form (solution or powder), vial size (from 1 to 100 mL) and manufacturer. Among the vial tested, four different types of vials were evaluated: glass vials, plastic vials, glass protected by plastic shrink-wrap and glass vials in break-proof plastic containers. None of the vials evaluated in this work showed any signs of damage.

Results are described in detail in Table 2 and are summarized in Table 3 according to the type of vials.

The following key points can be discussed:

- Vial contaminations

It seems that a relationship between the contamination levels on surface of the vial and the amount of drug contained inside the vial for the same manufacturer only exists in two cases (DOX from Ebewe and ETO from Bristol Myers Squibb). The highest contamination by the active ingredient was observed for the vials containing 1.136 g of dried ETO (ETO5-6-7), and lower amounts of ETO were detected on the vials from the same manufacturer containing lower amounts of ETO (ETO1-2-3 contained 10 times less ETO than ETO5-6-7). Similar observations were found for vials DOX4 through DOX12, where the highest contamination levels were found on the vials containing the highest amount of DOX (200 mg in DOX10-11-12). However, this finding was not observed for CYT1-11, IRI1-12 and MTX12-20 where the highest contamination was not found on the vials with the largest content.

As regards the formulation form, the highest contaminations were observed for solid products (ETO and IF). But all vials containing high amounts of powder did not show high contamination levels (GEM4-9, GEM13-15, CP1-4). Moreover, few liquid formulations have also demonstrated external contaminations superior to 100 ng per vial (IRI26 and IRI 27). Consequently, it appears that the contamination levels depended on not only the drug form (liquid or solid) but also on the drug type and certainly also of the production process.

As shown in Table 3, results observed for glass vials (without protection system) and glass vials in break-proof plastic container were similar. This was not surprising given the both types of vials were subjected on the same filling and washing procedures in the production chain, only the further step of packaging in a plastic container differs. The vial breakage prevention offered by the secondary packaging (break-proof plastic container) is only ensured at the end of production chain (for the storage, transport and handling by healthcare professionals) compared to plastic vials present from the beginning of the production chain. Also, the range of contamination level was much smaller in the case of glass vials in break-proof plastic container. A possible explanation is that the chemical contamination is due to a vial breakage at the end of the production chain. Therefore, the main role of break-proof plastic container is not only to establish a physical barrier between the operator and the vial but also to avoid the presence of traces of cytotoxic drugs on vials. The plastic (polypropylene) vials, which were all from the same manufacturer, had very low levels of chemical contamination (CYT1 to CYT7, DOX16-17-18, EPI10-11-12, IRI19-20-21, MTX6-7-8 and VRC1-2-3 vials). An adequate decontamination of vials after the filling step by the manufacturer, the nature of the container, which may prevent the deposition of drug and the prevention of vial breakage in the production chain are potential explanations for the low contamination observed on the plastic vials. The vials protected by plastic shrink-wrap had external contamination in lower amounts (less than 1 ng per vial). These results are consistent with those of Schierl et al.[14]

A variability of contamination levels was observed for different vials from the same batch number; which have already been demonstrated by the study of Touzin et al.[7] Contamination levels with the active ingredient varying from <LOQ to 47.15 ng per vial were detected

Table 3. Summary of surface contamination results obtained for the evaluation of 133 cytotoxic vials available on a Swiss market.

Vials	Number of tested vial	With chemical contamination (amount range in ng per vial)	With chemical contamination superior to 10 ng per vial (amount range in ng per vial)	With chemical contamination on septum (amount range in ng per vial)	With chemical cross-contamination (amount range in ng per vial)
Glass vial (without protection)	49	65% (<LOQ- 1896.66)	35% (12.74-1896.66)	47% (<LOQ- 368.50)	41% (<LOQ- 169.32)
Glass vial in break-proof plastic container	35	63% (<LOQ- 63.77)	17% (20.54-63.77)	43% (<LOQ- 24.76)	37% (<LOQ- 17.81)
Vial protected by a plastic shrink vial	33	52% (<LOQ- 47.15)	9% (10.53-47.15)	36% (<LOQ- 47.15)	24% (<LOQ- 0.70)
Plastic vial	16	81% (<LOQ- 8.63)	0	0	38% (<LOQ- 8.63)
Total	133	63% (<LOQ- 1896.66)	20% (10.53-1896.66)	38% (<LOQ- 368.50)	35% (<LOQ- 169.32)

NF: not found; LOQ (limit of quantification) were reported in experimental section for each compound.

on a batch of 3 vials of 1 g of GEM. A factor of 40 was observed between the contamination levels by IF on the walls of 3 vials from the same batch (vials of 2 g of IF).

A difference in term of contamination levels was also observed according to the manufacturer. As already mentioned, the highest rate of contamination with the active ingredient was detected on vials of 1.136 g of ETO from Bristol Myers Squibb. A high amount of active ingredient has been detected on one of Baxter products (IF). Ebewe products (DOX, EPI, GEM and IRI) have shown high concentration levels for not only the active ingredient but also for other cytotoxic drugs except for vials containing low amounts of doxorubicin (no contamination). At least one of the tested products supplied by Teva, Labatec and Sandoz has shown contamination level by the active ingredient superior to 10 ng per vial. Amounts inferior to 5 ng per vial of active ingredient were determined on Pfizer (CYT, DOX, EPI, IRI, MTX and VCR), Actavis (DOX, EPI, GEM and IRI), Orion Pharma (IRI and MTX) and Lilly (GEM) products. No contamination by the active ingredient was detected on the only tested product from Janssen Cilag (DOX) and Mepha (EPI).

- Septum contamination

The septum wiping procedure was chosen in this study because the vial washing step involved usually at the end of the production chain (after placing the capsule and the cap cover) did not allow a decontamination of the septum. Moreover, the presence of cytotoxic drugs on the injection area of the vial may affect the safety of the medicine, especially in the case of a cross-contamination. As shown in Table 3, the protection systems of vials, such as break-proof plastic containers and plastic shrink vials, have not prevented the presence of cytotoxic drugs (GEM7-8-9, GEM10-11-12 and GEM13-14-15). Similar results were observed for no protected glass vials. This confirmed the hypothesis that the decontamination procedures were not performing well enough and/or were applied at the end of the production chain after placing the capsule and the cap cover on the vial in these cases. On the other hand, no contamination was detected on the septum of plastic vials. Given plastic vials were supplied by the same manufacturer and the low external contamination detected on the walls of vials, the use of an adequate decontamination of vials after the filling step by the manufacturer and the prevention of vial breakage in the production chain seemed to be the main explanations.

The presence of others cytotoxic drugs other than the active ingredient were detected on the septum of 15 vials (e.g. 9 glass vials, 5 glass vials in break-proof plastic containers and 1 plastic shrink vial) with cross-contaminations inferior to 5 ng per vial.

- Cross-contamination

Traces of cytotoxic drugs other than the active ingredients were detected on 47 vials, whoever the manufacturer. Ten vials showed cross-contaminations superior to 10 ng per vial (21% of vials with a cross contamination detected). In this case, the most detected cytotoxic compounds were drugs administered at high concentration and, thus, present in high amount (in the order of g) in cytotoxic vials (e.g. gemcitabine and cytarabine). The highest cross-contamination was observed for IRI10 vial supplied by Ebewe with a contamination by cytarabine (101.72 ng), gemcitabine (14.28 ng) and methotrexate (52.8 ng); which represented between 0.01 ppm and 0.1 ppm from the amount of the drug in the commercialised vials for each compound. Even if the presence of different cytotoxic compounds on the vials may be surprising, the detected amounts were inferior to the upper limits recommended by the authorities.[23] It can be noteworthy that the presence of other drugs, such as platinum compounds and 5-fluorouracil, which are not detectable by the method used herein, cannot be excluded. In most cases, the cytotoxic drugs detected corresponded to compounds available in other formulations produced by the same manufacturer and commercialized in Swiss and / or other countries' markets.

This cross-contamination certainly occurs during the production or the packaging process.

- Vial decontamination and handlers protection

Given the chemical contamination observed on the tested vials, a suitable decontamination step and the application of recommendations to reduce the risk of exposure to cytotoxic drugs for healthcare professional[24] are of utmost importance. Even if the levels of contamination are low, it cannot be excluded that chronic exposure may have negative effects on the health of the handlers. The use of personal protective equipment, especially suitable gloves, is essential for the protection of the healthcare professionals who handle these vials. The decontamination step must remove chemical contamination using a method that must be considered independent of microbiological decontamination, which primarily requires the use of alcoholic solutions that promote environmental dispersion of organic compounds. Nowadays, the use of decontamination agents with a degradation action on the cytotoxic drugs,[18,25–27] such as sodium hydroxide diluted solutions or oxidising solutions, and the use of less stronger decontamination agents,[7,28] such as dishwashing liquid, constitute interesting strategies to reduce the cytotoxic contamination.

Conclusions

An evaluation of the external chemical contamination of more than 100 cytotoxic vials that were available on the Swiss market was performed. A method for the simultaneous analysis of 10 cytotoxic compounds by LC-MS/MS allowed for the detection of cross-contaminations. In all, 63% of the tested vials exhibited contamination by a cytotoxic drug and cross-contamination was detected on 35% of the tested vials. The contaminations by a cytotoxic compound ranged between 0 ng and 1896.66 ng per vial. No contamination or very low levels of cytotoxic drugs were detected on the vials protected by plastic shrink-wrap. Traces of cytotoxic compounds were also found on the septa of 50 vials, including protected vials. This study shows that contamination rates were usually low, but vials with contamination at microgram levels of cytotoxic drugs still exist. Consequently, it is important to handle vials containing cytotoxic drugs with gloves and to use an appropriate procedure for the decontamination of vials before their use in preparing chemotherapies. It is also important to limit the dispersion of organic compounds to the environment (e.g., by spilling ethanol for disinfection) to avoid potential cross-contamination. In addition, manufacturers must enhance their efforts to implement validated decontamination procedures to ensure the supply of uncontaminated and safe vials.

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Article VIII

« Evaluation of decontamination efficacy of cleaning solutions on stainless steel and glass surfaces contaminated by 10 antineoplastic agents.»

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Evaluation of Decontamination Efficacy of Cleaning Solutions on Stainless Steel and Glass Surfaces Contaminated by 10 Antineoplastic Agents

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Objectives: The handling of antineoplastic agents results in chronic surface contamination that must be minimized and eliminated. This study was designed to assess the potential of several chemical solutions to decontaminate two types of work surfaces that were intentionally contaminated with antineoplastic drugs.

Methods: A range of solutions with variable physicochemical properties such as their hydrophilic/hydrophobic balance, oxidizing power, desorption, and solubilization were tested: ultrapure water, isopropyl alcohol, acetone, sodium hypochlorite, and surfactants such as dishwashing liquid (DWL), sodium dodecyl sulfate (SDS), Tween 40, and Span 80. These solutions were tested on 10 antineoplastic drugs: cytarabine, gemcitabine, methotrexate, etoposide phosphate, irinotecan, cyclophosphamide, ifosfamide, doxorubicin, epirubicin, and vincristine. To simulate contaminated surfaces, these molecules (200 ng) were deliberately spread onto two types of work surfaces: stainless steel and glass. Recovered by wiping with a specific aqueous solvent (acetonitrile/HCOOH; 20/0.1%) and an absorbent wipe (Whatman 903®), the residual contamination was quantified using high-performance liquid chromatography (HPLC) coupled to mass spectrometry. To compare all tested cleaning solutions, a performance value of effectiveness was determined from contamination residues of the 10 drugs.

Results: Sodium hypochlorite showed the highest overall effectiveness with 98% contamination removed. Ultrapure water, isopropyl alcohol/water, and acetone were less effective with effectiveness values of 76.8, 80.7, and 40.4%, respectively. Ultrapure water was effective on most hydrophilic molecules (97.1% for cytarabine), while on the other hand, isopropyl alcohol/water (70/30, vol/vol) was effective on the least hydrophilic ones (85.2% for doxorubicin and 87.8% for epirubicin). Acetone had little effect, whatever the type of molecule. Among products containing surfactants, DWL was found effective (91.5%), but its formulation was unknown. Formulations with single surfactant non-ionics (tween 40 and span 80) or anionic (SDS) were also tested. Finally, solutions containing 10^{-2} M anionic surfactants and 20% isopropyl alcohol had the highest global effectiveness at around 90%. More precisely, their efficacy was the highest (94.8%) for the most hydrophilic compounds such as cytarabine and around 80.0% for anthracyclines. Finally, the addition of isopropyl alcohol to surfactant solutions enhanced

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their decontamination efficiency on the least hydrophilic molecules. Measured values from the stainless steel surface were similar to those from the glass one.

Conclusion: This study demonstrates that all decontamination agents reduce antineoplastic contamination on work surfaces, but none removes it totally. Although very effective, sodium hypochlorite cannot be used routinely on stainless steel surfaces. Solutions containing anionic surfactant such as SDS, with a high efficiency/safety ratio, proved most promising in terms of surface decontamination.

Keywords: decontamination/methods; detergents; equipment contamination/prevention and control; hazardous substances/analysis; occupational exposure/analysis

INTRODUCTION

Nowadays, antineoplastic drugs are widely used in cancer therapies. Given their high toxicity, these substances represent a potential risk for professionals at each step of the healthcare process. The National Institute for Occupational Safety and Health has estimated that around 5.5 million healthcare workers are potentially exposed to hazardous drugs in the USA. Despite publications of guidelines describing handling protocols and the use of biology safety cabinets (BSCs) or barrier isolators, surface contamination still exists in hospital pharmacy units (Acampora *et al*., 2005; Crauste-Manciet *et al*., 2005; ISOPP, 2007). Environmental monitoring has indicated that all surfaces could be potentially contaminated (Turci *et al*., 2003; Bussières *et al*., 2006; Heinemann *et al*., 2008; Käslin *et al*., 2010). Biological monitoring has proved that genotoxic effects can be detected by the Ames test or SOS chromo tests (urine mutagenicity, chromosomal aberrations, sister chromatid exchanges, and micronuclei) in the urine of nurses and pharmacy technicians (Poyen *et al*., 1988; Sessink *et al*., 1994; Cavallo *et al*., 2005; Quillardet and Hofnung, 2009). Physical effects such as skin rashes, adverse reproductive effects (abortions, stillbirths, and congenital malformations), leukaemia, or cancers can occur (Skov *et al*., 1990; Connor and McDiarmid, 2006). Traces of contamination have been described in patients' rooms and hospital effluents, among operating theatre personnel, pharmacy technicians, and pharmacists (Mahnik *et al*., 2004, 2006; Sottani *et al*., 2010, 2011). Several papers have reported antineoplastic drug contamination on vials, surfaces, floors, countertops, carts, storage bins, waste containers, tabletops, chairs, and linen and in the atmosphere of pharmacy units (Mason, 2003; Connor and McDiarmid, 2006; Touzin *et al*., 2008). The main exposure routes have been by dermal contact with contaminated surfaces and by inhalation of particles (Kromhout *et al*., 2000; Fransman *et al*., 2005; Connor and McDiarmid, 2006). To confront this challenge, the pharmacist strategy is first to confine contamination in specific pharmacy areas within closed working areas (biosafety cabinets and isolators) and secondly to reduce the risk of contamination on pharmacists and on pharmacy technicians by using specific devices such as containment safety devices rather than needles for example. However, there is still a risk of accumulation over time. Efficient decontamination of surfaces is therefore of the utmost importance.

Several studies are available on the impact of decontamination procedures to reduce chemical contamination by cytotoxic agents. Raghavan *et al.* studied a water rinsing method on cisplatin decontamination using liquid chromatography (Raghavan *et al*., 2000). Chlorine-based agents reduced the mutagenicity of methotrexate (MTX) by inactivating it (Wren *et al*., 1993). Earlier studies described various other solutions for cytotoxic agents on different surfaces. Multiple compounds [carmustine (BCNU), lomustine (CCNU), chlorozotocin, N-[2-chloroethyl]-N'-[2,6-dioxo-3-piperidinyl]-N-nitrosourea (PCNU), 1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosoure (Methyl-CCNU), mechlorethamine, melphalan, chlorambucil, cyclophosphamide, ifosfamide, uracil mustard, and spiromustine] were degraded using nickel aluminium in a potassium hydroxyde solution without any toxic degradation (Lunn *et al*., 1989). Barek *et al.* proposed two methods for surface decontamination: the first reported almost total degradation of melphalan based on its oxidation by potassium permanganate in a sodium hydroxide solution, and the second degraded multiple compounds (amsacrine, azathioprine, asparaginase, and thiotepa) using sodium hypochlorite and a Fenton reagent [an oxidizing solution based on hydrogen peroxide oxidized by catalyst ferrous iron (II)] (Barek *et al*., 1987, 1998). Oxidizing agents had already been tested on antineoplastic agents (Hansel *et al*., 1996; Castegnaro *et al*., 1997; Roberts *et al*., 2006) and assessments established on different antineoplastic classes: oxazophosphorine or anthracycline molecules, using

hydrogen peroxide and sodium hypochlorite, which proved to be effective. Vaporized hydrogen peroxide and detergents were also investigated with positive results on 5-Fluorouracil, doxorubicin (DOX), and cyclophosphamide. Despite all previous studies, to date, no clear, effective, and evidence-based cleaning recommendations for daily practice exist. The aim of this paper is to evaluate the surface decontamination efficacy of different cleaning solutions through a step-by-step controlled study, to provide advice for cleaning steps in pharmacy units.

The first part of this experimental work was performed on stainless steel, where aqueous solutions, aqueous alcohol solutions, or organic solutions were first screened. An improvement was then made on selected solutions and finally the optimal volume required for decontamination was determined. The second part was performed on glass to test the effectiveness of selected solutions. The final objective is to provide an effective and clear review of cleaning solutions for the periodic decontamination of work areas.

EXPERIMENTAL

Chemicals and reagents

Antineoplastic agents. The study was performed with the following commercially available cytotoxic drugs (Table 1). Reconstitution of etoposide phosphate (Etopophos®), gemcitabine (Gemcitabine Teva®), and ifosfamide (Holoxan®) was obtained with water for injection (Bichsel Laboratories, Interlaken, Switzerland). 5% sterile glucose (Sintetica, Bioren SA, Couvet, Switzerland) was used for the reconstitution of Endoxan®.

Liquid chromatography–mass spectrometry/mass spectrometry. Lichrosolv® HPLC grade acetonitrile (ACN) and ultrapure water were purchased from Merck (Darmstadt, Germany), and formic acid (FA) came from Biosolve (Valkenswaard, the Netherlands).

Wiping and desorption material. Filter paper (Protein Saver TM 903 Card) was from Whatman (Dassel, Germany), and 1.5 ml polyethylene (PE) safe-lock tubes were from Eppendorf AG (Hamburg, Germany). Texwipe 3210 cleaning wipers, used as received as desorption material, were from ITW Texwipe (Kernersville, USA).

Cleaning solutions. Products used in cleaning solution formulations are summarized in Table 2. Simple solutions were tested as decontamination procedures. The choice of these solutions was based on current pharmaceutical practice and on scientific publications. Two kinds of solutions were tested: "elimination-type" solutions whose main action is to dissolve chemical products on the surface and "degradation-type" solutions that react with the chemical structure of compounds, leading to their degradation and the formation of expected non-cytotoxic compounds. Among "elimination-type" solutions, ultrapure

Table 1. Commercially available cytotoxic drugs used in the study.

Molecules (acronym)	Brand name	Hydrophilic (H)/ Hydrophobic (h)	Concentration	Manufacturer	Town, country
Molecules in test					
Irinotecan (IRI)	Campto®	h	20 mg ml^{-1}	Pfizer AG	Zürich, Switzerland
Cytarabine (CYT)	Cytosar®	H	20 mg ml^{-1}		
Gemcitabine (GEM)	Gemcitabin Teva®	H	20 mg ml^{-1}	Teva Pharma AG	Aesch, Switzerland
Vincristine (VI)	Vincristine Teva®	h	1 mg ml^{-1}		
Ifosfamide (IF)	Holoxan®	H	40 mg ml^{-1}	Baxter AG	Volketswil, Switzerland
Cyclophosphamide (CP)	Endoxan®	H	20 mg ml^{-1}		
Methotrexate (MTX)	Methotrexate Farmos®	H	2.5 mg ml^{-1}	Orion Pharma	Zug, Switzerland
Etoposide phosphate (ETO)	Etopophos®	h	20 mg ml^{-1}	Bristol-Myers Squibb SA	Baar, Switzerland
Doxorubicin (DOX)	Doxorubine Ebewe®	h	2 mg ml^{-1}	Ebewe Pharma	Cham, Switzerland
Epirubicin (EPI)	Epirubicin Actavis Solution®	h	2 mg ml^{-1}	Actavis	Regensdorf, Switzerland
Internal standard					
IS	[^{13}C, $^{2}H3$]-MTX	H	—	Alsachim	Illkirch, France

Table 2. Products used in formulations of cleaning solutions tested.

Products (acronym)	International name	Manufacturer	Commentaries	Abbreviation	Experimental phase	Concentrations tested
Acetone	Propane-2-one	Merck (Darmstadt, Germany)	Analysis quality	—	Screening	—
Kleralcohol (IPA)	IPA/water	Ecolab (Farmham, UK)	Guidelines reference	IPA	Screening	70/30% (vol/vol)
DWL	—	Migros (Zurich, Switzerland)	Contains anionic and non-ionic surfactants (<30%)	DWL	Screening, Optimization	5%, 10 and 20% in ultrapure water (vol/vol)
Sodium hypochlorite 5%	Sodium hypochlorite	Tempia (Carouge-Geneve, Switzerland)	oxidative agent	NaClO	Screening, Optimization	Diluted at 0.5% (vol/vol) in ultrapure water
SDS	Sodium lauryl-sulfate	Merck (Hohenbrunn, Germany)	CMC: 0.82×10^{-3}M (Mukerjee and Mysels, 1971)	SDS	Optimization	10^{-4}M, 0.5×10^{-3}M, 10^{-3}M, 10^{-2}M and 10^{-1}M in ultrapure water
Tween 40	Polysorbate 40	Hänseler AG (Herisau, Switzerland)	non-ionic surfactant	—	Optimization	10% in ultrapure water (vol/vol)
Span 80	Oleatesorbitan 80 or sorbitan-(Z)-mono-9-octadecanoate		non-ionic surfactant	—	Optimization	10% in ultrapure water (vol/vol)

water was tested single as a cleaning solution reference and as solvent when mixed with surfactants such as dishwashing liquid (DWL), span 80, tween 40, and sodium dodecyl sulphate (SDS). Isopropyl alcohol (IPA) 70/30 was also studied because of recommendations from guidelines for microbiological decontamination in chemotherapy production units (Le Garlantezec *et al*., 2011). Hydrophobic solvents such as acetone were used to determine its expected efficacy on the more hydrophobic compounds. Finally, among "degradation-type" solutions, a sodium hypochlorite solution, the most currently used solution to wash surfaces today, was also tested.

Preparations of compound stock solutions, calibration standards, and internal standard

All solutions (i.e. drug reconstitutions and sample dilutions) were prepared in appropriate conditions (BSC, individual protection) for handling hazardous compounds such as cytotoxic agents. The preparation of solutions and standards was performed with brand drugs to avoid any direct contact of the operator with cytotoxic powder and to minimize contamination risk during the preparation of solutions. Aliquots of the internal standard (IS; 250 $\mu g.ml^{-1}$) were prepared with a mixture of ACN and water (75/25, vol/vol) and stored at −22°C for 12 months with no sample degradation observed. Stock solutions of IS were diluted daily to 50 ng ml^{-1} in 20% ACN (vol/vol) with 0.1% FA (vol/vol) and were kept stable for at least 2 weeks at 2–8°C. A main stock solution containing the 10 cytotoxic drugs was prepared by diluting at 20 $\mu g.ml^{-1}$ concentration each cytotoxic compound in water. This solution was further diluted to obtain five independent stock solutions at 20, 40, 200, 1 000, and 4 000 ng ml^{-1} in 20% ACN (vol/vol) and 0.1% FA (vol/vol). For calibration standards (CS), stock solutions were diluted with the IS solution at 50 ng ml^{-1} to obtain five CS at 1, 2, 10, 50, and 200 ng ml^{-1}.

Equipment and liquid chromatography–mass spectrometry/mass spectrometry conditions

Analyses were carried out with the Accela liquid chromatography system from Thermo Fisher Scientific Inc. (Waltham, MA, USA) consisting of a quaternary pump equipped with an online degasser, an auto sampler and a solvent platform. The chromatographic system was coupled to a Quantum Discovery MS from Thermo Fisher Scientific Inc. equipped with Ion Max electrospray ionization (ESI) interface and a triple quadrupole. The liquid chromatography–mass spectrometry/mass spectrometry system was monitored with Xcalibur software (Thermo Fisher Scientific). Separations were obtained on a ZORBAX SB-C18 RR column with an inner diameter of 2.1 mm, a length of 10 cm, and a particular diameter of 3.5 µm from Agilent Technologies (Waldbronn, Germany). The liquid chromatography–mass spectrometry/mass spectrometry conditions and method validation have been described in detail elsewhere (Nussbaumer *et al*., 2010).

Decontamination

All tests were performed under a laminar airflow hood. The surface to be investigated (10 × 10 cm) was contaminated with 50 µl of stock solution sprayed on surface (solution containing all 10 cytotoxic agents at 4000 ng ml^{-1}) using an adjustable volume micropipette. This voluntary contamination was repeated 10 times for each cleaning solution. For the drying step, contaminated surfaces were protected from light in a laminar airflow hood for a period of 1 h. After drying, different cleaning solutions were applied. These were prepared extemporaneously and used directly. 300 µl of each cleaning solution was poured onto a 100 cm^2 Texwipe 3210 wipe. A single standard motion from top to bottom was adopted to clean each surface.

Wiping and analytical procedure

The wiping step can recover the residual contamination present on the surface after the decontamination step. A validated wiping procedure was performed to reclaim remaining cytotoxic compounds (Nussbaumer *et al*., 2012). To do so, a 1-cm^2 blotting paper (Whatman 903®) was soaked with 100 µl of an aqueous desorbing solution [ACN: water, 20/80 (vol/vol) with 0.1% FA]. The contaminated surface was then wiped for 30 s, turning the blotting paper regularly. Blotting papers were placed in PE safe-lock tubes, and 1 ml IS solution at 50 ng ml^{-1} was added. Then samples were ultrasonicated for 20 min and centrifuged at 4000 rd min^{-1} for 5 min. All samples were immediately placed in the LC auto sampler at 15°C and analysed within the day.

Decontamination evaluation

Data extracted from the analytical procedure correspond to residual contamination ($RC_{i,m}$) of each antineoplastic agent. For each molecule, an efficiency index was generated ($Eff_{i,m}$; Equation 1). Then, to be able to compare cleaning solutions with each other, an overall effectiveness index was calculated (Eff_i). It was the average of the 10 efficiency indexes (Equation 2). So, this Eff_i corresponded to the overall effectiveness of a solution on the 10 antineoplastic agents, during a single attempt. To validate the overall effectiveness of a solution, each cleaning procedure was tested 10 times. As a conclusion, in this paper, the median value of those 10 attempts (EP_{value} or Efficiency performance value) was used to compare cleaning solutions (Equation 3). Results were presented as follow: median value [minimum value − maximum value].

$$Eff_{i,m} = 100 - RC_{i,m}(\%) \quad (1)$$

$$Eff_i = \frac{\sum Eff_{i,m}}{n_i} \quad (2)$$

$$EP_{value} = \text{median value } (Eff_i)n \quad (n = 10) \quad (3)$$

Standard deviation (SD) per compound for each cleaning solution was also calculated on 10 attempts. It indicated the reproducibility of the cleaning solution on each compound. Due to the numerous manual steps throughout the procedure, its acceptance threshold was arbitrarily set at 10%.

Sequence of experiments

In the first part of the study, tests were performed on stainless steel. The first "Screening" step involved screening solutions with various physicochemical characteristics. Working on a solubility procedure, tests were carried out with ultrapure water, aqueous-alcoholic solutions and organic solvents such as ACN. An oxidative solution was assessed using an aqueous solution of 0.5% sodium hypochlorite. At last, complex micellar formulations such as DWL diluted in ultrapure water were tested to focus on surfactant molecules. In the "Optimization" step, other detergent solutions were also tested using single anionic and neutral surfactants. SDS was especially focused on to consider the impact of its concentration on decontamination efficacy. Different formulations of aqueous-alcoholic solutions with stable SDS concentration were also tested to reduce surfactant deposit. Up to this point, tests were performed with normalized surfaces and volumes, non-representative of current decontamination activity. So, in the "Practical" step, additional tests were carried out over a 0.2 m^2 surface area with different volumes of optimized solution to simulate current cleaning methods. Finally, the second part involved tests on glass to validate the effectiveness of our solution on the most commonly used materials in closed working areas. All data are summarized in Table 2.

Statistical analysis

Statistical analysis was performed by analysis of variance on ranks following the method of Conover and Iman (Conover and Iman, 1981). This method was used to compare the effectiveness performance value of cleaning solutions. When this analysis revealed a significant P value ($P < 0.05$), contrasts were established with the Tukey–Kramer test to detect significant differences between couples of cleaning solutions with a statistical threshold of 5%. Analysis was performed with XLSTAT® software (Addinsoft).

RESULTS

Screening phase

Considering physicochemical properties of the 10 antineoplastic agents, two groups of molecules can be distinguished: first one corresponding to the most hydrophilic substances with cytarabine (CYT), gemcitabine (GEM), MTX, etoposide phosphate (ETO), cyclophosphamide (CP), and ifosfamide (IF) and second one to more hydrophobic compounds with irinotecan (IRI), DOX, vincristine (VI), and epirubicin (EPI). All data and statistical analyses performed on the stainless steel surface are summarized in Fig. 1 and Table 3.

Ultrapure water, aqueous alcohol, and organic solvents. Ultrapure water effectiveness was considered to be insufficient (Fig. 1). It was effective to remove CYT, GEM, IF, CP, and VI, but for MTX, ETO, IRI, DOX, and EPI, $Eff_{i,m}$ values were between 39 and 73%. Reproducibility was low on hydrophobic molecules (e.g. DOX 15.5%), except for VI. For IPA/water 70/30 (vol/vol), EP_{value} was slightly higher than that of ultrapure water ($P = 0.041$). $Eff_{i,m}$ values for hydrophilic molecules (CYT, GEM, MTX, ETO, IF, and CP) were lower than for ultrapure water and inferior to 90.0%. On the other hand, efficacy on the most hydrophobic molecules (IRI, DOX, and EPI) was superior to that obtained with ultrapure water (Fig. 1). Acetone EP_{value} was significantly lower than ultrapure water and IPA (both with $P < 0.0001$).

Sodium hypochlorite. The 0.5% sodium hypochlorite had the highest EP_{values} (97.5%) and was significantly superior to all other solutions (all $P < 0.0001$). All removal values were superior to 90.0% Table 3. For CYT, GEM, MTX, IRI, and VI, $Eff_{i,m}$ were even superior to 99.0%. For these compounds, SDs were inferior to 5%. The lowest $Eff_{i,m}$ were found for ETO, IF, and CP.

Surfactants. As shown in Fig. 1, 10% DWL obtained a 91.5% EP_{value}. Results are reported in Fig. 2.

Optimization phase: focus on surfactants molecules

Complex surfactants assessments (DWL). During screening phase, 10% DWL reached a promising 91.50% EP_{value}. Two more concentrations (5 and 20%) were also tested in order to observe the potential of DWL concentration on the antineoplastic removal. No significant difference was observed between the three DWL concentrations tested. 20% DWL obtained an EP_{value} (89.7%) significantly

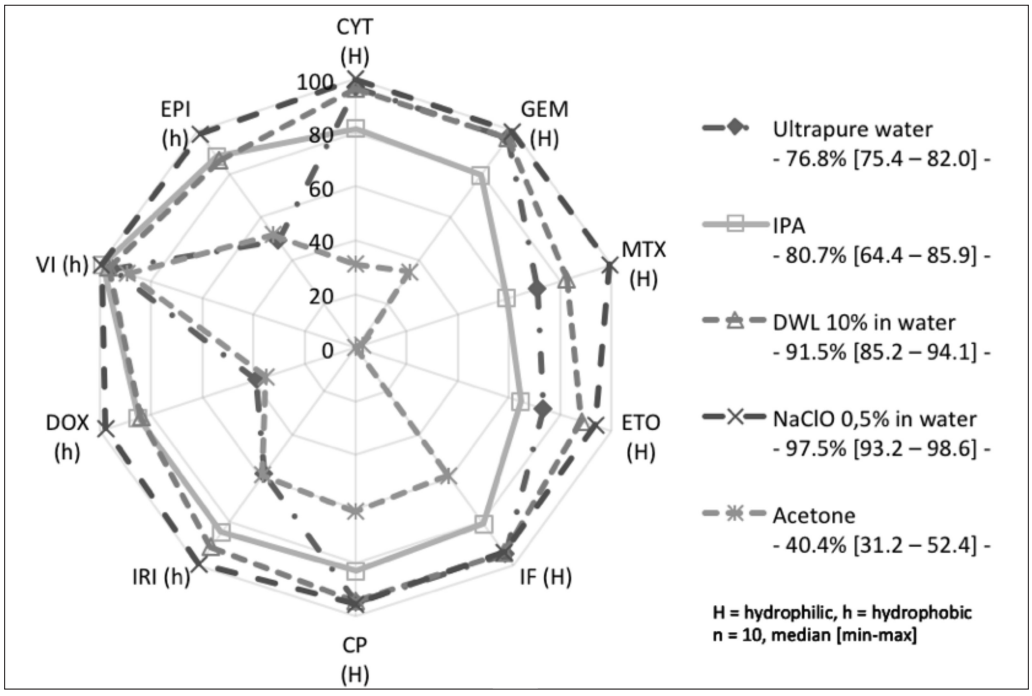

Fig. 1. Efficacy per compound and effectiveness performance of each cleaning solution on ten antineoplastic agents during tests on stainless steel surface

Table 3. Efficacy per compound of surfactant solutions on 10 antineoplastic agents and on stainless steel surface.

Modalities	CYT	GEM	MTX	ETO	IF	CP	IRI	DOX	VI	EPI	EP_{value}
Ultrapure water	97,10	96,60	71,27	73,35	94,94	94,49	58,10	38,76	95,40	49,13	76.8
	2,21	2,28	7,89	9,49	2,30	2,74	7,85	15,50	2,27	19,65	—
Acetone	31,04	34,69	2,90	−16,10	58,98	61,02	58,62	34,82	89,10	51,93	40.4
	16,58	15,38	18,07	22,79	12,50	11,18	10,45	21,42	3,64	14,26	—
IPA	81,43	79,40	59,24	64,54	81,08	83,16	85,07	85,21	99,08	87,76	80.7
	19,40	21,81	24,30	27,86	13,81	14,71	12,45	15,19	3,23	11,15	—
NaClO 0.5%	99,78	99,34	99,61	93,88	94,35	95,35	99,38	97,63	99,36	98,00	97.5
	1,15	2,01	0,78	6,04	13,09	8,45	4,06	2,58	0,05	2,35	—
5% DWL	96,88	96,95	88,00	81,77	94,99	94,91	86,71	80,17	94,52	79,59	86.1
	3,18	2,72	3,99	5,84	4,85	4,80	4,81	12,44	2,49	10,40	—
10% DWL	96,50	96,48	82,70	88,45	94,36	94,20	91,66	83,86	96,68	85,97	91.5
	3,93	4,23	7,33	7,92	4,38	4,09	10,51	15,21	1,96	14,36	—
20% DWL	95,54	95,46	87,13	77,86	92,42	92,33	86,27	76,94	93,28	73,84	89.7
	10,54	8,92	10,33	17,32	10,91	10,37	15,83	19,99	7,22	18,12	—
Span 80	82,36	80,33	66,42	65,82	79,13	80,40	45,47	54,82	79,28	55,06	76.8
	16,25	18,67	26,38	42,38	16,19	15,79	28,35	32,19	8,47	29,79	—
Tween 40	92,59	91,48	74,98	66,58	88,67	89,13	84,05	64,68	99,01	63,43	82.7
	8,88	9,27	10,96	17,27	9,25	9,38	5,52	19,20	0,57	7,73	—
10^{-1}M-SDS	96,91	97,33	92,10	89,78	95,02	94,98	100,00	93,28	99,72	95,90	95.4
	5,32	4,33	6,56	9,27	5,02	4,80	0,00	5,96	0,05	4,14	—
10^{-2}M-SDS	96,57	95,91	84,36	87,38	89,45	89,36	86,92	74,73	96,13	78,03	87.8
	4,44	4,13	6,12	6,41	5,06	5,17	6,57	16,93	1,73	7,94	—
0.5 × 10^{-2}M-SDS	94,90	94,63	81,23	81,55	92,41	91,91	82,79	79,20	92,28	81,60	87.5
	4,83	4,78	8,78	12,64	5,18	5,43	8,86	10,68	4,84	9,49	—
10^{-3}M-SDS	94,85	94,13	77,54	73,85	92,16	91,68	75,20	67,92	91,54	70,67	82.6
	6,69	7,10	14,84	19,22	7,19	7,55	14,55	13,06	5,93	15,73	—
10^{-2}M-SDS + 5% IPA	95,83	95,94	90,13	89,66	94,05	93,65	87,77	79,29	96,49	79,42	90.3
	7,08	5,70	6,33	6,56	6,23	6,31	5,87	8,20	2,16	10,26	—
10^{-2}M-SDS+ 20% IPA	95,13	95,47	90,03	92,35	94,32	94,20	89,56	77,77	96,67	80,43	89.6
	3,71	3,19	2,76	4,56	4,23	4,55	5,72	10,67	3,41	10,98	—
10^{-2}M-SDS + 30% IPA	93,24	93,38	89,25	89,18	92,63	92,06	91,60	80,92	98,31	81,59	89.9
	6,53	5,67	7,35	7,28	6,42	6,27	5,95	10,43	1,41	11,36	—

Notes: $n = 10$.
Results expressed in median values and SD in percentage.

higher than ultrapure water (all $P < 0.001$), but not 5% DWL (86.1%, $P = 0.001$). $Eff_{i,m}$ values for CYT, GEM, IF, CP, and VI were superior to 90.0% and SD values close to 10%, whatever the dilutions tested. On the other hand, for IRI, DOX, and EPI, the highest $Eff_{i,m}$ values were obtained using 10% DWL (Table 3).

Single surfactant assessments. Attempts realized on non-ionic surfactants (Tween 40 and Span 80): Fig. 3 reports results obtained with surfactant solutions. Span 80 effectiveness was not significantly different from ultrapure water. It was significantly inferior to 10% DWL ($P = 0.0001$) and to 10^{-2}M SDS ($P > 0.0002$). All of its $Eff_{i,m}$ values were inferior to the 90.0% threshold, whatever the polarity of the molecules. On the other hand, Tween 40 EP_{value} was significantly superior to Span 80 ($P = 0.018$) but not significantly different from ultrapure water ($P > 0.0610$). Its $Eff_{i,m}$ values were superior to 90.0% for CYT, GEM, and VI. Its lowest $Eff_{i,m}$ values were obtained for DOX and EPI. 10^{-2}M-SDS was significantly superior to Span 80 ($P = 0.0002$) but not to Tween 40 ($P = 0.0610$) and to 10% DWL ($P = 0.9276$; Fig. 3). Statistically as effective as 10^{-2}M-SDS, Tween 40 had nevertheless an SD superior to 10^{-2}M-SDS values for CYT, GEM, MTX, ETO, IF, CP, and DOX. As a result, subsequent evaluations were made with SDS on a concentration range of 10^{-4}–10^{-1}M.

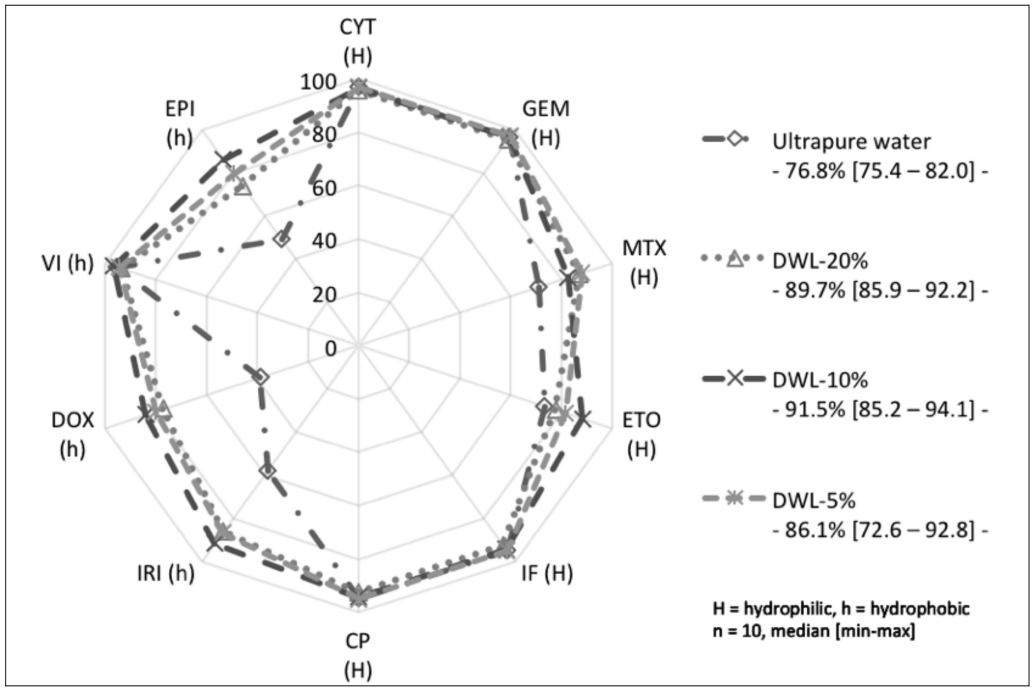

Fig. 2. Efficacy per compound and effectiveness performance of DWL dilutions on ten antineoplastic agents and on stainless steel surface

Attempts realized on anionic surfactant (SDS): Results obtained for decontamination solutions containing SDS at different concentrations are shown in Fig. 4. The effectiveness of SDS increased proportionally to concentration. Indeed, the lowest EP_{value} was obtained with 10^{-3}M-SDS. These results were significantly lower than 10^{-1}M-SDS ($P < 0.0001$) and 10^{-2}M-SDS ($P = 0.026$) but not significantly different from 0.5×10^{-3}M-SDS ($P = 0.062$). For both concentrations around the critical micellar concentration (CMC) value (10^{-2}M and 0.5×10^{-2}M), no significant difference was found [87.8% (83.9 – 92.3) and 87.5% (83.9 – 92.3); $P = 0.997$]. For both concentrations, all $Eff_{i,m}$ values were close to each other. For CYT, GEM, and VI, they were superior to 90.0% but slightly higher with "10^{-2}M-SDS". However, for DOX and EPI, efficacy was slightly lower with 10^{-2}M-SDS. The highest effectiveness was obtained with 10^{-1}M-SDS. Despite results significantly superior to 10^{-2}M-SDS ($P < 0.0001$), this concentration presented a major drawback. Indeed, a thin surfactant film appeared from time to time on the surface after the cleaning step. Microbiological contamination could appear inside, making it necessary to reduce the risk of formation of the residual film.

Attempts realized with improved anionic surfactant (SDS + IPA): To overcome the problem of surfactant deposit and to increase solution evaporation, the formulation was tested with the addition of IPA. Despite containing as much as 20% IPA, a large deposit of surfactant still remained on the stainless steel surface when "10^{-1}M-SDS + 20% IPA" was spread over it. Therefore, an SDS concentration of 10^{-2}M was selected for further experiments. Results are reported in Fig. 5. IPA ranging from 5 to 30% was diluted in an aqueous solution and mixed with 10^{-2}M-SDS. For all aqueous alcohol mixtures, EP_{values} were significantly higher than those obtained for an ultrapure water solution ($P < 0.0001$ except with "10^{-2}M-SDS + 10%-IPA", $P = 0.031$). However, no significant difference was found between 30%-IPA, 20%-IPA, 5%-IPA (and 10^{-2}M-SDS without IPA; Fig. 5). EP_{values} around 90% were obtained in all cases. Nevertheless, the mixture containing 20%-IPA was the most suitable solution, thanks to SD values inferior to our threshold of 10% and lower than those of other mixtures (Table 3). As already mentioned, IPA improved the decontamination efficacy of the most hydrophobic compounds, while SDS acted on hydrophilic molecules. More precisely, $Eff_{i,m}$ obtained with "10^{-2}M-SDS + 20%-IPA" were superior to SDS alone as far as the most hydrophobic molecules were concerned, but they were slightly lower for the two most hydrophilic molecules, CYT and GEM (Table 3).

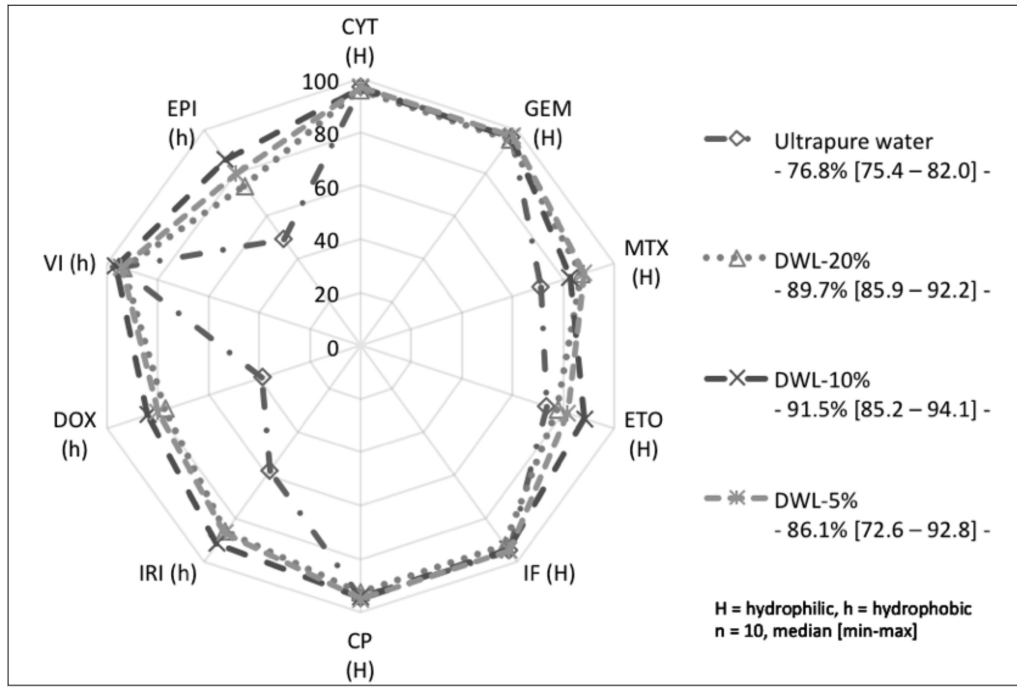

Fig. 3. Efficacy per compound and effectiveness performance of surfactant solutions on ten antineoplastic agents and on stainless steel surface

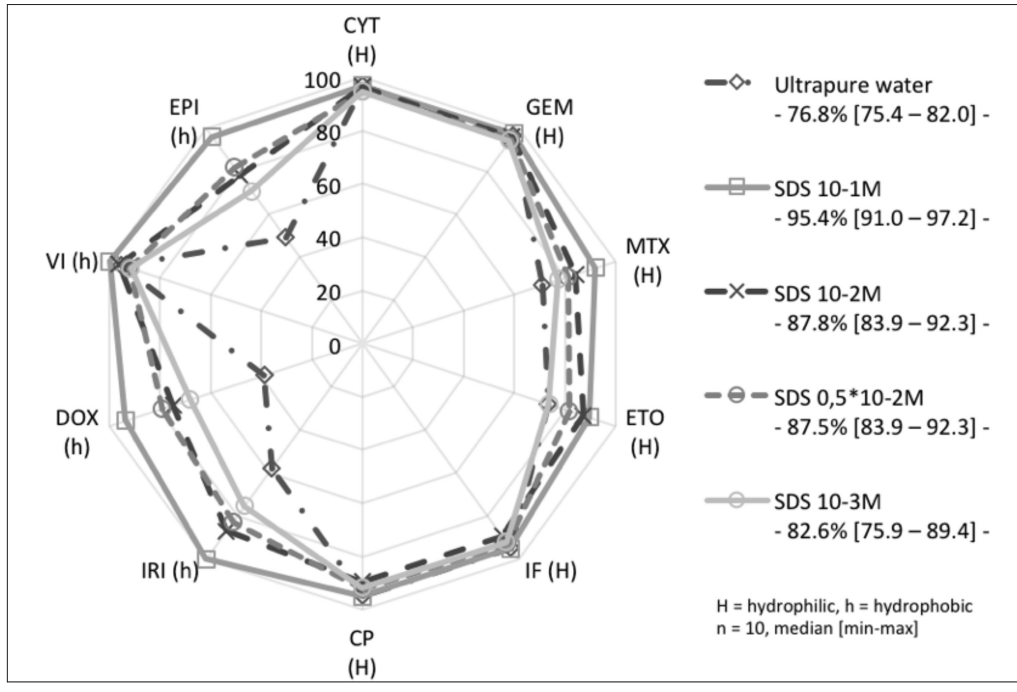

Fig. 4. Efficacy per compound and effectiveness performance of SDS range concentration on ten antineoplastic agents and on stainless steel surface

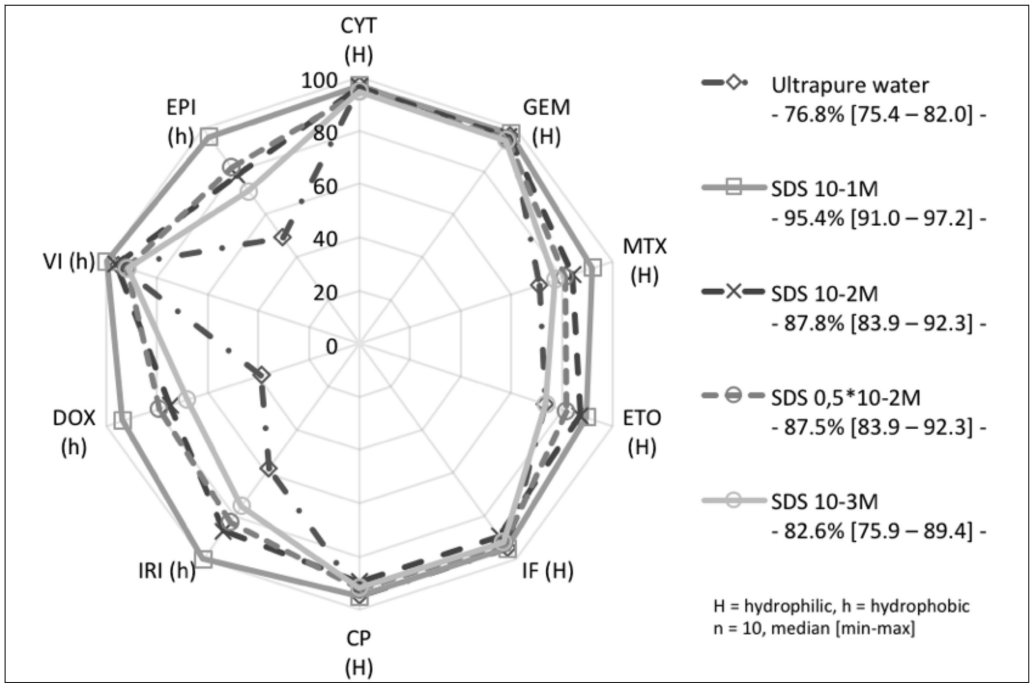

Fig. 5. Efficacy per compound and effectiveness performance of concentration range of optimised solutions, on ten antineoplastic agents and on stainless steel surface

Practical phase: volume sprayed and decontamination effectiveness

In this test, the solution was sprayed directly on the contaminated surface (0.2 m²), and a new whole Texwipe 3210 was used for each trial. Results are presented in Table 4. Whatever the volume of mixture used ("10^{-2}M-SDS + 20 %-IPA"), EP_{value} was superior to 90% and even to 93%. Effectiveness with "6 ml" was significantly higher than with "3 ml" [97.3% (96.5 – 98.1) versus 96.12% (92.77 – 97.27); $P = 0.008$] or with "1 ml" [97.3% (96.5 – 98.1) versus 93.9% (78.4 – 94.4); $P < 0.0001$]. Moreover, SD was inferior to the 10% threshold (Table 4).

Decontamination procedure glass versus stainless steel surface

Results are presented in Fig. 6, and all data are compiled in Table 5. Similar to results found on stainless steel, IPA was the least effective on glass. With $Eff_{i,m}$ values superior to the 90% threshold except for IF and CP, sodium hypochlorite was the most effective and was significantly different from IPA ($P < 0.0001$) on glass. Nevertheless, it was not statistically different from "10^{-2}M-SDS", unlike its use on stainless steel. With an EP_{value} of 88.3%, "10^{-2}M-SDS" had the same effectiveness on glass as on stainless steel. The dispersion of its values was less important than those of sodium hypochlorite, which may explain the non-statistical difference between the two modalities.

DISCUSSION

As the most widely used solvent in cleaning solutions, ultrapure water had to be evaluated. Its performance highlighted the minimal performance required for all other aqueous cleaning solutions. According to our results, its effectiveness is not sufficient and optimization was required, especially for hydrophobic compounds. IPA/water 70/30 (vol/vol) was expected to improve decontamination of the most hydrophobic compounds. In practice, an improvement was found on IRI and anthracyclin compounds, but at the same time, deterioration was measured on the most hydrophilic ones. Acetone was no more suitable at improving the decontamination process. Despite a lower polarity than IPA, hydrophobic molecules were not more effectively removed than with IPA. Furthermore, as far as hydrophilic molecules are concerned, acetone was the least effective solution tested. As a result, evaluating the solubility of single solvents did not seem to be the proper solution for decontamination procedure. Two other

Table 4. Efficacy per compound of "10^{-2}M-SDS + 20% IPA" solutions on 0.2 m² stainless steel surface.

Modalities (ml)	CYT	GEM	MTX	ETO	IF	CP	IRI	DOX	VI	EPI	EP_{value}
1	94,68	94,82	94,57	90,52	93,79	93,58	94,47	91,51	96,64	91,76	93.9
3	97,47	97,77	97,35	93,72	97,35	97,19	96,44	93,83	97,11	93,96	96.1
6	97,46	97,65	97,91	95,96	97,69	97,47	99,00	95,22	98,36	95,23	97.3

Notes: $n = 2$. Results expressed in median values.

hypotheses were considered: oxidative action and modification of solvents' solubility by adjunction of surfactants. For sodium hypochlorite, results are in accordance with those obtained by Hansel *et al*. who reported degradation efficacy for CP and an IF superior to 98.0% (Hansel *et al*. 1996). Nevertheless, despite its high decontamination potential, the use of sodium hypochlorite solutions has major drawbacks. First of all, the possibility of cytotoxic agents to be degraded in mutagenic residues has already been mentioned (Barek *et al*., 1987, 1998). To avoid this phenomenon, a time gap after cleaning (minimum 1 h) should be respected, but this delay is not feasible in everyday pharmacy routine (Castegnaro *et al*., 1997). It is necessary to clean the surface after use with a soaked wipe, otherwise corrosion phenomena appear on metals such as stainless steel. Nowadays, most barrier isolators and BSCs are made of stainless steel and manufacturers do not recommend the use of sodium hypochlorite. Finally, according to United States Pharmacopeia (USP) (797, Table 2), sodium hypochlorite can cause side effects such as skin, eye, and respiratory irritations or systemic toxicity. To overcome these inconveniences, another decontamination method as surfactant should be considered. Already available on the food market, DWL could be convenient. The poorer efficacy of 5% DWL can be explained by an insufficient concentration of surfactants to remove hydrophobic compounds properly because of the lack of micelle structures. With 20% DWL, a residual film was observed on the stainless steel surface, which persisted after the wiping step. This was probably the reason for the higher residual contamination observed (Table 3). This film can be removed with a large volume of water spilled over the surface, but this solution is not suitable within BSCs. None of these limits were found with the intermediate dilution 10% DWL. Even if its results were less effective than those obtained with 0.5% sodium hypochlorite, the main advantage of 10% DWL was undoubtedly its safety not only for humans but also for work surfaces. These results confirmed a previous work that studied cyclophosphamide chemical contamination on a glass vial surface (Touzin *et al*., 2008). Nevertheless, as already mentioned, the exact composition of DWL was unknown and depending on the supplier tested. DWL formulations are usually based on mixtures of anionic and non-ionic surfactants. For a better understanding of DWL action and to simplify formulation of cleaning solutions, subsequent experiments were focused on a single surfactant. Span 80 did not appear to be efficient, so attempts were not pursued further. Tween 40 and SDS were both effective on stainless steel surface. Nevertheless, after a brief literature review, SDS appeared to be the most widely employed surfactant on decontamination products. An additional benefit of SDS is that it is commercially available in certified laboratory quality powdered form. The use of a standardized formulation would allow users to guarantee the quality of the cleaning agent. Despite its high effectiveness, SDS 10^{-1}M was not selected because a residual film was noticed after each decontamination procedure. This residual film was similar to the one observed with 20% DWL. CMC is the main characteristic to take into account when using surfactants. This is found in our results. With a concentration 10 times inferior to CMC (SDS 10^{-3}M), the effectiveness of the cleaning solution decreased. The highest ratio "effectiveness/residual film on surface" was found for concentrations around CMC (10^{-2}M and 0.5×10^{-2}M). To promote the formation of micelles, concentration has to be superior to CMC. So, SDS 10^{-2}M was selected as the cleaning solution for further experiments. To further minimize the risk of residual film in everyday use, adjunction of IPA in SDS formulation was tested. Deposit of surfactants was less serious on a stainless steel surface, and its removal by evaporation was found to be especially fast with the 20%-IPA concentration. Moreover, no decrease of effectiveness (compared with SDS 10^{-2}M) was noticed using the "10^{-2}M-SDS + 20%-IPA" mixture. Finally, the "10^{-2}M-SDS + 20%-IPA" mixture presented the best balance between decontamination profile and reduced deposit and so was selected for further trials.

In our research so far, effectiveness has been tested on 100 cm² surfaces with 300 µl of decontamination solution, which is not representative of current decontamination in an isolator or a laminar airflow hood. Simulations of practical decontamination on larger surfaces with different volumes were

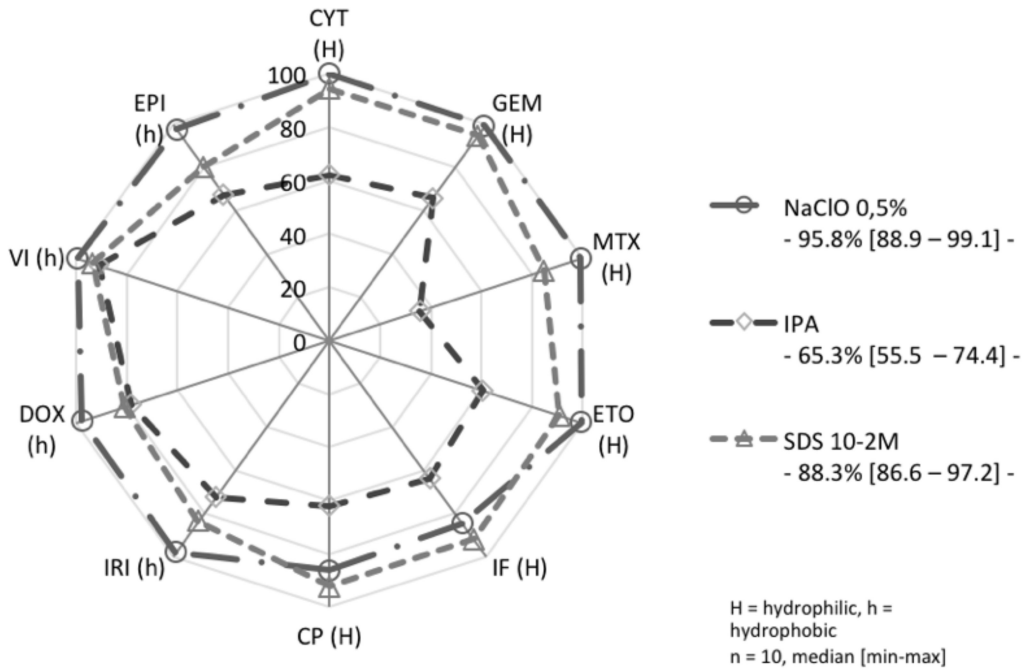

Fig. 6. Efficacy per compound and effectiveness performance of hypochlorite, IPA and SDS 10-2M on glass surface

needed to validate both the effectiveness of the solution and to quantify the volume needed to clean a large surface properly. The best results were obtained when the largest volume was used. Even when using "6 ml", no deposition of surfactant film was found after the wiping procedure. Consequently, a calculation by proportionality between the standardized surface (0.2m²) and a theoretical surface of 1 m² can be evaluated. As a result, ratio of cleaning agent to surface area between 20 and 30 ml m^{-2} should be recommended as an informal rule to clean a stainless steel surface properly.

The most frequently encountered materials in BSCs and isolators are stainless steel and glass. Consequently, further experiments were performed on a glass surface with only three selected aqueous mixtures. IPA/water 70/30 (vol/vol) is recommended by guidelines and has microbiological decontamination effectiveness. During our study, 0.5% sodium hypochlorite, a "degradation-type" solution, presented the best overall EP_{value}, and finally, "10^{-2}M-SDS", developed and tested by ourselves, presented the best ratio between performance and safety. IPA had the lowest EP_{value} (65.3%) and presented no more advantage on glass than on stainless steel. NaOCl effectiveness (95.8%) was equivalent on both stainless steel and glass surfaces. The variations observed between the two surfaces can be accounted for by their physicochemical characteristics. Glass as a more hydrophilic surface has high wettability, and stainless steel as metal has higher hydrophobicity and lower wettability. Finally, 10^{-2}M-SDS with an

Table 5. Efficacy per compound of cleaning solutions on glass surface.

Modalities	CYT	GEM	MTX	ETO	IF	CP	IRI	DOX	VI	EPI	EP_{value}
0.5% NaClO	99,74	99,02	99,23	99,53	84,91	86,34	98,22	97,58	99,42	97,35	95.8
	8,33	8,41	5,83	6,66	19,08	17,61	7,81	8,60	0,68	6,88	—
10^{-2}M-SDS	94,11	94,69	84,58	90,84	92,36	92,21	83,76	80,68	93,46	80,14	88.3
	5,74	4,82	10,12	6,51	7,33	7,71	10,49	16,07	5,62	18,58	—
IPA	62,11	65,85	35,70	60,67	64,09	62,08	72,52	78,11	91,15	67,36	65.3
	16,44	18,08	23,97	15,91	19,02	27,45	11,38	10,36	0,96	9,47	—

Notes: $n = 6$.
Results expressed in median values and SD in percentage.

88.3 EP_{value} was as effective on glass as on stainless steel surfaces.

CONCLUSION

The "degradation-type" solution represented by sodium hypochlorite was very attractive because of oxidation. However, stainless steel as a building material in isolators and BSC prohibits its use. Moreover, this recommendation conveyed by manufacturers themselves is reinforced by the risk of producing agents with unknown chemical structures and cytotoxic potential. The "elimination-type" solutions demonstrated promising results. The use of surfactants such as DWL proves to be efficient and reproducible. Previous studies had already proved its efficiency on glass surface, but those studies were performed with a single antineoplastic agent, the cyclophosphamide or the carboplatin (Lê *et al*., 2012). In our study, the DWL efficiency was again found on 10 antineoplastic agents. Approved on both hydrophilic and hydrophobic agents, DWL had nevertheless a major drawback. Indeed, many manufacturers are on the household cleaning market with their own unknown formulation. Nevertheless, DWLs were very convenient and practical products, and further tests will be performed to evaluate the relevance of their use in daily practical conditions. During our study, surfactants used alone have proved to be effective especially the SDS. Moreover, they have the advantage of being available with the pharmaceutical certification, which eases their use and approval in pharmacy units. SDS allows the desorption of antineoplastic agents and reinforces their solubility. Their effectiveness was successfully proved on both stainless steel and glass surfaces. However, the appearance of residual film at high concentrations can be a potential source of cross and bio contamination. To overcome this problem, IPA was added into the formulation. With a quantity of 20–30 ml m^{-2}, the "10^{-2}M-SDS + 20% IPA" formulation was efficient when sprayed on both stainless steel and glass surfaces with an efficacy superior to 97% on a single run. This effectiveness confirms its suitable use in current practice. Nevertheless, this study was performed on standardized surfaces that cannot be substituted for production units used daily by healthcare workers, where additional parameters have to be taken into account. The best rated decontamination solvents will have to be tested further in real environments, as well as on other materials (transparent thermoplastic such as poly-methylmethacrylate and polycarbonate) and on molecules (platine derivatives and 5-fluorouracil) for which no data is available. The decontamination procedure could also be tested on various supports such as cytotoxic packaging which is known to be contaminated in its industrial area.

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Article IX

« Efficacy of two cleaniong solitions for the decontamination of 10 antineoplastic agents in the biosafety cabinets of a hospital pharmacy.»

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Efficacy of Two Cleaning Solutions for the Decontamination of 10 Antineoplastic Agents in the Biosafety Cabinets of a Hospital Pharmacy

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ABSTRACT

Objective: This study aimed to evaluate two cleaning solutions for the chemical decontamination of antineoplastic agents on the surfaces of two biosafety cabinets routinely used for chemotherapy preparation in a hospital pharmacy.

Methods: For almost 1 year (49 weeks), two different solutions were used for the weekly cleaning of two biosafety cabinets in a hospital pharmacy's centralized cytotoxic preparation unit. The solutions evaluated were a commercial solution of isopropyl alcohol (IPA) and water (70:30, vol:vol), and a detergent solution constituted by 10^{-2} M of sodium dodecyl sulfate (SDS) with 20% IPA. Seven areas in each biosafety cabinet were wiped 14 times throughout the year, before and after the weekly cleaning process, according to a validated procedure. Samples were analyzed using a validated method of high-performance liquid chromatography coupled to mass spectrometry. The decontamination efficacy of these two solutions was tested for 10 antineoplastic agents: cytarabine, gemcitabine, methotrexate, etoposide phosphate, irinotecan, cyclophosphamide, ifosfamide, doxorubicin, epirubicin, and vincristine.

Results: Overall decontamination efficacies observed were 82 ± 6% and 49 ± 11% for SDS solution and IPA, respectively. Higher contamination levels were distributed on areas frequently touched by the pharmacy technicians—such as sleeves and airlock handles—than on scale plates, gravimetric control hardware, and work benches. Detected contaminations of cyclophosphamide, ifosfamide, gemcitabine, and cytarabine were higher than those of the others agents. SDS solution was almost 20% more efficient than IPA on eight of the antineoplastic agents.

Conclusion: Both cleaning solutions were able to reduce contamination levels in the biosafety cabinets. The efficacy of the solution containing an anionic detergent agent (SDS) was shown to be generally

higher than that of IPA and, after the SDS cleaning procedure, biosafety cabinets demonstrated acceptable contamination levels.

KEYWORDS: antineoplastic analysis; cleaning; decontamination; detergents; hospital; occupational prevention and control; pharmacy service

INTRODUCTION

Antineoplastic agents used in cancer therapy are substances that nonspecifically inhibit or stop cell development. These molecules are potentially hazardous because they do not distinguish diseased cells from healthy ones, creating undesirable side effects in patients. Healthcare professionals, such as pharmacists, oncology nurses, physicians, and technicians, there forerun a real risk of being contaminated by antineoplastic agents during their daily routines, if they work with these compounds. From the early 1980s, several studies conducted in hospitals, industries, and pharmacies demonstrated that those occupationally involved in the preparation, transport, administration, and elimination of antineoplastic materials were exposed to the risk of being contaminated by them (Benhamou *et al.*, 1986; Sorsa *et al.*, 1988; Kiffmeyer *et al.*, 2013). Biological monitoring studies to evaluate the effects of antineoplastic agent contamination on healthcare personnel have been published for the last 30 years, providing evidence of the exposure of healthcare professionals to antineoplastic agents. They have reviewed the effects caused by acute or prolonged exposition (Sorsa *et al.*, 1988; Sessink *et al.*, 1994; Suspiro and Prista, 2011). Biological monitoring studies, combined with environmental studies, could be effective in investigating either the causes of contamination or the effects of preventive measures (Sessink *et al.*, 1997; Turci *et al.*, 2011; Yoshida *et al.*, 2013). Results of environmental studies in hospital pharmacies highlighted the presence of antineoplastic agent contamination of work surfaces (benches, tables, and fridge doors), materials (vials, gloves, infusion bags), and floors, but also in logistical rooms outside the background clean room (Touzin *et al.*, 2009; Käslin *et al.*, 2010; Kiffmeyer *et al.*, 2013). Based on these results, the professional associations and authorities of different countries have published guidelines to limit healthcare professionals' exposure to contamination by hazardous agents (NIOSH, 2004; Marcel *et al.*, 2004; ASHP, 2006).With the objective of confining contamination, the preparation of antineoplastic treatments should be carried out at separate workstations, such as in biological safety cabinets (BSC) or isolators. It is of utmost importance that an effective post drug-preparation cleaning procedure is carried out in these workstations in order to limit the accumulation of residual contamination, both chemical and microbiological. Several studies have been published on different cleaning procedures for surfaces contaminated by antineoplastic agents (Roberts *et al.*, 2006; Lee *et al.*, 2009; Queruau Lamerie *et al.*, 2013; Le *et al.*, 2013). Decontamination protocols involving sodium hypochlorite were considered effective for a variety of active ingredients, but they could damaged cleaned surfaces (need for rinsing after use) and were potentially genotoxic (Lee *et al.*, 2009; Sharma *et al.*, 2013). Hydrogen peroxide, whether liquid or vaporized (VHP®), showed good decontamination and degradation action on 5-Fluorouracil, doxorubicin (DOX), and cyclophosphamide (CP) (Roberts *et al.*, 2006). The recently published efficacies of cleaning procedures involving different products highlighted the importance of the presence of a surfactant in the cleaning solution (Le *et al.*, 2013; Queruau Lamerie *et al.*, 2013). Until now, to the best of our knowledge, no clear, practical recommendations about the decontamination procedures to be adopted with antineoplastic agents have been available in the literature. A recent systematic evaluation of the efficacy of several cleaning solutions on 10 antineoplastic agents on different surfaces was performed in experimental conditions (Queruau Lamerie *et al.*, 2013).

The present work aimed to evaluate the efficacy of two cleaning solutions on the decontamination of 10 antineoplastic agents in a real-world setting. The first was an isopropyl alcohol hydroalcoholic solution (IPA, brand name Klercide®) which has long been used for routine BSC cleaning procedures in our

centralized cytotoxic preparation unit. The second was a sodium dodecyl sulfate (SDS) solution, at a concentration of 10^{-2} M and with 20% IPA. The latter solution was chosen because of the results previously obtained from the decontamination of antineoplastic agents on stainless steel and glass surfaces (Queruau Lamerie *et al.*, 2013). Data were provided from the results of a validated global analytical procedure involving a wiping step (Nussbaumer *et al.*, 2012) followed by a liquid chromatography mass spectrometry (LC-MS/MS) analysis (Nussbaumer *et al.*, 2010, 2012).

MATERIALS AND METHODS

Setting

The Geneva University Hospitals (HUG) centralized the preparation of antineoplastic agents in its pharmacy in 2000. Two class III biosafety cabinets (BSC, CDC-D-2GR from Envair, Rossendale, England) are installed in a GMP class C (ISO 7) background clean room, producing antineoplastic preparations daily. The staff of the pharmacy's cytotoxic unit produces more than 17 000 oncology products annually.

Chemicals and reagents

Antineoplastic agents

This study was carried out using the following commercially available antineoplastic preparations: Vincristin Teva® (vincristine 1 mg·ml^{-1}, VIN) and Methotrexat Teva® (methotrexate 2.5 mg·ml^{-1}, MTX), purchased from Teva Pharma AG (Basel, Switzerland); Adriblastin® (doxorubicine 2 mg·ml^{-1}, DOX), from Pfizer AG (Zurich, Switzerland); Epirubicin Actavis Solution® (epirubicin 2 mg·ml^{-1}, EPI), from Actavis (Regensdorf, Switzerland); Endoxan® (cyclophosphamide reconstituted in glucose 5% at 20 mg·ml^{-1}, CP), from Baxter AG (Volketswil, Switzerland); Etopophos® (etoposide phosphate reconstituted in water at 20 mg·ml^{-1}, ETO), from Bristol-Myers Squibb SA (Baar, Switzerland); Cytosar® (cytarabine 20 mg·ml^{-1}, CYT), from Pfizer AG (Zürich, Switzerland); Gemzar® (Gemcitabine, reconstituted in water at 20 mg·ml^{-1}, GEM), from Eli Lilly (Verbier, Switzerland); Irinotecan Fresenius® (irinotecan 20 mg·ml^{-1}, IRI), from Fresenius Kabi AG (Stans, Switzerland); and Holoxan® (ifosfamide reconstituted in water at 40 mg·ml^{-1}, IFO), purchased from Ebewe Pharma (Cham, Switzerland).

The reconstitutions of Etopophos®, Gemzar®, and Holoxan® were carried out with water for injection purchased from Bichsel Laboratories (Interlaken, Switzerland). Glucose 5% for the reconstitution of Endoxan was from Sintetica-Bioren SA (Couvet, Switzerland). The internal standard (IS), [13C, 2H3]-methotrexate, was purchased from Alsachim (Illkirch, France).

Other products

The two cleaning solutions were:

1. Commercially available, sterile, hydroalcoholic solution, Klercide® (IPA: water, 70:30, vol:vol; named IPA), from Shield Medicare (Farnham, UK), was used directly in a spray form;
2. SDS purchased from Sigma-Aldrich (Steinhein, Germany) and Klercide® were used to produce the detergent cleaning solution constituted by SDS 10^{-2} M with 20% of Klercide® (vol:vol), and then conditioned in a spray bottle.

The LC-MS/MS analysis was performed using the following solvents and chemicals: Lichrosolv® HPLC grade acetonitrile (ACN) and ultrapure water from Merck (Darmstadt, Germany), and formic acid (FA) from Biosolve (Valkenswaard, the Netherlands).

Preparation of solutions

All solutions of antineoplastic agents (i.e. drug reconstitutions and sample dilutions) were prepared in appropriate conditions (i.e. personal protective equipment and BSC) for handling hazardous compounds. Aliquots of the IS were prepared with a mixture of ACN and water (75:25, vol:vol) at 250 µg·ml^{-1} and stored at −22°C for a maximum of 12 months. Stock solutions of IS were diluted on the day of analysis at 50 ng·ml^{-1} in ACN 20% with FA 0.1%. A main stock solution containing the 10 antineoplastic agents was prepared by diluting each component in water at a concentration of 20 µg·ml^{-1}. This solution was diluted further to obtain five independent stock solutions at 20, 40, 200, 1000, and 4000 ng·ml^{-1} in ACN 20% with FA 0.1%. For calibration standards (CS), these solutions were

diluted using the IS solution at 50 ng·ml^{-1}, to obtain five CS at 1, 2, 10, 50, and 200 ng·ml^{-1}. LC-MS/MS analyses were performed using three mobile phases: ultrapure water (A), ACN (B), and FA 1% (C). The needle and the injection loop were washed using 5% ACN in water after each injection.

LC-MS/MS analysis

Analyses were carried out using an Accela LC-MS/MS system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The operating system—consisting of a quaternary pump equipped with an online degasser, an autosampler, and a solvent platform—was coupled to a quadrupole (TSQ) Quantum Discovery mass spectrometer (MS) (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with Ion Max electrospray ionization (ESI). Separations were carried out on a ZOBRAX SB C18 RR 2.1 × 100 mm, 3.5 µm particle diameter column (Agilent Technologies, Waldbronn, Germany). The chromatographic system coupled to the MS operated with Xcalibur® software (Thermo Fisher Scientific Inc.). The LC-MS/MS conditions are described in detail elsewhere (Nussbaumer *et al.*, 2010).

Wiping and desorption material

The wiping was performed using Protein Saver TM 903 Card filter paper (Whatman, Dassel, Germany). Desorption was performed in 1.5 ml polyethylene (PE) safe-lock tubes (Eppendorf AG, Hamburg, Germany). The wiping solution was a 20% ACN solution with 0.1 % FA. The validated wipe sampling procedure is described in detail elsewhere (Nussbaumer *et al.*, 2012). Recoveries of the sampling procedure on the different surfaces are presented in Table 1.

BSC decontamination and wiping procedure

Pharmacy technicians are responsible for the manipulation, reconstitution, and production of antineoplastics preparations and clean the two BSC in the hospital pharmacy's centralized cytotoxic preparation unit two to four times a day, at the end of the morning and afternoon work sessions. This post-preparation cleaning procedure is performed without opening the BSC, using a sterile solution of IPA and TX612 TechniCloth wipes (TexWipe, Kernersville, NC, USA) on all inside surfaces. Gloves are changed after the post-preparation cleaning procedure.

Once a week, the two BSC are opened and cleaned in depth by trained cleaning technicians in charge for the cleaning and preparation of BSC before the work sessions. This study focused on this weekly cleaning procedure, in order to evaluate the efficacy in routine conditions of a SDS solution in comparison with the usual IPA solution. Both solutions were used with an identical cleaning protocol, as follows: (i) the front panel was opened; (ii) all materials inside the BSC were taken out [e.g. scale, stainless steel work bench, gravimetric control (CATO®) hardware]; (iii) the cleaning solution (see after) was sprayed on all the interior surfaces (also inside the airlock box, and the insides of sleeves) and wiped using TX612 TechniCloth wipes; (iv) materials taken out were also sprayed and wiped with the same solution; (v) the exterior surfaces (also outside the airlock box and the outsides of sleeves) were cleaned as described previously; (vi) all the cleaned objects were replaced in the BSC; and (vii) the BSC was closed and air was circulated for 15 min before a new work session could start. In total, surfaces were wiped between 13 and 23 times a week in BSC 1 and between 11 and 21 times a week in BSC 2, depending on the quantity of chemotherapies produced.

In order to compare the efficacy of the two solutions tested, a specific weekly cleaning procedure was applied to each BSC:

1. For BSC 1, a three-step cleaning procedure was applied to surfaces and materials: (i) surfactant cleaning solution (SDS10^{-2} M + 20% IPA); (ii) sterile water (to rinse residues of SDS); and (iii) IPA (to guarantee microbiological decontamination). Each step was followed by a wiping step for all surfaces.
2. For BSC 2, the usual one-step procedure was applied using IPA on all surfaces and materials.

The two BSC were decontaminated on the same days by the same cleaning technician, following instructions to frequently change the wipes used for the cleaning procedure. Around 12–15 wipes were used for the entire cleaning procedure for one BSC. Time required to complete the cleaning procedure in BSC1 was 1 h. BSC 2 cleaning procedure took 30 min to be completed. Time required completing the cleaning procedure were operator-dependent.

In order to compare the efficacy of the two cleaning procedures, seven spots inside the BSC (Fig. 1) were

Table 1. Quantitative performance of the wiping method for the 10 antineoplastic drugs on different surfaces, adapted from (Nussbaumer *et al.*, 2012).

	Surface material	Stainless steel	Polypropylene	Computer mouse
CYT	Recovery (%)	81	79	69
	Intermediate precision (%)	8.3	7.8	8.8
GEM	Recovery (%)	82	79	81
	Intermediate precision (%)	9.5	8.8	6.4
MTX	Recovery (%)	63	85	64
	Intermediate precision (%)	9.8	5.1	9.8
ETO	Recovery (%)	45	82	81
	Intermediate precision (%)	7.8	8.2	22.6
IFO	Recovery (%)	82	91	98
	Intermediate precision (%)	10.4	8.2	24.8
CP	Recovery (%)	86	94	77
	Intermediate precision (%)	10.8	4.8	20.4
IRI	Recovery (%)	57	84	45
	Intermediate precision (%)	11.8	11.9	12.0
DOX	Recovery (%)	46	54	35
	Intermediate precision (%)	5.1	6.1	12.2
VIN	Recovery (%)	46	58	19
	Intermediate precision (%)	5.2	6.2	11.1
EPI	Recovery (%)	50	55	22
	Intermediate precision (%)	12.0	11.9	12.3

wiped for sampling, both before and after the cleaning procedure described here. Wiping spots were: 100 cm^2 of sleeves (polypropylene), 100 cm^2 of the left side of the work bench (stainless steel), the scale plate (stainless steel), the gravimetric control (CATO®) hardware (mouse in BSC 1, keyboard in BSC 2, plastic), and airlock handles (polyester).Wiping was performed following a validated procedure (Nussbaumer *et al.*, 2012). After wiping, the samples were placed in a PE safe-lock (Eppendorf AG, Hamburg, Germany) and stored at −22°C until LC-MS/MS analysis. Sampling was performed once a week for the first 3 weeks (W) of the study, then every 4 or 5 weeks over nearly a year (49 weeks in total). During this entire period, the two BSC were cleaned weekly, as described earlier; in total, measurement of contamination was performed 14 times.

Decontamination overview and efficacy calculation

Total contamination and impact of the introduced quantities of antineoplastics

For each wiping spot, the cumulative contamination of the 10 antineoplastic agents (GEM, CYT, CP, VIN, MTX, DOX, EPI, IFO, ETO, and IRI) was expressed in terms of total quantity (= Q in ng). Mean of Q($\bar{Q}$) across the 14 contamination measurements was calculated. Total contamination level was expressed as the sum of Q (Σ_Q) of all wiping samples during a wiping

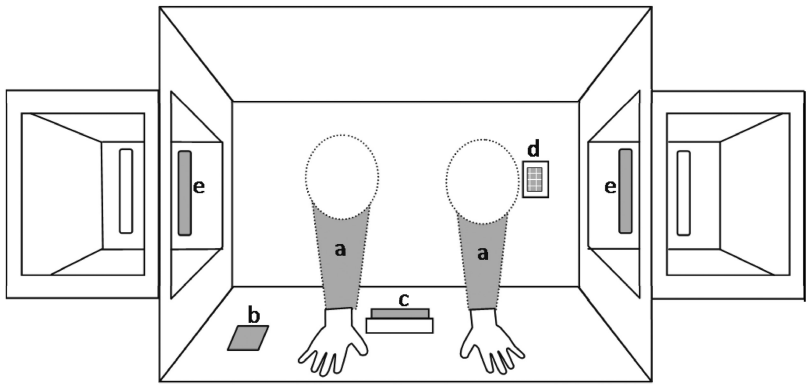

Figure 1 Representation of a biosafety cabinet with seven sampling spots highlighted: (**a**) left and right sleeves; (**b**) work bench; (**c**) scale plate; (**d**) CATO mouse or keyboard; (**e**) left and right airlock handles.

campaign for each BSC. The relationship between the quantity of the 10 antineoplastic agents introduced into each BSC during the week before the cleaning procedure and the $\Sigma_{Q\text{before cleaning procedure}}$ was studied using a linear regression. This was in order to evaluate whether the quantity of antineoplastic agents handled in each BSC influenced Σ_Q.

Decontamination efficacy

The contamination overview was expressed by Σ_Q at the time of wiping (W_1, W_2,..., W_{49}).The difference between $\Sigma_{Q\text{before cleaning procedure}}$ and $\Sigma_{Q\text{after cleaning procedure}}$ was expressed by Δ_Q. Positive and negative values of Δ_Q were observed. Efficacy (Eff_Q) was calculated from Σ_Q values of all seven spots, for the two BSC at the time of wiping, using Equation 1.

$$Eff_Q = \left(1 - \frac{\sum Q_{\text{after cleaning procedure}}}{\sum Q_{\text{before cleaning procedure}}}\right)\% \qquad (1)$$

The average of Eff_Q was calculated. Results of $Eff_Q < 0$ were considerate as 0% (no decontamination had occurred).

Analysis of the contamination on the wiping areas for the 10 selected antineoplastic agents

Contamination by each separate cytotoxic agent was expressed in terms of quantity (= q in ng). The distribution of the contaminations in each BSC was calculated using the mean values of q($\bar{q}$) of all wiping samples from a selected spot. Means were calculated to evaluate the general trends in the decontamination procedures and to highlight any accidental contamination during the study.

The decontamination efficacy (Eff_q) of the two cleaning solutions on the 10 cytostatic agents was calculated from the q values of all seven spots for the two BSC at the time of wiping using, Equation 2.

$$Eff_q \text{ of selected antineoplastic agents} = \left(1 - \frac{q_{\text{after cleaning procedure}}}{q_{\text{before cleaning procedure}}}\right)\% \qquad (2)$$

Efficacy of the cleaning solutions was evaluated by calculating the mean of all Effq of a selected antineoplastic agent according to the cleaning solution employed during the cleaning procedure. When $\Sigma q_{\text{after cleaning procedure}}$ was higher $\Sigma q_{\text{before cleaning procedure}}$ a negative Eff_q result had occurred. To evaluate the efficacy of the cleaning solution on antineoplastic agents, results of $Eff_q < 0$ were considered as 0%, as no decontamination had occurred. Fisher–Student tests ($\alpha < 0.05$) were carried out to compare the average efficacy of the two cleaning solutions, and to evaluate whether a difference of efficacy on antineoplastic agents existed between the two cleaning solutions.

RESULTS

During this study, 390 wiping samples (195 for BSC 1 (SDS + IPA) and 195 for BSC 2 (IPA)) were collected.

Total contamination and impact of the introduced quantities of antineoplastic agents

$\bar{Q}_{BSC\,1}$ was 3557.6 ± 2700.5 ng for $\bar{Q}_{before}$ and 402.3 ± 333.4 ng for $\bar{Q}_{after}$. The $\bar{Q}_{BSC\,2}$ levels

detected were 2997.1 ± 2239.4 ng for $\overline{Q}_{before}$ and 3168.2 ± 3261.4 ng for $\overline{Q}_{after}$. Results showed higher values of $\overline{Q}_{before\ cleaning\ procedure}$ in BSC 1 than in BSC 2. No linear relationship was evident between the detected Q and the total quantity of the 10 antineoplastic agents treated in the BSC in the week before the analyses ($R^2 < 0.06$ for both BSC) (Fig. 2).

Decontamination efficacy

Eff_Q for each BSC was calculated using Equation 1, in order to evaluate the general efficacy of the cleaning solutions on the 10 selected antineoplastic agents; this is represented by the histograms in Fig. 3. For BSC 1 (SDS), an average Eff_Q value of 82% ± 6% [relative standard deviation (RSD) 13%] was observed. At each wiping campaign, a positive Δ_Q was measured, indicating a decrease in the contamination level after a cleaning procedure. An average Eff_Q value of 49 ± 11% (RSD 29%) was obtained for BSC 2 (IPA), and three samples (W1, W2, and W44) were found to present negative values of Δ_Q.

Analysis of the contamination of wiping areas for the 10 selected antineoplastic agents

Mean values of q ($\overline{q}$), for the 10 cytostatic agents, were plotted according to the wiping areas. Results of the contamination distribution are shown in Table 2. For both BSC, the most contaminated areas were sleeves and airlock handles. On two occasions (during weeks 1 and 2), in the BSC 2 airlock, handles were contaminated with high q_{CYT} (over 1200 ng) both before and after the cleaning procedure. In both BSC, values of $\overline{q}_{CP}$, $\overline{q}_{IFO}$, $\overline{q}_{GEM}$, and $\overline{q}_{CYT}$ were higher than the $\overline{q}$ *of* the other cytostatic agents. Efficacy of the two cleaning solutions used during the cleaning procedure for the 10antineoplastic agents was expressed in terms of means values of Eff_q (Fig. 4). Efficacy was calculated as an evaluation of the percentage of antineoplastic agents washed away during the cleaning procedure. A high value of Eff_q meant that the contamination had been reduced during the cleaning procedure. The efficacy of the SDS solution was higher than that of IPA on eight of the 10antineoplastic agents (CYT, GEM, MTX, ETO, IFO, CP, IRI, and DOX). SDS solution was almost 20% more effective than IPA on almost all antineoplastic agents. IPA was more effective on VIN and EPI, but both cleaning solutions showed efficacies lower than 20%. As shown in Fig. 4, significant differences in the efficacy of the cleaning solutions were only observed for CYT, GEM, and CP ($P < 0.05$, Fisher–Student test).

DISCUSSION

This study aimed to compare the efficacy of two cleaning solutions for the chemical decontamination of two class III BSC. These BSC were used daily to produce chemotherapies in the centralized cytotoxic preparation unit in a hospital pharmacy. Over the course of a year, each of BSC was cleaned with a different solution: a solution of 10^{-2} M of SDS containing 20% IPA (BSC 1), and a solution of IPA (BSC 2). SDS solution was chosen based on results of a previous experimental study (Queruau Lamerie *et al.*, 2013),

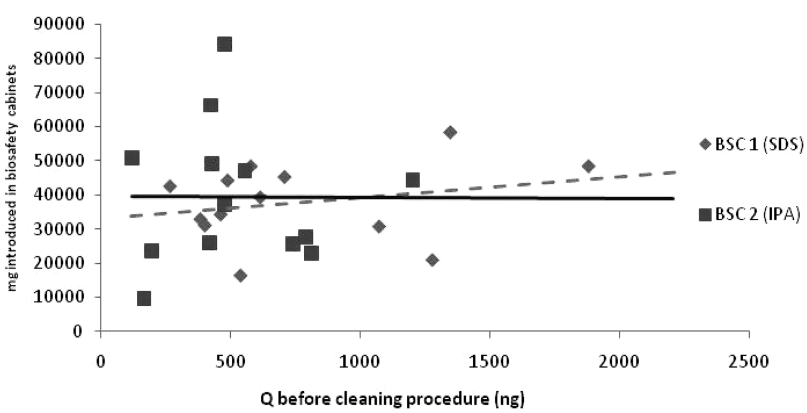

Figure 2 Relationship between the quantities of the 10 antineoplastic agents introduced in the BSC for the chemotherapy preparations and the quantities detected (Q) before the cleaning procedure. BSC 1) $y = 5.9948x + 33218$, $R^2 = 0.059$; BSC 2) $y = -0.3824x + 39772$, $R^2 = 0.0003$.

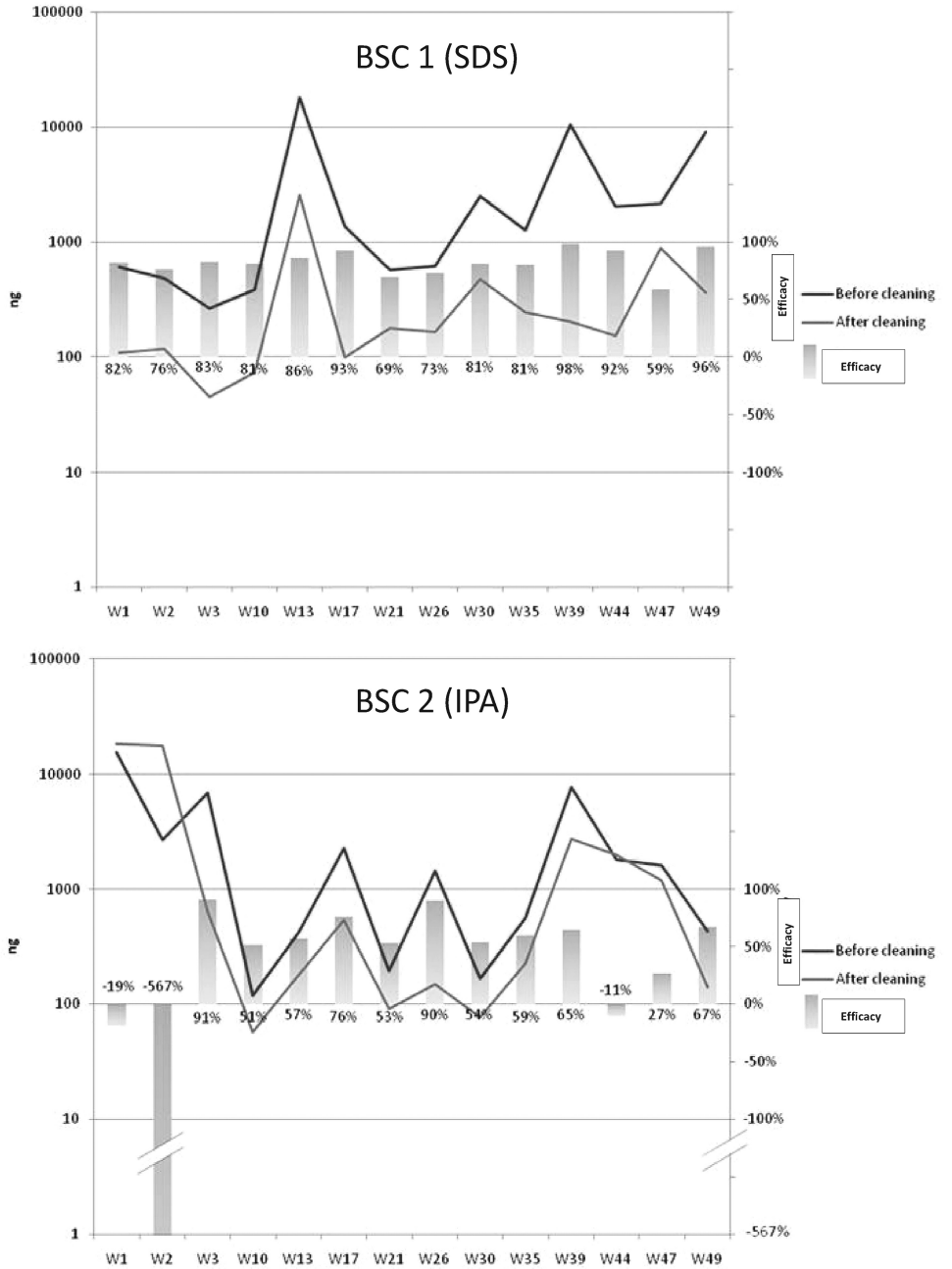

Figure 3 Contamination rate over view in terms of Σ_Q of all antineoplastics and efficacy histograms are plotted according to the wiping campaigns.

while IPA is the disinfectant conventionally used in routine microbiological cleaning of BSC in numerous pharmacy hospitals. Throughout the duration of the study, contaminations by antineoplastic agents were detected in both BSC both before and after the cleaning procedures.

Total contamination and the impact of introduced quantities of antineoplastic agents

Higher total quantities of antineoplastics were detected for $\bar{Q}_{BSC\,1}$ than for $\bar{Q}_{BSC\,2}$. Several factors, including the quantity of introduced antineoplastic agents, were investigated to explain this difference, and

Table 2. Mean *q* values of antineoplastic tested, at wiping spot. Values in bold denote samples collected before the cleaning procedure and values in brackets denote samples collected after the cleaning procedure.

	Sleeve L	Sleeve R	Airlocks handles L	Airlocks handles R	CATO® mouse	Working bench	Scale plate
BSC 1 (SDS)							
CYT	**49.7** (1.9)	**108.2** (11.2)	**18.5** (3.5)	**40.1** (5.0)	**27.6** (1.0)	**8.9** (1.0)	**9.8** (1.2)
GEM	**115.3** (1.3)	**104.8** (2.2)	**64.2** (6.9)	**43.7** (5.0)	**19.7** (2.2)	**18.1** (20.9)	**37.9** (0.9)
MTX	**110.4** (0.9)	**30.0** (1.7)	**6.0** (0.6)	**7.4** (2.1)	**8.0** (3.3)	**16.7** (0.7)	**386.6** (0.6)
ETO	**9.9** (0.4)	**64.9** (0.00)	**2.0** (0.0)	**0.7** (1.3)	**5.8** (0.1)	**0.2** (0.2)	**0.2** (0.0)
IFO	**132.4** (77.9)	**675.0** (30.2)	**643.2** (78.0)	**257.3** (28.6)	**23.3** (6.6)	**6.7** (2.0)	**5.3** (0.9)
CP	**19.3** (4.2)	**51.8** (10.4)	**11.2** (3.5)	**29.3** (2.7)	**131.9** (15.5)	**4.0** (1.8)	**26.4** (0.9)
IRI	**1.7** (0.2)	**0.4** (0.2)	**1.9** (0.3)	**1.4** (0.3)	**0.6** (0.3)	**1.7** (1.7)	**0.7** (0.1)
DOX	**0.3** (0.1)	**0.2** (0.4)	**1.0** (0.4)	**0.6** (0.4)	**0.2** (0.2)	**0.4** (0.3)	**0.4** (0.2)
VIN	**26.0** (4.9)	**23.3** (5.8)	**27.9** (5.5)	**16.7** (9.4)	**15.8** (11.3)	**13.8** (0.2)	**22.7** (3.0)
EPI	**22.7** (1.6)	**10.9** (2.8)	**18.7** (1.6)	**12.3** (4.2)	**15.2** (5.5)	**8.1** (0.6)	**13.4** (1.1)
BSC 2 (IPA)							
CYT	**329.8** (8.7)	**189.6** (52.3)	**1613.4** (1464.4)	**108.6** (1292.7)	**8.8** (5.4)	**5.4** (5.8)	**23.4** (4.9)
GEM	**98.8** (13.4)	**104.5** (18.3)	**21.5** (13.7)	**20.0** (11.8)	**39.6** (8.1)	**7.8** (10.6)	**18.5** (4.0)
MTX	**12.9** (2.4)	**10.3** (2.0)	**6.1** (1.2)	**2.7** (0.8)	**1.0** (0.5)	**2.7** " (6.1)	**15.2** (0.5)
ETO	**0.0** (0.0)	**0.0** (83.6)	**0.4** (0.1)	**0.0** (0.0)	**0.6** (0.0)	**0.0** (0.0)	**0.7** (0.0)
IFO	**29.4** (12.5)	**24.8** (9.7)	**97.2** (10.7)	**13.9** (23.1)	**4.1** (2.1)	**3.5** (3.8)	**3.5** (1.2)
CP	**13.2** (4.3)	**19.2** (4.5)	**13.7** (5.1)	**14.0** (5.2)	**14.6** (2.7)	**2.0** (0.8)	**4.2** (1.1)

Table 2. *Continued*

	Sleeve L	Sleeve R	Airlocks handles L	Airlocks handles R	CATO® mouse	Working bench	Scale plate
IRI	**4.9** (5.2)	**3.9** (3.0)	**23.7** (1.4)	**19.2** (3.8)	**9.1** (1.1)	**3.1** (0.4)	**4.0** (0.5)
DOX	**0.3** (0.3)	**0.5** (0.3)	**0.4** (0.5)	**0.5** (0.4)	**0.3** (0.4)	**0.4** (0.3)	**0.2** (0.4)
VIN	**13.7** (0.2)	**0.1** (0.2)	**0.6** (6.2)	**0.2** (7.5)	**0.1** (0.2)	**0.3** (9.5)	**0.7** (9.9)
EPI	**13.4** (0.5)	**0.5** (0.5)	**0.4** (3.6)	**0.5** (5.7)	**0.4** (4.0)	**0.5** (1.8)	**0.3** (2.0)

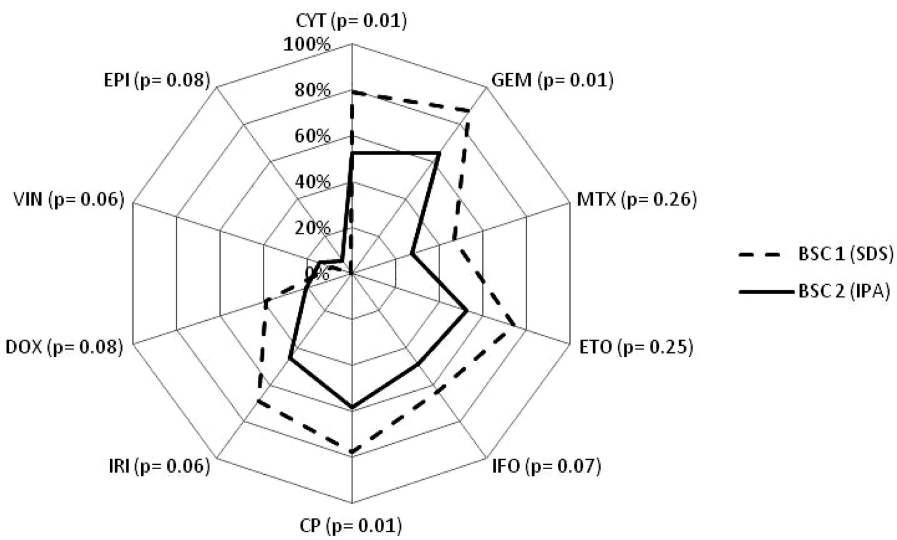

Figure 4 Efficacy of cleaning solutions on 10 antineoplastic agents in BSC 1 and BSC 2 during the study, with *P* value results of Fisher–Student test in brackets.

no significant relationship was observed. Literature describes several factors which can have an impact on contamination levels—such as operators training, work practices, materials, and facilities (Marcel *et al.*, 2004; Käslin *et al.*, 2010; Le *et al.*, 2013)—these were beyond the scope of this study.

Independent guidelines have suggested a reference value of 1 ng cm^{-2} (Käslin *et al.*, 2010), based on the experimental 90th percentile of the contamination load detected during the MEWIP study (Kiffmeyer *et al.*, 2013). In this study, 10 antineoplastic agents are detected in seven areas, each of about 100 cm^2, which gave a total contamination reference value of about 7000 ng. Target contamination levels in the MEWIP study were 0.1 ng cm^{-2}, corresponding roughly to a 700 ng contamination level in this study. $\bar{Q}$ values before the cleaning procedure ranged between 7000 and 700 ng. $\bar{Q}_{BSC\,1}$ values were lower than 700 ng after the cleaning procedure whereas $\bar{Q}_{BSC\,2}$ values were always higher than this target value. These results suggest that the SDS solution (BSC 1) had indeed decreased contamination to acceptable levels (700 ng in our case), but that IPA had not (BSC 2). Moreover, some $\bar{Q}_{BSC\,2}$ values for after the cleaning procedure were higher than $\bar{Q}_{BSC\,2}$ values before it, suggesting either that IPA was less effective than the SDS solution or that a dispersion of antineoplastic agents occurred during the cleaning procedure.

Decontamination efficacy

According to experimental conditions results showing a better decontamination efficacy for a SDS 10^{-2} M solution with 20% IPA than for an IPA solution alone (Queruau Lamerie *et al.*, 2013), a larger Δ_Q could have been expected in the BSC cleaned using the SDS solution (BSC 1). A decrease in contamination level was indeed observed for all the BSC 1 samples after a cleaning procedure (Δ_Q always positive). During the W1, W2, and W44 wiping campaigns in BSC 2, however, Δ_Q values were negative. This was probably due to a dispersion of antineoplastic agents during the IPA solution cleaning procedure in that BSC. Similar effects were discussed in another study on the efficacy of cleaning solutions, which showed a higher dispersion potential for alcoholic solutions than for detergent solutions (Le *et al.*, 2013). It should be noted that traces of contamination remained after the cleaning procedures whichever solution was used. This residual contamination was also observed in other studies investigating the efficacy of cleaning solutions based on a desorption phenomenon (Le *et al.*, 2013; Queruau Lamerie *et al.*, 2013).Desorption-type cleaning procedures are not able to completely eliminate contamination. Better efficacy was obtained with decontamination protocols involving destructive agents such as sodium hypochlorite or hydrogen peroxide (Castegnaro *et al.*, 1997; Hansel *et al.*, 1997; Roberts *et al.*, 2006). However, due to several major drawbacks, including surface corrosion (ISOPP, 2007) and the production, by oxidation, of mutagenic residues (Castegnaro *et al.*, 1985; Barek *et al.*, 1998), these products are not suitable for routine use in BSC cleaning procedures. The present study's results for cumulative contamination levels demonstrated that even if the SDS solution were more effective than IPA, both experimental and routine conditions, the total decontamination of a BSC cannot be reached. The cleaning product itself is only one of several factors—including cleaning procedures, pharmacy technicians awareness, and training of cleaning technicians—which need to be evaluated and optimized in order to decrease contamination levels in BSC (Roberts *et al.*, 2006; Käslin *et al.*, 2010; Le *et al.*, 2013).The effect of the number of mechanical cleaning was neglected between the two methods because no significant difference in the efficiency was observed between a one-step procedure and a three-step cleaning procedure using IPA only (Table 3). Indeed to assess whether the mechanical action affects the efficacy of the cleaning procedure, BSC 2 was once cleaned three times with IPA (W13). Eff_Q value of a three steps cleaning procedure (57%) was included in the confidence interval (49% ± 16%), calculated from the Eff_Q values of one-step cleaning procedures.

Table 3. Mean Eff_Q values of BSC 2 (IPA) according to the number of cleaning steps. Confidence interval was included from 33 to 65%.

	Eff_Q BSC 2 (IPA)	
Step count	1	3
W1	0%	
W2	0%	
W3	91%	
W10	51%	
W13		57%
W17	76%	
W21	53%	
W26	90%	
W30	54%	
W35	59%	
W39	65%	
W44	0%	
W47	27%	
W49	67%	
n	13	
Mean	49%	
SD	31%	
CI	49 ± 16%	

Analysis of the contamination by wiping area for the 10 selected antineoplastic agents

The higher contamination levels detected on sleeves and airlock handles were probably due to a high frequency of contact with gloves, as observed elsewhere (Sessink *et al.*, 1992; Chu *et al.*, 2012). Moreover, sleeves are voluminous and relatively difficult to clean

by the pharmacy technicians themselves during the post-preparation cleaning procedure, leading to only a partial elimination, or a dispersion, of contamination. Sleeves are also permanently close to vials of products in the preparation area, increasing the risk of contamination by dispersion of the contaminant present on the outside of vials (Favier *et al.*, 2003; Connor *et al.*, 2005; Fleury-Souverain *et al.*, 2014) or by drops, spills, or aerosols generated during drug manipulation(Vyas *et al.*, 2013).Dispersion might also occur during the cleaning procedure, as observed in BSC 2, when airlock handles were contaminated with elevated q_{CYT} both before and after the cleaning procedure. Lower contamination levels were observed on the scale plate, CATO® hardware and the work bench. Indeed, only materials such as syringes or infusion bags touch the scale plate. Lower contamination levels on work benches could be explained by the use of a disposable preparation mats, placed in the center of the preparation area, which protect the stainless steel surfaces: when preparation sessions are over (or if visible contamination is observed), the disposable preparation mat is carefully folded, discarded into a waste bag, sealed, and taken out using the left airlock (and replaced) to be destroyed. No sampling was performed on the disposable mats. The use of preventive protective measures (like the work bench mat) could decrease the risk of contamination by antineoplastic agents. In order to limit cross-contamination, a glove changes could occur between preparations when a different antineoplastic agent is used (Mason *et al.*, 2003; Fleury-Souverain *et al.*, 2014). To prevent spillage or drops during the manipulation of antineoplastic agents, the HUG pharmacy uses vented needles containing a hydrophobic filter or a chemo-dispensing pin. Although these devices improve safety during the chemotherapy preparation session (Siderov *et al.*, 2010; Favier *et al.*, 2012) and are useful for reducing contamination levels, their use does not appear to have been sufficient to eliminate the risks of spills or drops when the syringe is disconnected from the device during preparation sessions(Guillemette *et al.*, 2014). In both BSC, contamination values for $\overline{q}_{CP}$, $\overline{q}_{IFO}$, $\overline{q}_{GEM}$, and $\overline{q}_{CYT}$ were higher than the $\overline{q}$ for other cytostatic agents. The higher contamination levels for these four antineoplastic agents (CP, IFO, GEM, and CYT)was probably due to their higher therapeutic doses and the risk of spills or drops associated with the reconstitution step of the freeze-dried drugs (IFO and GEM). SDS solution was more effective than IPA on hydrophilic molecules (CYT, GEM, MTX, ETO, IFO, and CP), but also on two hydrophobic molecules (IRI and DOX), due to the presence of an anionic surfactant promoting the formation of micelles, as previously demonstrated elsewhere(Le *et al.* 2013; Queruau Lamerie *et al.* 2013). During this study, results for the efficacy of SDS showed a greater potential for the decontamination of eight antineoplastic agents. Using SDS solution instead of IPA solution during the cleaning procedure reached a statistically significant better decontamination on three of these antineoplastic agents: CYT, GEM, and CP. The results obtained in the present routine use study were sometimes different from experimental conditions studies; this could be explained by the fact that contamination levels of the 10 antineoplastic agents were unpredictable compared to standardized simulated contamination. This difference had an impact on efficacy (i.e. high levels of contamination were more difficult to clean up), leading to higher residual contamination after the cleaning procedure. Other potential sources of differences concerned the contact time which antineoplastic agents had with the air, their exposition to light. Moreover heterogeneity of contaminated surfaces (stainless steel, polypropylene, polyester, and plastic) could explain the differences between the experimental and routine conditions results of the efficiency on CYT, GEM, or CP. The inherent variability of different cleaning technicians' ways of applying procedures could also have had an impact on the efficacy of decontamination. Before the study, the cleaning method to be applied was decided upon with reference to the routine procedure. Although the sequence for this procedure was followed by all the cleaning technicians and did not change throughout the study, some differences, such as the quantity of cleaning product sprayed or the wiping path, were observed. These differences were dependent on the individual cleaning technicians and could be reduced by the highly detailed training of these personnel in BSC cleaning procedures (Käslin *et al.*, 2010; Le *et al.*, 2013).

CONCLUSIONS

This study evaluated the efficacy of two cleaning solutions for the chemical decontamination of 10 antineoplastic agents (GEM, CYT, CP, VIN, MTX, DOX,

EPI, IFO, ETO, and IRI) on the surfaces of two BSCs in a real-world context. The efficacy of a solution containing a surfactant agent—the SDS solution—was shown to be higher than the efficacy of the IPA solution alone, thus confirming experimental conditions studies. Neither cleaning solution was able to totally remove the contamination, but the efficacy of the cleaning solution containing a surfactant was sufficient to reduce the contamination of each individual antineoplastic agent to under a level corresponding to the 0.1 ng cm^{-2}, the acceptable limit proposed by the MEWIP study (Kiffmeyer *et al.*, 2013).The present study's results also suggested that the decontamination of BSC depends on such important factors as the cleaning products used, cleaning procedures, the awareness of pharmacy technicians, and the training of cleaning technicians. Measures such as standardized cleaning protocols and regular training of the cleaning technicians must be undertaken in order to make cleaning procedures more effective. Additional measures, such as the use of a second pair of gloves, the decontamination of external vial surfaces or the use of closed system drug transfer devices, should be considered in an effort to reduce initial contamination. The cleaning procedure using the SDS solution could be easily transferred and applied to other contaminated surfaces presents in pharmacy or health care units working with antineoplastic agents. However, future studies are required to carry out a detailed investigation of glove contamination levels, e.g. to look at the impact of standardized cleaning protocols on cleaning efficacy and to analyze the decontamination of further antineoplastic agents.

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DISCLAIMER

Its contents, including any opinions and/or conclusions expressed, are solely those of the authors.

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Chapitre 5 : Effluents hospitaliers

L’exposition aux agents anticancéreux ne s’effectue pas uniquement auprès du personnel hospitalier. En effet, ces substances se retrouvent, au même titre que les autres molécules médicamenteuses, dans les effluents. Leur présence dans l’environnement aquatique représente un réel risque pour la santé des écosystèmes et par conséquent pour l’homme. Etant donné que les agents anticancéreux sont principalement administrés à l’hôpital, les HUG se sont intéressés au contenu réel de ces effluents. Un projet visant à évaluer les molécules médicamenteuses présentes dans les effluents des HUG a été conduit sur la base d’une étude de priorisation (article X) et de mesures réelles du contenu des effluents (article XI). Si l’étude de priorisation, basée sur les données de consommation ainsi que sur les profils de devenir de ces molécules dans les effluents et stations d’épuration, ne met pas en exergue les agents anticancéreux (principalement du au manque de données sur ces molécules), l’analyse des effluents révèle leur présence. Une attention particulière a été portée sur les dérivés du platine en raison de la présence de l’élément métallique connu pour son accumulation dans les écosystèmes et, par conséquent, son action délétère sur l’environnement et l’homme. Le dosage des dérivés du platine a été effectué par ICP-MS au cours de ce travail.

Article X

« Priorization methodology for the monitoring of active pharmaceutical ingredients in hospital effluents.»

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Research article

Prioritization methodology for the monitoring of active pharmaceutical ingredients in hospital effluents

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ABSTRACT

The important number of active pharmaceutical ingredients (API) available on the market along with their potential adverse effects in the aquatic ecosystems, lead to the development of prioritization methods, which allow choosing priority molecules to monitor based on a set of selected criteria. Due to the large volumes of API used in hospitals, an increasing attention has been recently paid to their effluents as a source of environmental pollution. Based on the consumption data of a Swiss university hospital, about hundred of API has been prioritized following an OPBT approach (Occurrence, Persistence, Bioaccumulation and Toxicity). In addition, an Environmental Risk Assessment (ERA) allowed prioritizing API based on predicted concentrations and environmental toxicity data found in the literature for 71 compounds. Both prioritization approaches were compared. OPBT prioritization results highlight the high concern of some non steroidal anti-inflammatory drugs and antiviral drugs, whereas antibiotics are revealed by ERA as potentially problematic to the aquatic ecosystems. Nevertheless, according to the predicted risk quotient, only the hospital fraction of ciprofloxacin represents a risk to the aquatic organisms. Some compounds were highlighted as high-priority with both methods: ibuprofen, trimethoprim, sulfamethoxazole, ritonavir, gabapentin, amoxicillin, ciprofloxacin, raltegravir, propofol, etc. Analyzing consumption data and building prioritization lists helped choosing about 15 API to be monitored in hospital wastewaters. The API ranking approach adopted in this study can be easily transposed to any other hospitals, which have the will to look at the contamination of their effluents.

1. Introduction

Active pharmaceutical ingredients (API) are continuously released into the aquatic environment and are thus considered as 'pseudo-persistent' pollutants (Daughton, 2003; Kümmerer, 2009a). Their inputs are diverse and may come from human and animal use, waste disposal and/or manufacturing (Daughton, 2003; Kümmerer, 2010). Generally, urban wastewater treatment plants (WWTP) are the main contributors of API residues inputs into the aquatic ecosystems (Götz et al., 2010b; Kümmerer, 2010; Michael et al., 2013). A small proportion of this point source pollution comes however from hospitals and health care facilities, which differentiate itself from domestics ones by the nature of administrated molecules (Kümmerer, 2001; Mullot, 2009). Globally, hospitals represent indeed only a small proportion of the urban API load source found at the watershed outlet: <10% (Kümmerer, 2010), <15% (Ort et al., 2010; Le Corre et al., 2012), 20–25% (Helwig et al., 2013). But, this fraction can vary from 3 to 74% according to the compound type and the hospital beds/inhabitants ratio of the watershed (Santos et al., 2013).

Once in the environment, API residues can cause some adverse effects in wildlife, such as fish feminization by synthetic hormones (Fent et al., 2006; Santos et al., 2010), or fish and birds kidney impairment by the non-steroidal anti-inflammatory drug diclofenac (Oaks et al., 2004; Hoeger et al., 2005). The environmental toxicity of API is generally appreciated by ecotoxicological tests, which give dose–response curves, from which water quality

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criteria are derived, such as predicted no effect concentrations (PNEC) (European Commision, 2003). Unfortunately, experimental values are scarce (Chèvre, 2014). They cannot be replaced by theoretical ones, such as the ones modeled by quantitative structure–activity relationships (QSAR) approaches (Escher et al., 2011; Jean et al., 2012; Orias and Perrodin, 2013). Therefore, PNEC values found in the literature are scarce, often modeled, and can vary as much as three orders of magnitude between studies according to conditions and endpoints (Helwig et al., 2013).

Prioritization methods applied to pharmaceuticals are generally based on consumption data and a simplified risk assessment for the environment and/or human health (EMEA, 2006; de Jongh et al., 2012; Le Corre et al., 2012). Nevertheless, other parameters are most of the time also considered such as environmental persistence, bioaccumulation factor, and mode of action and/or analytical feasibility (Besse and Garric, 2008; Perazzolo et al., 2010; Jean et al., 2012; Ortiz de Garcia et al., 2013). Thus, the elaboration of a priority list of pharmaceuticals strongly depends on the pertinence of the chosen criteria, and also on the exhaustiveness and the quality of the available data (Mullot, 2009; Coutu et al., 2012). Among the numerous approaches applied for prioritizing substances, the persistence, bioaccumulation and toxicity (PBT) approach is used in Europe in the framework of the registration, evaluation, authorization and restriction of chemicals (REACH), as well as in specific studies dealing with pharmaceuticals (Wennmalm and Gunnarsson, 2009; Ortiz de Garcia et al., 2013). It consists of giving a ranking of concern according to their PBT properties, but these latter are not available for many compounds leading researchers to use QSAR models to predict them (Pavan and Worth, 2008).

Another method for prioritizing chemicals, such as pharmaceuticals, is proposed by the European Medicines Agency. It consists in a tier-based environmental risk assessment procedure for API which comprises two phases (EMEA, 2006): the estimation of exposure (phase I) and the environmental fate and effects analysis (phase II). Phase I comprises a PBT approach and the calculation of the predicted environmental concentrations (PEC): if the API shows a bioaccumulation tendency (Log K_{ow} > 4.5) or a PEC > 0.01 μg/L, then a phase II is needed. Phase II deals with the calculation of the environmental risk quotient (RQ) as the ratio between exposure (PEC) and effects (PNEC). This procedure has been adopted by several authors and adapted according to the study's specific needs (Besse and Garric, 2008; Mullot, 2009; Perazzolo et al., 2010; Coutu et al., 2013).

The objectives of the present study are manifold. First, it aims at elaborating a prioritization list of API based on consumption data of a university hospital and on their environmental persistence, bioaccumulation potential and ecotoxicity data found in the scientific literature. A weighting of the PBT properties is done according to the quality of data. Second, predictions of API concentrations in the hospital effluents and in surface waters, as well as of their environmental risk quotients are calculated, under the assumptions of on-site total consumption and mass conservation into the sewers. The results of both methods are compared and discussed. In the end, these prioritization and predictions should help choosing priority compounds to be measured in the hospital effluents and taking decisions for the hospital managers in order to reduce the inputs of pharmaceutical residues into the urban network and, subsequently, their potential adverse effects for the aquatic ecosystems.

2. Material and methods

2.1. Setting and consumption data collection

The Geneva University Hospitals (HUG) is one of the most important hospitals of Switzerland. It comprises eight hospitals and about 40 other health care facilities, providing both primary and tertiary care. In 2012, 8443.2 full-time equivalent collaborators and a total of 671′709 days of hospitalization were registered, for 1908 beds, 48′112 inpatients and over 860′000 outpatient consultations. The average daily water consumption was 762 m^3.

Aggregated data of drugs dispensed in both the inpatient and outpatient settings in 2012 were first obtained from the hospital pharmacy database using "Business Object®" software. These data correspond to the drugs ordered by the different medical units to the pharmacy to treat their patients, as well as returns (stock and delivery errors, leaving or deceased patients, etc.). The data gives an approximation of the yearly inpatient consumption of API by transforming the overall unit doses (UD) in grams of active ingredient while considering their dosages (Jean et al., 2012). It is worth to stress that only drugs purchased as a total package was taken into account. Also, the stock of each medical unit was considered but the stock difference between years was neglected. All confidential health information was removed to create anonymous analytic datasets in conformity with Swiss data protection regulations.

2.2. PBT prioritization

The prioritization procedure applied to active pharmaceutical ingredients (API) consumed in the Geneva university hospitals as a whole was adapted from previous studies (Besse and Garric, 2008; Perazzolo et al., 2010; Jean et al., 2012). Among the about 1000 API delivered by the hospital pharmacy in 2012, only about 150 API with more than 10′000 unit doses (UD) were first retained. Then, after conversion from UD to grams of API, only 84 API sold at more than 1 kg in 2012 were kept. To these, antineoplastic and immunomodulant drugs (Code L according to the Anatomical Therapeutic Classification; ATC) with more than 10′000 UD were added due to their inherent toxicity, giving a total of a hundred of API for the prioritization. Each API has been attributed 4 ranks, from 1 to 5, based on 4 criteria (Table 1): Occurrence (O), Persistence (P), Bioaccumulation (B) and Environmental Toxicity (T).

The occurrence (O) criteria was based on the excreted amounts in the hospital effluents, which was obtained by multiplying the consumed mass (M) by the excretion factor (F_{excr}), assuming that 100% of the amounts delivered by the pharmacy were used. Excretions factors (F_{excr}) in urine and feces as unchanged drugs were found in databases (www.uptodate.com; www.compendium.ch). When different values were reported, a mean value was calculated. The thresholds of 0.05, 0.5, 5 and 20 kg were chosen for the scoring according to the distribution of the excreted mass values and observed natural thresholds. Pro-drugs consumption was added to the one of its related drug: capecitabine – 5-fluorouracil; valaciclovir – aciclovir, etc. Indeed, when metabolized, pro-drugs end mainly as their related compound in the hospital sewers.

For the persistence (P) criteria, values of WWTP removal efficiency were found in the scientific literature for only 32 API with a

Table 1
Criteria thresholds for the ranking of API.

Rank	Occurrence	Toxicity	Bioaccumulation	Persistence
criteria	$Mass_{excr}$	PNEC[a] [μg/L]	Log K_{ow}	WWTP removal [%]
1	≤0.05 kg	>100	<1	≥80
2	≤0.5 kg	≤100	≥1	≥60
3	≤5 kg	≤10	≥2	≥40
4	≤20 kg	≤1	≥3	≥20
5	>20 kg	≤0.1	≥4.5	<20

[a] PNEC = Predicted no-effect concentration.

great variability (Verlicchi et al., 2012b; Margot et al., 2013). Thresholds of 20, 40, 60 and 80% were chosen to give a rank, and a safety value of 20% was attributed arbitrarily to the missing data. The removal efficiency varies often between 10% and 90% for compounds with measured values, because the efficiency of biological treatments depends on many factors and varies among localities (Verlicchi et al., 2012b).

For the bioaccumulation (B) criteria, only 14 experimental values of log K_{ow} were available in the databases (www.drugbank.ca, www.chemspider.com). Therefore 90 values were modeled based on a Quantitative Structure Activity Relationship (QSAR) approach. Two values were unavailable. The thresholds of 3 and 4.5 were selected to discriminate API with a high bioaccumulation potential based on Perazzolo et al. (2010) and EMEA (2006), respectively - which are themselves based on OECD (Organization for Economic Co-operation and Development) and OSPAR Convention (Convention for the protection of the marine environment of the North-East Atlantic) values. Thresholds of 2 and 1 are arbitrary and represent compounds with low bioaccumulation potential.

Regarding the environmental toxicity (T), 69 PNEC data were found in the scientific literature (Fent et al., 2006; Perazzolo et al., 2010; Santos et al., 2010; Escher et al., 2011; Verlicchi et al., 2012b; Orias and Perrodin, 2013). When several values were available, the lower experimental ones (Orias and Perrodin, 2013) were preferred to the QSAR modeled ones (Escher et al., 2011). For the 27 API with unknown PNEC values, a mean rank of 3 (corresponding to PNEC values between 1 and 10 μg/L) was attributed arbitrarily. Regarding the 11 antineoplastic drugs for which no values could be found, a security rank of 5 (PNEC < 0.1 μg/L) was given due to the inherent toxicity of this kind of molecules. Thresholds of 0.1, 1, 10 and 100 μg/L were chosen according to the distribution of the PNEC values.

The final ranking was then obtained by the addition of the ranks of the four criteria, and also weighted according to the data quality. In order to take into account the data quality, the ranks of the different criteria are multiplied by a quality factor: this factor is equal to 1 if no data was available, to 2 if the PNEC or the Log K_{ow} were modeled with a QSAR approach, and to 3 if experimental values were available.

2.3. Environmental risk assessment

The environmental risk assessment (ERA) was calculated by determining risk quotients (RQ) using the predicted environmental concentrations (PEC) and the predicted no effects concentrations (PNEC) values found in the literature. The predicted environmental concentrations in the hospital wastewater (PEC_{HWW}) were obtained by dividing the excreted mass, i.e. the consumed mass (M) multiplied by the excretion factor (F_{excr}), by the volume of wastewater (V) (European Commision, 2003; Mullot, 2009; Perazzolo et al., 2010; Escher et al., 2011):

$$PEC_{HWW} = M \times F_{excr}/V \quad \text{(Eq.1)}$$

PEC_{HWW} were calculated assuming that 100% of drug consumption occurs on site and that no wastes are produced. Moreover, the volume of wastewater (V) was assumed to be equal to the known volume of consumed water (see Section 2.1). It is worth to stress that excretion factors are the sum of excretion in urine and feces as unchanged drugs but do not take into account metabolites. Even though some authors take into account the glucuronide conjugates as they assume that they are cleaved in the environment (Besse et al., 2008), we chose to neglect these fractions. Indeed, although glucuronide bonds are known to be unstable and can be easily cleaved, either by microbial activity or photolysis, many uncertainties remain and for some compounds glucuronide conjugates were detected in surface waters (Bonvin et al., 2012).

In order to predict concentrations in receiving surface waters (PEC_{SW}), the removal rates in WWTP (R) and the dilution (Dil) that hospital effluent undergo in the watershed have to be taken into consideration (Mullot, 2009; Perazzolo et al., 2010; Escher et al., 2011):

$$PEC_{SW} = M \times F_{excr} \times (1 - R)/(V \times Dil) \quad \text{(Eq.2)}$$

The dilution factor (Dil) is generally fixed to 10 for pharmaceuticals in WWTP (European Commision, 2003; EMEA, 2006). However, this factor concerns the dilution of municipal wastewaters into the receiving aquatic ecosystem, but hospital effluents are first diluted into urban sewer network. Kümmerer (2009b) considers that dilution of hospital effluents in municipal wastewaters is more important than the dilution of these latter into rivers or lakes and suggest a dilution factor of 100, which is more close to reality when calculated (Escher et al., 2011). In our case, the dilution factor for hospital wastewaters in the urban network was 296 (volume of urban wastewaters/volume of hospital water consumption) in 2012, and a factor of 10 was applied for its second dilution in receiving waters (Dil = 2960). Thus, the PEC_{SW} are representative of the hospital contribution and do not take into account the domestic consumption.

Finally, Risk Quotients (RQ) were calculated using the PEC and the PNEC values according to Equation (3) (European Commision, 2003; EMEA, 2006):

$$RQ = PEC/PNEC \quad \text{(Eq.3)}$$

Though it is not realistic to calculate a risk for hospital effluents, because the exposure of living organisms is null in hospital sewers, risk quotients are useful tools to evaluate the potential risk of hospital effluents once they reach the aquatic environment. Thus, a Hazard Quotient (HQ) was calculated for hospital wastewaters (HQ_{HWW}) and a Risk Quotient for surface waters (RQ_{SW}). Due to the PNEC values availability, only 71 RQ could have been calculated. Finally, a $RQ_{SW} \geq 1$ would mean that the considered API poses a high risk for the aquatic ecosystems, a $RQ_{SW} \geq 0.1$ a medium risk and a $RQ_{SW} < 0.1$ a low risk (Al-Aukidy et al., 2014). A $HQ_{HWW} \geq 1$ would however only mean that the considered API contributes to the environmental hazard of the hospital effluent.

A sensitivity analysis was performed for 34 API in order to assess the variability range of the predicted risk quotients associated with the different variables of the Equations (2) and (3), with the exception of Dil which was set fixed. The influence of API consumption was tested according to the monthly variation during the year 2012. It is worth to stress that annual variability may have an influence but this was neglected here. The minimum and maximum values of excretions factors (F_{excr}) found in databases (www.uptodate.com; www.compendium.ch) were tested for their differences as compared to the retained mean values. The influence of the uncertainty of R values was assessed according to the different values found in literature data (Verlicchi et al., 2012b, Margot et al., 2013). For the influence of V on RQ values, the variability of the daily and the hourly variation of the water consumption was tested.

3. Results and discussion

3.1. Consumption data

According to the university hospital data 4301 kg of API were delivered in 2012. Under the hypothesis that 100% of the administrated drugs are consumed in hospital, this gives a ratio of 90 g/

patient. When taking into account outpatient consultations a much more realistic ratio of 4.8 g/patient is obtained. The most consumed pharmaceutical classes were laxatives (36.8%), analgesics (31.3%), antibiotics (11.4%), antiviral (6.4%) and anti-inflammatory (4.9%) (Fig. 1A). Laxative macrogol was the most consumed drug (with 1.5 tons in 2012): this is not surprising as its defined daily dose is 10 g, in comparison to 1 g for oral ciprofloxacin for example (www.whocc.no). In general, antibiotic drugs are the most consumed class of drugs in hospitals (Kümmerer, 2001; Le Corre et al., 2012), but in our case analgesics are more important. The 'no pain' policy of the studied hospital could explain the important use of paracetamol (1180 kg in 2012). It is followed by ibuprofen (172 kg), and the antibiotics piperacillin (162 kg) and amoxicillin (132 kg). When taking into account the excretion ratio, the most excreted classes were antibiotics (39%), followed by antiviral (17.8%), analgesic (10.2%) and antidiabetic drugs (7.3%) (Fig. 1B). Anti-epilepsy (4.7%), antineoplastic (4.5%) and anti-inflammatory (3.1%) drugs are also excreted in non negligible proportions. It is worth to stress that laxatives and antidiabetics classes are constituted of only one molecule present in our selection, macrogol and metformin respectively.

Compared to another Swiss hospital (Escher et al., 2011; McArdell et al., 2011), antiviral and antibiotic drugs are excreted in higher proportion in our case, whereas a lower fraction of iodinated X-ray contrast media and laxatives is excreted. This is probably due to the size difference between the two hospitals (338 vs. 1908 beds) and also to the difference in prescriptions and activities between a cantonal hospital and a university hospital. Outpatient treatments can also represent an important fraction of the consumption depending on the nature of the compounds. Indeed, Weissbrodt et al. (2009) showed that 50% of iodinated X-ray contrast media and 70% of antineoplastic agents prescribed in the studied hospital were excreted at home. Concerning systemic antiviral drugs (ATC code J05), they are specifically prescribed and delivered in the HUG, as for in a city pharmacy, but are likely to be excreted at home by outpatients (McArdell et al., 2011; Le Corre et al., 2012). Moreover, the pharmacy data are delivery data which can differ from real consumption in the services due to lack of patient compliance, outside consumption for leaving patients, etc (Jean et al., 2012).

3.2. OPBT prioritization

The adopted OPBT approach leads to the prioritization of the 100 most consumed API in the studied hospital (Supplementary data S1). The 20 most priority compounds without any weighting and with a weighting according to the data quality are presented in Table 2. 55% of the compounds are present in the top 20 of both weighting methods. This means that the weighting according to the data quality changes the order of importance for about half of the compounds.

Three non-steroidal anti-inflammatory drugs (NSAID; ATC code M01) were ranked among the 10 first molecules with both methods: ibuprofen, diclofenac and mefenamic acid. Ibuprofen and diclofenac have been previously identified as priority compounds in studies with different methodologies (Lienert et al., 2007; de

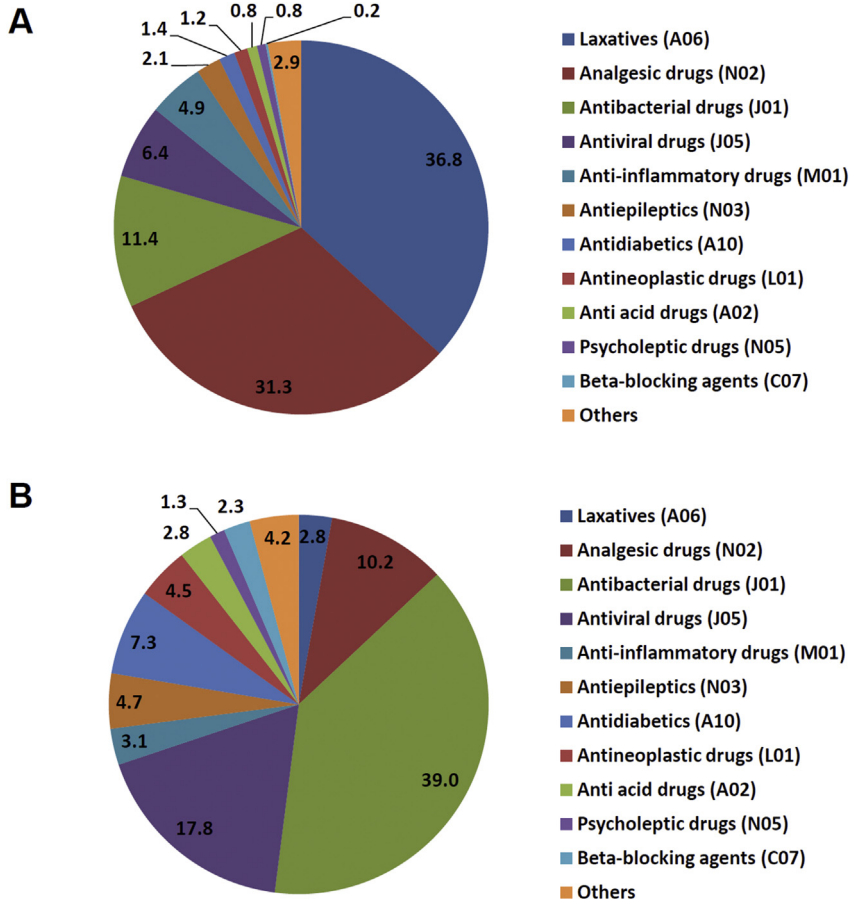

Fig. 1. Proportion of the therapeutic classes (ATC code) of (A) the most consumed and (B) the most excreted pharmaceuticals in 2012, based on the hypothesis that 100% was excreted at the hospital.

Table 2
Top 20 API ranked according to the different prioritization methods: The Occurrence, Persistence, Bioaccumulation, Toxicity approach (OPBT), weighted or not according to the data quality, and the environmental risk assessment (ERA), as well as the combination of both methods.

#	OPBT No weighting	OPBT data quality	ERA	OPBT + ERA
1	Sertraline	Ibuprofen	Ciprofloxacine	Ibuprofen
2	Lopinavir	Sertraline	Amoxicillin	Sertraline
3	Ibuprofen	Diclofenac	Trimethoprime	Diclofenac
4	Mefenamic acid	Mefenamic acid	Fluorouracile/Capecitabine	Mefenamic acid
5	Ritonavir	Sulfamethoxazole	Sulfamethoxazole	Sulfamethoxazole
6	Propofol	Gabapentin	Ritonavir	Gabapentin
7	Efavirenz	Ritonavir	Ibuprofen	Trimethoprime
8	Atazanavir	Trimethoprime	Lidocaïne	Ritonavir
9	Etravirine	Valsartan	Gabapentin	Amoxicillin
10	Diclofenac	Amoxicillin	Lopinavir	Valsartan
11	Valsartan	Mycophenolic acid	Propofol	Ciprofloxacine
12	Mycophenolic acid	Metoprolol	Ifosfamide	Oxazepam
13	Raltegravir	Oxazepam	Oxazepam	Mycophenolic acid
14	Citalopram	Propofol	Clozapine	Metoprolol
15	Amiodarone	Ciprofloxacine	Raltegravir	Propofol
16	Asparaginase	Furosemide	Citalopram	Furosemide
17	Paclitaxel	Carbamazepine	Piperacillin	Carbamazepine
18	Sulfamethoxazole	Raltegravir	Mycophenolic acid	Raltegravir
19	Gabapentin	Metronidazole	Diclofenac	Metronidazole
20	Clozapine	Lidocaïne	Efavirenz	Lidocaïne

Voogt et al., 2008; Coutu et al., 2012). This was not the case for mefenamic acid, the importance of which was not revealed in previous studies. This can be explained by a relatively high prescription of this drug in Switzerland, in a similar range as ibuprofen (Tauxe Würsch, 2005).

Without any weighting, five antiviral drugs (ATC code J05) are listed among the top ten ranked API (lopinavir, atazanavir, ritonavir, etravirine and efavirenz), due mainly to their high bioaccumulation potential (Log Kow > 3.9). When taking into account the data quality, only ritonavir, which was previously identified as a potent hospital compound (Escher et al., 2011), remains in the top 10.

The antidepressant sertraline (ATC code N06) is ranked #1 without any weighting, and #3 with the data quality weighting. This high rank is not related to its level of use (2.1 kg in 2012), but to its high environmental toxicity (PNEC = 0.09) and bioaccumulation potential (log K_{ow} = 5). It shows adverse effects in aquatic organisms (Santos et al., 2010), and is considered as priority compound by several authors (Besse and Garric, 2008; Ortiz de Garcia et al., 2013). With the exception of citalopram, ranked 15 without weighting, other antidepressant drugs (mirtazapine, trazodone and venlafaxine) received much lower ranks. Although antidepressant are generally detected at the ng/L level in surface waters and may not represent isolated threats to aquatic organisms, exposure of these latter to antidepressant mixture may induce chronic toxicity since they exert similar effects (Santos et al., 2010; Yuan et al., 2013).

Seven anesthetics and analgesics (ATC codes N01 and N02) were ranked among the first 30 API, considering data quality: propofol (#17), lidocaine (#19), tramadol (#21), paracetamol (#22), codeine (#26), acetylsalicylic acid (#27) and morphine (#30). When considering no weighting, only propofol remains in the top 30.

Among antibiotic drugs (ATC code J01), sulfamethoxazole, trimethoprime, amoxicillin, ciprofloxacin and metronidazole are present in the top 20. Others exhibit much lower ranks (piperacillin #33, cefuroxime #40, etc.). Piperacillin has been previously identified as a hospital-specific compound by Le Corre et al. (2012), whereas ciprofloxacin and amoxicillin have a wide domestic use (Coutu et al., 2013). Due to their antimicrobial properties and their role in the propagation of resistance, antibiotics remain one of the most hazardous pharmaceutical classes for the aquatic environment (Besse et al., 2008; Kümmerer, 2009b).

Among drugs for the cardiovascular system (ATC code C), valsartan is ranked #11 and amiodarone #17 without any weighting, whereas valsartan is ranked #10 and metoprolol #16 with the data quality weighting.

Antineoplastic drugs (ATC code L01) are not consumed in high amounts (15–990 g/y) with the exception of asparaginase (34 kg/y) and 5-fluorouracil and its prodrug capecitabine (11 kg/y). Only paclitaxel is ranked in the top 20 without any weighting, but with an arbitrarily low PNEC of 1 ng/L as its environmental toxicity was unknown.

The immunomodulant mycophenolic acid (ATC code L04) is ranked #14 with both weighting methods.

3.3. Environmental risk assessment

3.3.1. Predicted environmental concentrations

Predicted environmental concentrations were calculated in hospital effluents (PEC_{HWW}; Fig. 2a) and surface waters (PEC_{SW}; Fig. 2b) according to Eqs. (1) and (2) respectively. The five API with the highest PEC_{HWW} are macrogol (4.5 mg/L), piperacillin (408 μg/L), amoxicillin (285 μg/L), metformin (212 μg/L) and paracetamol (212 μg/L; Fig. 2a). Among the 20 API with the highest PEC_{HWW}, 8 antibiotic and 5 antiviral drugs were counted. The NSAID ibuprofen has a PEC_{HWW} of 96 μg/L. It is worth to stress that PEC_{HWW} are certainly over-evaluated due to the fractions excreted by outpatients (Le Corre et al., 2012).

The calculation of predicted environmental concentrations in surface waters (PEC_{SW}) after treatment in WWTP highlighted the persistence character of metformin (32 ng/L) and the high probability of finding antibiotic drugs piperacillin (69 ng/L) and amoxicillin (33 ng/L) in the freshwater environment (Fig. 2b). These results confirm the previously demonstrated low removal and degradation rates of metformin, both in WWTP and in surface waters respectively (Scheurer et al., 2009; Blair et al., 2013; Margot et al., 2013). Result for piperacillin should be taken with caution because its WWTP removal rate was unknown and an arbitrarily value of 0.5 was attributed. It would therefore be interesting to measure this hospital-specific compound in the influents and effluents of WWTP to refine this result. Measured concentrations of some API in the River located downstream of the studied hospital (17–18 May 2010; 24 h time-proportional sampling) were one order of magnitude higher than PEC_{SW}: metformin (370 ng/L; PEC_{SW} = 32), paracetamol (95 ng/L; PEC_{SW} = 2), gabapentin (56 ng/

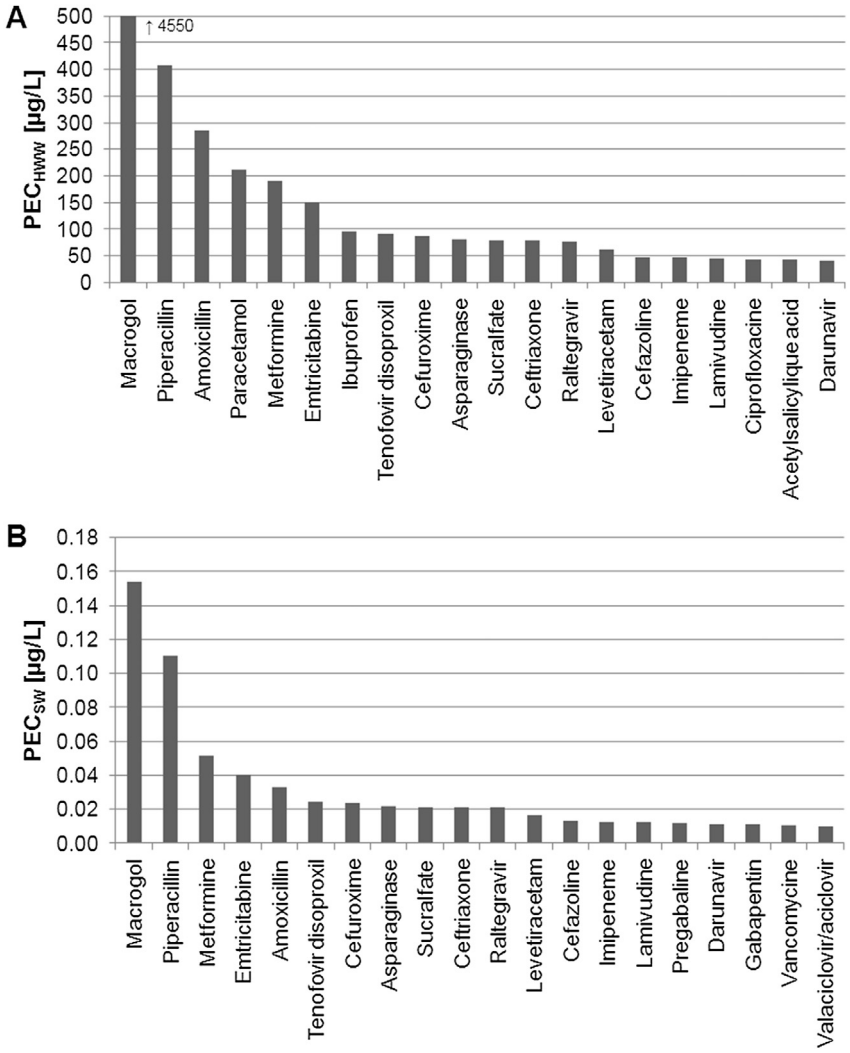

Fig. 2. Predicted Environmental Concentrations of active pharmaceutical ingredients in (A) hospital wastewaters (PEC_{HWW}) and (B) surface waters (PEC_{SW}) according to pharmacy consumption data. Only the 20 first API are represented.

L; $PEC_{SW} = 11$) and ibuprofen (19 ng/L; $PEC_{SW} = 8$) (Ortelli et al., 2011). Calculated PEC_{SW} are lower than measured concentrations as they derived from hospital consumption without considering domestic one, and are thus only representative of the API hospital fraction.

PEC in both hospital wastewaters and surface waters can thus be of help during the selection process for monitoring campaign, but they comprise strong limitations. It is indeed built upon several assumptions: the total consumption of the delivery data, the conservative mass transfer of substances during their transport in the urban wastewater network and surface waters, as well as the accuracy of the known removal data. Due to all these uncertainties, predicted concentrations are thought to be overestimated, which has been previously proven (Le Corre et al., 2012; Ortiz de Garcia et al., 2013). For instance, amoxicillin or paracetamol are likely to be rapidly degraded in surface waters (Besse et al., 2008; Benotti and Brownawell, 2009). Moreover, the seasonal consumption has not been taken into account, and can, for some compounds, highly influence the resulting concentrations (Coutu et al., 2013). Nevertheless, PEC represent useful tools and allow calculating risk quotients.

3.3.2. Risk quotient

The hazard quotient calculated for hospital wastewaters (HQ_{HWW}; Supplementary data S2) varies widely (from 10^{-3} to 10^{3}). On the 71 calculated HQ_{HWW}, 32 were above 1. The 10 most hazardous compounds were: ciprofloxacin, amoxicillin, trimethoprim, 5-fluorouracil, ibuprofen, lidocaine, sulfamethoxazole, paracetamol, ritonavir and lopinavir. These results are consistent with previous studies (Verlicchi et al., 2012a; Orias and Perrodin, 2014). Indeed, ibuprofen, amoxicillin, paracetamol, gabapentin and sulfamethoxazole were listed among the 10 most hazardous compounds in common with Orias and Perrodin (2014). Although prescribed drugs can differ between hospitals, largely consumed API, such as these latter, are likely to participate to the environmental hazard. HQ_{HWW} can thus help hospital manager to focus on priority compounds and to elaborate strategies to reduce their input into the urban network.

The environmental risk quotient calculated for surface waters (RQ_{SW}; Fig. 3) revealed that *a priori* only the hospital fraction of ciprofloxacin is likely to pose a high risk to the aquatic ecosystems ($RQ > 1$). This confirms the previous results obtained in another Swiss hospital (Chèvre et al., 2013). The antibiotics amoxicillin, trimethoprime, sulfamethoxazole, the cytostatic fluorouracil and the antiviral ritonavir are characterized by a medium risk ($RQ > 0.1$). The 20 most hazardous compounds, listed in Table 2, are consistent with previous studies. Sulfamethoxazole, ciprofloxacin and ibuprofen were indeed identified as high priority

pharmaceuticals for the water cycle by de Voogt et al. (2008). Ritonavir was identified as potent hospital compound by Escher et al. (2011), and lidocaine, amoxicillin, ciprofloxacin and sulfamethoxazole were selected as typical hospital compounds for monitoring by Helwig et al. (2013), and are likely to pose problems when reaching the aquatic ecosystem (Valcarcel et al., 2011; Verlicchi et al., 2012b; Orias and Perrodin, 2014). Trimethoprim is generally administrated in combination with sulfamethoxazole, and it has also been identified as problematic by Valcarcel et al. (2011). Fluorouracil (5-FU) and capecitabine were predicted to have low concentrations in European surface waters (Johnson et al., 2013), but they were not considered together by these latter authors. Capecitabine is a pro-drug which is enzymatically transformed into 5-FU in the body and thus should be considered together with 5-FU. Though it was not among priority compounds according to the OPBT approach, as it is not excreted in high amounts, it participates to the environmental risk to aquatic species ($RQ_{SW} = 0.2$).

3.3.3. Sensitivity analysis

Results of the sensitivity analysis are summarized in Table 3, and presented in details for the 34 selected API in Supplementary data S3. On average, uncertainties of human metabolism (F_{excr}) and ecotoxicological data (PNEC) are likely to influence the most RQ values. API consumption (M) and removal rates (R) variability had moderate consequences and water consumption pattern a small influence. The F_{excr} variability is likely to enhance the risk quotient values, up to one order of magnitude, whereas the uncertainty associated to PNEC values may in contrary diminish the risk, down to three orders of magnitude (Supplementary data S3). High F_{excr} uncertainties are mainly concerning cytostatic and antiviral drugs, and cytostatics and antibiotics for PNEC variability. Furthermore, due to the selection of the lowest PNEC values according to the precautionnary principle, the RQ variability range maximum is always 100%. The influence of the monthly variability of API consumption on RQ values is mass-dependent: largely consumed API such as anti-inflammatory (ibuprofen) or analgesic drugs (paracetamol) exhibited much lower variations than the lowest consumed cytostatic drugs (methotrexate, epirubicine). In the end, according to the worst scenario (maximum values for M, F_{excr}, and minimum values for V, R and PNEC), 5 compounds may exhibit a high risk and 4 a moderate risk, compared to 1 and 5, respectively, according to the mean scenario.

3.4. Methods comparison and compounds selection

3.4.1. Methods comparison

The comparison of the 20 first API resulting from the different prioritization approaches - the environmental risk assessment, the OPBT with and without weighting – highlighted that 8 of them was revealed by the three methodologies (40%), and 12 by two different methodologies (60%) (Table 2). The ERA highlighted more antibiotics (4 in the top 5), whereas OPBT ranked more NSAID (3 in the top 5). This difference can be explained by the fact that the ERA does not take into account the bioaccumulation potential (Log K_{ow}). Furthermore, PNEC values were not available for 27 molecules and thus, these latter were not taken into account with the ERA. Both approaches are complementary and combined results should therefore be considered. When summing ranks of both OPBT and ERA approaches, the NSAID ibuprofen is the most priority compound and diclofenac and mefenamic acid, the antidepressant sertraline and the antibiotic sulfamethoxazole are in the top 5 (Table 2). Antiviral drugs (ritonavir and raltegravir), analgesics (lidocaine and propofol) and antibiotics (trimethoprime, amoxicillin, ciprofloxacin and metronidazole) were also highlighted in the top 20 with the rank combination of both methods. Nine compounds present in Table 2 (ibuprofen, paracetamol, diclofenac, ciprofloxacin, sulfamethoxazole, trimethoprim, metronidazole, metoprolol and carbamazepine) were previously highlighted as priority compounds in at least 2 different studies as reported by Al Aukidy et al. (2014).

It is worth to stress that these prioritization have some drawbacks: they deal only with the most consumed drugs (>1 kg/y), many PNEC and Log K_{ow} values are obtained by QSAR models, and excretion factors were often mean values. Nevertheless, and even though it is a theoretical approach, the highlighted priority compounds - NSAID, antiviral drugs, the antidepressant sertraline, the sedative propofol and/or the antibiotics sulfamethoxazole, trimethoprime, ciprofloxacin and amoxicillin - are consistent with previous works (Besse and Garric, 2008; Escher et al., 2011; Le Corre et al., 2012; Santos et al., 2013).

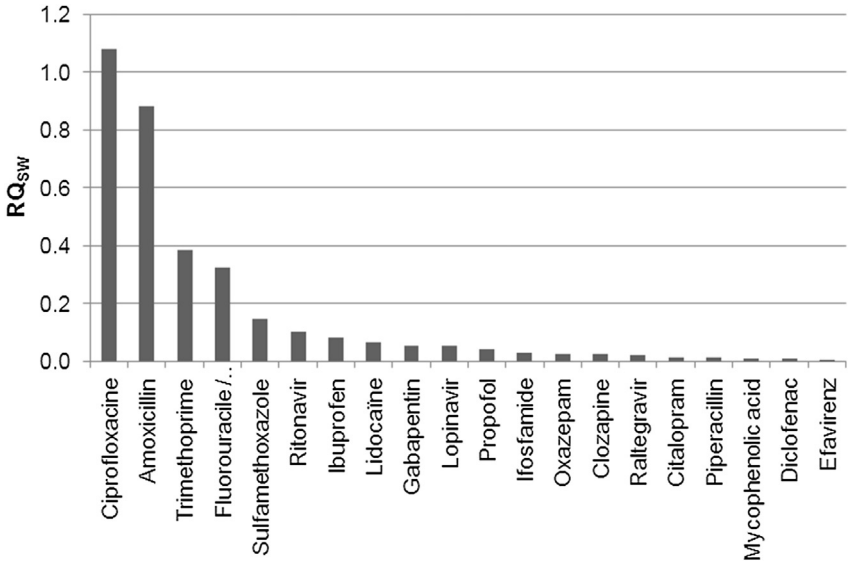

Fig. 3. Predicted risk quotients in surface water (RQ_{SW}) for the 20 first active pharmaceutical ingredients.

Table 3
Range of percentage variation in predicted risk quotients for surface water (RQ_{SW}) associated to the different variables used for their calculation for 34 API. Range of RQ variation associated with water volume (V) refers to the hourly and daily variations of hospital water consumption, for the first and the second line respectively.

Variable influence on risk quotients	Mass consumption (M)	Excretion factor (F_{excr})	Water volume (V)	WWTP removal (R)	PNEC
Range of variation [%]	2–234	20–1000	77–143	1–364	0.004–100
Mean range of variation [%]	64–141	80–237	91–107	78–121	12.1–100

3.4.2. Compounds selection

About 15 API and some of their associated human metabolites were selected to be monitored in the hospital wastewaters. This was based on the results of the adopted prioritization approach, but also according to the diversity in drug classes, the antecedent detection in local surface waters or effluents of other Swiss hospitals, the analytical feasibility and most importantly the probability to find residues in the sewer of the main building. Thus, even though they exhibited relative high ranks, antiviral drugs and antidepressants were not considered as they are thought to be consumed in other buildings or at home.

Non-steroids anti-inflammatory drugs (NSAID) ibuprofen, diclofenac and mefenamic acid have been selected because of their high ranks. Though it is not consumed in high amounts at the hospital, diclofenac was also chosen because it was proposed as a model compounds to monitor in surface water (Götz et al., 2010b). The hydroxyl and carboxyl metabolites of ibuprofen were selected because of their importance. Though they are not considered as active metabolite, they account for 25% and 37% of the parent compound, respectively (Besse and Garric, 2008).

Among antibacterial drugs, ciprofloxacin, piperacillin, sulfamethoxazole and metronidazole were selected. Ciprofloxacin exhibits the highest rank according to environmental risk assessment, whereas piperacillin was identified as a hospital-specific compound (Le Corre et al., 2012). Sulfamethoxazole was selected because it exhibits relatively high rank and was proposed as a representative API to follow in Swiss surface waters (Götz et al., 2010b). Furthermore, it has been found in drinking water (Daughton and Ruhoy, 2011), and is considered as mutagenic (Isidori et al., 2005). The antibiotic metronidazole was retained though it is not really a hospital-specific molecule (Coutu et al., 2013), but its contribution to the total load of urban micropollution can be important (Verlicchi et al., 2012a). Moreover, it has been proved to be genotoxic and not biodegradable (Kümmerer et al., 2000), and it was detected in the effluents of another Swiss university hospital (Bonnefin, 2011). Its main associated metabolite hydroxyl-metronidazole was also selected.

Among analgesics, paracetamol was selected due to its major consumption. Morphine and codeine were also selected because of their relatively important toxicity.

The antiepileptic drug gabapentin was selected because of its environmental persistence and its antecedent detection in the regional surface waters, whether the Lake of Geneva, the Arve River and/or the Rhone River (Götz et al., 2010a; Ortelli et al., 2010; Bonvin et al., 2011). Carbamazepine was also selected despite its low consumption, because, like diclofenac and sulfamethoxazole, it as has been proposed as a representative API to follow in Swiss surface water (Götz et al., 2010b).

4. Conclusions

The OPBT prioritization of the hundred most consumed API in the studied hospital allows identifying several NSAID and antiviral drugs as the most priority compounds. Predicted concentrations in the hospital effluents were in the ng-μg/L range in accordance with previous studies dealing with hospital wastewaters. Their calculation allowed performing an environmental risk assessment (ERA) by determining risk quotients dividing predicted environmental concentrations (PEC) by predicted no effects concentrations (PNEC), which were available for 71 API in the literature. According to this ERA, antibiotic drugs represented the most problematic class of medicines for the aquatic ecosystems, but only the hospital fraction of ciprofloxacin was predicted to pose a risk for aquatic organisms. This study shows that results can differ markedly according to the prioritization method, as well as when applying a weighting on the quality of the data. The comparison of the two methods (OPBT and ERA) revealed, however, that 45% of the compounds were among the 20 top-priority of both methods, independently of the weighting method. Based on these prioritization lists, about 15 API and human metabolites were chosen to be monitored in hospital wastewaters. Appropriate analytical tools can now be developed to allow a confrontation of the predicted concentrations with real measurements in hospital wastewaters. The approach adopted in this study can be easily transposed to any other hospitals, which have the will to look at the contamination of their effluents by API.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2015.06.037.

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Article XI

« Dynymics of active pharmaceutical ingredients loads in a Swiss university hospital wastewaters and prediction of the related environmental risk for the aquatic ecosystems.»

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Dynamics of active pharmaceutical ingredients loads in a Swiss university hospital wastewaters and prediction of the related environmental risk for the aquatic ecosystems

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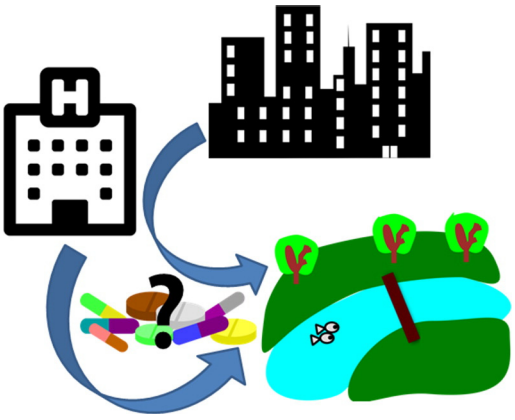
HIGHLIGHTS

- Pharmaceutical residues were studied in Geneva hospital wastewaters, Switzerland.
- Measured concentrations were compared to predictions based on consumption data.
- Risks for the aquatic ecosystems were assessed for hospital and urban contributions.
- The hospital fraction of ciprofloxacin and sulfamethoxazole threatened aquatic life.
- Urban consumption was likely more problematic with 7 risky compounds out of 14.

GRAPHICAL ABSTRACT



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ABSTRACT

The wastewater contamination of a Swiss university hospital by active pharmaceutical ingredient (API) residues was evaluated with a three months monitoring campaign at the outlet of the main building. Flow-proportional samples were collected with an automatic refrigerated sampler and analyzed for 15 API, including antibiotics, analgesics, antiepileptic and anti-inflammatory drugs, by using a validated LC–MS/MS method. The metals Gd and Pt were also analyzed using ICP-MS. Measured concentrations were compared to the predicted ones calculated after the drug average consumption data obtained from the hospital pharmacy. The hospital contribution to the total urban load was calculated according to the consumption data obtained from city pharmacies. Lastly, the environmental hazard and risk quotients (RQ) related to the hospital fraction and the total urban consumption were calculated. Median concentrations of the 15 selected compounds were ranging from 0.04 to 675 μg/L, with a mean detection frequency of 84%. The ratio between predicted and measured environmental concentrations (PEC/MEC) has shown a good accuracy for 5 out of 15 compounds, revealing over- and under-

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PEC
Risk assessment

estimations of the PEC model. Mean daily loads were ranging between 0.01 and 14.2 g/d, with the exception of paracetamol (109.7 g/d). The hospital contribution to the total urban loads varied from 2.1 to 100% according to the compound. While taking into account dilution and removal efficiencies in wastewater treatment plant, only the hospital fraction of the antibiotics ciprofloxacin and sulfamethoxazole showed, respectively, a high (RQ > 1) and moderate (RQ > 0.1) risk for the aquatic ecosystems. Nevertheless, when considering the total urban consumption, 7 compounds showed potential deleterious effects on aquatic organisms (RQ > 1): gabapentin, sulfamethoxazole, ciprofloxacin, piperacillin, ibuprofen, diclofenac and mefenamic acid. In order to reduce inputs of API residues originating from hospitals various solutions can be envisioned. With results of the present study, hospital managers can start handling this important issue.

1. Introduction

Active pharmaceutical ingredients (API) are continuously released into the aquatic environment through urban wastewater treatment plants (WWTP) (Götz et al., 2010; Kümmerer, 2010; Michael et al., 2013). A non-negligible proportion of this point source pollution comes from hospitals and health care facilities, which differentiate itself from domestic ones by the nature of administrated molecules (Kümmerer, 2001; Mullot, 2009), as well as a higher proportion of antibiotic-resistant bacteria (Czekalski et al., 2012; Lyko et al., 2012). In general, the main pharmaceutical classes consumed in hospitals are X-ray contrast media, laxatives, analgesics and anti-inflammatory drugs, as well as antibiotics (McArdell et al., 2011; Le Corre et al., 2012). Other classes of pharmaceuticals mainly administrated at the hospital are of environmental concerns, such as cytostatic agents (Weissbrodt et al., 2009; Ferrando-Climent et al., 2014). On average, hospital contribution represents 10 to 25% of the urban API load found at the watershed outlet (Kümmerer, 2010; Le Corre et al., 2012). But, this fraction can vary from 3 to 74% according to the compound and the hospital beds/inhabitants ratio of the watershed (Santos et al., 2013).

Once in the environment, API residues can cause some adverse effects in wildlife (Fent et al., 2006; Santos et al., 2010). For example, the non-steroidal anti-inflammatory drug (NSAID) diclofenac has been shown to alter the kidney functions in fishes and birds (Oaks et al., 2004; Hoeger et al., 2005). Therefore, environmental risk assessment (ERA) of API is recommended at the pre-commercial stage already (EMEA, 2006). In ERA approach, risk quotients (RQ) are calculated according to the ratio between organisms exposure, by means of predicted or measured environmental concentrations (PEC or MEC), and water quality criteria or potential hazards, usually their predicted no effect concentrations (PNEC) (European Commision, 2003; Cooper et al., 2008). These latter are derived from dose–response curves obtained with ecotoxicological tests, usually performed on the three trophic levels of aquatic ecosystems (algae, daphnia, fish). PNEC values found in the literature are scarce, often modeled, and can vary as much as three orders of magnitude between studies according to test conditions and endpoints (Santos et al., 2010; Helwig et al., 2013).

In Switzerland, several studies have been recently conducted to evaluate the environmental risk (Escher et al., 2011), and the possible treatment options regarding hospital wastewaters (Kovalova et al., 2012, 2013). Concerning API environmental risk assessment, 30–40 API contributed to the mixture risk quotient (Escher et al., 2011). However, this was done based on predicted concentrations, and many uncertainties remained about ecotoxicological data for several compounds. Concerning treatment options, there is still an active debate whether or not a dedicated treatment of the hospital effluents is necessary, and if so which kind of treatment (Verlicchi et al., 2015). In the end, possible options are site-specific and should be evaluated case-by-case (Kümmerer, 2010).

In this context, the present study aimed at evaluating the wastewater contamination of a university hospital in western Switzerland by API residues, as well as the risk for the aquatic ecosystems. A monitoring campaign of 3 months of the main building's sewer was implemented, and the comparison of measured concentrations with predictions based on the pharmaceutical consumption data was performed. Daily loads were calculated based on flow measurements, and the hospital contribution to the total urban load was estimated according to pharmacy sales data. Lastly, the environmental risk of API residues in hospital effluents was assessed based on measurements and PNEC values found in the literature. This work intended to help the hospital managers to find a solution to reduce the inputs of pharmaceutical residues into the urban network and, subsequently, their potential deleterious effects for the aquatic biota.

2. Material and methods

2.1. Study site

The Geneva University Hospitals (HUG) are among the most important hospitals of Switzerland. They comprise eight hospitals and about 40 other health care facilities, providing both primary and tertiary care. In 2014, 9068 full-time equivalent collaborators and a total of 656,598 days of hospitalization were registered, for 1781 beds, and over 960,000 outpatient consultations. Mean daily water consumption was almost 800 m^3/d in 2014, which gives 450 L/bed × day. The effluents of the HUG main site (1200 beds) as well as those of other health care facilities (approx. 300 beds) are ending in the Geneva main WWTP (Fig. 1). This latter can treat wastewaters from 600,000 population equivalent, and discharge up to 2.5 m^3/s wastewaters in the Rhône River. Treatments consist of classic stages of screening, grit and grease removal and primary settling, biological treatment and clarification. Thus, the bed density of the studied catchment is about 3.75 beds/1000 inhabitants.

2.2. Sampling

In most of the studies dealing with APIs in wastewaters, 24 h time-proportional composite samples are reported, where a flow-proportional sampling is desirable, especially in the case of hospital effluents (Weissbrodt et al., 2009; Ort et al., 2010). Thus, the sewer pipe of the HUG main building (741 beds) was equipped with a sharp-crested rectangular weir for the flow rate determination. An ultrasonic flow meter device (ISCO 4210) was installed upstream of this latter, and the wastewater height measurements was done every 2 min and checked for accuracy at least every 2 weeks. The flow rate was then calculated according to the Kindsvater–Carter equation (Kindsvater and Carter, 1959):

$$Q = C_e \frac{2}{3}\sqrt{2g}\, b_e\, (h_e)^{3/2} \quad (1)$$

where Q = discharge [m^3/s], C_e = discharge coefficient [$m^{1/2}$/s], g = acceleration of gravity [m/s^2], b_e = effective width [m], h_e = effective height [m].

The uncertainty with this kind of measurements was estimated at 5% by the metrology section of the Water Ecology Service of the Geneva State (SECOE-GE). It is worth to stress that rain water contributes to the waste water flow as roof gutters are not connected to separate

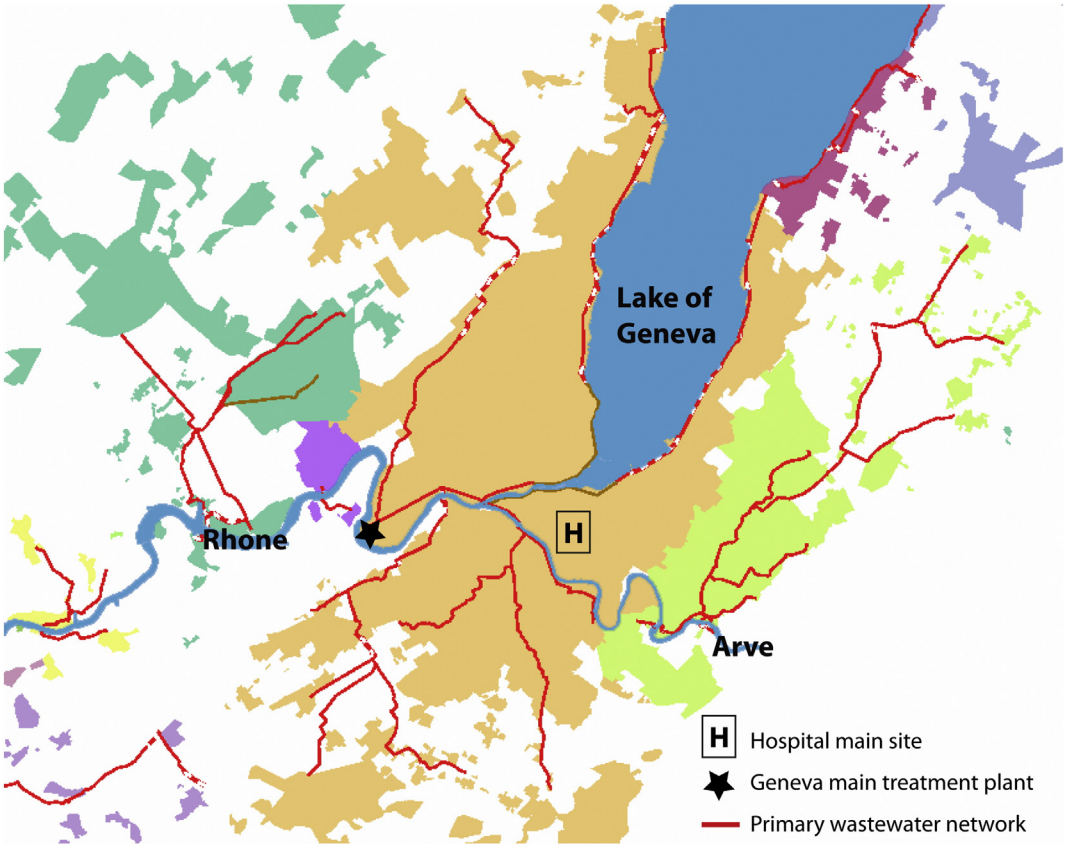

Fig. 1. Location of the studied hospital and of the primary urban wastewater network and the wastewater treatment plant watershed (Source: Geneva territory information service (SITG); http://ge.ch/sitg, accessed July 2015).

pipes. During the sampling period (October–December 2014), a total of 296 mm of cumulated precipitations were measured, resulting in a contribution of 1075 m^3 according to the roof surface. This latter contribution was equivalent to 6.3% of the measured total flow. The flow meter was coupled to a refrigerated automatic sampler (ISCO 6712, Avalanche) in order to collect flow-proportional samples. A total of 140 composite samples were obtained from October to December 2014 by mixing 8 samples of 50 mL after every 10 m^3 approximately (Table S1, Supplementary data). All samples were kept at 5 °C until their analysis.

2.3. Analytical methods

2.3.1. General parameters

General hospital wastewater characterization was performed on 24 h time-proportional composite samples, representative of 10 days of sampling with a 3 h frequency. Conductivity and pH measurements were performed using a multi-probes system (SevenMulti™, Mettler Toledo). Dissolved organic carbon (DOC) was quantified after a combustion catalytic oxidation and a NDIR detection (TOC-V, Shimadzu). Chemical oxygen demand (COD) was determined by colorimetric detection using a spectrophotometer (photoLab® 6100, WTW). Total Nitrogen (N_{tot}) was determined after a combustion catalytic oxidation and by electrochemical detection (Multi N/C® 3100, Analytik Jena), and total Phosphorous (P_{tot}) by chemical oxidation and colorimetric detection (Ganimede P®, Hach-Lange).

2.3.2. LC–MS/MS

An analytical method using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was previously developed and validated for the quantification of selected pharmaceuticals and metabolites (Daouk et al., 2015b). The analyzed compounds are listed in Table 1, along with their therapeutic classes and their annual consumption in the HUG and in the whole Geneva canton. Their selection was made according to a previous prioritization work based on these consumption data (Daouk et al., 2015a). Briefly, 50 mL of hospital wastewater samples are filtered (0.45 μm) and diluted twice with

Table 1
Analyzed compounds with their different therapeutic classes, and the hospital and urban consumption data used in this study.

Compound	Therapeutic class	Hospital consumption 2012 [g]	Urban consumption 2008 [g]
Metronidazole	Antibiotics	25,429	38,676
Ciprofloxacin		15,841	150,391
Sulfamethoxazole		17,706	76,791
Piperacillin		162,185	460
Codeine	Analgesics	15,119	4619
Morphine		7884	12,913
Paracetamol		1,180,121	7,162,022
Gabapentin	Anti-epileptics	15,635	212,445
Carbamazepine		4067	102,042
Ibuprofen	Anti-inflammatories	177,954	1,455,116
Diclofenac		1333	112,653
Mefenamic acid		24,192	895,629
Carboplatine[a]	Anti-cancer drugs	297	1350
Oxaliplatine[a]		51	2300
Cisplatin[a]		60	0
Gd[b]	Contrast agent	17.2	0
Mefenamic acid-OH	Metabolites	–	–
Metronidazole-OH		–	–
Ibuprofen-OH		–	–

[a] Analyzed as platinum (Pt) by ICP-MS.
[b] Gadolinium (Gd) is representative of the sum of Gd contained in MRI contrast agents: gadopentetic acid, gadoteric acid, gadobutrol and gadoxetic acid.

ultrapure water acidified to pH 3, and internal standards were added. Then, samples are passed through Strata-X polymeric reversed phase cartridges (200 mg, 6 mL), previously conditioned with 5 mL of methanol (MeOH) and 5 mL of ultrapure water acidified to pH 3, and then rinsed with 3 mL of acidified water and air dried for 20 min. Analytes were eluted with 4 mL of MeOH and evaporate to dryness and retrieved in 1 mL of mobile phase, before being injected in the LC–MS/MS system. The method limits of quantification (LOQ) vary between 0.05 and 0.2 μg/L according to the compound. Precision of the method varied between 71% and 123% with an overall variability below 20%. Recoveries of hospital wastewaters spiked between 0.1 and 100 μg/L varied between 67% and 115%.

2.3.3. ICP-MS

Besides organic API, the metals gadolinium (Gd) and platinum (Pt) were analyzed using inductively coupled plasma mass spectrometry (ICP-MS 5500, Agilent technologies). Gd-based contrast agents are used in magnetic resonance imaging (MRI), while Pt was tested to be representative of the sum of the 3 Pt-based cytostatic drugs cisplatin, oxaliplatin and carboplatin.

The three platinum anticancer drugs were obtained from the production unit of the Geneva University Hospital (HUG) pharmacy. Briefly, five samples spiked with 0.05, 0.1, 0.5, 1 and 2 μg/L of each cytostatic were prepared in both ultrapure and hospital wastewater under controlled conditions, and total Pt theoretical concentration was calculated. They were then quantified using an external calibration and recoveries were satisfactory, as the difference between nominal and measured concentrations were not higher than 5.5% (Table S2, Supplementary data). For samples quantification, external calibration curves made of five standards (2, 5, 10, 20 and 50 μg/L) were used, as well as certified quality control (QC) samples of 0.05 and 0.1 μg/L. Method LOD was set equal to the lowest QC of 0.05 μg/L, for which a signal-to-noise ratio of 3 was observed as compared to blank samples.

2.4. Consumption data

Aggregated data of drugs dispensed in the different medical units of the main building in 2014 were obtained from the hospital pharmacy database using "Business Object®" software. These data correspond to the drugs ordered by the different medical units to the pharmacy to treat their patients, as well as returns (stock and delivery errors, leaving or deceased patients, etc.). Outpatient consultations wards were not considered. The data gives an approximation of the yearly inpatient consumption of API by transforming the overall unit doses in grams of active ingredient while considering their dosages (Jean et al., 2012). It is worth to stress that only drugs purchased as a total package was taken into account. Also, the stock of each medical unit was considered but the stock difference between years was neglected. All confidential health information was removed to create anonymous analytic datasets in conformity with Swiss data protection regulations.

Urban consumption data from the whole canton of Geneva were obtained from OFAC (Professional Cooperative of Swiss Pharmacist), and corresponded to the sales data of all city pharmacies for the year 2008. The data gives an approximation of the API consumption of the population by transforming the overall unit doses in grams of active ingredient considering their dosages (Table 1).

2.5. Predicted concentrations

The predicted environmental concentrations in the hospital wastewater (PEC_{HWW}) were then calculated by dividing the excreted mass, i.e. the consumed mass (M) multiplied by the excretion factor (F_{excr}), by the volume of wastewater (V) (European Commision, 2003; Mullot, 2009; Perazzolo et al., 2010; Escher et al., 2011):

$$PEC_{HWW} = \frac{M \times Fexcr}{V} \quad (2)$$

PEC_{HWW} were calculated according to the annual mean consumption and by considering that 100% of drug consumption occurs on site and that no wastes are produced. Moreover, the volume of wastewater (V) was assumed to be equal to the mean volume of consumed water in the main building: 303 m^3/d. It is worth to stress that excretion factors are the sum of excretion in urine and feces as unchanged drugs, but do not take into account metabolites.

2.6. Loads

Daily loads were estimated from the measured concentrations with a basic numeric integration (US EPA, 2003):

$$Load = k\sum c_i\, q_i\, t_i \quad (3)$$

where k is a unit conversion factor, c_i the concentration of the sample i, q_i the discharge at its sampling time (t_i), and $t_i = 0.5\,(t_{i+1} - t_{i-1})$. In order to assess the temporal variability of API inputs into urban sewage network, mean normalized mass flows were calculated during one week (27 Oct–01 Nov) by dividing the obtained load with the mean weekly load (Coutu et al., 2013b). The contribution of the studied hospital to the total urban load was then estimated according to Le Corre et al. (2012): the ratio between the API consumed in the whole hospital during the year 2012 and the sum of hospital and urban API consumption was calculated.

2.7. Environmental risk assessment

The environmental risk of pharmaceutical residues was assessed by calculating risk quotients (RQ) using the measured concentrations (MEC) and the predicted no effects concentrations (PNEC) values found in the literature (European Commision, 2003):

$$RQ = MEC/PNEC. \quad (4)$$

Though it is not realistic to calculate a risk for hospital effluents, because the exposure of living organisms is null in hospital sewers, risk quotients are useful tools to evaluate the potential risk of hospital effluents once they reach the aquatic environment. Thus, hazard quotients (HQ) were calculated for hospital wastewaters according to Eq. (4). $HQ \geq 1$ would only mean that the considered API contributes to the environmental hazard of the hospital effluent. Then RQ were calculated for surface waters, by taking into account the dilution of hospital effluents into the urban network and the receiving water body (Dil), as well as removal efficiencies in WWTP (R):

$$RQ = \frac{MEC \times Dil \times (1 - R)}{PNEC}. \quad (5)$$

In our case, the dilution factor for hospital wastewaters in the urban network was 285 (volume of urban wastewaters/volume of hospital water consumption) in 2014, and a factor of 10 was applied for its second dilution in receiving waters (EMEA, 2006). Thus, the value of Dil in Eq. (5) was 2850. Values of PNEC and R were taken from literature data (Table 2). Then, according to the framework developed by Al Aukidy et al. (2014), minimum and maximum RQ values were calculated, as well as first and third quartile and median RQ values. Finally, a $RQ \geq 1$ would mean that the considered API poses a high risk for the aquatic ecosystems, a RQ between 0.1 and 1 a medium risk, and a $RQ < 0.1$ a low risk (Al Aukidy et al., 2014). Moreover, the mixture toxicity (RQ_{mix}) was evaluated as the sum of individual RQ calculated for each sample according to the concentration addition model (Escher et al.,

Table 2
Excretion factors (F_{excr}), predicted no effects concentrations (PNEC) and WWTP removal efficiencies (R) values from the literature used in this study.

	F_{excr}[a]	PNEC [μg/L]	Reference	R	Reference
Metronidazole	0.2	2.5	Bonvin et al. (2011)	0.85	Verlicchi et al. (2012b)
Paracetamol	0.05	0.5	Perazzolo et al. (2010)	0.95	Verlicchi et al. (2012b)
Gabapentin	0.63	0.196	Orias and Perrodin (2013)	0.09	Margot et al. (2013)
Codeine	0.1	0.06	Orias and Perrodin (2013)	0.82	Verlicchi et al. (2012b)
Morphine	0.1	0.03	Perazzolo et al. (2010)	0.86	Postigo et al. (2008)
Sulfamethoxazole	0.3	0.027	Verlicchi et al. (2012b)	0.38	Margot et al. (2013)
Carbamazepine	0.03	2.5	Valcarcel et al. (2011)	0.076	Margot et al. (2013)
Ibuprofen-OH	0.26	0.1	Perazzolo et al. (2010)	0.2[b]	
Metronidazole-OH	0.75	2.5	Bonvin et al. (2011)	0.2[b]	–
Ciprofloxacin	0.76	0.005	Bonvin et al. (2011)	0.63	Margot et al. (2013)
Ibuprofen	0.15	0.1	Perazzolo et al. (2010))	0.75	Verlicchi et al. (2012b), Margot et al. (2013)
Piperacillin	0.7	0.06	Kümmerer and Henninger (2003)	0.2[b]	
Mefenamic acid	0.05	0.43	Verlicchi et al. (2012b)	0.33	Margot et al. (2013)
Diclofenac	0.15	0.02	Orias and Perrodin (2013)	0.09	Margot et al. (2013)
Mefenamic acid-OH	0.7	0.43	Verlicchi et al. (2012b)	0.2[b]	
Gd	1	56.9	González et al. (2015)	0.01	Telgmann et al. (2013)
Pt	0[c]	1.22	Orias and Perrodin (2013)	0.01	Goullé et al. (2011)

[a] Mean values of excretions factors (Fexcr) in urine and feces as unchanged drugs were found in databases (www.uptodate.com; www.compendium.ch).
[b] Arbitrary value (Daouk et al., 2015a).
[c] Excretion factors for Pt-based anticancer drugs were considered separately: carboplatin (0.7), oxaliplatin (0.5) and cisplatin (0.4).

2011). Thus, values of $RQ_{mix} \geq 1$ mean that the sum of API contained in the hospital effluent is likely to affect aquatic species.

3. Results and discussion

3.1. Effluent characterization

The effluent average flow at the sampling site of the studied hospital, i.e. at the outlet of its main building, was 2.5 L/s with a standard deviation of 1.25. Three maximum flow peaks were generally observed during a normal day: 5–6 L/s at 8 AM, and about 4 L/s at 3 and 8 PM (Fig. S1, Supplementary data). The same pattern was observed with a lower intensity during the week-ends. Minimum night flows between 11 PM and 5 AM were ranging between 1 and 2 L/s. The same wastewater flow dynamics was previously observed in other hospitals (Boillot et al., 2008; McArdell et al., 2011). Surprisingly, a large difference was observed between the measured mean daily flow (219 m^3/d) and water consumption (303 m^3/d) during the studied period (Apr–Dec 2014), with a recovery rate of 70% (Fig. S1, Supplementary data). This can be explained by the possible presence of a water transfer to another building and of water cooling systems. This is of particular importance considering the calculation of the predicted environmental concentrations (PEC), which is often based on water consumption data (McArdell et al., 2011; Le Corre et al., 2012; Santos et al., 2013).

The classical parameters measured in effluent samples are presented in Table 3. Results showed similar values than like in other hospitals (Verlicchi et al., 2010; McArdell et al., 2011; Verlicchi et al., 2015), and some parameters (conductivity, chemical oxygen demand) showed higher values than the ones generally found in urban wastewater samples (Verlicchi et al., 2010; Margot et al., 2013). A difference can be observed between week and week-end values, those latter being of lower magnitude (Fig. S2, Supplementary data).

Table 3
General characteristics of wastewater composite samples from the main building of Geneva university hospital (n = 10). DOC = dissolved organic carbon; COD = chemical oxygen demand; N_{tot} = total nitrogen; P_{tot} = total phosphorus; Std dev = standard deviation.

Parameter	Mean	Std dev
pH	8.22	0.32
Conductivity [μS/cm]	924	75
DOC [mg/L]	164.4	37.8
COD [mg/L]	692.5	218.6
N_{tot} [mg/L]	61.8	10.2
P_{tot} [mg/L]	12.4	5.1

3.2. API concentration dynamics

The studied API exhibited a high detection frequency (>90%), with the exception of metronidazole-OH (81%), diclofenac (76%), piperacillin (32%), ibuprofen-OH (36%) and mefenamic acid (27%) (Mean = 84%; Fig. 2). This confirms the usefulness of the previous selection work based on the consumption data from the hospital pharmacy (Daouk et al., 2015a).

On average, API measured concentrations (MEC) were in the same order of magnitude than those measured in previous studies dealing with hospital effluents (Verlicchi et al., 2010; Oliveira et al., 2015). The most concentrated API was the analgesic paracetamol with a median concentration of 675 μg/L (Fig. 2). This result is in good agreement with previous studies, even if its average concentration was higher than in wastewater of other hospitals (Perrodin et al., 2013; Santos et al., 2013; Mendoza et al., 2015). The NSAID ibuprofen and the antibiotic ciprofloxacin had median concentrations of about 60 μg/L, while for the antibiotic sulfamethoxazole, the analgesic morphine, as well as the anti-epileptic gabapentin and the rare earth gadolinium they were varying between 18 and 29 μg/L (Fig. 1). Then, some compounds had median concentrations at the μg/L level (1.4–6.5 μg/L; metronidazole, carbamazepine, mefenamic-OH, metronidazole-OH, codeine), while others were only detected at trace amounts (0.04–0.82 μg/L; piperacillin, diclofenac, mefenamic acid, Pt).

The concentrations dispersion was little for some compounds (paracetamol, morphine, ibuprofen, sulfamethoxazole, ciprofloxacin), and much higher for others (metronidazole, gabapentin, hydroxyl metabolites, Gd). This can be explained by the different consumption patterns of the compounds. Indeed, some antibiotics are consumed regularly such as ciprofloxacin, while consumption of others like metronidazole may vary from month to month (Coutu et al., 2013a).

According to the compound, significant differences in concentration levels were observed with hospitals from other countries. While widely consumed drugs such as paracetamol and ibuprofen are found at similar levels, more specific ones, such as the antibiotics sulfamethoxazole and metronidazole, were found at higher level than in American (Oliveira et al., 2015) or Portuguese (Santos et al., 2013) hospitals. This is likely explained by the differences in sampling period: October–December in our case, July–October for Oliveira et al. (2015) and February–May for Santos et al. (2013). However, many other parameters may explain differences between countries, such as hospital type and size, water

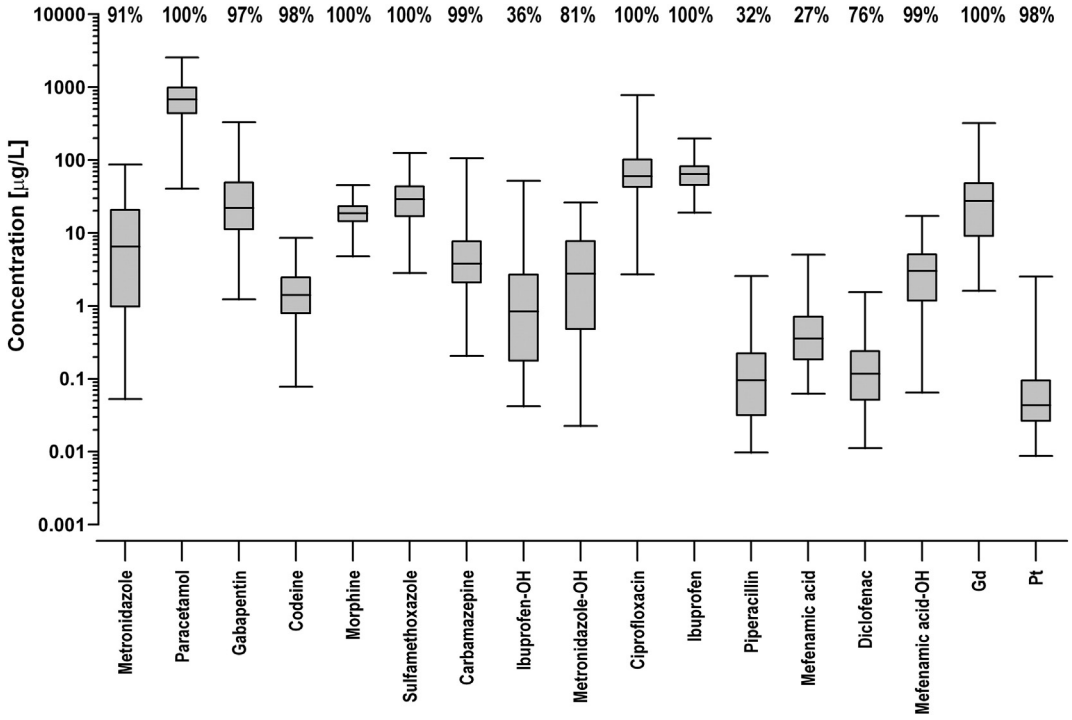

Fig. 2. Box-whisker plots of the measured concentration of API and the metals Pt and Gd in the effluent of the main building of the Geneva university hospitals (n = 140). The box represents the first and third quartile, the line the median value, and whiskers representing the resting 50% with minimum and maximum values. Note the logarithm scale. The detection frequencies expressed as percentage are shown at the top of the graph.

consumption and differences in API prescriptions (Le Corre et al., 2012; Mendoza et al., 2015). In general, the antiepileptic drug gabapentin was found at similar level than in other hospitals (Verlicchi et al., 2010), but at lower concentration range than in American hospitals (Oliveira et al., 2015). While Gd was found at similar concentrations than in other hospitals, Pt was found at much lower ones (Verlicchi et al., 2010).

The comparison of measured API concentrations (MEC) with predicted ones (PEC), through the PEC/MEC ratio (Verlicchi et al., 2014), showed a good accuracy (0.5 < PEC/MEC < 2) for 5 out of the 15 molecules (Fig. 3): ibuprofen, gabapentin, metronidazole, sulfamethoxazole and ciprofloxacin. PEC for piperacillin, Pt and hydroxyl metabolites were overestimated, what can be explained by the uncertainties of the PEC model which is based on average values of excretion factors and consumption data in the studied hospital building, and do not take into account degradation and/or adsorption phenomena. The β-lactam antibiotic piperacillin was indeed expected to be found at high concentrations (PEC_{HWW} = 628 µg/L) due to its high excretion factor (70%), but was only detected at trace level. Piperacillin was found to be unstable in urban wastewater kept at 4 °C (Rossmann et al., 2014), which can be explained by the possible hydrolysis of the β-lactam ring (Längin et al., 2009). Even though this is likely to happen relatively slowly, many uncertainties remain concerning favoring conditions for biotic and non-biotic hydrolysis of β-lactam antibiotics (Kümmerer, 2011). Hydroxyl-metabolites are likely to undergo further degradation processes (Celiz et al., 2009; Bonvin et al., 2012). A significant fraction of Pt-based anti-cancer drugs can be readily adsorbed on particulate matter (Lenz et al., 2005), and/or consumed and excreted at home (Weissbrodt et al., 2009).

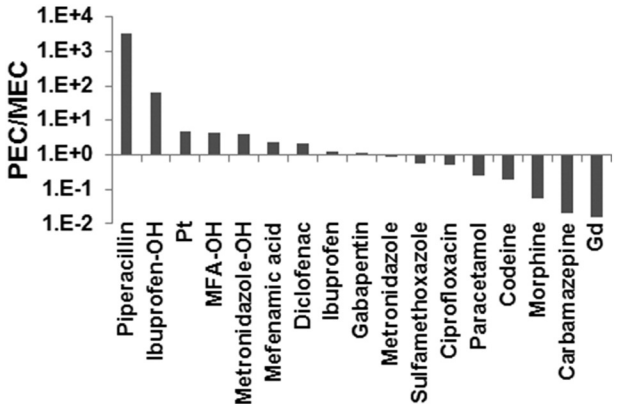

Fig. 3. Ratio between predicted and measured API concentrations (PEC/MEC) in the effluent of the main building of the Geneva university hospitals. Note the logarithm scale.

PEC for paracetamol, morphine, codeine, carbamazepine and Gd were on the contrary underestimated. A fraction of the consumption of drugs for chronic diseases or pain killing, such as carbamazepine or paracetamol respectively, can be brought by patients and some collaborators from home. Indeed, concerning carbamazepine for example, hospital contribution to the total WWTP influent was found very limited, between 0.2 and 1.8% (Le Corre et al., 2012). Another explanation can be that glucuronide conjugates can be transformed back into the parent compound by enzymatic activities in wastewaters, like for carbamazepine (Zhang et al., 2008). Concerning opiates, the illicit consumption of drugs of abuse by people coming to hospital can explained, for a little part, their PEC underestimation. Indeed, biological transformations are likely to occur, such as heroine being transformed into morphine (Evgenidou et al., 2015).

3.3. Loads and dynamics

Mean daily loads, calculated according to Equation 3, were ranging mainly between 0.1 and 14.2 g/d (Fig. S3, Supplementary data), with the exception of paracetamol (143), piperacillin (0.08), diclofenac (0.04) and Pt (0.01). Mean normalized mass flows were calculated to assess the temporal variability of API residues during one week (Coutu et al., 2013b). Main results are summarized in Fig. 4. The daily load was remaining in the range of 50–150% of the mean for compounds which are widely consumed and on a regularly basis, such as the pain killers paracetamol, morphine and ibuprofen. Drugs consumed at a

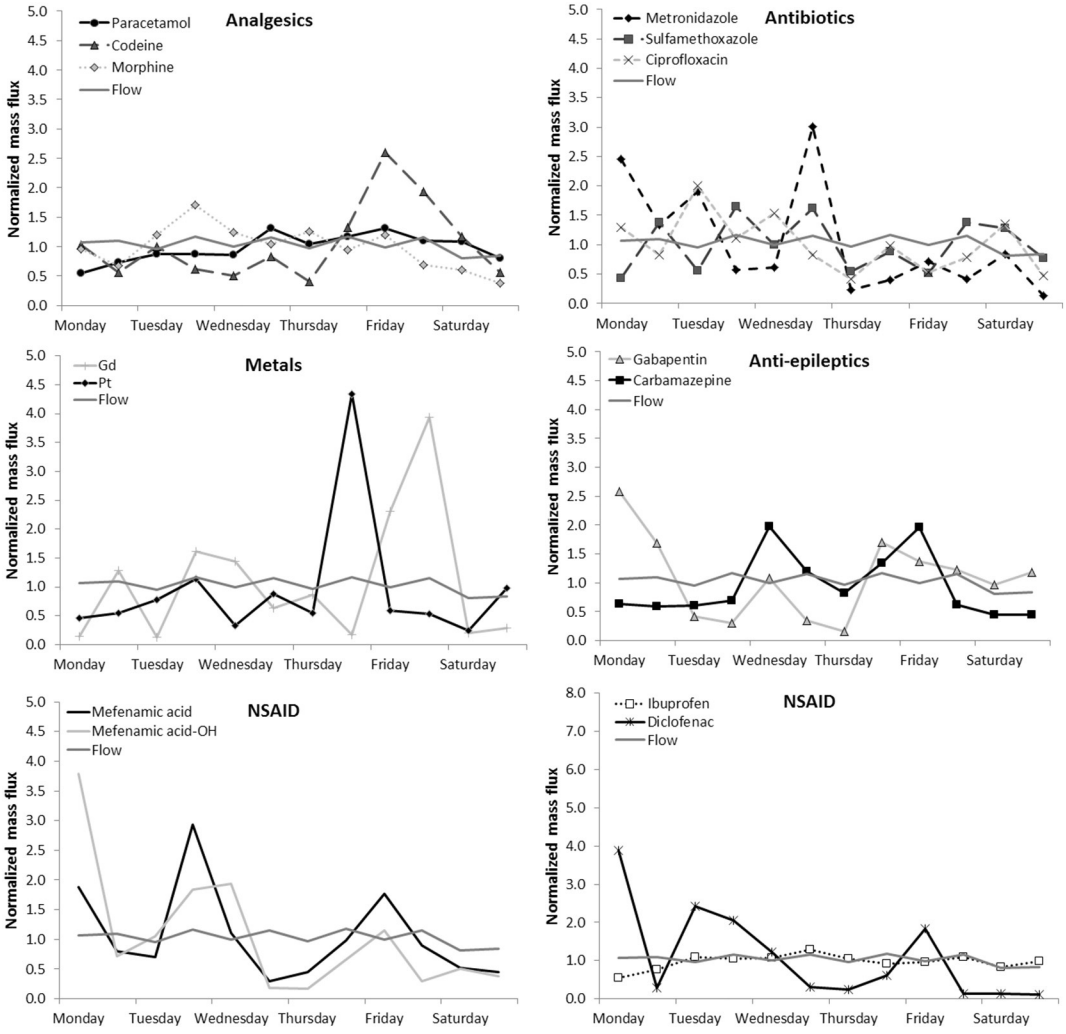

Fig. 4. Dynamics of mass flows during a week at the outlet of the sewer of the main building for selected compounds. API mass flows from 12 h-flow proportional samples are normalized by the mean value of the week.

lower extent, such as the NSAID diclofenac, mefenamic acid or the anti-epileptics gabapentin and carbamazepine, showed on contrary a higher variability, up to 400% the mean value. Concerning antibiotics, a higher variability was observed for metronidazole than for sulfamethoxazole and ciprofloxacin, which has been similarly observed in WWTP influent by Coutu et al. (2013b). The metals Gd and Pt showed an important deviation from the mean at the end of the week, suggesting daily variations in consumption of Pt-based chemotherapeutic and Gd-based MRI contrast agents. This has been previously observed for both metals in hospital and urban wastewaters in Germany (Kümmerer et al., 1999; Knappe et al., 2005). Metabolites are likely to have the same temporal dynamics than the parent-compound, as showed for the hydroxyl-metabolite of mefenamic acid (Fig. 4).

In order to compare daily loads with results from other hospitals, their normalization by the number of beds is recommended (Mendoza et al., 2015). On average, API loads in the sewer of the main building of the studied university hospital were ranging between 0.02 (Pt) and 186 mg/bed day (paracetamol) (Table S3, Supplementary data). Mean daily loads calculated according to measured concentrations are in good agreement with results of a previous study in another Swiss hospital (McArdell et al., 2011). However, they were in general higher than in Spanish and Italian hospitals, with the exception of codeine, mefenamic acid and diclofenac (Table S3, Supplementary data).

These comparisons should be taken with precaution because the methodology differs. Indeed, in the case of Mendoza et al. (2015) and Verlicchi et al. (2012a), the mean concentrations were multiplied by

Table 4
Fraction of the studied API consumed at the Geneva University Hospitals as compared to the urban consumption in Geneva, expressed as percentage, as well as the predicted concentration (PEC_{SW}) and risk quotient (RQ_{SW}) in surface water for the whole Geneva canton.

API	Hospital fraction [%]	PEC_{SW} [μg/L]	RQ_{SW}
Metronidazole	39.67	0.06	0.02
Paracetamol	14.15	0.31	0.63
Gabapentin	6.86	0.26	1.32
Codeine	76.60	0.004	0.07
Morphine	37.91	0.002	0.07
Sulfamethoxazole	18.74	0.07	2.71
Carbamazepine	3.83	0.12	0.05
Ciprofloxacin	9.53	0.08	15.38
Ibuprofen	10.90	0.51	5.10
Piperacillin	99.72	0.20	3.39
Mefenamic acid	2.63	0.77	1.79
Diclofenac	1.17	0.09	4.27
Gadopentetic acid	100	<0.001[a]	<0.001[a]
Carboplatin	18.03	0.001[a]	<0.001[a]
Oxaliplatin	2.17		
Cisplatin	100		

[a] Gadopentetic acid is representative of Gd-based MRI contrast agents, and carboplatin, oxaliplatin and cisplatin are Pt-based anticancer drugs. PEC_{SW} and RQ_{SW} are based on Gd and Pt measurements.

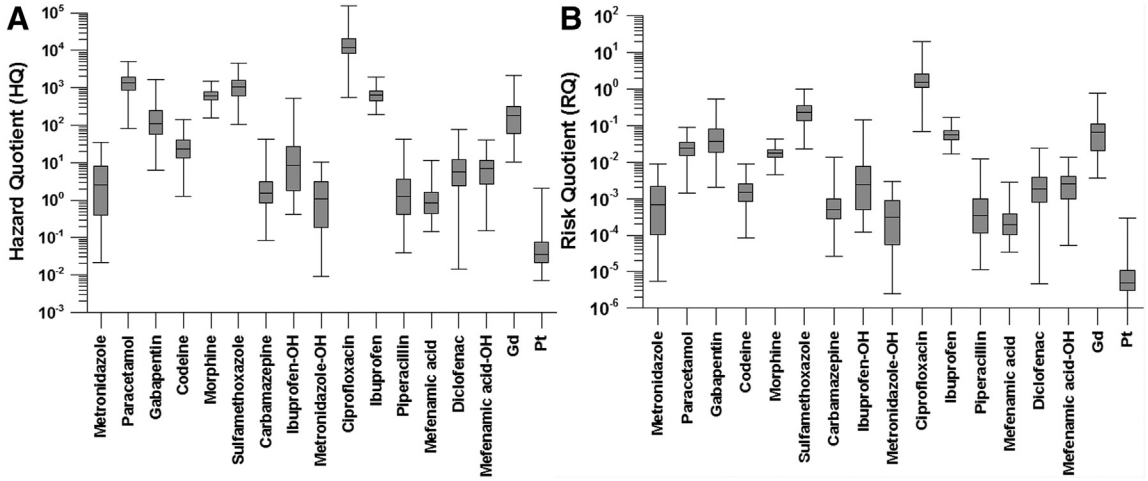

Fig. 5. Box-whisker plots of A) Hazard Quotients (HQ) and B) Risk Quotients (RQ) calculated after the API concentrations measured in the sewer of the hospital main building and the predicted no effect (PNEC) values found in the literature. Note the logarithm scale.

the consumed water per day and bed, whereas in the present study loads were obtained by multiplying concentrations by the measured flow rate for each sample (Eq. (3)), and the mean was calculated. As observed in our case, a significant difference was observed between the measured mean flow and the water consumption in the main building of the studied hospital (see Fig. S2, Supplementary data).

3.4. Contribution to the urban load

According to the consumption data obtained for the whole Geneva canton, the hospital contributions of the studied API were ranging between 1.2% and 76.6% of total urban load, with the exception of piperacillin (99.7%), cisplatin (100%) and gadopentetic acid (100%), which are only consumed at the hospital (Table 4). The mean contribution was 29%, which is higher than in other context (Le Corre et al., 2012). Some compounds were mainly consumed in households (mefenamic acid, diclofenac, carbamazepine, etc.), while the hospital contribution of some analgesics (codeine and morphine) and antibiotics (metronidazole, sulfamethoxazole) was relatively important.

3.5. Environmental risk assessment

According to the hazard evaluation of the measured API concentrations in the hospital wastewater, all API participate to the environmental hazard (HQ > 1), except the metal Pt (Fig. 5A). However, after dilution in both urban network and the receiving water body, and removal in classical activated sludge WWTP, only the antibiotics ciprofloxacin and sulfamethoxazole showed, respectively, a high (RQ > 1) and moderate (1 > RQ > 0.1) risk for the aquatic ecosystems (Fig. 5B). This result is in good agreement with previous studies which also identified these two compounds as hazardous for aquatic species (Santos et al., 2013). Indeed, it has been shown that these two compounds can inhibit photosynthesis and disturb carbon assimilation in aquatic algae (Liu et al., 2011; Johansson et al., 2014). In 95% of samples the API mixture toxicity (RQ_{mix}) was above 1, and thus the other API measured in hospital effluents are also participating to the potential deleterious effects for aquatic species (Escher et al., 2011). Furthermore, many other compounds were not measured but may also participate to the effluent mixture toxicity, such as other API (Escher et al., 2011), detergents and disinfectants (Kümmerer et al., 1998; Boillot et al., 2008), and contrast agents (Weissbrodt et al., 2009; Mendoza et al., 2015).

These considerations should however be taken with precaution as high uncertainties remain concerning PNEC and R values, as well as applied dilution factors (Daouk et al., 2015a). The risk posed by mixture toxicity can also be over-estimated due the application of concentration addition model (Escher et al., 2011). Thus, an environmental risk assessment based on real measurements in the aquatic system would give more reliable answers. Furthermore, it is worth to stress that calculated RQ are representative of the API hospital fraction, to which should be added the urban contribution in order to get a realistic picture. Thus, when considering the total consumption for the whole Geneva canton, 7 compounds showed potential deleterious effects on aquatic organisms (RQ > 1): gabapentin, sulfamethoxazole, ciprofloxacin, piperacillin, ibuprofen, diclofenac and mefenamic acid (Table 4).

4. Conclusion

This work provides some new insights on pharmaceutical residues dynamics in hospital effluents in Western Switzerland, as well as on their contribution to aquatic pollution. It allowed to estimate the contamination of the effluents of the Geneva university hospitals by newly detected API residues, such as piperacillin or hydroxyl metabolites, and to calculate loads emitted daily into the Geneva wastewater network. The comparison of the measured concentrations with consumption data allowed discussing prediction models used within the framework of the environmental risk assessment. The entire monitored API contributed to the environmental pollution and hazard in a significant way, but more specifically the two antibiotics ciprofloxacin and sulfamethoxazole represented a potential risk to aquatic biota. The mixture toxicity of API contained in the studied effluent was preoccupant but should be assessed in more details. With the results of the present study, the hospital managers possess decision-making tools to reduce the environmental pollution by API of downstream Rivers. Various solutions can be envisioned, such as implementation of on-site advanced treatments, but the choice will have to be made in dialog with the other stakeholders in order to coordinate the efforts supplied within the same territory.

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Chapitre 6 : Conclusions et perspectives

Conclusions et perspectives

Malgré leur ancienneté et leur grande utilisation, les agents anticancéreux conventionnels sont des molécules suscitant un faible intérêt pour la communauté scientifique. Leur haute toxicité (instabilité chimique) n'est pas étrangère à cet état de fait. Qu'importe le domaine concerné, l'utilisation de méthodes analytiques est incontournable pour pouvoir répondre aux différentes interrogations soulevées par les agents anticancéreux (contrôle qualité, stabilité, exposition, toxicité…). Or le développement de méthodes pour analyser ces « molécules à part » est un véritable challenge aussi bien d'un point de vue analytique (instabilité des molécules et similarité structurelle pour des agents d'une même famille) mais aussi d'un point de vue de la sécurité des opérateurs et de l'environnement. L'avènement de la LC-MS, caractérisée par une haute sélectivité et sensibilité, a bouleversé la situation en devenant une technique de choix pour l'analyse des agents anticancéreux. En effet, la LC-MS permet d'analyser la plupart des agents anticancéreux conventionnels sans recourir à des étapes longues et laborieuses de dérivatisations souvent indispensables pour réaliser une séparation par GC ou une détection par UV, et par conséquent, réduisant ainsi la manipulation de ces agents toxiques. Toutefois, malgré la grande popularité et la vulgarisation dont elle a jouit ces dernières années, la LC-MS demeure encore aujourd'hui une technique s'adressant à des laboratoires avertis et peine encore à franchir les portes des laboratoires des pharmacies hospitalières. En outre, l'utilisation d'un tel outil doit se faire dans des conditions garantissant la protection de l'opérateur et de l'environnement comme pour tout autre équipement en contact avec des agents anticancéreux. Au Laboratoire de Contrôle Qualité de la Pharmacie des HUG, la mise en place d'une structure séparée et adaptée à la manipulation des agents anticancéreux comprenant une CE-UV et une LC-UV-MS a permis l'accomplissement d'une grande partie des travaux présentés.

Au cours de ce travail, différentes méthodes analytiques ont été développées pour la détermination de plusieurs agents anticancéreux dans l'environnement et dans les formulations pharmaceutiques. Leurs applications à la pharmacie hospitalière, et plus particulièrement à la Pharmacie des HUG, ont, certes permis de répondre à certaines questions (stabilité, contrôle qualité) mais en ont soulevé d'autres. En effet, la

détection d'agents anticancéreux sur des surfaces de travail, des équipements/matériels manipulés et dans les effluents suscite un bon nombre de discussions quant aux précautions utilisées lors de la manipulation, la conservation et la destruction de ces molécules. Le premier exemple, illustré par le projet mené sur la détermination des niveaux de contamination des sites de préparation hospitaliers suisses, a démontré la faisabilité et l'intérêt de disposer d'une méthode LC-MS générique pour l'analyse d'agents anticancéreux. La mise au point d'une méthode plus rapide permettant l'analyse simultanée d'un plus grand nombre d'agents anticancéreux, reflétant au mieux la réalité, se profile comme la seconde étape de ce travail. Son application périodique non seulement aux sites de préparation des chimiothérapies mais aussi en l'élargissant aux unités de soins oncologiques constitue une perspective intéressante dans la mise en place de suivis du taux de contamination et des actions qui pourraient en découler (implémentation de nouvelles procédures de travail, de décontamination, sensibilisation des opérateurs…) visant à garantir la sécurité des manipulateurs et de l'environnement vis-à-vis des agents anticancéreux. Cette méthode générique LC-MS se révèle également un outil particulièrement intéressant pour l'analyse des effluents et des eaux de surface dans le but de discuter des performances des différents traitements appliqués aujourd'hui dans les stations d'épuration. Ceci n'est rendu possible qu'en agrémentant la méthode analytique d'éventuels métabolites ou produits de dégradation des agents anticancéreux recherchés. Ces différentes démarches s'inscrivent alors dans la volonté politique menée ces dernières années en Suisse et, plus largement, en Europe par les autorités sanitaires afin de protéger l'homme (médecine du travail) et l'environnement (lutte contre les micropolluants). Toutefois, il est important de signaler que si des réflexions entamées par les professionnels dans le but de sécuriser le cycle de vie de ces agents anticancéreux constituent la conséquence positive de ces mesures analytiques, le manque de données sur les conséquences d'une exposition chronique demeure. En effet, aujourd'hui, aucune limite d'exposition aux agents anticancéreux n'est clairement établie malgré leur haute toxicité. L'alimentation des données renseignant les taux de contaminations, accompagnée d'études toxicologiques plus systématiques et approfondies pourrait combler en partie ce vide réglementaire.

La volonté de poursuivre ce travail par le développement de méthodes génériques permettant l'analyse simultanée et rapide de plusieurs agents anticancéreux ne doit pas se limiter à des considérations environnementales et d'exposition humaine. En effet, disposer de méthodes séparatives pour l'analyse d'agents anticancéreux au sein d'un laboratoire de contrôle qualité d'une pharmacie hospitalière, représente un atout indéniable. Ainsi, le contrôle qualité des produits finis et la mise en place d'études de stabilité deviennent possibles, constituant alors un gage de qualité et de sécurité supplémentaires dans le flux de production des chimiothérapies en milieu hospitalier. Le développement et la validation d'une méthode LC-MS indicatrice de stabilité pour le busulfan, effectué au cours de ce travail, ouvre la voie à d'autres agents anticancéreux caractérisés par une logistique délicate (multitude de dosages prescrits, spécialité coûteuse) pouvant être liée à une instabilité établie et/ou sujet à controverse. En outre, l'introduction du concept de « dose-banding » en chimiothérapie conduisant à la production en série de formulations pharmaceutiques d'agents anticancéreux renforce le besoin de disposer de méthodes analytiques applicables dans le cadre d'études de stabilité (définition de la durée de stabilité et de conservation) et de contrôle qualité du produit fini. Si la LC-MS se révèle un outil puissant dans les études de stabilité en raison de sa haute sélectivité, dans le contrôle qualité des formulations pharmaceutiques, elle ne constitue pas la panacée. En effet, sa haute sensibilité n'est pas adaptée à l'analyse de composés à hautes concentrations (ce qui est le cas pratiquement de tous les agents anticancéreux en chimiothérapies) ; ce qui implique de multiples dilutions de l'échantillon et donc des manipulations supplémentaires des agents anticancéreux. Une des perspectives de ce travail est de se retourner vers des techniques séparatives analytiques plus simples et adaptées pour le contrôle qualité des chimiothérapies comme la LC-UV ou la CE et de recourir à des automates de préparation d'échantillons limitant ainsi l'intervention humaine.

L'introduction de nouvelles substances anticancéreuses avec une action ciblée (sélective) sur les cellules cancéreuses semble non seulement prometteuse d'un point de vue thérapeutique (diminution des effets collatéraux) mais aussi d'un point de vue environnemental. Toutefois, l'analyse de ces molécules issues de la « thérapie ciblée » reste complexe, surtout dans le cas des anticorps monoclonaux

non pas d’un point de vue toxicité et réactivité (excepté les anticorps monoclonaux conjugués) mais d’un point de vue purement structurel.