Analyse de médicaments produits en milieu hospitalier : applications aux composés non-UV absorbants et cytotoxiques

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THÈSE

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par

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" Analyse de médicaments produits en milieu hospitalier : applications aux composés non-UV absorbants et cytotoxiques "

La Faculté des sciences, sur le préavis de Messieurs J.-L. VEUTHEY, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), P. BONNABRY, professeur associé et codirecteur de thèse (Section des sciences pharmaceutiques), Ph. HUBERT, professeur (Service de chimie analytique, Institut de pharmacie, Université de Liège, Belgique) et de Madame V. SAUTOU, docteure (Laboratoire de contrôle - développement, Service pharmacie, Centre Hospitalier Universitaire de Clermont-Ferrand, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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N.B.- La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

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Avant propos

L'analyse de médicaments produits en milieu hospitalier revêt un grand intérêt du point de vue qualité et sécurité des traitements médicamenteux non seulement pour le patient mais aussi pour les personnes manipulant ces médicaments. Au cours de cette thèse, différentes méthodes d'analyse ayant pour objectif la détermination de composés non-UV absorbants et de substances cytotoxiques contenus dans des formulations produites en milieu hospitalier ont été développées pour le contrôle qualité et le contrôle de l'environnement. Plusieurs techniques ont été étudiées:

- l'électrophorèse capillaire (CE) couplée à des systèmes de détection de type UV/Vis ou conductimétrique sans contact avec le capillaire (C^4D) a permis de déterminer plusieurs substances actives contenues dans des formulations produites à l'hôpital;
- la chromatographie liquide couplée à la spectrométrie de masse (LC-MS) a, quant à elle, été employée pour déterminer la contamination par des cytotoxiques sur différentes surfaces de locaux de production de pharmacies hospitalières.

Le travail a été effectué au sein du laboratoire de contrôle qualité (LCQ) de la pharmacie des Hôpitaux Universitaire de Genève (HUG) sous la supervision du Docteur Sandrine Fleury-Souverain et la direction du Professeur Jean-Luc Veuthey et du Professeur Pascal Bonnabry. Il s'agit de la première thèse effectuée au sein du LCQ. Entre autres, la thèse présentée a permis le développement de différentes applications dont une en particulier, à savoir l'analyse par CE-C^4D du suxaméthonium contenu dans des seringues prêtes à l'emploi. La méthode a contribué à une étude de stabilité figurant dans le travail de thèse du Docteur Cyril Stucki effectué au sein de la pharmacie des HUG sous la direction du Professeur Pascal Bonnabry (''Contribution à la sécurisation du processus de préparation des médicaments en anesthésiologie'', thèse de l'Université de Genève, n° 4220). Pour les aspects fondamentaux de l'analyse, ce travail repose sur des thèses effectuées sous la direction du Professeur Jean-Luc Veuthey, notamment les travaux du Docteur Julie Schappler sur le couplage de la CE à différents systèmes de détection, dont la CCD ("Analyse de composés pharmaceutiques par électrophorèse capillaire couplée à des techniques de détection alternatives", thèse de l'Université de Genève n° 3937) et du Docteur Laurent Geiser sur les analyses rapides en CE ("Développement et validation de méthodes analytiques pour l'analyse de composés

pharmaceutiques par électrophorèse capillaire couplée à un spectrophotomètre UV ou à un spectromètre de masse", thèse de l'Université de Genève n° 3442). Pour la chromatographie liquide couplée à la spectrométrie de masse, la thèse se base sur les travaux du Docteur Sandrine Souverain (''Extraction en ligne sur support solide pour l'analyse de composés pharmaceutiques contenus dans des matrices biologiques par chromatographie liquide – spectrométrie de masse'', Université de Genève, Thèse n° 3520) et du Docteur Didier Ortelli (''Utilisation de la chromatographie liquide couplée la spectrométrie de masse pour l'analyse de médicaments dans les fluides biologiques'', Université de Genève, Thèse n°3296).

Structure de la thèse:

La thèse présentée se divise en quatre parties. La première (''Chapitre 1'') est constituée d'une bibliographie résumant l'importance de l'analyse des médicaments produits en milieu hospitalier, en discutant plus particulièrement les aspects cliniques et analytiques des substances étudiées (composés cytotoxiques et non-UV absorbants). Les techniques analytiques utilisées (CZE-C^4D, MEKC-UV, MEEKC-UV, HPLC-MS/MS) et la validation sont également présentées. La deuxième partie (''Chapitre 2'') reprend les principaux résultats et expériences effectuées au cours de cette thèse ainsi que les résumés en français des articles publiés. Le ''Chapitre 3'' regroupe les différents articles rédigés en anglais durant ce travail de thèse, qui sont déjà publiés, acceptés pour publication ou soumis à publication. Pour terminer, le ''Chapitre 4'' discute des perspectives et conclusions de ce travail de thèse.

Les principaux sujets ainsi que les articles illustrant ce travail sont reportés ci-dessous :

<u>Contrôle qualité de médicaments produits en milieu hospitalier</u>

Tout d'abord, les intérêts de la CE dans le contrôle qualité en milieu hospitalier sont présentés (**article II**), suivi de plusieurs applications. Le contrôle qualité de préparations considérées à haut risque est illustré avec l'analyse des nutritions parentérales (**article III**). Pour déterminer les électrolytes contenus dans ces formulations (Na^+, K^+, Ca^{2+}, Mg^{2+}), une méthode CE-C^4D a été développée et validée (**article IV**). Pour le contrôle qualité d'une production de série, une autre méthode CE-C^4D permettant de quantifier le suxaméthonium dans une formulation injectable a été mise au point (**article V**). Cette méthode est utilisée non seulement dans le cadre d'un test de stabilité pour développer une nouvelle formulation prête à l'emploi (CIVAS)

mais aussi pour le contrôle qualité de la production de cette formulation en routine (**article VI**). Finalement le contrôle qualité de préparations à base de composés cytotoxiques est illustré à l'aide de l'analyse des complexes de platine par MEKC-UV et MEEKC-UV (**article VII**) et par l'analyse de 10 autres composés cytotoxiques par LC-MS/MS (**article VIII**).

Toxicologie : analyse de cytotoxiques sur des surfaces

L'analyse des composés anticancéreux introduite au ''Chapitre 1'', est très importante pour le contrôle qualité de formulations produites en milieu hospitalier, et pour le contrôle environnemental (**article I**). Pour ce dernier point, une méthode LC-MS/MS comprenant une étape de prélèvement de surface a été développée et validée (**articles VIII** et **IX**) pour la détermination de 10 substances cytotoxiques. Finalement, cette méthode a été appliquée avec succès pour mesurer la contamination de surface au sein de l'unité cytotoxique de la pharmacie des HUG (**article IX**).

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.

Publications

I. **Analysis of anticancer drugs: a review**
Nussbaumer S, Fleury-Souverain S, Bonnabry P, Veuthey JL**.**
Talanta 85 (2011) 2265-2289

II. **Interest of capillary electrophoresis for the quality control of pharmaceutical formulations produced in hospital pharmacy**
Fleury-Souverain S, Nussbaumer S, Schappler J, Bonnabry P, Veuthey JL.
European Journal of Hospital Pharmacy Practice 17 (2011) 32-34

III. **Compounding of parenteral nutrition: usefulness of quality control methods**
Fleury-Souverain S, Nussbaumer S, Bouchoud L, Sadeghipour, F, Bonnabry P.
Hospital Pharmacy Europe 53 (2010) 52-54

IV. **Determination of potassium, sodium, calcium and magnesium in total parenteral nutrition formulations by capillary electrophoresis with contactless conductivity detection**
Nussbaumer S, Fleury-Souverain S, Bouchoud L, Rudaz S, Bonnabry P, Veuthey JL.
Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 130-136

V. **Determination of suxamethonium in a pharmaceutical formulation by capillary electrophoresis with contactless conductivity detection (CE-C^4D)**
Nussbaumer S, Fleury-Souverain S, Rudaz S, Bonnabry P, Veuthey JL.
Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 333-337

VI. **Development of ready-to-use succinylcholine syringes for safe use in general anesthesia**
Stucki C, Nussbaumer S, Fleury-Souverain S, Sautter AM, Sadeghipour F, Bonnabry P.
European Journal of Hospital Pharmacy Science (2010) In Press

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

Nussbaumer S, Fleury-Souverain S, Schappler J, Rudaz S, Veuthey JL, Bonnabry P.

Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 253-258

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

Nussbaumer S, Fleury-Souverain S, Antinori P, Sadeghipour F, Hochstrasser D F, Bonnabry P, Veuthey JL, Geiser L.

Analytical and Bioanalytical Chemistry 398 (2010) 3033-3042

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

Nussbaumer S, Geiser L , Sadeghipour F, Hochstrasser D F, Bonnabry P, Veuthey JL, Fleury-Souverain S.

Analytical and Bioanalytical Chemistry (2011) DOI 10.1007/s00216-011-5157-2

Présentations orales

- **Contamination des surfaces par des agents anticancéreux?**

  Nussbaumer S.

  Séminaire « La Chimie Analytique au service de la Santé » Maîtrise universitaire d'études avancées en pharmacie hospitalière, 26-28 mai 2011, Pharmacie HUG Genève

- **Etudes de stabilité des chimiothérapies**

  Nussbaumer S.

  Séminaire « La Chimie Analytique au service de la Santé » Maîtrise universitaire d'études avancées en pharmacie hospitalière, 26-28 mai 2011, Pharmacie HUG Genève

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

  Nussbaumer S, Fleury-Souverain S, Geiser L, Antinori P, Sadeghipour S, Bonnabry P, Veuthey JL.

  Drug Analysis, 21-24 septembre 2010, Anvers

- **Quality control of parenteral nutrition: analysis of inorganic ions by capillary electrophoresis**

  Fleury-Souverain S, Nussbaumer S, Bonnabry P, Rudaz S, Veuthey JL.

  EAHP, 24-26 mars 2010, Nice

- **Use of capillary electrophoresis for the quality control of pharmaceutical formulations produced in hospital pharmacy**

  Nussbaumer S, Fleury-Souverain S, Rudaz S, Bonnabry P, Veuthey JL.

  CEPharm, 12 octobre 2009, Boston

- **Production de médicaments en milieu hospitalier : quels tests effectués ?**

  Nussbaumer S.

  Séminaire « La Chimie Analytique au service de la Santé » Maîtrise universitaire d'études avancées en pharmacie hospitalière, 26-28 mai 2008, Pharmacie HUG Genève

Affiches

- **Quality control of parenteral nutrition: analysis of inorganic ions by capillary electrophoresis**

  Fleury-Souverain S, Nussbaumer S, Bonnabry P, Rudaz S, Veuthey JL.

  EAHP, 24-26 mars 2010, Nice

- **Analysis of cytostatic traces in chemotherapy production/use areas**

  Nussbaumer S, Fleury-Souverain S, Geiser L, Antinori P, Sadeghipour S, Bonnabry P, Veuthey JL.

  Journée d'inauguration SCAHT, 10 novembre 2009, Genève

- **Use of CE-C4D for quality control of pharmaceutical formulations produced in hospital pharmacy**

  Nussbaumer S, Fleury-Souverain S, Rudaz S, Bonnabry P, Veuthey JL.

  13th RDPA, 9-12 septembre 2009, Milan

- **Développement de préparations injectables prêtes à l'emploi (CIVAS) en ophtalmologie**

  Nussbaumer S, Dobrinas M, Fleury-Souverain S, Sadeghipour F, Bonnabry P.

  15èmes JFSPH, 2-3 avril 2009, Dijon

Abréviations

Les abréviations utilisées au cours de ce travail sont répertoriées ci-dessous. La plupart d'entre elles sont exprimées avec les acronymes anglais et traduites en français.

ACN	Acétonitrile
APCI	Ionisation chimique à pression atmosphérique
API	Ionisation à pression atmosphérique
APT	Alimentation parentérale totale
BPF	Bonne pratique de fabrication
BGE	Solution électrolytique
CCD	Conductimétrie sans contact
C^4D	« Capacitively coupled contactless conductivity detection »
CD	Conductimétrie
CE	Electrophorèse capillaire
CV	Coefficient de variation
CZE	Electrophorèse capillaire de zone
DAD	Diodes en réseau
EOF	Flux électro-osmotique
ESI	Ionisation par électrospray
FIA	« flow injection analysis»
5-FU	5-fluorouracile
FS	Silice fondue
FT-IR	Infrarouge par transformée de Fourier
HIBA	Acide hydroxyisobutyrique
His	Histidine
HUG	Hôpitaux Universitaire de Genève
GC	Chromatographie gazeuse
IARC	Centre international de recherche sur le cancer
ICH	Conférence internationale sur l'harmonisation des méthodes
ICP	Ionisation par plasma inductif
ID	Diamètre interne

LC	Chromatographie liquide
LCAP	Laboratoire de chimie analytique pharmaceutique
LCQ	Laboratoire contrôle qualité
LIF	Fluorescence induite par laser
LOD	Limite de détection
LOQ	Limite de quantification
m/z	Rapport masse-sur-charge
ME	Microémulsion
MEKC	Chromatographie électrocinétique micellaire
MEEKC	Chromatographie électrocinétique en microémulsion
MES	Acide 2-(N-morpholino)ethanesulfonique
MS	Spectrométrie de masse
MS/MS	Spectrométrie de masse en mode tandem
NMR	Résonance magnétique nucléaire
NP	Nutrition parentérale
PD	Pharmacodynamie
PK	Pharmacocinétique
PPI	Eau pour préparation injectable
RF	Radiofréquences
SDS	Sodium dodécyl sulfate
SFSTP	Société française des sciences et techniques pharmaceutiques
SI	Standard interne
SIM	« Selected ion monitoring »
SMC	Succinylmonocholine
SRM	« Selected reaction monitoring »
SUX	Suxaméthonium
TDM	Suivi thérapeutique (« Therapeutic drug monitoring »)
TRIS	Tris(hydroxymethyl)aminomethane
UV/Vis	Ultraviolet/Visible
µapp	Mobilité apparente
µeff	Mobilité électrophorétique
µeof	Mobilité du flux électro-osmotique

Chapitre 1 : Bibliographie

Préface: Intérêt et rôle du laboratoire de contrôle qualité de la pharmacie des HUG

Ce travail de thèse s'est déroulé au laboratoire de contrôle qualité (LCQ) de la pharmacie des HUG sur l'analyse de médicaments produits en milieu hospitalier. Pour saisir le contexte et l'objectif de cette thèse, il faut d'abord comprendre une des missions de la pharmacie hospitalière des HUG, à savoir la production de formulations pharmaceutiques. Ensuite, le rôle joué par le LCQ dans ce circuit des médicaments fabriqués en milieu hospitalier est exposé.

La plupart des médicaments utilisés en milieu hospitalier sont disponibles sur le marché. Mais pour répondre aux besoins cliniques d'une manière optimale ainsi que pour améliorer la sécurité des traitements médicamenteux, des préparations hospitalières sont mises à disposition par la pharmacie des HUG. Les raisons principales pour une production en milieu hospitalier sont les suivantes:

- le médicament n'est pas disponible sur le marché;
- le médicament est disponible, mais pas sous une forme adaptée;
    - dosage non adapté, p.ex. à la pédiatrie ou à la gériatrie;
    - risque d'erreur lors de la préparation (p.ex. nécessité de dilution, notamment en situation d'urgence);
    - risque de contamination microbienne lors de la préparation, notamment pour des voies d'administration à haut risque (intrathécal, intraophtalmique);
    - produit toxique, devant être manipulé dans une enceinte protégée (ex. cytostatiques).
- médicaments pour la recherche, dans le cadre d'essais cliniques.

Pour diminuer le risque d'erreur lié à la dilution ou à une mauvaise préparation en situation d'urgence, des médicaments prêts à l'emploi sont mis à disposition par la pharmacie hospitalière. Pour les voies d'administration critiques (injectables), une contamination microbienne peut être évitée en préparant le médicament en milieu aseptique à la pharmacie en respectant les bonnes pratiques de fabrication (BPF) [1]. En ce qui concerne la préparation des chimiothérapies aux HUG, cette dernière est centralisée à la pharmacie afin de réduire l'exposition du personnel soignant à ces substances, tout en confinant leur préparation dans

des enceintes protégées et en confiant leur manipulation à des opérateurs formés et expérimentés.

La fabrication des médicaments en pharmacie hospitalière est régie par les BPF de médicaments en petites quantités de la Pharmacopée helvétique [1]. Lorsque le produit présente un risque important, défini par une grille d'évaluation, ou que la production est prévue pour d'autres hôpitaux [2, 3], ce sont les BPF industrielles qui prennent le relais. Selon les BPF, toutes les formulations pharmaceutiques (injectables, solutions orales, suspensions, collyres, émulsions, suppositoires, capsules…) fabriquées en série, doivent être soumises à un contrôle qualité avant leur administration au patient. Pour les préparations sur prescription magistrale, c'est-à-dire fabriquées de manière individualisée, un contrôle libératoire n'est pas requis selon la loi.

Les activités du LCQ de la pharmacie des HUG sont les suivantes:

- l'analyse basée principalement sur la mise en évidence de l'identité des matières premières utilisées pour la fabrication des formulations hospitalières;
- l'analyse des produits finis à l'aide de tests physico-chimiques (comme le pH, l'osmolarité, le comptage de particules, l'identification des principes actifs et des excipients et la quantification du principe actif) et microbiologiques (test de stérilité et détermination des endotoxines);
- le contrôle des salles blanches utilisées pour la production des médicaments stériles pour vérifier la qualité de l'environnement de travail;
- la gestion des études de stabilité permettant la détermination des conditions de stockage et la durée de validité de nouvelles formulations développées;
- le développement de tests d'incompatibilités entre différents médicaments pouvant être demandés par certaines unités de soins, pour établir des recommandations d'utilisations de ces médicaments.

Ceci représente environ 23'000 analyses effectuées par année au LCQ. Tous ces contrôles ont pour objectif d'assurer la qualité des formulations produites par la pharmacie des HUG et de garantir la sécurité du traitement médicamenteux pour le patient. Plusieurs méthodes analytiques utilisant des techniques de séparation différentes et permettant le contrôle de

formulations produites par la pharmacie des HUG ont été développées dans le cadre de cette thèse. En outre, ces dernières années, le LCQ s'est engagé dans une politique de sécurisation des procédures de fabrication des chimiothérapies au sein de la pharmacie des HUG. Dans ce contexte, le travail de thèse présente également une méthode analytique validée pour définir l'exposition des opérateurs aux substances cytotoxiques présentes sur les surfaces de production des chimiothérapies. L'objectif final est de pouvoir appliquer des mesures visant la réduction d'une contamination éventuelle (p.ex. modification des procédures de décontaminations chimiques).

1. Médicaments produits en milieu hospitalier : aspects cliniques et analytiques

1.1 Introduction

Aujourd'hui, une grande variété de formulations pharmaceutiques est fabriquée en série ou sur prescription magistrale par la pharmacie des HUG, afin de fournir aux soignants le médicament prêt à l'emploi, autrement dit ne nécessitant aucune autre manipulation avant son administration. Les principales catégories de médicaments concernés sont les suivantes:

- Nutrition parentérale (NP);
- Chimiothérapies anticancéreuses injectables;
- Seringues ou poches de solutions de médicaments dilués à la concentration souhaitée, qualifiées de prêtes à l'emploi;
- Autres préparations stériles comme les solutions intrathécales ou ophtalmiques;
- Préparations non stériles (capsules, sirops, suspensions, solutions, gels, pommades…).

La Figure 1 illustre les différentes formulations produites à la pharmacie des HUG en 2010. Deux catégories de préparations peuvent être distinguées : les productions planifiables (en grande majorité il s'agit des productions de série) et les préparations non planifiables (les préparations magistrales).

Parmi ces préparations, ce travail s'est focalisé sur l'analyse des NP, des chimiothérapies et d'une seringue prête à l'emploi de suxaméthonium. Les composés analysés sont soit des substances non-UV absorbantes, soit des composés cytotoxiques. Les aspects cliniques et analytiques de ces médicaments sont discutés dans ce premier chapitre, et leur rôle aux HUG, sera reporté au 2ème chapitre, avec les expériences réalisées.

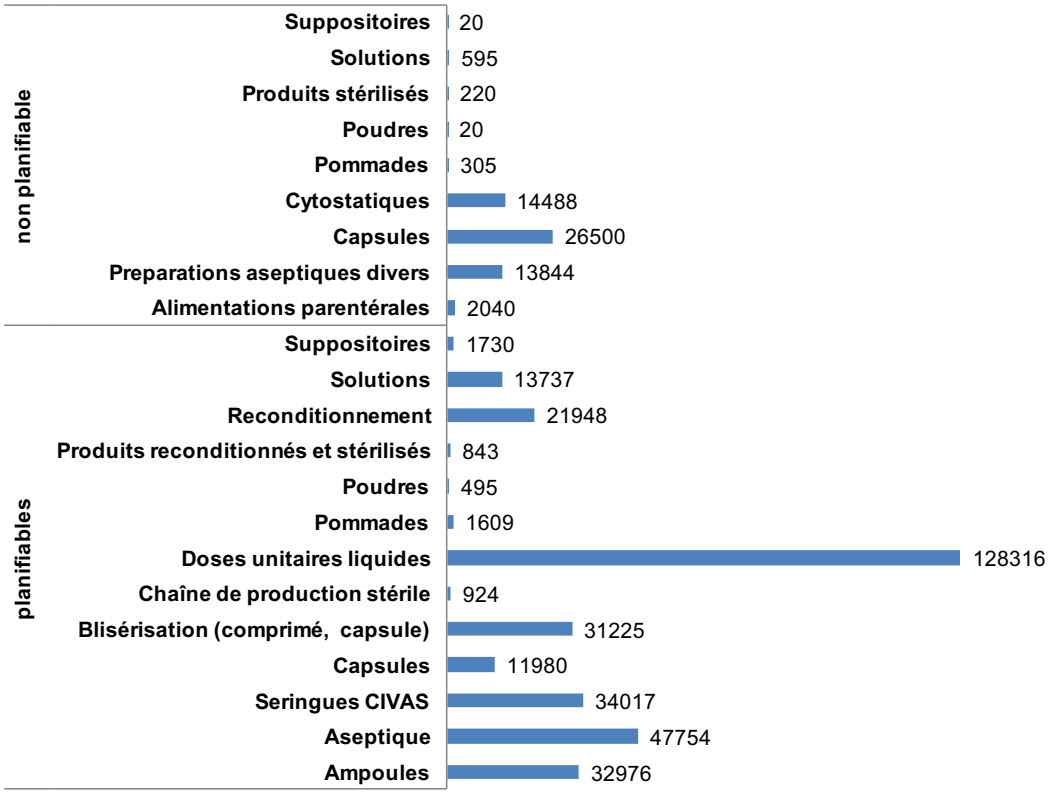

Figure 1: Formulations pharmaceutiques produites par la pharmacie des HUG en 2010 par nombre d'unités produites

1.2 Composés non-UV absorbants

Environ 80% des substances médicamenteuses possèdent un groupement chromophore et peuvent être détectées par spectrophotométrie ultraviolet/visible (UV/Vis). Pour les substances non-UV absorbantes, différentes stratégies peuvent être utilisées comme l'UV-indirect ou un autre système de détection adapté comme la spectrométrie de masse ou la conductimétrie. Pour pouvoir discriminer les principes actifs, des produits de dégradation ou des excipients présents dans une formulation, il est important d'utiliser des méthodes séparatives. Le temps de rétention (en LC) ou le temps de migration (en CE) peuvent ainsi donner des indications sur l'identité de la substance détectée et confirmer la présence d'autres composés présents dans l'échantillon. Dans ce travail de thèse, l'utilisation de la CE couplée à un conductimètre était l'approche de choix pour la détermination de composés non-UV absorbants dans différentes formulations.

1.2.1 Electrolytes: K, Na, Ca, Mg

Parmi les médicaments utilisés en milieu hospitalier, les solutions contenant des électrolytes, comme les solutions pour perfusion ou solutions nutritionnelles, sont essentielles pour l'amélioration de l'état clinique du patient. L'apport de liquide et d'électrolytes est nécessaire pour compenser les pertes via les urines, la peau et les selles. Le but de l'hydratation est de maintenir un volume et une tonicité des fluides corporels et les électrolytes sodium et potassium sont fortement liés au bilan hydrique. Le potassium est l'électrolyte le plus abondant dans le compartiment intracellulaire alors que le sodium est le principal ion positif du compartiment extracellulaire. Un bon équilibre entre les deux ions est important pour garantir une bonne répartition des volumes intra et extracellulaire. A part leur importance dans l'équilibre hydrique, ils sont importants pour la transmission de l'influx nerveux et la contraction musculaire. Le magnésium agit comme stabilisateur des membranes cellulaires et influe sur l'irritabilité neuromusculaire et sur le système cardio-vasculaire. Il intervient également dans la régulation de l'homéostasie du calcium. Un apport adapté en calcium et phosphore est primordial pour contribuer à une bonne minéralisation osseuse [4-7]. Le calcium est également nécessaire aux contractions musculaires, à la propagation de l'influx nerveux et à la coagulation du sang [8].

Les nutritions parentérales (NP) contiennent à part des électrolytes, du glucose, des acides aminés, des lipides, des vitamines et des oligoéléments, pour maintenir les fonctions des organes et la masse musculaire, traiter et prévenir une malnutrition et éviter les effets secondaires d'une dénutrition. La voie parentérale est indiquée si le tube digestif est non fonctionnel et quand aucune autre voie d'administration n'est possible. Pour les adultes, des solutions standardisées et fabriquées par l'industrie, sont en général utilisées. Pour les enfants et prématurés, une standardisation est plus difficile, car le besoin nutritionnel varie fortement entre chaque enfant. Lorsque l'usage d'une formulation standard n'est pas possible, la prescription se fait à la carte et les nutritions sont fabriquées tous les jours à la pharmacie de l'hôpital, soit en mélangeant manuellement les différents composés, soit en utilisant un système de mélange automatique, comme montré dans la Figure 2.

Figure 2 : Préparation d'une nutrition parentérale par un mélangeur automatique BAXA MM12 (pharmacie des HUG)

Il s'agit de formulations à haut risque, car des erreurs, avec des conséquences graves pour le patient, peuvent survenir pendant toutes les étapes du processus (prescription, fabrication et administration). Une contamination microbiologique peut avoir des conséquences mortelles pour le patient, surtout s'il s'agit d'un prématuré. Un tel incident a causé la mort de plusieurs enfants sous NP en Allemagne en août 2010, car la solution contenant les acides aminés était contaminée [9]. De même, les erreurs dans le dosage des électrolytes (surtout pour le potassium et sodium) ou des confusions de solutions concentrées, peuvent mettre la vie du patient en danger. L'utilisation d'un automate de remplissage permet d'améliorer la sécurité de préparation, mais des erreurs, par exemple des inversions de flacons, demeurent possibles [10]. Pour sécuriser ce processus, un contrôle ultime de ces préparations avant administration au patient peut être introduit. La situation et la mise en place d'un contrôle qualité des NP aux HUG sont décrites au chapitre 2.

La détermination des électrolytes (Na, K, Ca, Mg) dans les nutritions parentérales représente un défi, car la composition de ces solutions varie avec chaque prescription. Souvent la quantification des cations est faite par absorption atomique, comme par exemple dans les denrées alimentaires [11]. Mais dans le cas des NP, d'autres composés présents en grandes concentrations (acides aminés, glucose, vitamines) peuvent interférer avec l'analyse des ions et obstruer le système d'injection de l'instrumentation. Un lavage complet de cette partie du système est alors nécessaire après quelques injections, même si des formulations diluées sont analysées. En chimie clinique, la potentiométrie est fréquemment employée pour la

détermination des électrolytes dans les échantillons biologiques [12, 13]. La chromatographie ionique avec une détection par conductimétrie a aussi été reportée dans la littérature [14]. Plusieurs méthodes basées sur l'utilisation de la CE avec une détection UV indirecte ont été développées pour l'analyse des cations inorganiques en solution aqueuse [15-21] et pour déterminer les électrolytes (sodium, potassium, calcium et magnésium) dans des nutritions parentérales [22, 23]. En comparaison avec l'absorption atomique ou la chromatographie ionique [15, 23], ces méthodes possèdent des performances quantitatives plus faibles en termes de précision, mais peuvent être considérées comme des alternatives acceptables. La conductivité élevée des électrolytes permet aussi de coupler la CE avec une détection par conductimétrie [16-18, 24-37]. Dans la plupart des méthodes publiées, la solution tampon (''background electrolyte'' ou BGE) utilisée est constituée de 2-(N-morpholino)ethane-sulfonique acide et histidine (MES/His) pour séparer l'ammonium des métaux alcalins et alcalino-terreux [16-18, 24-33, 37]. Pour améliorer la séparation des différents cations ou changer la sélectivité, des agents complexant faibles peuvent être ajoutés dans le BGE, comme l'acide α-hydroxyisobutyrique (HIBA) [18, 21, 30] ou des solvants organiques [33, 38].

1.2.2 Ammoniums quaternaires: suxaméthonium

Un autre composé non UV-absorbant couramment rencontré en milieu hospitalier est le suxaméthonium (ou succinylcholine, SUX), faisant partie du groupe des curarisants. Les curarisants sont des agents pharmacologiques puissants, qui affectent les fonctions vitales et exigent pour leur utilisation clinique des précautions rigoureuses (comme un milieu logistique protégé, des connaissances théoriques et une grande expérience clinique). Utilisé depuis plus de 50 ans, le SUX est encore à ce jour un des seuls curarisants dépolarisants de brève durée d'action. Le mécanisme implique une dépolarisation persistante de la jonction neuromusculaire presque immédiatement après son injection (30-45 secondes). Il est indiqué comme traitement adjuvant en anesthésie générale pour faciliter l'intubation endotrachéale, et pour aider la relaxation des muscles squelettiques pendant la chirurgie. Le SUX peut provoquer une relaxation musculaire profonde, entraînant une dépression respiratoire jusqu'à l'apnée [39]. Imitant les effets de l'acétylcholine sur les récepteurs cholinergiques, des bradyarythmies sont couramment observées chez le patient. Malgré ces effets indésirables

fréquents, le SUX offre les avantages de procurer une myorelaxation d'installation rapide, profonde et de brève durée, donc contrôlable, et optimale pour une intubation [40].

Le SUX étant utilisé dans un contexte d'urgence, une mise à disposition immédiate du médicament est nécessaire. Par précaution, le médicament est préparé avant la plupart des anesthésies générales et il doit souvent être jeté, car non utilisé. Ceci génère des pertes inutiles en termes de temps et d'argent. En plus, cette molécule est souvent citée dans la littérature comme un des médicaments les plus impliqués dans les erreurs médicamenteuses en anesthésie [41], sujet étudié par Cyril Stucki, lors de sa thèse intitulée ''La sécurisation de processus de préparation des médicaments en anesthésiologie'' [42]. La mise à disposition d'une formulation prête à l'emploi aux HUG sera discutée au chapitre 2, partie 1.3.

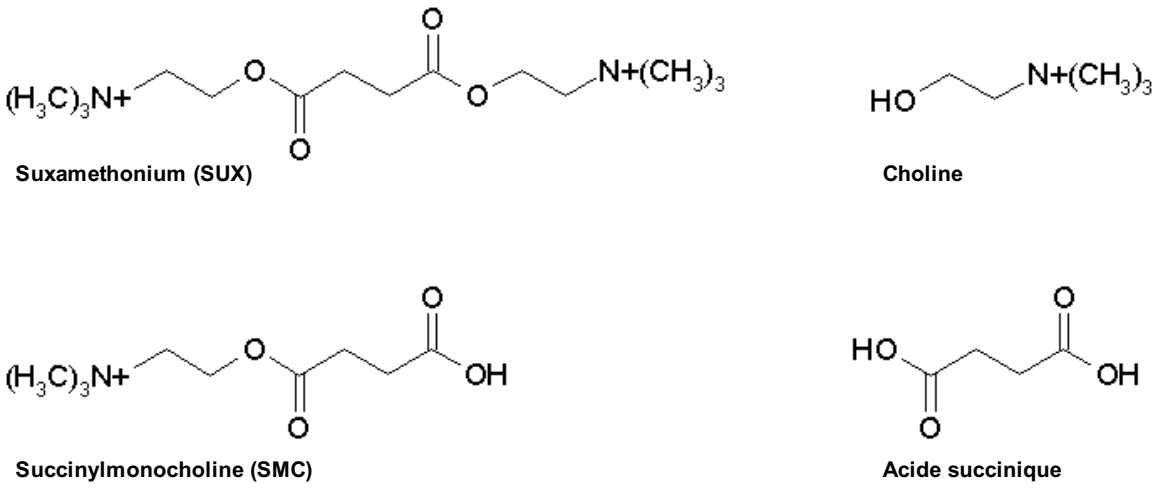

Figure 3: Structure du suxamethonium et de ses produits de dégradation

Le SUX possède deux groupements ammonium quaternaire qui contribuent à sa polarité élevée (Figure 3). En solution alcaline, il est rapidement hydrolysé pour former le succinylmonocholine (SMC) et la choline. Le SMC a tendance à se dégrader en choline et acide succinique [43]. Plusieurs méthodes analytiques ont été décrites pour la détermination du suxaméthonium [39, 43-47]. Vu qu'il ne possède pas de groupement chromophore, une détection par UV direct n'est pas possible. Des méthodes LC avec une détection électrochimique [39, 44] ont été publiées, mais la séparation du SUX et des produits de

dégradation a souvent été insuffisante, et des temps d'analyse de plus de 20 min ont été observés. Pour contrebalancer la faible résolution du SUX et de ses produits de dégradation avec une séparation chromatographique, des détecteurs plus sélectifs, comme la MS [43, 45], ou la résonance magnétique nucléaire (NMR) [48] peuvent être utilisés. La CE est une technique alternative pour analyser les petites molécules possédant un ammonium quaternaire [49-52]. Une première approche pour déterminer le SUX par CE a été réalisée en couplant la CE avec un spectrophotomètre infrarouge par transformée de Fourier (FT-IR) [47]. Finalement, le SUX possède une conductivité élevée à cause de la présence des groupements ammonium quaternaire qui permettent de le quantifier par CE couplée à une détection conductimétrique [53].

1.3 Substances cytotoxiques

L'intérêt de l'analyse de ces substances et les méthodes publiées pour la détermination de ces composés sont discutés dans **l'article I**, qui propose une large revue de la littérature sur le sujet. Ici, un résumé de cette revue est présenté en plus de quelques aspects cliniques.

Introduction

Le cancer décrit un ensemble de maladies causées par une croissance cellulaire anormale et non contrôlée (tumeur) qui peut se disséminer dans d'autres organes, formant ce qu'on appelle des métastases. Le cancer est une cause majeure de décès dans le monde, soit 7,6 millions de décès en 2008 (13% de la mortalité mondiale) [54]. Les traitements possibles reposent sur la chirurgie, la chimiothérapie et la radiothérapie [55]. La chimiothérapie classique utilise des substances de petits poids moléculaires pour détruire la tumeur ou réduire sa croissance (substances cytotoxiques). Ces médicaments n'agissent pas sélectivement sur les cellules cancéreuses et les cellules saines sont souvent atteintes, engendrant de nombreux effets secondaires (myélosuppression, nausée et vomissement, mucites, alopécie, néphrotoxicité, …) et le développement de résistances [56]. L'utilisation des composés cytotoxiques a débuté en 1940 avec des agents alkylants (les moutardes azotées) et les antimétabolites [55]. Depuis le succès de ces médicaments, de nombreuses substances ont été développées, et font partie des traitements standards pour combattre le cancer.

Les agents cytotoxiques peuvent être classés selon leur mode d'action [56]:

- Les antimétabolites inhibent la synthèse et la transcription de l'ADN en bloquant des enzymes essentielles pour la synthèse des nucléotides (ex. méthotrexate) ou en s'incorporant dans les acides nucléiques (ex. 5-fluorouracile) (Figure 4).

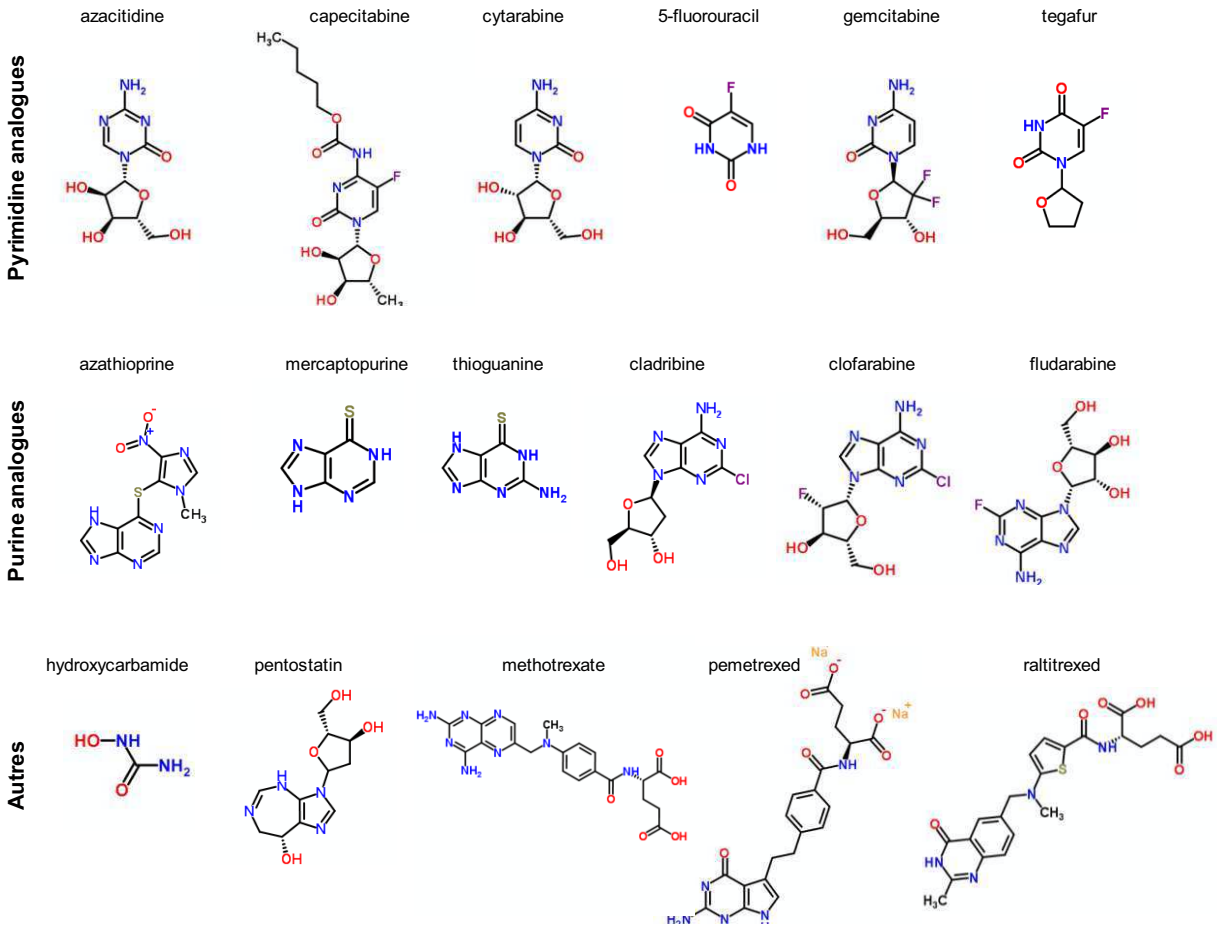

Figure 4: Structures chimiques des antimétabolites

- Les agents interagissant avec l'ADN possèdent différents mécanismes d'action (Figures 5-6):
  - Agents alkylants (ex. dacarbazine, temozolomide)
  - Agents se liant à l'ADN (''cross-linking agent'') (ex. complexes de platines, cyclophosphamide)
  - Agents intercalants : qui se lient entre des paires de bases (ex. anthracylines)
  - Inhibiteurs des topoisomérases, les enzymes qui contrôlent la superstructure de l'ADN (ex. irinotecan, étoposide)
  - Agents scindant l'ADN : se lie à l'ADN et libère des radicaux libres qui vont couper les brins d'ADN (ex. bléomycine)

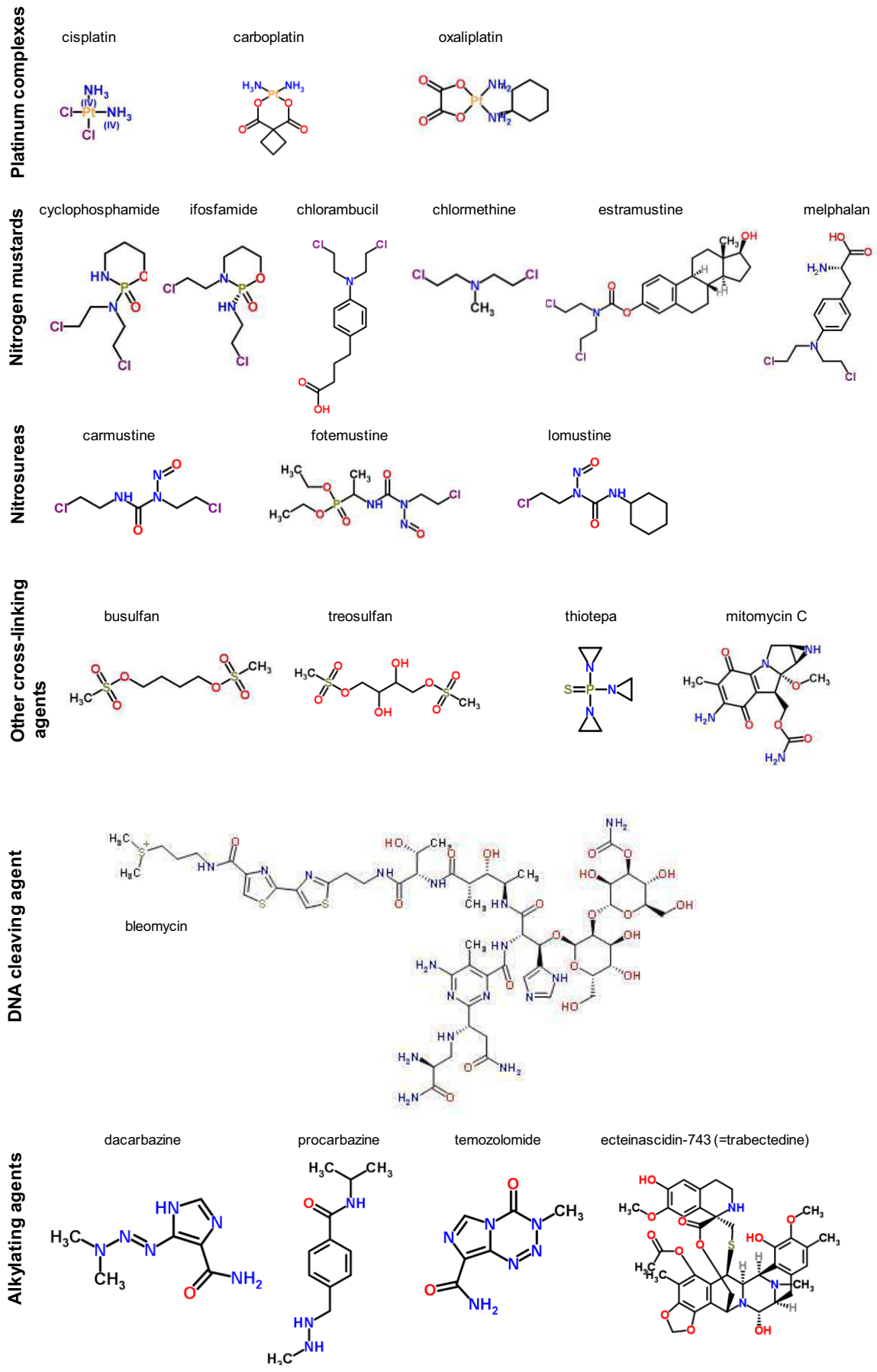

Figure 5 : Structures chimiques des agents alkylants, agents se liant entre les brins d'ADN et agent scindant l'ADN

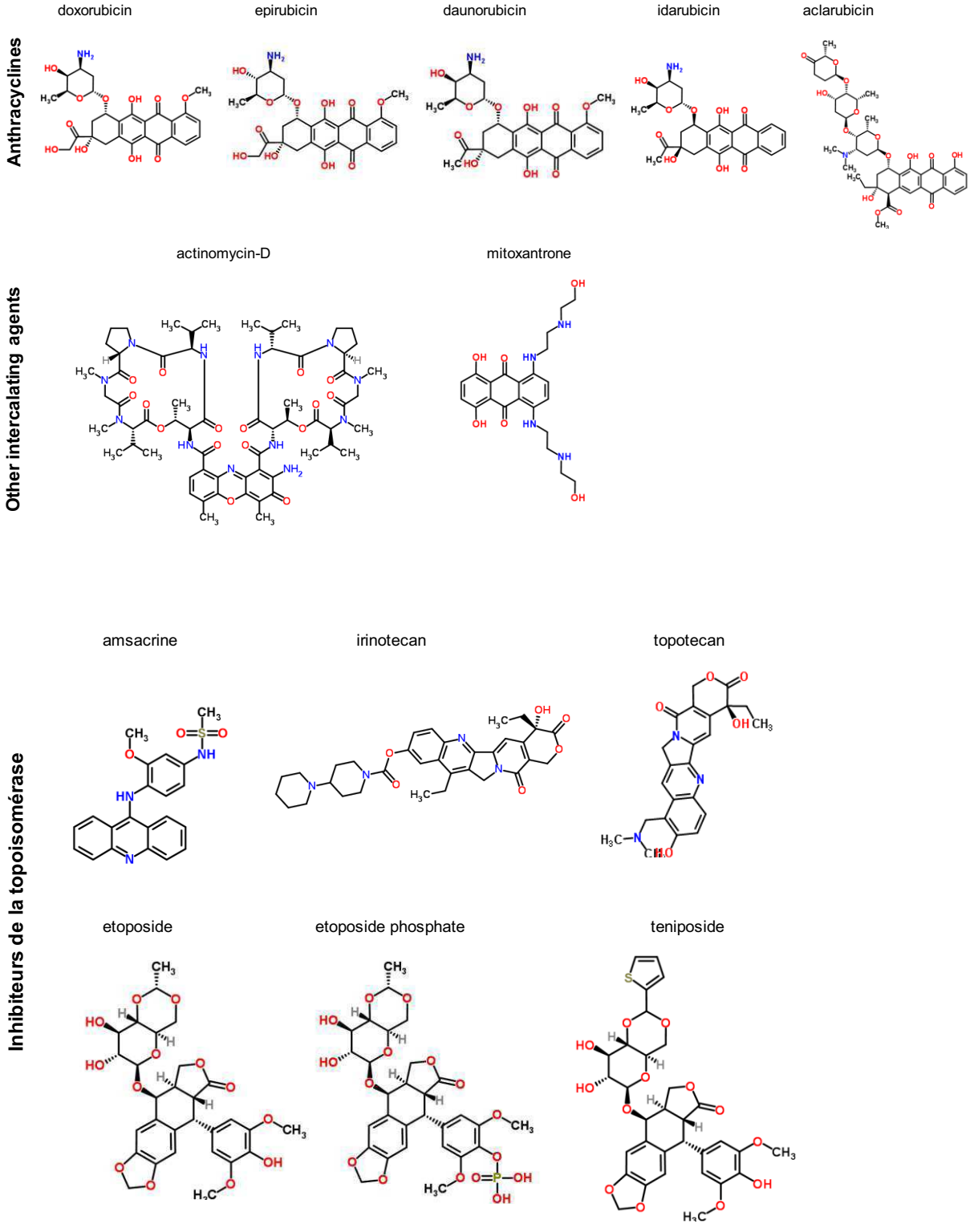

Figure 6 : Structures chimiques des agents intercalants et inhibiteurs de la toposisomérase

- Les agents de type antitubuline bloquent la polymérisation (vinca-alcaloïdes) ou la dépolymérisation (taxanes) de la tubuline (Figure 7). La tubuline polymérise pour former les microtubules, qui sont responsables du mouvement des chromosomes lors de la mitose.

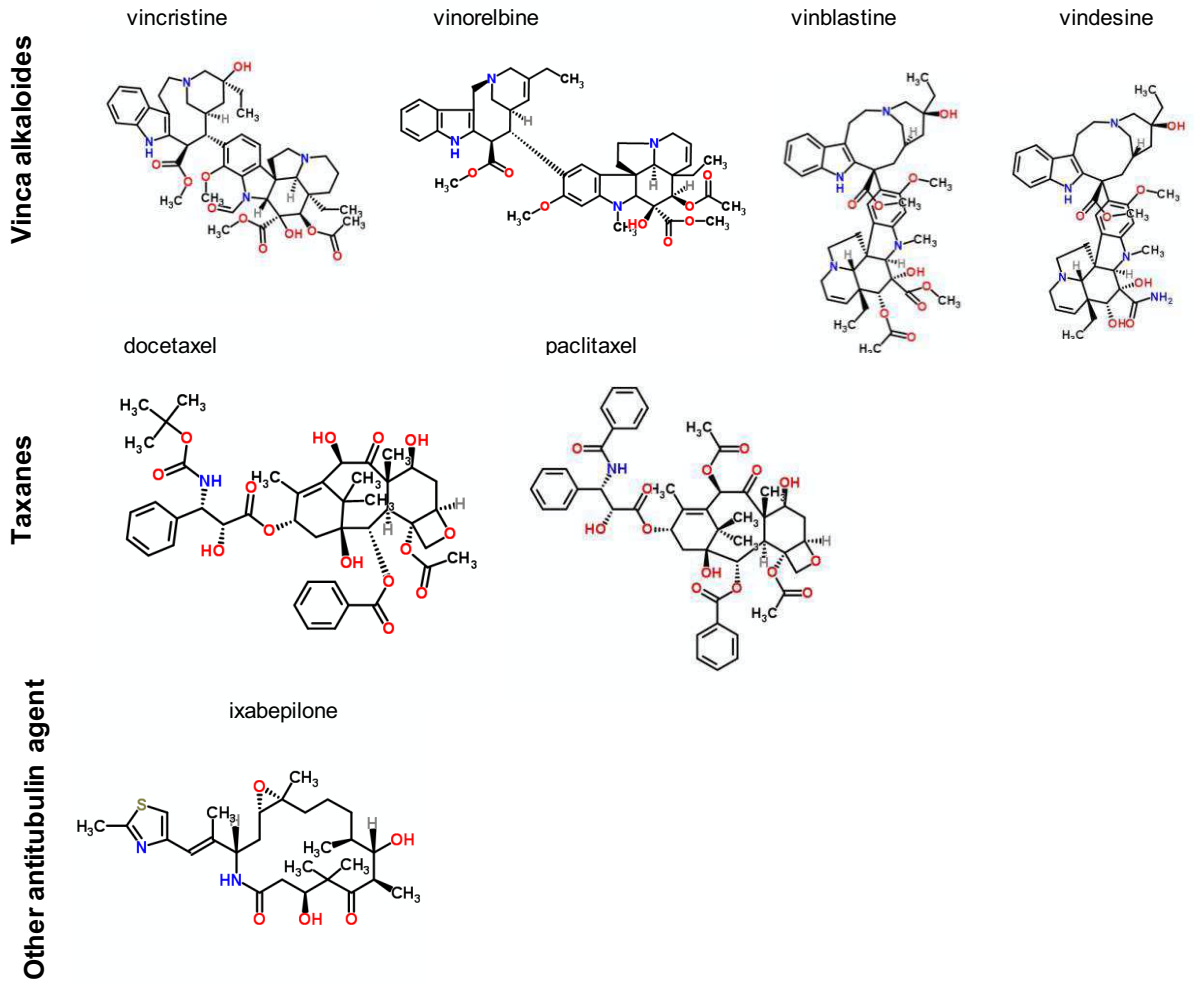

Figure 7: Structures chimiques des agents antitubulines

Les chimiothérapies classiques à base de substances cytotoxiques font toujours partie de la plupart des protocoles. Les nouveaux traitements avec un mode d'action plus ciblé au niveau moléculaire, les agents biologiques (anticorps monoclonaux) ou encore les vaccins sont fréquemment testés dans des études cliniques et s'instaurent comme médicaments adjuvants des chimiothérapies classiques.

Avec le vieillissement de la population, la susceptibilité de développer un cancer augmente, de sorte que l'incidence du cancer est en croissance. Les prestations demandées à une pharmacie d'hôpital vont donc fortement augmenter pendant les prochaines années [57]. Etant donné la forte toxicité des chimiothérapies pour l'être humain (pas seulement pour le patient, mais aussi pour les professionnels de la santé), le développement de méthodes analytiques pour déterminer ces substances est nécessaire dans plusieurs domaines. L'analyse de cytotoxiques contenus dans des formulations pharmaceutiques, des échantillons biologiques et environnementaux est de la plus haute importance et les différents types d'analyse sont présentés ci-dessous.

Analyse de cytotoxiques dans des formulations pharmaceutiques

Des méthodes analytiques sont nécessaires pour le contrôle qualité de matières premières et de formulations commercialisées (i), pour le contrôle qualité de formulations diluées avant administration au patient (ii), et pour des études de stabilité et d'incompatibilité (iii).

Contrôle qualité de matières premières et de formulations commercialisées :

Une méthode pour le contrôle qualité de matière première et de produits pharmaceutiques, doit être en mesure de déterminer simultanément la molécule mère, ses impuretés et produits de dégradation, afin de vérifier la qualité de la formulation pharmaceutique et de répondre aux normes en vigueur concernant les produits pharmaceutiques. Dans la plupart des cas, des techniques séparatives, comme la LC ou la CE sont utilisées, offrant une grande sélectivité. Parmi les systèmes de détection les plus couramment utilisés, la MS peut être considérée comme une technique de choix. Sa sélectivité et sa sensibilité permettent de détecter de très faibles concentrations d'impuretés ou de produits de dégradation. Par exemple, Jerremalm et coll. ont étudié la stabilité de l'oxaliplatine en présence de chlorure et ils ont identifié un nouveau produit de transformation (monochloro, monooxalato complexe) par LC-MS/MS [58]. Toutefois, c'est très souvent la spectrophotométrie UV qui est couplée à une technique de séparation. Dans ce cas, la sélectivité et la sensibilité de la méthode peuvent être suffisantes pour étudier la dégradation ou établir un profil d'impureté. Mallikarjuna et coll. ont développé une méthode LC-UV pour déterminer la stabilité du docétaxel dans des

formulations pharmaceutiques [59]. Un autre exemple est l'étude de stabilité chimique du teniposide [60] ou de l'étoposide [61] dans différentes formulations.

Contrôle qualité de chimiothérapies avant administration au patient :

Avant l'administration de la chimiothérapie au patient, les produits pharmaceutiques commercialisés sous forme de poudres lyophilisées ou de solutions à des concentrations élevées de cytotoxiques, sont dissous et/ou dilués avec du NaCl 0.9% ou du glucose à 5% pour obtenir la dose du médicament prescrite par le médecin à une concentration adéquate. La stabilité de ces formulations diluées est souvent limitée (ou inconnue) et la préparation doit être faite juste avant administration. Même si les réglementations pharmaceutiques ne requièrent pas de contrôle final pour les préparations individualisées, une analyse peut assurer la bonne concentration et l'identité du médicament. Ainsi les risques de morbidité et de mortalité pour le patient liés à des erreurs de préparation sont réduits [62]. Dans la plupart des cas, les méthodes développées pour cette application donnent des informations approximatives sur la concentration et l'identification de la substance cytotoxique contenues dans la formulation. Etant donné le nombre important de préparations cytotoxiques produites par jour et la forte contrainte de temps de quelques heures entre la prescription, la préparation et l'administration, des techniques analytiques simples et rapides sont nécessaires pour un contrôle qualité avant administration au patient.

Une première approche consiste à utiliser une analyse par « flow injection » (FIA) avec une détection UV-DAD. Selon Delmas et coll., 80% des préparations cytotoxiques (correspondant à 21 substances cytotoxiques) ont pu être déterminées en moins de 3,5 min dans une unité de préparation centralisée [62]. Compte tenu de l'absence de séparation avant la détection, la présence d'excipients dans la formulation peut interférer avec l'analyse FIA-UV/DAD et des composés de structure similaire ne peuvent pas être distingués.

Un contrôle des chimiothérapies a également été développé par couplage d'un spectromètre infrarouge à transformée de fourrier (FTIR) avec un spectrophotomètre UV/Vis [63, 64], ce qui a permis d'augmenter la sélectivité par rapport à la spectrophotométrie UV/Vis seule. L'identification et la détermination de la concentration du principe actif, ainsi que les excipients ont été réalisées dans un temps d'analyse court sans préparation d'échantillon. Comme pour le FIA-UV/DAD, des additifs dans les formulations ou des contaminations

croisées dans le système d'analyse peuvent être sources d'interférence. En plus, les données sur les performances quantitatives pour le contrôle qualité d'agents cytotoxiques sont très rares avec cette méthode et aucune validation n'a été publiée à notre connaissance.

Une autre alternative plus sélective serait la spectrométrie Raman. C'est une méthode non-invasive, non-destructive et rapide, permettant l'identification et la quantification de principes actifs et d'excipients dans des formulations pharmaceutiques [65, 66]. L'analyse est possible sans échantillonnage avec une excellente protection des techniciens. A l'instar des deux précédentes stratégies, aucune information sur les performances quantitatives n'a été publiée.

En conclusion, lors de la mise en place d'un contrôle qualité des chimiothérapies en routine quotidienne avant l'administration aux patients, des méthodes FIA-UV/DAD, FTIR-UV/DAD ou spectrométrie Raman constituent des approches intéressantes en termes de temps et de simplicité. Néanmoins, le manque de sélectivité et de données quantitatives sont les points faibles de ces techniques.

Etudes de stabilité et d'incompatibilité :

La compatibilité des médicaments cytotoxiques avec les matériaux des emballages est très importante pour éviter une adsorption ou dégradation des principes actifs, ce qui pourrait avoir des conséquences graves pour le patient [67]. Des méthodes analytiques permettant la séparation de la substance active et de ses produits de dégradation sont nécessaires pour réaliser des tests de stabilité et établir des recommandations concernant la conservation des médicaments dans différents contenants. Dans les années 80, des données de stabilité d'agents anti-tumoraux contenus dans des emballages en verre et en plastique [68] ou dans des systèmes de libération médicamenteux implantables [69] ont été établies. En 1992, un autre article sur les données de stabilité des agents cytotoxiques a été publié [70]. Dans la revue de Benizri et coll., plusieurs publications sur des études de stabilité ont été discutées. Des agents cytotoxiques avec une stabilité chimique et physique suffisamment longue ont été sélectionnés pour instaurer un traitement à domicile [71]. Dans toutes ces études, la LC-UV a été la technique analytique la plus couramment utilisée.

Analyse de cytotoxiques dans des échantillons biologiques

La plupart des méthodes sont destinées à la quantification des médicaments cytotoxiques dans des matrices biologiques dans le cadre d'études fondamentales de nouveaux médicaments, d'études pharmacocinétiques (PK) et pharmacodynamiques (PD), de suivi thérapeutique (TDM) et de contrôles de l'exposition professionnelle.

Développement de nouveaux médicaments et formulations

Dans la découverte et le développement pharmaceutique, l'interaction entre le médicament et l'ADN est un des aspects les plus importants des études biologiques. Une revue sur les différentes techniques utilisées pour étudier les interactions entre les anticancéreux et l'ADN a été publiée et mentionne les techniques suivantes: empreinte ADN, NMR, MS, FT-IR et la spectroscopie RAMAN, des techniques de modélisation moléculaire et CE [72]. L'approche électrochimique peut donner des idées pour la conception rationnelle de médicaments et permettrait une meilleure compréhension du mécanisme d'interaction entre les médicaments anticancéreux et l'ADN [72]. Des études PK et PD ont souvent initié le développement de nouvelles méthodes analytiques afin de déterminer les agents cytotoxiques dans des échantillons biologiques (urine, sérum, plasma, intracellulaire, tissus). Par exemple, une méthode LC-MS/MS pour déterminer le docétaxel dans du plasma a été publiée [73]. Cette méthode présente de meilleures performances que les anciennes méthodes en termes de sensibilité et semble être prometteuse pour une étude de pharmacologie clinique de grande taille.

Suivi thérapeutique (ou TDM, '' therapeutic drug monitoring'')

Le suivi thérapeutique des agents anticancéreux est rarement utilisé en routine, étant donné le manque de concentrations thérapeutiques recommandées. La mise en place de concentrations cibles est difficile, car la relation concentration-effet dépend souvent des traitements [74]. Cependant, le TDM a le potentiel de réduire quelques effets secondaires graves et d'améliorer l'utilisation clinique de certains médicaments de chimiothérapie [75]. Par exemple, pour le méthotrexate, un suivi thérapeutique est régulièrement réalisé [75-78]. Une revue sur le TDM des cytotoxiques a été publiée par Eksborg et Ehrsson en 1985 [79] et par Guetens et coll. en 2002 [80, 81].

Suivi biologique du personnel travaillant avec des cytotoxiques

Les substances cytotoxiques sont reconnues comme dangereuses pour les professionnels de la santé depuis 1970 [82-86]. Selon la classification du centre international de recherche sur le cancer (IARC), les cytotoxiques sont considérés comme cancérogènes, mutagènes ou tératogènes [87]. Cependant, la relation directe entre l'exposition à une contamination cytotoxique et les effets nocifs est difficile à établir, et aucune concentration maximale n'a pu être déterminée jusqu'à aujourd'hui. Le suivi biologique exige des méthodes très sensibles et sélectives pour analyser des traces de cytotoxiques dans les échantillons d'urine ou de sang et un manque de méthodes standardisées et validées a été constaté [88, 89]. Généralement, des méthodes par chromatographie gazeuse couplée à la spectrométrie de masse (GC-MS) ou LC-MS ont été utilisées [90, 91], mais en fonction de l'analyte, d'autres techniques peuvent être intéressantes (par exemple ICP-MS ou voltamétrie pour les dérivés du platine [92, 93]). Même si des recommandations pour manipuler des cytotoxiques sont appliquées, des traces de ces substances sont trouvées dans les urines ou le sang des professionnels de la santé dans plusieurs études [94-98]. Selon les principes de précaution, l'exposition doit être maintenue au niveau le plus bas possible [99] et un contrôle environnemental peut se révéler utile.

Analyse de cytotoxiques dans l'environnement

Deux types de contrôles environnementaux peuvent être distingués: l'analyse de contaminations cytotoxiques dans des locaux de production ou d'administration de chimiothérapies (surface, air) et dans les eaux usées (ex. à la sortie des hôpitaux).

Contamination cytotoxique sur des surfaces et dans l'air

Une revue complète sur les méthodes d'analyse utilisées pour le suivi environnemental des agents antinéoplasiques a été publiée en 2003 par Turci et coll. [94]. Des méthodes d'analyse pour la quantification d'un (ou deux) agent(s) cytotoxique(s), considéré(s) comme marqueur(s) ou des méthodes génériques pour la détermination de plusieurs médicaments ont été développées. Lors de l'utilisation de marqueurs, les échantillons ont été obtenus par une procédure spécifique, suivie par une technique analytique adaptée (ex. la voltamétrie pour les complexes de platine [100]). Ces méthodes ont montré de très bonnes performances quantitatives et de très faibles limites de quantification (0.05 $ng \cdot cm^{-2}$). Il est à noter que ces

méthodes ne donnent qu'une estimation d'une potentielle contamination par d'autres cytotoxiques [100-105]. En effet, dans les unités de cytotoxiques des hôpitaux, une grande variété de formulations de chimiothérapies avec différentes procédures de préparation sont produites tous les jours. Pour obtenir un bon aperçu des contaminations, des méthodes multi-composés sont nécessaires avec des procédures de prélèvement génériques. Pour obtenir une sélectivité et une sensibilité suffisantes, des méthodes LC-MS/MS sont souvent utilisées [106-110].

Eaux usées

Etant donné la toxicité de ces substances, il est pertinent de se poser la question de leur élimination après administration et de leur impact sur l'environnement en tant que micropolluants. Des médicaments cytotoxiques et leurs métabolites ont été détectés dans les eaux usées, par exemple dans les effluents d'un hôpital [111-113]. Diverses techniques d'analyse peuvent être utilisées: ICP-MS pour les composés de platine [112], CE-UV pour le 5-fluorouracile [111], LC avec une détection par fluorescence pour les anthracyclines [113] et LC-MS/MS pour des antimétabolites [114] et d'autres agents cytotoxiques [115, 116].

Méthodes analytiques par composés cytotoxiques

L'**article I** donne un aperçu des méthodes analytiques publiées jusqu'à aujourd'hui pour les différentes substances cytotoxiques. Les composés sont regroupés selon leur mode d'action (antimétabolites, agents interagissant avec l'ADN, agents antitubuline) et pour chacun, une brève introduction reprenant les indications cliniques, suivie par des aspects techniques pour l'analyse des différentes matrices est reportée (formulation pharmaceutique, échantillon biologique, contamination de surface, eaux usées).

Conclusion

Au cours des trente dernières années, de nombreuses méthodes analytiques pour la détermination de substances cytotoxiques dans des formulations pharmaceutiques, des échantillons biologiques et environnementaux ont été publiées dans la littérature. D'abord, des méthodes LC-UV, ont permis d'établir les bases pour l'utilisation de chimiothérapies pour

traiter le cancer chez l'homme, en se focalisant sur les interactions avec l'organisme, le développement de formulations pharmaceutiques et la toxicité de ces composés. Comme pour toutes les substances pharmaceutiques, des études plus élaborées de PK, PD, TDM ont ensuite été publiées grâce à des techniques de détection bénéficiant d'une haute sélectivité et sensibilité telles que la MS. Pendant les dernières années, une attention particulière a été portée sur la manipulation sécurisée des médicaments cytotoxiques et sur la protection de l'environnement. En effet, plusieurs études ont trouvé des cytotoxiques dans les eaux usées, sur des surfaces de travail et dans des échantillons biologiques de professionnels de la santé.
Aujourd'hui, avec les nouveaux traitements de chimiothérapie (anticorps monoclonaux, agents de ciblage moléculaire, vaccin..), le développement d'autres méthodes appropriées constitue la prochaine étape dans l'analyse des médicaments anticancéreux.

1.4 Discussion

Les composés étudiés durant ce travail de thèse possèdent des propriétés particulières, dont l'analyse peut constituer un défi. Dans un premier temps, des composés non-UV absorbants ont été analysés. Comme mentionné précédemment, la mise à disposition de méthodes d'analyse capables de quantifier ces composés, que se soit les électrolytes ou le suxaméthonium, était incontournable étant donné leur importance clinique. Différentes techniques analytiques étaient envisageables. Toutefois notre choix s'est porté sur la CE avec un détecteur par conductimétrie étant donné que la CE-UV était déjà couramment utilisée au LCQ pour les analyses de routine. L'implantation d'un détecteur par conductimétrie a permis d'élargir la gamme de composés analysés par CE (à côté d'autres avantages présentés plus loin).
Pour l'analyse des cytotoxiques, la propriété particulière liée à leur toxicité et l'intérêt d'analyser ces substances ont été largement décrits ci-dessus. De plus, différentes précautions doivent être prises lors de la manipulation de ces substances, afin de minimiser l'exposition de l'analyste. La CE-UV et la LC-MS/MS apparaissent comme des outils intéressants, car pour le premier, la séparation se fait en système clos, ce qui est parfait pour le contrôle qualité. Pour le 2ème, les concentrations analysées sont de l'ordre des ng·mL^{-1}, donc la LC-MS/MS apparaît particulièrement adaptée pour l'analyse de traces.

2. Principes fondamentaux des techniques analytiques utilisées

Dans cette partie, les différentes techniques analytiques utilisées durant ce travail de thèse sont brièvement présentées. Il s'agit de l'électrophorèse capillaire et de la chromatographie liquide. Ce sont des techniques complémentaires basées sur des mécanismes de séparation complètement différents : la CE se base sur une séparation de molécules en présence d'un champ électrique en fonction de leur charge et de leur taille, tandis que la séparation des composés en LC repose sur la distribution de ces derniers entre une phase mobile et une phase stationnaire.

2.1 Electrophorèse capillaire

La séparation électrophorétique s'effectue dans un capillaire, dont les extrémités sont immergées dans des récipients remplis de solutions riches en électrolytes (tampon de séparation, BGE). Le BGE doit également être présent dans le capillaire afin de garantir des conditions constantes de force ionique et de pH durant la séparation. L'échantillon est introduit dans le capillaire par pression (hydrodynamique) ou par un champ électrique (électromigration). Le volume injecté est normalement compris entre 1 et 2% du volume total du capillaire, correspondant à 5 - 50 nL. Une différence de potentiel pouvant aller jusqu'à 30 kV est appliquée au moyen de deux électrodes plongeant dans les récipients de BGE (Figure 8).

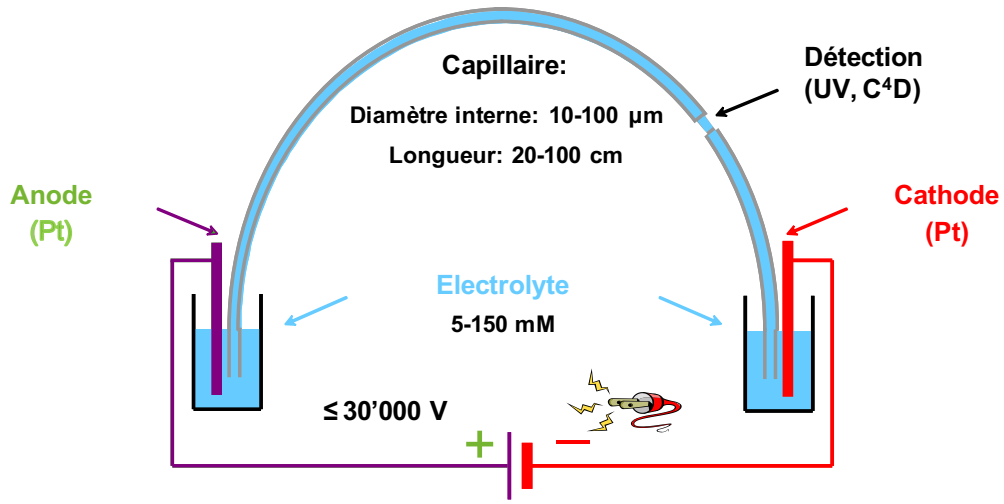

Figure 8: Schéma d'un instrument CE

Le couplage de la CE à différents détecteurs est possible, tels la spectrophotométrie UV/Vis, l'électrochimie (conductimétrie, ampérométrie), la fluorescence induite par laser ou la spectrométrie de masse. Le détecteur le plus courant est la spectrophotométrie UV/Vis qui permet la détection « in situ ».

Les principaux avantages de la CE sont une grande efficacité, un développement de méthode rapide, un coût d'analyse raisonnable et une grande versatilité. Il existe différents modes en CE, permettant d'analyser une grande variété de composés (allant des petits ions inorganiques aux protéines) et le principe de séparation est toujours basé sur la différence de migration des particules en fonction de leur rapport charge sur taille sous l'influence d'un champ électrique. Les modes CE utilisés dans cette thèse (CZE : électrophorèse capillaire de zone, MEKC: la chromatographie électrocinétique micellaire, et MEEKC: chromatographie électrocinétique en microémulsion) sont expliqués ci-dessous. De plus, le petit diamètre des capillaires utilisés permet une faible consommation d'échantillon et de solvant, ce qui rend cette méthode aussi attractive pour des raisons écologiques. Le petit diamètre des capillaires peut par contre engendrer des problèmes de sensibilité avec une détection UV/Vis, car la réponse est directement proportionnelle à la longueur parcourue, donc au diamètre du capillaire.
Aujourd'hui la CE possède un large domaine d'applications, comme par exemple la pharmacie, la biologie et l'environnement. De plus, c'est une méthode de séparation orthogonale (principes de séparation des analytes différents) et complémentaire aux techniques chromatographiques.

2.1.1 Electrophorèse capillaire de zone (CZE)

Les premières applications par CZE ont été effectuées par Jorgenson et Lukacs en 1981 [117-120] permettant de séparer des composés ionisés ou ionisables selon leur charge et leur taille. Le principe de base implique la migration d'une particule chargée dans une solution d'électrolytes, sous l'influence d'un champ électrique. Les différentes vitesses de migration des composés permettent de les séparer.

La vitesse de migration (v ; cm s^{-1}) d'un composé dépend de sa mobilité électrophorétique et du champ électrique appliqué :

$$v = \mu_{app} E \qquad \text{[Equation 1]}$$

avec μ_{app} correspondant à la mobilité apparente ($cm^2V^{-1}s^{-1}$) et E au champ électrique (Vcm^{-1}) qui dépend de la tension appliquée et de la longueur du capillaire utilisé.

Les mobilités sont indépendantes de la tension et de la longueur du capillaire utilisé et sont ainsi préférées à la vitesse pour comparer le comportement de différents composés en CZE. La mobilité apparente est la somme de la mobilité effective (μ_{eff}) et de la mobilité du flux électroosmotique (μ_{EOF}):

$$\mu_{app} = \mu_{eff} + \mu_{EOF} \qquad \text{[Equation 2]}$$

La μ_{EOF} est identique pour toutes les particules pour un capillaire et un électrolyte utilisés et le principe de séparation repose donc sur μ_{eff}, qui est décrite par l'équation 3. Pour une petite molécule, elle dépend de la charge effective (q) et du rayon hydrodynamique (r) du composé ainsi que de la viscosité du milieu (η).

$$\mu_{eff} = \frac{q}{6 \cdot \pi \cdot \eta \cdot r} \qquad \text{[Equation 3]}$$

La mobilité du flux électroosmotique correspond au mouvement de l'électrolyte sous l'influence d'un champ électrique. En fonction du pH et de la force ionique de l'électrolyte, les groupements silanols présents sur les parois internes du capillaire, possèdent une charge plus ou moins négative. Ainsi les cations présents dans le BGE sont attirés et forment un système de double couche (couche liée et couche diffuse positive) à la surface des parois internes du capillaire. Avec le champ électrique, la couche diffuse migre vers la cathode et entraîne l'électrolyte, créant ainsi un écoulement à l'intérieur du capillaire, appelé flux électroosmotique (EOF).

Le pH et la force ionique du BGE sont les principaux paramètres influençant l'EOF et jouent donc un rôle central dans le développement de méthode par CZE. Vu que les différents groupements silanols peuvent varier d'un capillaire à l'autre, un conditionnement est préconisé avant utilisation d'un capillaire neuf pour atténuer les variations d'EOF [121].

L'ordre de migration en CZE en mode positif est illustré dans la Figure 9. Les cations sont attirés par la cathode (côté du détecteur) et leur ordre de migration est proportionnel à leur rapport charge sur taille. L'EOF généré se dirige aussi vers le détecteur, entraînant les neutres et les anions. Les espèces neutres ne sont pas séparées, mais les anions peuvent être détectés, si la μ_{EOF} est supérieure à leur μ_{eff}.

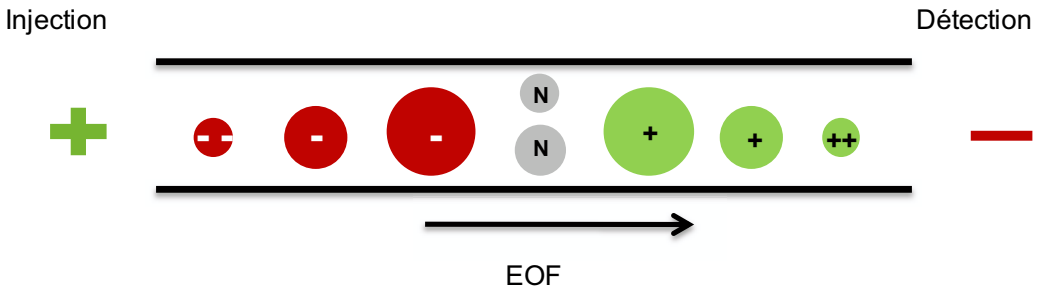

Figure 9: Ordre de migration de particules chargées et neutres par CZE en mode positif

En conclusion, la CZE permet, en présence d'un flux électro-osmotique (EOF), l'analyse simultanée de substances cationiques et anioniques. Les substances neutres migrent dans le flux mais ne sont pas séparées et une autre stratégie doit être utilisée comme par exemple la MEKC ou MEEKC.

2.1.2 Chromatographie électrocinétique micellaire (MEKC)

La MEKC est un autre mode de la CE, développé par Terabe et coll. [122] en 1984 pour pouvoir séparer des composés neutres. Des tensioactifs anioniques ou cationiques à une concentration supérieure à la concentration micellaire critique sont ajoutés au tampon. Le dodécylsulfate de sodium (SDS), utilisé dans ce travail de thèse pour l'analyse des complexes de platine, fait partie des tensioactifs les plus couramment utilisés. Ces micelles sont hydrophiles à l'extérieur et hydrophobes à l'intérieur et peuvent migrer dans le même sens ou dans le sens inverse du EOF (phase pseudo-stationnaire). Les composés neutres peuvent être retenus à l'intérieur de la micelle selon un mécanisme de partage et peuvent être séparés en fonction de l'interaction hydrophobe entre la micelle et le soluté. Il s'agit donc d'un hybride entre la CE et la chromatographie avec un mécanisme de séparation mixte entre mobilité et partage hydrophobe avec une phase pseudo-stationnaire. Les BGEs utilisés présentent souvent une stabilité courte et doivent être préparés extemporanément. Bien qu'à l'origine elle ait été développée pour la séparation d'espèces non-chargées, la MEKC est aussi largement utilisée pour l'analyse de composés ionisés, car elle peut augmenter la solubilité des analytes et séparer simultanément des composés neutres et chargés.

2.1.3 Chromatographie électrocinétique en microémulsion (MEEKC)

La MEEKC a également été développée par Terabe et coll. en 1992 [123]. C'est le même mécanisme de séparation que celui de la MEKC, sauf que la phase pseudo-stationnaire consiste en une microémulsion (ME): le BGE est composé d'une phase huileuse (n-octane, ou n-heptane), de tensioactif (SDS), de co-surfactant (butanol) et de la phase aqueuse (tampon phosphate, borate…) dans des proportions bien définies. Avec une détection UV/Vis, ces composés présents dans le BGE peuvent diminuer la sensibilité de la méthode. Souvent la MEEKC est plus sélective que la MEKC et une bonne corrélation avec la lipophilicité (logP) du composé peut être établie. A l'instar de la MEKC, la MEEKC permet l'analyse simultanée de composés neutres et chargés, et aide à solubiliser des composés hydrophobes. En plus, la stabilité du BGE en MEEKC est souvent meilleure qu'en MEKC.

2.1.4 Détection conductimétrique

La détection conductimétrique (CD) est une technique de détection quasi-universelle pour tous les composés chargés [32, 35] et elle est une alternative intéressante pour les composés non-chromogènes, pour lesquels une détection UV/Vis-directe n'est pas possible. La CD consiste à mesurer la différence de conductivité entre l'analyte et les co-ions présents dans le BGE. La réponse est d'autant plus grande et la détection plus sensible, si l'amplitude entre la conductivité de la zone de l'échantillon et celle du BGE est élevée. Ainsi, l'utilisation de tampons amphotères permet d'obtenir un maximum de sensibilité, car ils possèdent une conductivité faible. En outre, ils peuvent être utilisés à hautes concentrations pour assurer une bonne efficacité par effet « stacking » sans générer trop de courant [25, 29, 124].

Les premiers développements de détecteurs CD pour la CE étaient en contact avec la solution électrolytique [125, 126] ce qui a posé des problèmes d'encrassement et d'interférences entre le champ électrophorétique et la détection. En 1998, une nouvelle génération de détecteurs conductimétriques sans contact (CCD ou C^4D: Capacitively Coupled Contactless Conductivity Detector), introduit par le groupe de Zemann [25, 26, 127] et le groupe de Fracassi da Silva et do Lago [36, 128] a permis d'augmenter l'attractivité de ce mode de détection grâce à sa simplicité au niveau manipulation et maintenance, la possibilité de l'utiliser pour différentes tailles et natures de capillaires ainsi qu'au format miniaturisé, et son coût abordable [25, 27, 29]. La détection C^4D consiste en un couplage capacitif entre un champ électrique alternatif et la solution électrolytique à l'intérieur du capillaire, à l'aide de deux électrodes externes à la solution, sans établir de contact direct avec elle [129]. Un schéma du C^4D est montré dans la Figure 10.

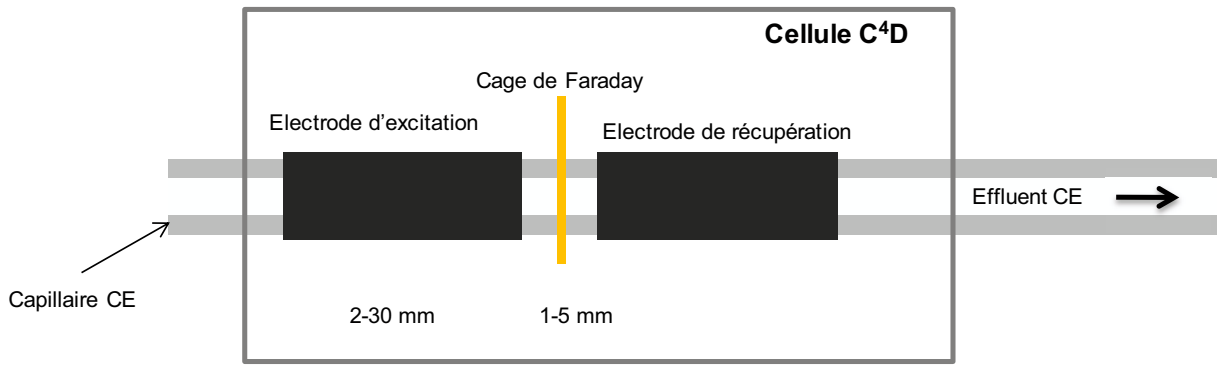

Figure 10 : Schéma de principe CE-C^4D [130]

Le détecteur est constitué de deux électrodes (en forme de tubes en acier inoxydable d'environ 2 à 30 mm de longueur) placées autour du capillaire CE avec un écart de 1 à 5 mm. Les électrodes sont séparées par une cage de Faraday constituée d'une feuille mince en cuivre, pour éviter qu'un courant soit directement établi entre les deux électrodes et afin de diminuer le bruit de fond [16]. Le montage peut être schématisé comme deux condensateurs en série (électrodes) séparés par une résistance (la solution d'électrolyte à l'intérieur du capillaire). Une tension oscillante est appliquée sur la première électrode (appelée électrode d'excitation) et un courant capacitif est établi entre cette électrode et le liquide à l'intérieur du capillaire. Le courant est conduit à travers la solution jusqu'à ce qu'il soit récupéré par la seconde électrode (appelée électrode de récupération). Les modifications de conductivité dans l'espace entre les deux électrodes (à l'intérieur du capillaire) sont enregistrées, car un changement de la conductivité est traduit par un courant récupéré différent [16, 131]. Les deux électrodes sont elles-mêmes placées dans une cellule permettant un positionnement stable et une distance répétable entre les électrodes. Cette cellule peut être placée à n'importe quel endroit sur le capillaire, selon la résolution et le temps d'analyse nécessaires et on peut en même temps ajouter d'autres types de détection (ex. UV/Vis, Fluo, LIF).

Depuis quelques années, le C^4D est devenu une alternative intéressante par rapport à des détecteurs optiques, et a montré de nombreuses applications dans la détermination de produits pharmaceutiques [131, 132]. Introduit depuis 2007 à la pharmacie des HUG, la CE-C^4D est utilisée au quotidien pour la détermination des électrolytes dans les nutritions parentérales et pour l'analyse de quelques productions de série (ex. seringues prêtes à l'emploi de suxamethonium, capsules de gluconate de calcium (0.5 mmol) et de glycérophosphate de calcium (0.5 mmol)).

2.2 Chromatographie en phase liquide - spectrométrie de masse (LC-MS/MS)

2.2.1 Principes fondamentaux de la LC

Un appareillage LC se compose d'une colonne chromatographique, d'un système d'injection, d'une pompe LC et d'un système de détection. La colonne qui contient la phase stationnaire est le cœur de la séparation et doit être choisie en fonction des analytes. Les pompes à haute pression permettent de percoler régulièrement l'éluant à travers la colonne. Différents détecteurs peuvent être utilisés, comme l'UV, la fluorescence, l'électrochimie ou la MS, permettant d'atteindre de grandes sélectivité et sensibilité. De plus, le grand choix de phases stationnaires commercialisées avec différents motifs greffés et la multitude de phases mobiles possibles rendent cette technique particulièrement sélective selon de nombreux mécanismes de séparation.

Dans ce travail, seule la chromatographie de partage à polarité de phase inversée a été utilisée: le motif greffé est une chaîne alkyle ou phényle possédant un caractère hydrophobe. Environ 80% des séparations chromatographiques en phases liquides sont effectuées par partage sur des phases inversées. Comme phase mobile, des mélanges hydro-organiques sont appliqués: eau-méthanol ou eau-acétonitrile et éventuellement un troisième composé (p.ex. un solvant, un tampon). La rétention des solutés apolaires est d'autant plus grande que la proportion en eau est importante. Pour la séparation de composés possédant des structures proches, il est recommandé de travailler en mode gradient : au début, il n'y a qu'un faible pourcentage de solvant organique dans la phase mobile et en augmentant le pourcentage du solvant organique, les composés sont élués selon leur hydrophobicité. Pour avoir une bonne séparation chromatographique entre les différents composés, le gradient est soigneusement étudié lors du développement de la méthode.

Le mécanisme de rétention se situe entre les deux hypothèses suivantes.

- La phase mobile imprègne les greffons et les solutés se partagent entre la phase mobile et la phase stationnaire imprégnée.
- Le soluté est retenu par interaction hydrophobe sur les motifs greffés et la phase mobile les déplace continuellement par partage.

2.2.2 Principes fondamentaux de la MS

La spectrométrie de masse (MS) est un outil puissant pour détecter une substance après une technique analytique séparative. Le détecteur MS ne nécessite aucune fonction particulière de la part de la substance à détecter, si ce n'est sa capacité à s'ioniser, c'est pourquoi il est qualifié de quasi-universel. Il permet d'effectuer une séparation en fonction du rapport masse sur charge (m/z) de la substance, offrant ainsi une grande sélectivité. La sensibilité offerte par la MS dépend du composé (de son potentiel d'ionisation) et du type d'analyseur de masse utilisé. De plus, la MS peut également apporter une information qualitative sur la substance. Dans ce travail de thèse, une source d'ionisation électrospray et un analyseur de type triple quadripôles ont été utilisés, et seront détaillés ci-dessous.

Source d'ionisation : Electrospray (ESI)

Pour pouvoir coupler la chromatographie liquide à la MS, différentes sources d'ionisation ont été développées. Ces sources doivent pouvoir introduire le haut débit de liquide en provenance de la LC à pression atmosphérique dans le haut vide de la MS. Les sources sous vide qui sont de moins en moins utilisées aujourd'hui sont remplacées progressivement par les sources à pression atmosphérique (API) plus performantes, robustes, et applicables à de nombreuses applications. Les sources API les plus fréquemment rencontrées sont l'électrospray (ESI) et l'ionisation chimique à pression atmosphérique (APCI). Il s'agit de techniques d'ionisation douces qui permettent de former majoritairement l'ion moléculaire protoné ou déprotoné en n'induisant qu'une fragmentation réduite. Actuellement, l'ESI constitue le mode d'ionisation le plus utilisé. Les principaux précurseurs de l'ESI sont Fenn [133, 134] et Bruins et coll. [135] pour le développement de l'ESI assistée pneumatiquement.

Le rôle de l'électrospray est donc la formation d'ions en phase gazeuse à partir de molécules en solutions en provenance de la LC. Une tension comprise entre 2 et 5 kV est appliquée sur le capillaire métallique à l'intérieur duquel se trouve une phase liquide en provenance de la LC, qui contient un solvant à caractère polaire et des électrolytes solubles. Sous l'action du haut champ électrique généré à l'extrémité du capillaire, les électrolytes positifs et négatifs s'orientent au sein du liquide de sorte que les charges positives sont entraînées vers le front du liquide, tandis que les charges négatives se dirigent vers les parois du capillaire (mode positif). Lorsque les forces de répulsions électrostatiques entre les ions positifs excèdent la

tension de surface du solvant, une dilatation de ce dernier survient et permet au liquide et aux charges positives de progresser vers l'extrémité du capillaire où se forme un cône de liquide (appelé cône de Taylor). Si le champ électrique est suffisamment important, des gouttes chargées positivement sont produites et se dirigent vers l'entrée de la masse (la contre électrode). Puis, ces gouttes diminuent progressivement en taille en perdant des molécules de solvant par des mécanismes de désolvatation et d'évaporation. En s'évaporant, la densité de charges électriques des gouttelettes devient trop grande, ce qui génère leur explosion coulombique et libère des ions en phase gazeuse [136, 137].

L'electrospray assistée pneumatiquement (ou ion spray) permet la formation de nébulisat par l'application d'un gaz de nébulisation coaxial au flux de la phase mobile [135]. Ceci est nécessaire pour pouvoir nébuliser de plus grandes quantités de liquide par unité de temps, donc en travaillant avec des débits plus élevés, à partir de 50 $\mu L \cdot min^{-1}$.

La plupart des sources ESI commercialisées font intervenir les paramètres suivants: le gaz de nébulisation, la tension appliquée, le gaz de séchage et la position du capillaire dans la source. Tous ces paramètres doivent être étudiés pour chaque composé lors du développement d'une méthode LC-ESI-MS.

- Le gaz de nébulisation a pour fonction d'assister la formation du nébulisat. Il s'agit d'azote délivré à des pressions différentes et proportionnelles au débit de la phase mobile et dans la même direction que le flux. Il permet également de focaliser le nuage de gouttes produit dans une zone restreinte de la source afin d'augmenter la quantité d'ions pénétrant dans le MS.

- La tension appliquée entre le capillaire métallique et l'entrée du MS se concentre principalement sur la charge portée par les gouttelettes. Elle permet également de diriger les gouttelettes chargées et les ions émis vers l'entrée du MS. Les solvants avec une haute tension de surface comme l'eau nécessitent de hautes tensions pour être dispersés en gouttelettes, surtout en ESI pur (non assisté pneumatiquement). La présence d'acétonitrile peut donc favoriser l'ionisation des composés.

- Le gaz de séchage facilite la désolvatation des gouttes. Généralement il s'agit d'azote chauffé introduit dans la chambre soit perpendiculairement, soit à contre-courant ou concourant à la nébulisation. Il permet également de limiter l'accès au MS des molécules neutres et d'éviter une contamination de l'entrée du spectromètre par du matériel non volatile.

- La position du capillaire dans la source est aussi un paramètre important dans le processus d'ionisation. Un angle entre 30° à 90° avec l'entrée du MS permet d'augmenter la stabilité du signal généré en réduisant l'accès au MS des gouttelettes et des molécules neutres. Ceci est particulièrement adapté à l'analyse d'échantillons complexes qui contiennent une multitude d'éléments susceptibles de contaminer la chambre d'ionisation.

L'ESI est une technique d'ionisation douce parfaitement adaptée pour l'analyse de molécules polaires et ioniques de masses moléculaires comprises entre 10^2 et 10^5 Dalton et se présente donc très favorablement pour l'analyse simultanée de différentes substances cytotoxiques. Elle engendre des molécules protonées $[M+H]^+$ ou déprotonées $[M-H]^-$, parfois multichargées $[M+nH]^{n+}$. Bien que les ions cités précédemment soient majoritairement formés, des adduits $(M+NH_4]^+$ ou des multimères ($[2M+H]^+$ sont parfois observés. En travaillant en mode positif, l'ionisation des composés basiques est favorisée en utilisant une phase mobile acide qui aide à la protonation des composés (ex. acide formique à 0.1% dans l'eau et acétonitrile).
L'intensité du signal ESI-MS peut être affectée par la présence d'autres électrolytes en provenance de différentes origines comme les additifs dans la phase mobile, d'impuretés de solvant, de co-analytes ou des interférents matriciels [138].

Analyseur de masse : triple quadripôle [139, 140]

Les ions générés par la source d'ionisation pénètrent dans le haut vide du spectromètre de masse où ils sont accélérés et focalisés jusqu'à l'analyseur qui opère leur discrimination en fonction de leur rapport m/z. L'analyseur de masse de type triple quadripôle est l'analyseur hybride le plus utilisé dans l'analyse de molécules de faibles poids moléculaires.

Un quadripôle est constitué de quatre barreaux métalliques cylindriques ou hyperboliques placés équidistants et parallèles qui servent d'électrodes. Par application d'une tension alternative (dans le domaine de radiofréquences RF) et continue entre les barres (DC), il y a création à l'intérieur du quadripôle d'un champ quadripolaire en deux dimensions (x, y). Pour un rapport RF/DC donné, seuls les ions d'une valeur m/z donnée pourront traverser le champ quadripolaire et atteindre le détecteur. Pour cette raison on décrit le quadripôle comme un filtre de masse. Pour obtenir un spectre de masses (m/z 100 à m/z 1000) on varie l'intensité de la tension alternative et continue en maintenant un rapport RF/DC constant (= balayage).

Le triple quadripôle consiste en un couplage de deux quadripôles en série séparés par une chambre de collision, comme représenté dans la Figure 11. Dans le premier quadripôle (Q1) une séparation des ions est effectuée. Le second quadripôle (Q2), ou chambre de collision, permet la transmission et la fragmentation des ions grâce à l'introduction d'un gaz de collision (argon). Le troisième quadripôle (Q3) permet d'effectuer une seconde séparation en fonction du rapport m/z. Dans une configuration SRM (= selected reaction monitoring), Q1 et Q3 sont à une valeur m/z fixe et permettent donc la quantification d'un composé donné. Les rapports m/z d'ion précurseur et d'ion fragmenté sont étudiés lors du développement d'une méthode en fonction de l'énergie de collision en Q2. Cette génération de fragments est spécifique à chaque molécule et ainsi une double sélectivité en masse peut être obtenue en mode tandem (MS/MS).

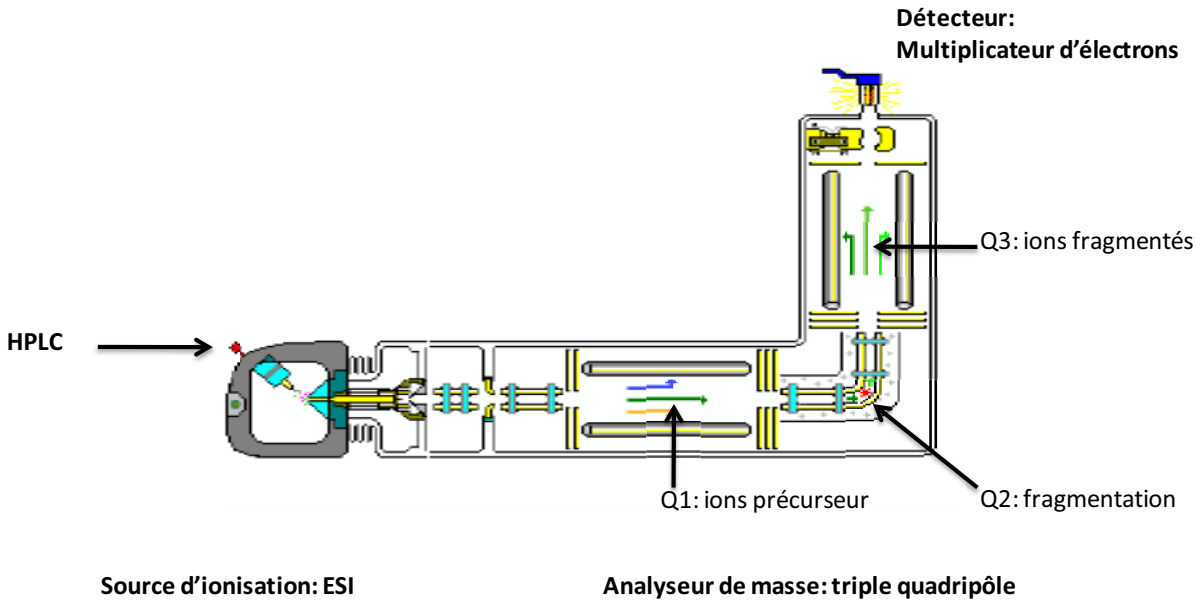

Figure 11 : Schéma d'une LC-ESI-MS/MS, en mode SRM utilisé lors de ce travail de thèse (Thermos TSQ Access Manuelle)

La très grande sélectivité et sensibilité des triples quadripôles en font l'analyseur de choix lorsque des limites de détection très basses doivent être obtenues, comme c'est le cas pour l'analyse de traces de cytotoxiques dans l'environnement.

3. Validation analytique

Toutes les méthodes développées dans ce travail de thèse ont été validées avant leur mise en pratique dans le laboratoire. La stratégie de validation utilisée pour le contrôle qualité des médicaments est basée sur le guide de la commission de la Société Française des Sciences et Techniques Pharmaceutiques (SFSTP, 2003) [141]. Ce guide propose un consensus sur les différentes normes existantes en incorporant la terminologie ISO.

3.1 Objectifs

Un résultat erroné pendant le contrôle qualité d'une préparation hospitalière peut avoir des conséquences très graves pour le patient. Ainsi, il faut d'abord s'assurer que la méthode utilisée permet de donner des résultats fiables en accord avec des limites acceptables demandées par l'usage du produit analysé. La validation d'une méthode analytique est requise pour passer de la phase de développement à celle de l'utilisation en routine et elle permet de fixer les spécifications de contrôle pour un principe actif ou pour un produit fini.
L'objectif de la validation d'une méthode analytique est de démontrer que chaque mesure réalisée ultérieurement en routine sera suffisamment proche de la valeur vraie ou dans les limites acceptables selon le besoin de l'analyse. Les critères de la validation sont fixés en fonction de la finalité de la procédure analytique.

3.2 Critères de validation

Les principaux critères de validation et termes utilisés sont brièvement expliqués ci-dessous avec des exemples concrets, rencontrés durant ce travail de thèse.

La spécificité d'une méthode analytique est sa capacité à déterminer de manière univoque la substance à analyser en présence d'autres composants. La spécificité correspond à une sélectivité maximale. Une méthode utilisée dans le cadre d'études de stabilité d'une formulation doit posséder une sélectivité suffisante pour discriminer le principe actif des impuretés, excipients et produits de dégradation, c'est-à-dire posséder une bonne séparation électrophorétique ou chromatographique entre les différents composés.

La réponse considérée (signal) doit être étudiée en fonction de la concentration de l'analyte dans un intervalle de dosages approprié. Une procédure est dite « sensible » si une faible variation de la concentration ou de la quantité d'analyte entraîne une variation significative de la réponse. Les courbes d'étalonnage sont construites à partir des rapports d'aires des analytes sur celles des standards internes pour la méthode LC. En CE, le rapport des aires est corrigé par le temps, car les temps de migrations peuvent bouger. Pour le contrôle qualité d'une formulation, la fonction réponse est étudiée dans un intervalle restreint autour de la valeur cible (ex. 80% - 120%), tandis que pour un contrôle environnemental, un intervalle beaucoup plus large est choisi, car on ne connait pas la valeur réelle de l'échantillon. L'intervalle de dosage d'une méthode analytique est donc la région entre les niveaux supérieur et inférieur (valeurs incluses) pour lesquels il a été démontré que la méthode est appropriée par rapport à son exactitude et sa linéarité.
La linéarité d'une procédure d'analyse est sa capacité à l'intérieur d'un certain intervalle de dosages d'obtenir des résultats directement proportionnels à la quantité (concentration) d'analyte dans l'échantillon.

La justesse exprime l'étroitesse de l'accord entre la valeur moyenne obtenue à partir d'une série de résultats d'essais et une valeur qui est acceptée soit comme une valeur conventionnellement vraie, soit comme une valeur de référence acceptée (standard pharmacopée). La mesure de la justesse est généralement exprimée en termes de recouvrement et de biais absolu ou relatif (erreur systématique). La précision ou fidélité fournit une indication sur les erreurs liées au hasard en exprimant le coefficient de variation (CV) entre une série de mesures provenant de multiples prises d'un même échantillon homogène (résultats d'essais indépendants) dans des conditions prescrites. La répétabilité concerne les mesures effectuées dans des conditions opératoires identiques (même opérateur, même appareil,...) pendant un court intervalle de temps tandis que la fidélité intermédiaire concerne les mesures effectuées à l'intérieur d'un même laboratoire, quand un ou plusieurs facteurs sont changés (jour, opérateur, équipements...) pendant un intervalle de temps donné.

Toutes les validations ont été effectuées sur trois séries indépendantes (incluant l'arrêt de l'appareillage, nouvelle préparation des solutions tampons et des standards de calibration et de validations, …). Pour une méthode de contrôle qualité (comme pour le suxaméthonium), chaque série contenait 4 standards de validation, préparés à 3 niveaux de concentration (80, 100 et 120%) qui permettait de calculer la justesse et la répétabilité ainsi que la fidélité intermédiaire à chaque concentration. La justesse est exprimée comme le rapport entre la concentration moyenne mesurée et la concentration théorique. La répétabilité est définie comme le coefficient de variation du rapport de l'écart type des résultats dans une même série sur la valeur théorique à chaque concentration. La fidélité intermédiaire est exprimée comme le coefficient de variation du rapport de l'écart type entre les différentes séries sur la valeur théorique à chaque concentration.

L'exactitude exprime l'étroitesse de l'accord entre la valeur trouvée et la valeur acceptée comme référence. Elle est la somme des erreurs systématiques et aléatoires, en d'autres termes l'erreur totale liée au résultat c'est-à-dire la somme de la justesse et de la fidélité. Dans ce travail de thèse, l'erreur totale acceptée pour une méthode utilisée pour la détermination du principe actif dans une formulation était 5 %, tandis que pour l'analyse de traces sur des prélèvements de surface, une erreur totale jusqu'à 30% était considérée comme acceptable. En effet, si 2 ou 3 ng par substance cytotoxique sont détectés sur une surface de 100 cm^2, la différence n'est pas considérée comme très importante et la contamination reste faible.

Pour chaque niveau de concentration, l'erreur totale est calculée, ce qui permet d'établir le profil d'exactitude [142]. Cet outil visuel de décision permet d'évaluer la capacité de la méthode à donner des résultats dans les limites d'acceptation. Dans la Figure 12, le profil d'exactitude pour l'analyse du cyclophosphamide par une méthode LC-MS/MS est donné (**article VII**).

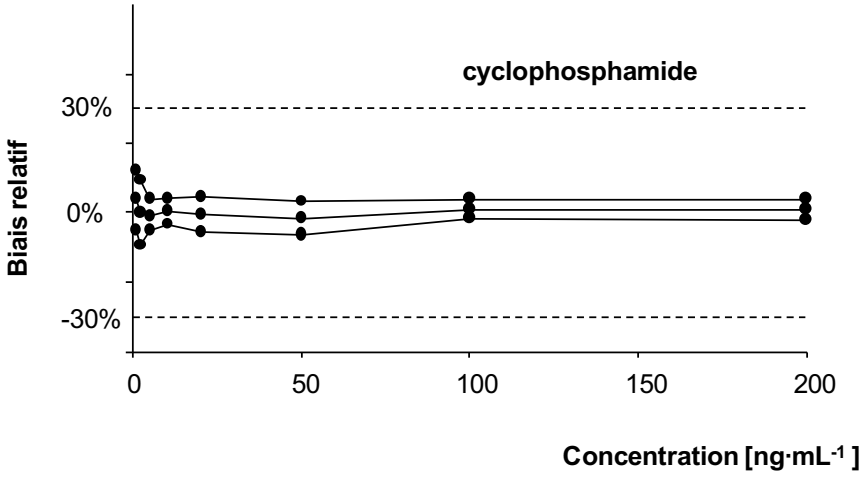

Figure 12: Profil d'exactitude du cyclophosphamide par LC-MS/MS (article VIII)

La limite de détection (LOD) d'une procédure d'analyse correspond à la plus petite quantité d'analyte dans un échantillon pouvant être détectée, mais non quantifiée. La limite de quantification (LOQ) est la plus petite quantité de l'analyte dans un échantillon pouvant être dosée dans les conditions expérimentales décrites avec une exactitude définie. Des valeurs de LOD et LOQ les plus basses possibles sont des critères indispensables lorsque l'analyse de très faibles concentrations est envisagée, comme dans le cas de l'analyse de traces de substances cytotoxiques dans l'environnement.

3.3 Conclusion

La validation d'une méthode analytique est indispensable avant de pouvoir l'utiliser en analyse de routine, car elle permet d'assurer que le résultat obtenu lors d'une analyse est fiable et dans des limites acceptables. Ceci est très important pour garantir la qualité du produit fini, ainsi que le rejet d'un produit non-conforme en ce qui concerne sa teneur. Les différents critères de validation sont étudiés en fonction de l'applicabilité de la méthode; les exigences diffèrent pour une méthode destinée au contrôle qualité d'une préparation pharmaceutique et pour une méthode utilisée pour la détermination de la contamination de surface d'une substance cytotoxique. La finalité de la méthode doit donc être prise en compte lors de la validation.

4. Références

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Chapitre 2: Résumé du travail de thèse

1. Contrôle qualité de médicaments produits en milieu hospitalier

Cette première partie concerne le contrôle qualité des médicaments produits en milieu hospitalier. Tout d'abord l'**article II** illustre l'intérêt de la CE en milieu hospitalier. Ensuite, deux méthodes CE-C^4D ont été développées (**article IV et V**) pour la détermination de composés non-UV absorbants. La première méthode permet l'analyse quotidienne d'une préparation à haut risque, les nutritions parentérales (**article III**), et la deuxième est utilisée pour le développement d'une nouvelle formulation prête à l'emploi de suxaméthonium, puis lors de son contrôle qualité de routine (**article VI**). Finalement le contrôle qualité de substances cytotoxiques a été élaboré avec l'analyse des complexes de platine par MEKC-UV (**article VII**) et pour d'autres cytotoxiques par LC-MS/MS (**article VIII**).

1.1 Intérêt de l'électrophorèse capillaire pour le contrôle qualité en milieu hospitalier

L'intérêt de la CE pour le contrôle qualité (QC) de formulations produites en milieu hospitalier et la mise en place de cette technique au laboratoire de contrôle qualité (LCQ) est décrit dans l'**article II**.

1.1.1 Contrôle qualité à la pharmacie des HUG

Les différentes activités du LCQ ont déjà été présentées dans la préface de cette thèse. La quantification du principe actif dans une formulation est une analyse importante pour le contrôle qualité de produits finis ainsi que pour des études de stabilité. Jusqu'en 2004, la LC-UV était la seule technique séparative utilisée dans ce but au LCQ. Avec les méthodes LC conventionnelles (colonne: diamètre interne 4 mm, longueur 15 cm; débit 1-1.5 mL/min; 1 colonne pour 1 - 2 substances), la grande consommation de solvant et le coût d'analyse élevé constituaient les points faibles.

1.1.2 Analyse CE aux HUG

En 2004, le LCQ a eu l'opportunité d'acheter un nouvel instrument. La CE a été sélectionnée, car c'est une technique analytique complémentaire à la LC, qui présente de nombreux avantages (grande efficacité, développement de méthode rapide, faible consommation de

solvant, voir chapitre 1, partie 2.1). En général, la concentration du principe actif dans une formulation est de l'ordre du $mg \cdot mL^{-1}$ et la préparation peut être analysée par CE-UV sans problème de sensibilité. De plus, la CE est en accord avec la politique de protection de l'environnement et les limitations budgétaires de l'institution.

Depuis 2004, plusieurs méthodes ont été développées et validées au LCQ et remplacent aujourd'hui des analyses effectuées par LC-UV souvent plus complexes et coûteuses (ex. adrénaline ou isoprénaline) [1]. Des formulations nécessitant au préalable une étape de purification, comme le sirop de codéine, peuvent être analysées après une simple dilution par une méthode séparative comme la CE-UV. Des méthodes CE génériques sont capables d'analyser plusieurs composés dans les mêmes conditions analytiques. Au LCQ, une seule méthode CE peut être utilisée pour la détermination de 9 composés différents. Avec la LC, 4 colonnes différentes étaient nécessaires pour l'analyse de ces mêmes composés. Pour des composés non-UV-absorbants, la CE-C^4D a été mise en place en 2007 (suxaméthonium [2] et cations inorganique [3]).

La CE est une technique complémentaire à la LC qui ne peut pas la remplacer complètement. Pour des composés à faible concentration (ex. fentanyl destiné à la pédiatrie), ou pas suffisamment stable (ex. vancomycine), la LC reste la technique de choix. Le pourcentage d'analyses séparatives effectuées par CE et LC entre 2000 et 2008 est représenté dans la Figure 13.

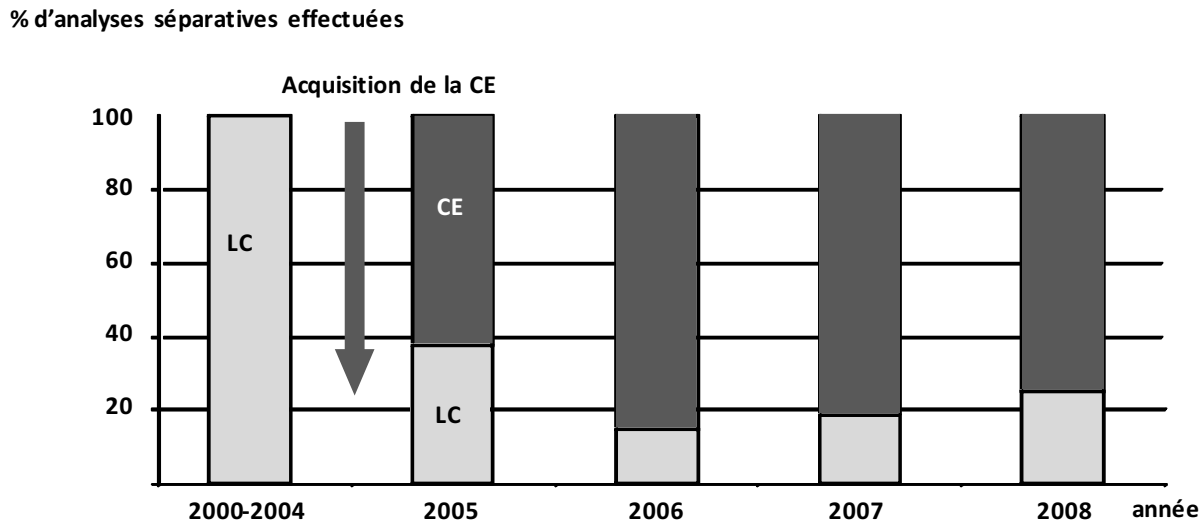

Figure 13: Proportion des analyses séparatives effectuées par LC (gris) et par CE (noir) au laboratoire contrôle qualité de la pharmacie des HUG

Toutes les méthodes CE utilisées au laboratoire ont été validées selon les recommandations SFSTP [4] et possèdent de bonnes performances quantitatives. Comme indiqué dans le Tableau 1, la consommation de solvant, le coût et le temps d'analyse ont été fortement réduits.

Tableau 1: Caractéristiques de l'analyse quantitative d'un lot d'une production de série fabriquée à la pharmacie des HUG.

Critères	***LC***	***CE***
Temps	5 h	3 h
Consommation de solvant organique	≥ 100 mL	0 - 5 mL
Coût* (capillaires ou colonnes, consommables, produits)	30 euro	7 euro
Performances quantitatives (justesse 100 ± 2%, CV <3%)	conforme	conforme

* L'instrumentation et le temps de travail ne sont pas inclus, car ils sont considérés équivalents dans notre cas.

1.1.3 Conclusion

La CE au sein d'un laboratoire de contrôle qualité d'une pharmacie hospitalière s'est révélée pertinente en termes de gain de temps et de respect de l'environnement avec des performances similaires à la LC conventionnelle pour l'analyse de formulations pharmaceutiques. Après 4 ans de mise en routine, 80% des préparations hospitalières sont analysées par CE.

1.2 Contrôle qualité de préparations à haut risque : nutritions parentérales

Depuis plusieurs années, de nombreux efforts sont engagés par les HUG pour améliorer la sécurité et la qualité de l'usage des médicaments, de la prescription à l'administration [5-8]. Une attention particulière a été portée sur la fabrication aseptique, considérée comme un processus à haut risque, surtout quand des formulations individuelles sont préparées, comme les nutritions parentérales (NP). Deux articles sur le contrôle qualité des NP ont été publiés : **l'article III** rapporte l'implémentation du contrôle qualité des NP à la pharmacie et **l'article IV** décrit le développement d'une méthode CE-C^4D pour la détermination des électrolytes dans ces formulations.

1.2.1 Contrôle qualité des NP aux HUG

Aux HUG, les NP individuelles sont principalement destinées à la pédiatrie et plus particulièrement à la néonatalogie. Le médecin saisit chaque jour sa prescription électronique avant 13 h, celle-ci est validée à la pharmacie, puis des opérateurs qualifiés produisent les NP sous une hotte à flux laminaire (classe A GMP / ISO 4,8) placée dans une salle blanche (classe B GMP / ISO 5) jusqu'à 15 h avec un automate de remplissage (MicroMacro^R MM12, Baxa Royaume-Uni). A 17 h, les NP sont transportées dans les unités de soins, afin d'être administrées au patient dès 18 h. En conséquence, le LCQ dispose de deux heures pour analyser les NP (Figure 14).

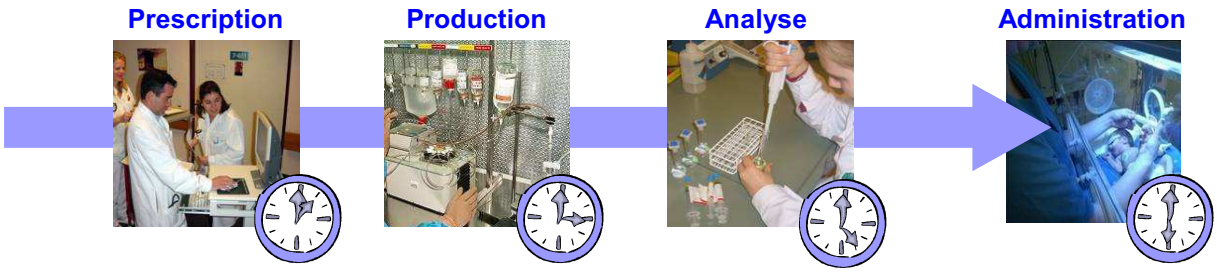

Figure 14: Le processus des nutritions parentérales aux HUG

1.2.1.1 Intérêt de l'analyse des NP avant administration

Les NP sont des mélanges complexes de près de 50 composants fabriqués à partir de plus de 10 solutions différentes. Il existe un risque d'erreur dans la composition ou dans la qualité

microbiologique du produit pouvant survenir lors du processus de fabrication. Comme démontré par l'analyse de risque de Bonnabry et coll., la prescription électronique et l'implémentation d'un automate de remplissage pour la fabrication des NP réduisent la criticité de ce processus [9]. Toutefois, cette analyse a également souligné que des erreurs peuvent apparaître lors de l'utilisation d'un tel système, comme par exemple l'inversion de composés lors du changement de bouteille des solutions. En conséquence, des actions supplémentaires, telles que le contrôle qualité, peuvent identifier des NP non-conformes avant leur administration.

1.2.1.2 Contrôles effectués

La concentration des principaux électrolytes (potassium, sodium, calcium et magnésium) et du glucose sont sans doute les paramètres les plus critiques. Une erreur dans la concentration de ces composés peut induire de graves problèmes cliniques, en particulier chez les prématurés et les nouveau-nés. La stérilité des NP semble également importante, car une contamination microbienne pendant la fabrication peut aussi avoir des conséquences dramatiques pour le patient. Les caractéristiques des méthodes chimiques utilisées sont présentées dans le Tableau 2.

Tableau 2: Caractéristiques des méthodes de contrôle chimique des NP

Caractéristiques	**Electrolytes (Na, K, Ca, Mg)**	**Glucose**
Technique analytique:	CE-C^4D [3]	Spectrophotométrie après réaction enzymatique (hexokinase) [10]
Préparation d'échantillon:	Dilution de l'échantillon et injection dans la CE	100 µl d'échantillon de NP diluée sont ajoutés à 900µL de réactif. La solution est mélangée et analysée après 5 minutes.
Temps d'analyse:	- séparation complète des 4 ions en moins de 4 min. - compatible avec la période des deux heures disponibles - possibilité d'analyser au maximum 15 NP par jour.	moins de 10 minutes (y compris un temps de réaction de 5 minutes).
Performances quantitatives:	Justesse : 99,7 et 101,9% Fidélité intermédiaire: < 2% pour tous les cations. Validation fiable	Justesse : 99,4 et 102,1%, Fidélité intermédiaire: < 2% Validation fiable

Aujourd'hui, aucun test reconnu par les pharmacopées ne peut contrôler la stérilité des préparations pharmaceutiques en moins de deux heures. Afin de garantir la stérilité du processus de fabrication, une validation complète du processus de remplissage aseptique a été réalisée durant la mise en place de l'automate BAXA. En outre, un test de stérilité selon la Pharmacopée européenne, est effectué sur des formulations de type «contrôle» produites le week-end, pour s'assurer, dans une approche d'assurance-qualité, du maintien de la stérilité au cours du processus de mélange aseptique [11]. Ces «poches de contrôle» contiennent des concentrations standards de glucose, chlorure de sodium, chlorure de potassium, chlorure de calcium et d'eau pour préparations injectables et sont produites de la même manière que les NP des patients. Jusqu'à présent, aucun échantillon positif n'a été révélé.

Tous les résultats sont rapportés dans un système informatique conçu en collaboration avec le service d'informatique médicale des HUG. Ainsi, la traçabilité et la sécurité du flux des NP peuvent être encore améliorées [9]. Des NP non-conformes sont éliminées, reproduites, analysées et libérées. Dans ce cas, les médecins sont avertis du retard et des mesures appropriées sont prises. En avril 2011, plus de 2000 NP ont été analysées et 15 NP ont été considérées comme non-conformes parce que la concentration des électrolytes ou du glucose n'était pas dans la limite acceptable de la concentration prescrite. C'est en particulier lorsque de très faibles quantités d'électrolytes doivent être ajoutées que des problèmes de précisions surviennent. Un étalonnage du système automatique de mélange ou le changement de quelques parties de ce système peuvent corriger les erreurs.

1.2.1.3 Conclusion

Le contrôle de qualité des NP a été mis en place à la pharmacie des HUG, avec une quantification des électrolytes et du glucose pour chaque NP avant administration au patient et un test de stérilité complémentaire pour vérifier la qualité microbiologique du processus de fabrication. Ce contrôle qualité est pratiqué avec succès dans la routine quotidienne du laboratoire depuis 2009 et a permis la détection de NP non-conformes. Ainsi, la mise en œuvre de ce contrôle qualité, en plus de la prescription électronique et du système de mélange automatique, a contribué à l'amélioration de la sécurité du processus des NP aux HUG.

1.2.2 Détermination de K, Na, Ca et Mg dans les NP par CE-C^4D

Pour quantifier les électrolytes (K, Na, Ca et Mg) dans les NP, une méthode CE-C^4D a été développée et validée (**article IV**). Même si la CE-C^4D est couramment utilisée pour l'analyse des ions inorganiques, c'est la première fois que cette technique a été appliquée à l'analyse des NP lors d'un contrôle de qualité quotidien.

1.2.2.1 Développement d'une méthode CE-C^4D

La méthode à développer doit être capable de déterminer les quatre électrolytes (K, Na, Ca et Mg) dans un minimum de temps, afin de pouvoir utiliser la méthode pour le contrôle qualité des préparations avant administration au patient. De plus, la concentration des électrolytes varie beaucoup d'une poche à l'autre et une sélectivité suffisante doit être obtenue pour l'analyse simultanée des composés.

Tout d'abord, différents BGE ont été comparés pour trouver la meilleure séparation des quatre cations. Le tampon MES/His à pH 6.1, qui est couramment utilisé pour l'analyse de ions inorganiques [12-25], et un tampon acétate/Tris à pH 4.5 ont donné de bons résultats. Toutefois, pour les deux BGE, la résolution entre Na, Ca et Mg doit être améliorée afin de pouvoir déterminer le Mg et le Ca en présence de haute concentration de Na, comme c'est généralement le cas dans les NP. Le tampon acétate / Tris a été retenu par la suite, car il a déjà montré avec succès son utilité dans l'analyse de suxaméthonium par CE-C^4D [2]. Il possède une faible conductivité et peut être utilisé à une concentration de 100 mM sans générer un courant trop élevé (ca. 20 µA). Le lithium a été choisi comme standard interne, car il n'est pas un constituant des NP et il présente une mobilité beaucoup plus faible que les autres quatre cations et pourra donc être clairement séparé des autres cations.

Pour améliorer la résolution entre les cations, différentes concentrations d'acétate dans le BGE ont été testées (10, 20, 30, 50, 75 et 100 mM). Les premiers essais ont été réalisés avec une concentration d'acétate faible afin de réduire sa conductivité. En augmentant la concentration d'acétate, l'ordre de migration des quatre cations a changé, probablement en raison d'interactions entre les acétates et les cations (figure 15)[26]. Les constantes de complexation de l'acétate avec Ca et Mg sont supérieures à celles du sodium ou du potassium [27], ce qui pourrait expliquer le changement de l'ordre de migration. Dans la littérature, plusieurs études ont montré une amélioration de la sélectivité en CE suite à des interactions entre les analytes et les composants du BGE [12, 24, 26, 28-31]. Un faible agent complexant

(par exemple l'acide α-hydroxyisobutyrique (HIBA)) a été ajouté au BGE, pour modifier la séparation des cations. Ainsi, la mobilité des ions Mg et Ca a diminué suite à une interaction avec ce composé [12, 28, 30].

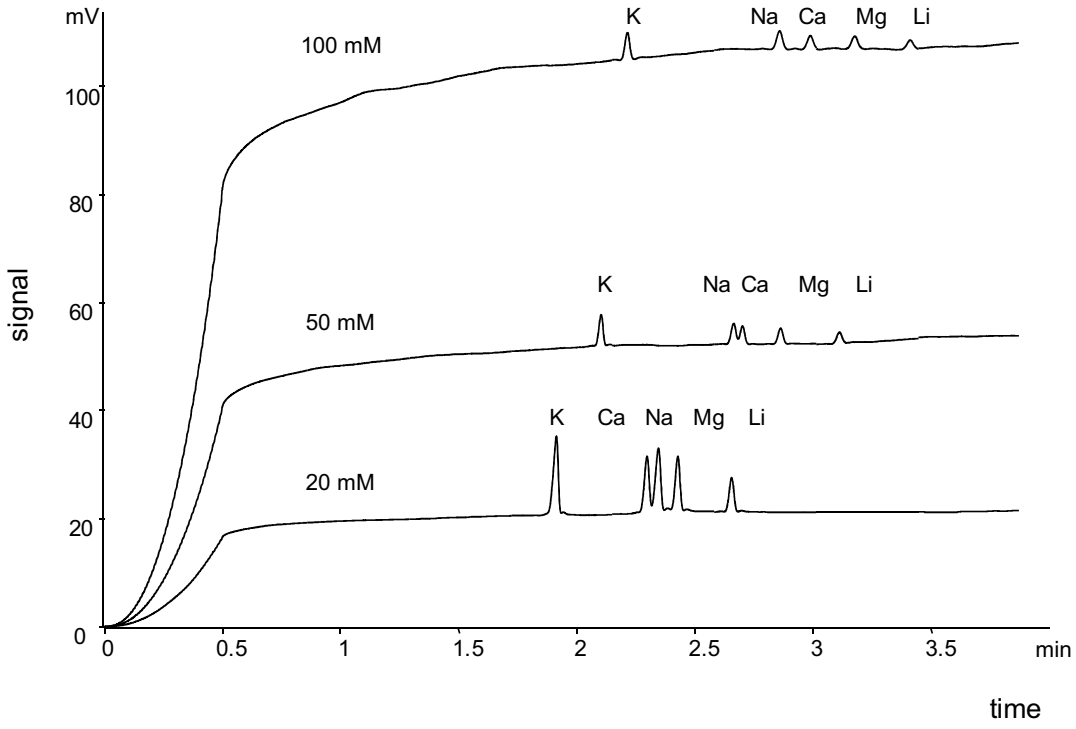

Figure 15 : L'ordre de migration des électrolytes en fonction de la concentration d'acétate dans le BGE (30 kV)

Le même phénomène lié au changement de sélectivité a été observé en modifiant le pH du BGE, car la proportion d'acétate change par rapport à l'acide acétique. La meilleure séparation a été obtenue avec un tampon de pH 4.9, mais le rapport signal sur bruit était plus faible suite à l'augmentation de la conductivité du BGE. Ainsi, un système tampon acétate/Tris (100 mM; pH 4.5) a été sélectionné.

Le dernier essai pour améliorer la résolution entre les quatre cations consistait à ajouter un solvant organique au tampon électrophorétique. La formation de paires d'ions peut être favorisée par la présence de solvants non aqueux en raison de leur faible constante de permittivité [32]. Les solvants organiques modifient les rayons de solvatation des ions contribuant ainsi à un changement de leur mobilité [33, 34]. Ils modifient également la viscosité du BGE, ce qui influence directement la mobilité des analytes. L'ajout de 10 à 30% de

méthanol n'a pas changé la séparation des cations dans les NP, alors qu'il a été utile pour changer la sélectivité dans d'autres études sur l'analyse de cations [34]. Toutefois, l'ajout de 20% d'ACN a amélioré la séparation de manière significative, sans modifier l'ordre de migration. En conséquence, le BGE acétate / Tris (100 mM, pH 4.5) avec 20% d'ACN (v: v) a été sélectionné pour la séparation des quatre cations. La résolution était supérieure à 1.5 pour tous les composés.

1.2.2.2 Validation

Les NP sont fabriquées tous les jours sur prescription médicale et la concentration des différents constituants varie dans chaque cas. La validation de la méthode ne pouvait pas inclure toutes les dilutions et compositions possibles. Pour cette raison, l'étude a été réalisée en considérant une poche test contenant une grande quantité de glucose et d'acides aminés. En général, le sodium est le cation le plus abondant dans les NP, alors que le magnésium est le moins concentré. Le calcium et le magnésium sont présents à des concentrations plus faibles que le sodium ou le potassium. Par conséquent, les solutions standards de Ca et Mg ont été choisies à la moitié de la concentration de Na, K.

La méthode développée a été validée selon les directives de l'ICH en suivant les recommandations SFSTP [4]. Les performances quantitatives ont été évaluées avec trois séries distinctes (j = 3) selon le protocole V1. Ce protocole se base sur un niveau de concentration (k = 1) à l'extrémité supérieure de l'intervalle étudié avec deux répétitions (n = 2) pour les standards de calibration (CS) et trois niveaux de concentration (k = 3) avec quatre répétitions (n = 4) pour les standards de validation (VS). Les VS sont des nutritions reconstituées obtenues par le mélange d'une matrice très dense, composée de fortes concentrations de glucose, acides aminés, héparine et oligoéléments et des concentrations habituelles des solutions d'électrolytes (Na, K, Ca, Mg) .

Les performances quantitatives obtenues en termes de justesse et de précision sont très satisfaisantes (Tableau 3) et l'erreur totale ne dépasse pas les limites acceptables de ± 5% pour tous les niveaux de concentration (Figure 16). Par conséquent, la méthode développée est considérée comme validée pour la détermination des quatre cations dans l'intervalle de dosages étudié et peut être appliquée en routine.

Tableau 3: Résultat de validation des électrolytes dans les NP par CE-C^4D

Concentration théorique [mM]	*Justesse*	*Répétabilité (CV)*	*Fidélité intermédiaire (CV)*
potassium			
1	100.6%	1.0%	1.3%
2	101.8%	1.2%	1.4%
4	101.6%	1.1%	1.1%
sodium			
1	100.9%	1.2%	1.5%
2	100.9%	1.1%	1.5%
4	99.7%	0.9%	1.2%
calcium			
0.5	100.5%	1.1%	1.1%
1	100.4%	1.3%	1.8%
2	99.0%	0.4%	1.1%
magnésium			
0.5	99.1%	1.0%	1.2%
1	99.2%	0.8%	1.1%
2	98.6%	0.8%	0.8%

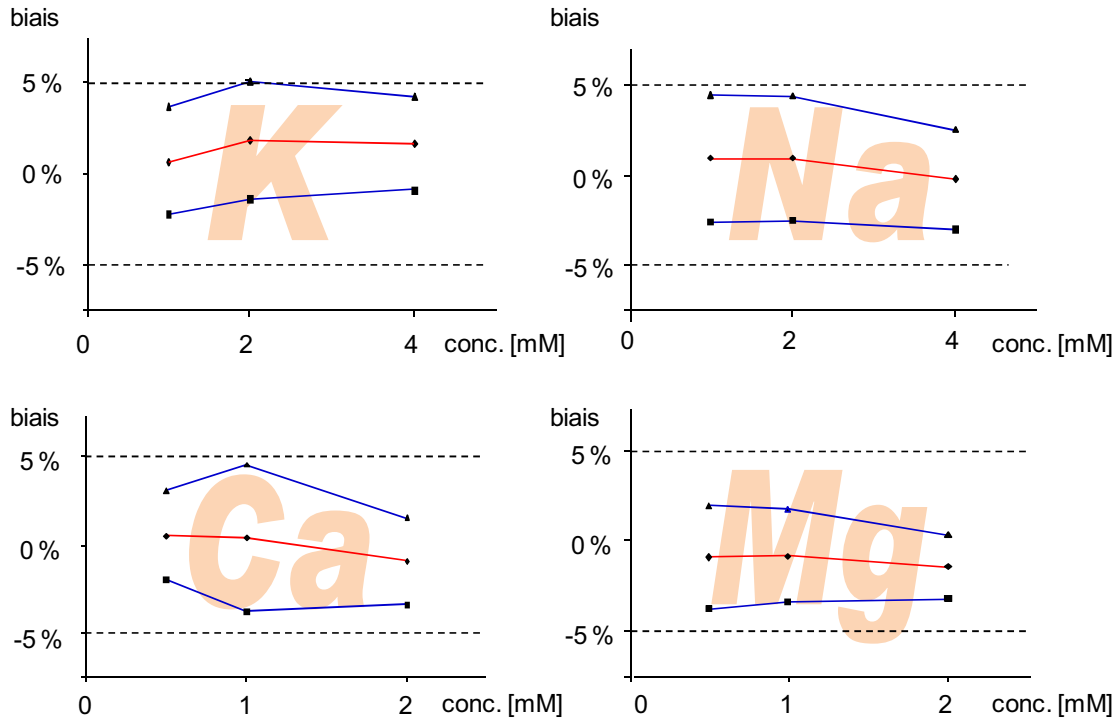

Figure 16: Profils d'exactitude des K, Na, Ca et Mg dans une NP obtenus lors de la validation de la méthode CE-C^4D

1.2.2.3 Analyse de NP aux HUG

Afin de démontrer l'applicabilité de la méthode CE-C^4D à des échantillons réels, la quantification des quatre cations a été effectuée sur plusieurs formulations préparées à la pharmacie des HUG. Pour toutes les formulations, les concentrations des quatre cations se retrouvent dans la limite autorisée de ± 15% de la valeur cible. Cette limite a été fixée dans l'institution par rapport au besoin clinique, en concertation avec les néonatologues. La Figure 17 montre des électrophérogrammes obtenus pour deux nutritions parentérales produites aux HUG.

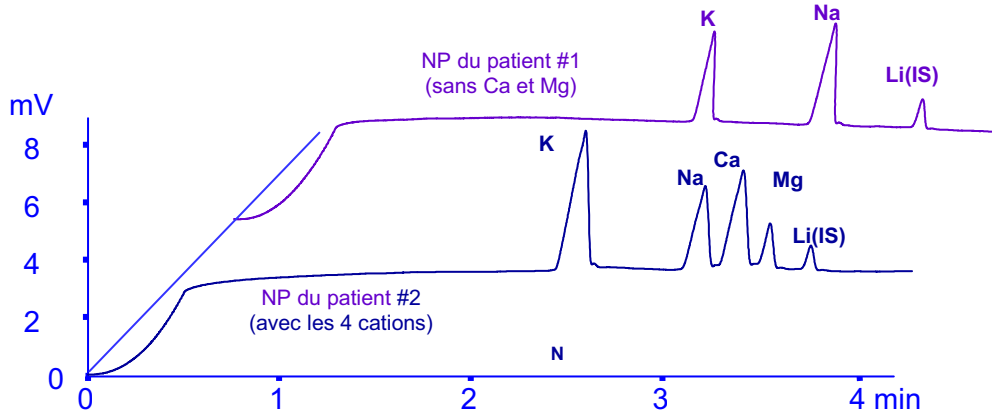

Figure 17: Electrophérogramme de la détermination des électrolytes dans une NP par CE-C^4D.
BGE: 100 mM Tris-acétate pH 4.5 : ACN (90:20, v/v). 30 kV

2.2.2.4 Conclusion

Une méthode CE-C^4D a été développée pour la détermination du potassium, sodium, calcium et magnésium dans les NP. Elle présente de très bonnes performances quantitatives en termes de justesse et fidélité avec un temps d'analyse de moins de 4 minutes pour tous les cations. Ainsi, la méthode peut être utilisée en routine pour analyser les NP avant administration au patient.

1.3. Contrôle qualité des productions de série : seringues prêtes à l'emploi de suxaméthonium

Lors du développement d'une nouvelle formulation, la stabilité de la préparation doit être étudiée afin de définir la date de péremption et les conditions de stockage de cette dernière. Pour ceci, des méthodes indicatrices de stabilité sont nécessaires, permettant la séparation du principe actif et de ses produits de dégradation. Une méthode CE-C^4D a été développée pour analyser le suxaméthonium (SUX) contenu dans une nouvelle formulation **(article V)** dans le cadre d'une étude de stabilité **(article VI)** ainsi que pour le contrôle de routine de cette dernière.

1.3.1 Détermination de suxaméthonium dans une formulation pharmaceutique par CE-C^4D

Comme déjà indiqué au chapitre 1, le suxaméthonium est utilisé en médecine d'urgence et en anesthésiologie comme bloquant neuromusculaire. Pour le contrôle qualité de produits pharmaceutiques de SUX, une méthode indicatrice de stabilité a été développée et validée (**article V**).

1.3.1.1 Développement d'une méthode CE-C^4D

Vu que le suxaméthonium est doublement chargé et ne possède pas de groupement chromophore, la CE a été couplée à un C^4D pour la quantification de ce composé. Ainsi, un BGE avec une faible conductivité a été choisi pour avoir un meilleur rapport signal sur bruit de fond. Le pH du BGE doit être inférieur à 7.0 pour éviter une hydrolyse du suxaméthonium [35]. Pour que la méthode soit indicatrice de la stabilité du suxaméthonium dans des formulations, une bonne sélectivité entre le suxaméthonium, les produits de dégradation et le Na (présent dans les formulations) doit être obtenue. Le potassium a été choisi comme standard interne, vu qu'il est bien séparé des autres composés et est absent de la formulation (SUX, succinylmonocholine (SMC), la choline et de Na).

Parmi différents BGE testés, le système acétate/Tris présente le meilleur compromis pour réaliser la séparation complète du suxaméthonium et de ses produits de dégradation. Même si le pouvoir tampon optimal se trouve à pH 4.8, la sensibilité à été améliorée en diminuant le pH à 4.2, parce que la conductivité du BGE diminue.

Pour améliorer la résolution entre le sodium et le SUX, différentes concentrations d'acide acétique dans le tampon (de 20 à 100 mM) ont été testées. L'acétate agit comme faible agent complexant du SUX et la séparation entre le sodium et le SUX est améliorée à 100 mM. Le système de tampon choisi (100 mM ; pH 4.2) possède une faible conductivité et peut être utilisé à une force ionique de 100 mM sans générer un courant élevé (~20 µA).

La choline a été détectée peu après le SUX et le SMC présente un pic à 6 minutes. Dans ces conditions, l'acide succinique n'interfère pas avec l'analyse des autres composés. L'analyse s'effectue en moins de 4 minutes comme présenté dans la Figure 18.

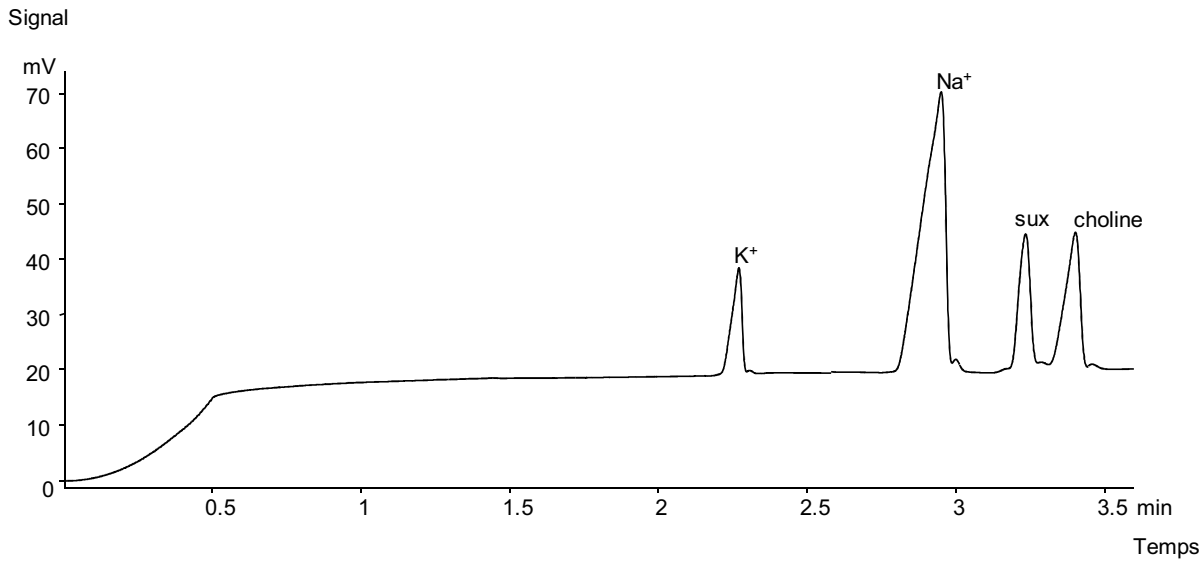

Figure 18: Electrophérogramme obtenu pour l'analyse CE-C^4D d'un échantillon contenant du SUX (0.2 mg/ml), de la choline (0.2 mg/ml) et du K (0.4 mM) en solution aqueuse (présence de Na à 3 mM). BGE: 100 mM Tris-acétate pH 4.2 : ACN (90:10, v/v). 30 kV

Les groupements ammonium quaternaire peuvent interagir avec les parois des capillaires [36-38], et une adsorption importante de SUX à la verrerie a été rapportée [35]. Avec le tampon 100 mM Tris-acétate à pH 4,2, une déformation du pic de SUX a eu lieu après plusieurs injections et la symétrie du pic se détériorait (0,54) (Figure 19A). Pour éviter une adsorption du composé à analyser sur la paroi des capillaires, différentes stratégies peuvent être choisies. L'utilisation de températures élevées ou des pH extrêmes pourrait aider à diminuer cette adsorption [39], mais en raison de la faible stabilité du suxaméthonium, cette approche n'a

pas été étudiée. L'utilisation de capillaires avec un revêtement en PVA (polyvinyle alcool) a été une solution pour surmonter les problèmes d'adsorption des protéines [39] et d'ammoniums quaternaires [40]. La forme du pic de SUX a aussi été améliorée en utilisant ces capillaires PVA. Néanmoins, les capillaires en PVA sont plus onéreux que les capillaires classiques. La dernière possibilité envisagée est l'utilisation de modificateurs organiques [36-38], car ils changent la viscosité du BGE et la solvatation de l'analyte, induisant souvent des meilleures formes de pic. Pour le SUX, l'ajout de 10% d'ACN dans le BGE a donné une bonne symétrie du pic (1.16) et la forme de ce dernier est restée stable durant une série d'injections (Figures 19B).

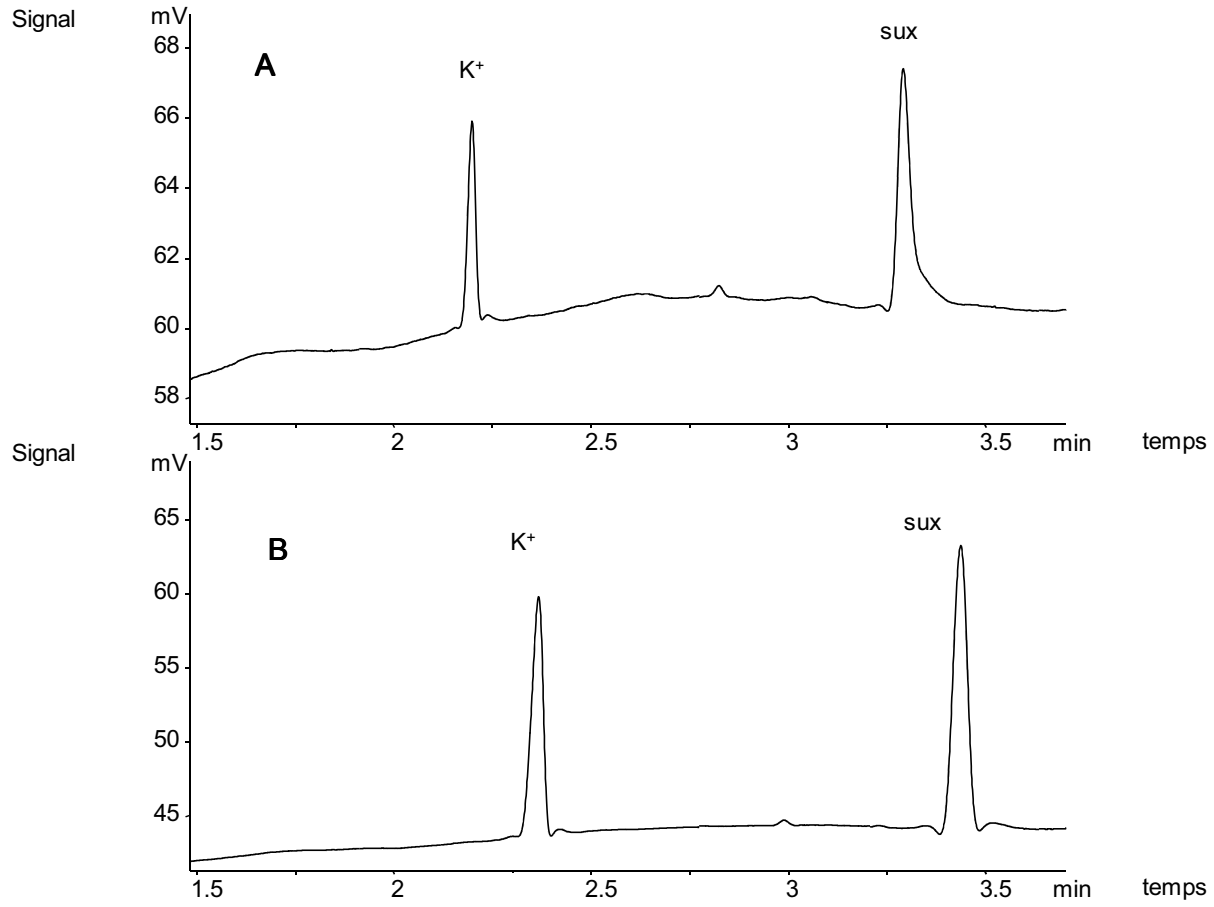

Figure 19: Electrophérogrammes obtenus pour une analyse CE-C^4D d'un échantillon contenant du SUX (0.2 mg/ml) et du K (0.4 mM) en solution aqueuse. 30 kV BGE
A BGE : 100 mM Tris-acétate pH 4.2 ; **B** BGE : 100 mM Tris-acétate pH 4.2 : ACN (90:10, v/v)

1.3.1.2 Validation

La méthode a été validée selon les recommandations SFSTP, avec trois séries indépendantes utilisant le protocole V2 [4]. Les résultats, comprenant la justesse, la répétabilité et la fidélité intermédiaire, sont présentés dans le Tableau 4 et la Figure 20 montre le profil d'exactitude de la méthode. L'erreur totale ne dépasse pas les limites d'acceptation de ± 5% pour les trois niveaux de concentration. Ainsi la méthode peut être considérée comme validée pour l'intervalle de dosages étudié.

Tableau 4: Résultat de validation du suxaméthonium par CE-C^4D

Concentration théorique de suxaméthonium	*Justesse*	*Répétabilité (CV)*	*Fidélité intermédiaire (CV)*
80%	98.8%	1.1%	1.2%
100%	100.2%	1.3%	1.3%
120%	101.1%	0.6%	1.6%

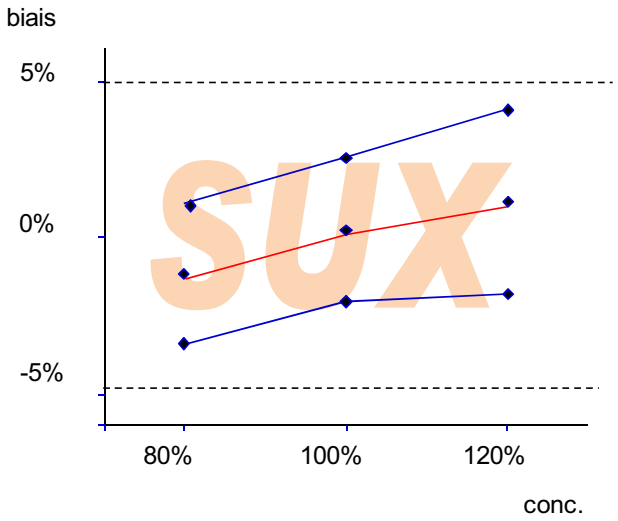

Figure 20: Profil d'exactitude pour l'analyse de SUX par CE-C^4D. Les lignes pointillées indiquent les limites d'acceptation de 5%.

1.3.1.3 Analyse de produits pharmaceutiques commerciaux

Afin de démontrer l'applicabilité de la méthode CE-C^4D à des échantillons réels, le SUX a été quantifié dans trois produits pharmaceutiques: Lysthenon® (2% et 5%) de Nycomed Pharma SA (Dübendorf, Allemagne) et Succinolin® (5%) de Amino AG (Neuenhof, Suisse). Le Lysthenon

(2%) contenait 20.2 ± 0.2 mg·mL^{-1}, Lysthenon (5%) 50.0 ± 0.5 mg·mL^{-1} et Succinolin 51.3 ± 0.5 mg·mL^{-1} de SUX. La concentration de toutes les formulations étaient donc conformes avec les limites autorisées de ± 5% de la valeur cible (19-21 mg·mL^{-1} et 47,5 à 52,5 mg·mL^{-1}, respectivement).

1.3.1.4 Conclusion

Une méthode CE-C^{4}D a été développée pour la détermination du suxaméthonium dans des formulations pharmaceutiques et a présenté de très bonnes performances quantitatives en termes de justesse et précision. Le SUX et ses produits de dégradation sont suffisamment résolus et la méthode peut être utilisée lors d'une étude de stabilité d'une nouvelle formulation et pour le contrôle de routine de cette dernière.

1.3.2 Seringues prêtes à l'emploi de suxaméthonium : étude de stabilité

Le développement d'une formulation prête à l'emploi de suxaméthonium a fait partie de la thèse de Cyril Stucki (thèse n°4220 de l'Université de Genève) dans le cadre de la sécurisation du processus de préparation des médicaments en anesthésiologie [41].

1.3.2.1 Intérêt d'une nouvelle formulation hospitalière

Comme indiqué au chapitre 1, le SUX fait partie des préparations à risque en anesthésiologie et le produit est fréquemment impliqué dans des erreurs médicamenteuses [42]. Pour sécuriser l'administration de ce curarisant, une nouvelle formulation de seringues prêtes à l'emploi de suxaméthonium a été développée à la pharmacie des HUG (Figure 21). Ces seringues prêtes à l'emploi peuvent diminuer le risque de contamination (particulaire et microbiologique) et le risque de mauvaise dilution ou de mauvaise sélection de spécialité pendant la préparation en salle d'opération. L'étiquetage et le conditionnement du produit peuvent en même temps être améliorés, afin de diminuer le risque de confusion entre deux seringues lors de l'administration [41].

La mise au point de la formulation a dû tenir compte de la dose usuelle administrée pour un bolus adulte (i.e. 0.3 à 1.1 mg·kg^{-1}) mais également d'un volume et d'une dilution adéquate pour la pédiatrie. La formulation finale contient 10 mg·mL^{-1} de suxaméthonium dans 10 mL de NaCl 0.9%.

Figure 21: Seringue prête à l'emploi de suxamethonium à 10 mg/mL dans 10 mL de NaCl 0.9%

1.3.2.2 Test de stabilité

Pour s'assurer de la stabilité du suxaméthonium dans son conditionnement final (la seringue), une étude de stabilité de cette formulation a été conduite pendant une année à trois températures différentes (i.e. 4 ± 2°C, 25 ± 2°C, et 40 ± 2°C). Différents paramètres ont été évalués immédiatement après la fabrication (t0), et après 4, 10, 40, 65, 180, 270, et 365 jours. Le dosage du principe actif par CE-C^4D permet de mettre en évidence une dégradation du SUX au cours du temps et l'apparition de produits de dégradation [2]. Le pH indique aussi des changements dans la solution, par exemple liés à l'apparition de produits de dégradation. Le comptage de particules met en évidence un relargage de la part du conditionnement ou une précipitation des composants de la solution. Les tests microbiologiques (test de stérilité et endotoxines) assurent que le conditionnement final conserve sa stérilité et apyrogénicité tout au long de l'étude.

1.3.2.3 Résultats et discussion

Les tests de stérilité et d'endotoxines étaient négatifs pour les 3 températures considérées à tous les temps testés et la formulation n'a pas montré une augmentation de la quantité de particules. L'évolution de la concentration du SUX au cours du temps est montrée dans la Figure 22.

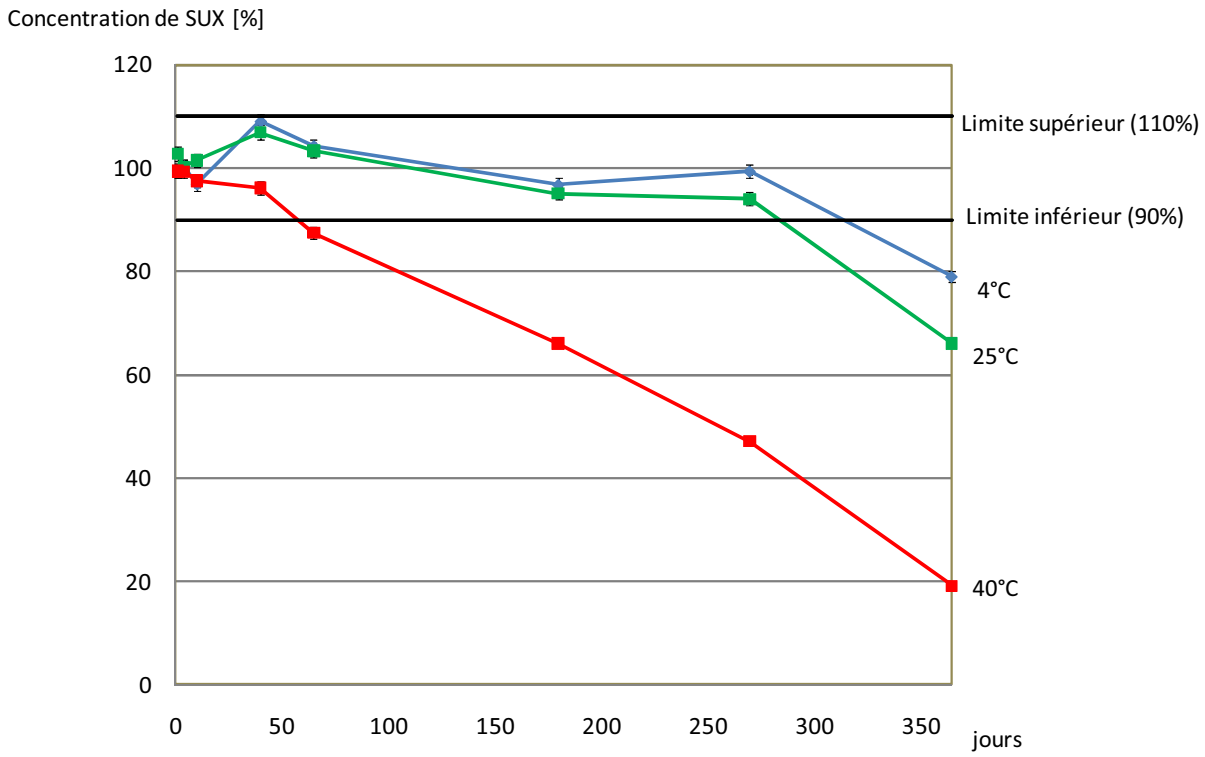

Figure 22: Stabilité du suxaméthonium à 10mg/mL (NaCl 0.9%) dans des seringues en polypropylène pendant 12 mois à 4, 25 et 40 °C

En combinant tous les résultats, le SUX conditionné en seringues prêtes à l'emploi peut être gardé pendant 9 mois au réfrigérateur. Pour des raisons pratiques en anesthésiologie, une conservation à température ambiante est préférée, et la durée a été raccourcie à 6 mois à 25°C, car le pH après 9 mois à 25°C avait diminué et la choline était détectée.

1.3.2.4 Conclusion

Ces seringues prêtes à l'emploi de SUX sont maintenant disponibles pour l'anesthésiologie et présentent une stabilité suffisante pour répondre de manière optimale aux besoins de ce service, tout en garantissant une production efficiente par grands lots. Aujourd'hui, cette formulation fait partie des plus importantes préparations prêtes à l'emploi produite par la pharmacie des HUG avec environ 10'000 seringues fabriquées par année.

1.4 Contrôle qualité de cytotoxiques

Aux HUG, la préparation des chimiothérapies est complètement centralisée à la pharmacie. La fabrication se fait dans des postes de sécurités biologiques de type 3, sous pression négative, afin de protéger l'opérateur (Figure 23).

Figure 23: Production de préparations cytotoxiques à la pharmacie des HUG dans un poste de sécurité biologique de type 3

Environ 50 préparations cytotoxiques sont fabriquées par jour. Parmi toutes ces chimiothérapies, plus de 20% sont des solutions à base de complexes de platine. Ainsi le contrôle qualité de ces formulations présente un intérêt particulier. Dans ce contexte, une méthode MEKC-UV destinée au contrôle qualité de ces préparations a été développée et validée (**article VII**). Pour la détermination d'autres substances cytotoxiques dans des formulations couramment préparées, une méthode LC-MS/MS a été employée (**article IX**).

1.4.1 Analyse des complexes de platine par MEKC et MEEKC

Le cisplatine, le carboplatine, et l'oxaliplatine sont les complexes de platine les plus importants du point de vue clinique (Figure 24). Le cisplatine a été le premier complexe de platine utilisé avec une activité marquée dans les cancers des testicules et des ovaires. Les deux analogues, à savoir le carboplatine et l'oxaliplatine, ont été développés plus tard pour réduire les effets secondaires problématiques du cisplatine (néphrotoxicité, ototoxicité, neuropathie périphérique...). Le carboplatine est utilisé dans le traitement du cancer avancé de l'ovaire et le cancer du poumon, tandis que l'oxaliplatine est indiqué pour le traitement du cancer colorectal métastatique, en association avec le 5-fluorouracile et l'acide folinique [43].

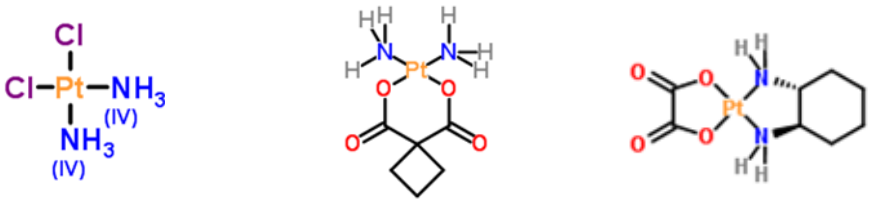

Figure 24: Structures chimiques du cisplatine, carboplatine et oxaliplatine

Durant les dernières années, la CE est devenue une technique de choix pour l'analyse des complexes de platines, en raison de sa haute efficacité et des conditions de séparations douces [44-46]. De plus, la CE permet une analyse en système quasiment clos et le volume de déchet cytotoxique est de l'ordre du µL, ce qui est particulièrement intéressant du point de vue de la sécurité du technicien et de l'environnement. Les complexes de platine sont des complexes de coordination non-chargés. Par conséquent, la CZE n'est pas une technique séparative adaptée, mais la MEKC ou MEEKC sont des approches possibles. Plusieurs méthodes ont été publiées pour l'analyse d'échantillons biologiques [47-57], mais seulement quelques-unes pour l'analyse de formulations [58, 59] sans validation complète. L'**article VII** présente le développement d'une méthode MEKC validée pour le contrôle qualité de ces formulations à l'hôpital.

1.4.1.1 MEKC et MEEKC

La méthode doit être capable de séparer les trois complexes de platine (cisplatine, carboplatine, oxaliplatine), afin de permettre l'identification des composés utilisés pendant la préparation. Vu que la MEEKC présente souvent une meilleure sélectivité que la MEKC, cette approche a été étudiée en premier lieu. Les différentes microémulsions (MEs) étudiées peuvent être consultées directement dans l'**article VII**. La meilleure séparation des trois composés a été obtenue avec une microémulsion de phosphate (20 mM; pH 7,0), 1-butanol 6.6% (w / v), SDS 3.3% (w / v), et heptane 0,78% (w / v) en appliquant une tension de 20 kV (Figure 25).

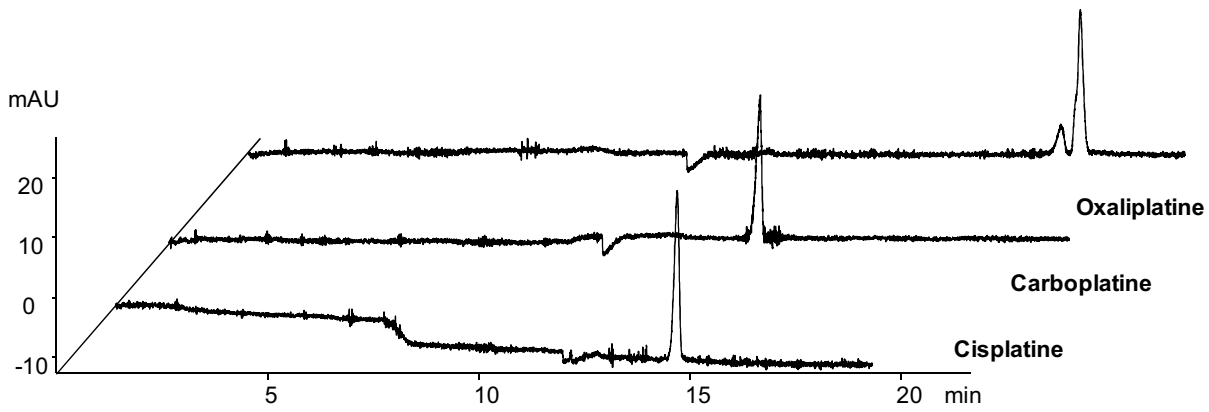

Figure 25: Electrophérogramme d'une analyse MEEKC-UV de solutions aqueuses de cisplatine, carboplatine, et oxaliplatine à 0.5 mg·mL^{-1}. BGE: 20 mM phosphate à pH 7.0 avec 6.6% (w/v) 1-butanol, 3.3% (w/v) SDS, et 0.78% (w/v) heptane. Voltage: 20 kV (article VII)

Pour l'oxaliplatine, deux pics ont été observés avec toutes les MEs testées. Les expériences ont été réalisées avec la spécialité Eloxatin® et le deuxième pic pouvait provenir d'un additif ou d'une impureté présente dans la formulation. Pour cette raison, l'analyse a été répétée avec la substance de référence et les impuretés (A, B, C, D) de la Pharmacopée : avec la substance de référence, exactement le même électrophérogramme a été obtenu. L'impureté A (acide oxalique) ne donne aucun pic et les impuretés B et C ont été complètement séparées de l'oxaliplatine et ne migrent pas avec le pic inconnu. Aucune résolution entre l'oxaliplatine

et l'impureté D, correspondant à l'énantiomère SS, a été observée. Ainsi, ces expériences ont démontré que le second pic observé pour l'oxaliplatine n'est pas dû à des impuretés ou additifs présents dans la formulation. Pour exclure une dégradation de l'oxaliplatine ou une instabilité de la ME dans les conditions de séparation utilisées, quelques paramètres ont été modifiés dans la méthode (voltage, température). Toutefois, aucune différence n'a été relevée. Les MEs ont également été préparées avec des solvants d'origines différentes, pour exclure une réaction entre l'oxaliplatine et une impureté contenue dans le BGE. Mais avec tous les MEs testées, les deux pics ont toujours été observés pour l'oxaliplatine. Etant donné que les expériences pratiques n'ont pas pu apporter une explication à la présence de ce deuxième pic, la littérature a été étudiée. Selon Tyagi et coll. [60], plusieurs conformères coexistent à la température ambiante pour la 1,2-diaminocyclohexane, qui fait partie de la structure de l'oxaliplatine. Les 2 pics pourraient donc éventuellement représenter deux conformères. Une autre étude traitait de la transformation intramoléculaire de certains complexes de platine et discutait de la possibilité de séparer des molécules à cycle ouvert ou fermé, induite par la formation d'adduits entre la SDS et les complexes de platine [61]. Mais à notre connaissance, ce phénomène n'a jamais été rapporté pour l'oxaliplatine.
Pour les formulations à très faible concentration de cisplatine, le rapport signal/bruit obtenu dans les conditions testées était insuffisant pour analyser ces formulations. Compte tenu du problème de détection pour le cisplatine et de la présence non-expliquée des deux pics pour l'analyse de l'oxaliplatine une stratégie alternative basée sur la MEKC a été adoptée.

Après avoir choisi un BGE phosphate à pH 7.0, différentes concentrations de phosphate et de SDS ont été comparées, afin d'améliorer la séparation entre les trois complexes de platine et l'EOF. Plus de détails sur le développement de la méthode peuvent être trouvés directement dans l'**article VII**. La meilleure séparation a été obtenue avec un BGE composé de phosphate 25 mM avec 80 mM de SDS. Dans ces conditions, le courant généré est encore acceptable (~ 50 µA) (Figure 26).

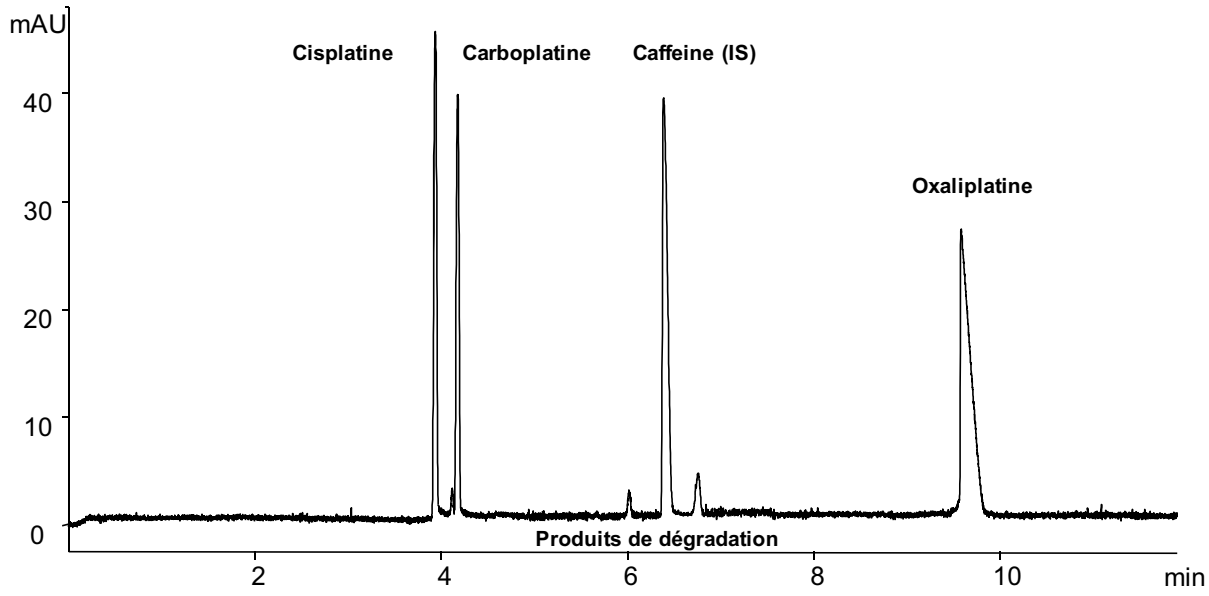

Figure 26: Electrophérogramme obtenu lors de l'analyse MEKC-UV d'une solution aqueuse de cisplatine, carboplatine, oxaliplatine à 0.5 mg·mL^{-1} et de caféine (IS) à 0.1 mg·mL^{-1} BGE: 25 mM phosphate à pH 7.0 avec SDS à 80mM. Voltage: 30 kV (article VII)

Dans les conditions choisies, les produits de dégradation obtenus lors des études de dégradation forcée (chauffage en présence de HCl 1 M, NaOH 1 M et H_2O_2 à 3% pendant 60 min à 80 °C) n'ont pas interféré avec l'analyse des platines. En comparaison avec la MEEKC, la méthode MEKC présente une meilleure sensibilité pour les trois composés et des formulations à faible concentration ont pu être analysées. Ainsi, cette méthode a été validée pour l'analyse des formulations.

1.4.1.2 Validation

Toutes les solutions ont été préparées dans des conditions appropriées pour la manipulation de composés dangereux comme les agents cytotoxiques (sous flux avec deux paires de gants, manchettes, masque et blouse) (Figure 27). En plus, le développement de la méthode a été réalisé avec des spécialités pharmaceutiques pour éviter le contact direct de l'opérateur avec la poudre cytotoxique et pour minimiser les risques de contamination pendant la préparation des solutions. Pour la validation, les solutions standard de cisplatine et oxaliplatine ont été comparées avec des standards de référence Pharmacopée et aucune différence n'a été observée.

Figure 27: Préparation des standards de calibration et de validation avec une protection adaptée

La méthode a été validée selon ICH en suivant les recommandations SFSTP, protocole V2 [4]. Les concentrations des chimiothérapies à base de platine prescrites aux HUG ont été prises en compte pour la détermination des concentrations des standards de validation et un intervalle de dosages de 0,05 à 1 $mg \cdot mL^{-1}$ a été fixé pour les trois composés.

Les résultats obtenus sont reportés dans le Tableau 5. En combinant la justesse et la précision, le profil d'exactitude a été construit (Figure 28).

Tableau 5: Résultat de la validation de la méthode MEKC pour les trois complexes de platine

Concentration théorique $[mg \cdot mL^{-1}]$	*Justesse*	*Répétabilité (CV)*	*Fidélité intermédiaire (CV)*
cisplatin			
0.05	100.6%	1.0%	1.7%
0.50	100.1%	0.7%	1.4%
1.00	100.8%	1.1%	1.1%
carboplatin			
0.05	100.7%	0.8%	1.6%
0.50	100.0%	0.7%	1.4%
1.00	99.7%	0.7%	1.3%
oxaliplatin			
0.05	100.2%	1.4%	1.4%
0.50	99.9%	0.9%	1.3%
1.00	100.3%	1.3%	1.4%

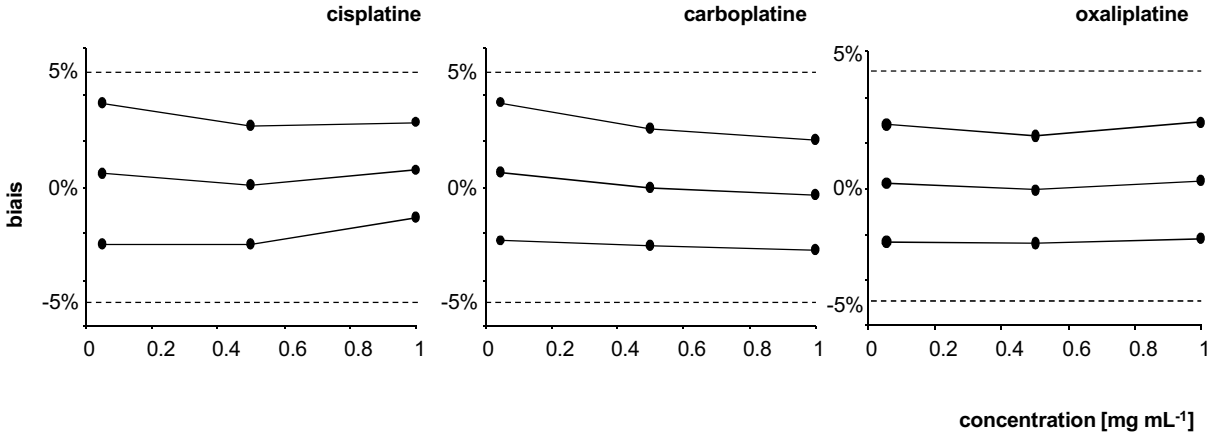

Figure 28: Profils d'exactitude de la méthode MEKC-UV pour le cisplatine, carboplatine, et oxaliplatine contenus dans une formulation pharmaceutique

L'erreur totale ne dépasse pas les limites acceptables de ± 5% et la méthode a été considérée comme exacte pour la détermination des trois composés de platine dans l'intervalle de dosages étudié.

1.4.1.3 Analyse de préparations fabriquées aux HUG

La méthode a été utilisée pour déterminer la concentration de différentes formulations hospitalières. Un prélèvement de 0.5 mL a été fait après fabrication et le SI a été rajouté. Dans quelques cas, la formulation a dû être diluée pour être dans la cible de 0.05 à 1 $mg \cdot mL^{-1}$. Chaque analyse a été répétée deux fois par formulation. Les résultats obtenus (Tableau 6) étaient conformes à la concentration prescrite, avec une limite d'acceptation classique de ± 10% de la valeur cible.

Tableau 6: Analyses par MEKC-UV de cisplatine, carboplatine et oxaliplatine dans des formulations pharmaceutiques préparées à la pharmacie des HUG.

Numéro de lot	**Concentration par rapport à la concentration prescrite**
CYT/10-123162: 104 mg cisplatine dans 604 mL de NaCl 0.9%	107.0% ± 2.8%
CYT/10-122999: 140 mg cisplatine dans 640 mL de NaCl 0.9%	100.8% ± 2.8%
CYT/10-121694: 40 mg cisplatine dans 540 mL de NaCl 0.9%	106.9% ± 2.8%
CYT/10-122599: 529 mg carboplatine dans 303 mL de glucose 5%	96.8% ± 2.8%
CYT/10-122482: 260 mg oxaliplatine dans 302 mL de glucose 5%	95.0% ± 2.0%
CYT/10-122846: 114 mg oxaliplatine dans 273 mL de glucose 5%	94.3% ± 2.0%
CYT/10-123120: 120 mg oxaliplatine dans 274 mL de glucose 5%	97.1% ± 2.0%

1.4.1.4 Conclusion

Une méthode en MEKC a été développée pour la détermination quantitative de cisplatine, carboplatine, et oxaliplatine dans des formulations pharmaceutiques. De très bonnes performances quantitatives en termes de justesse et de précision ont été obtenues avec un temps d'analyse de moins de 10 minutes pour les trois composés de platine. La préparation d'échantillon est très simple avec un minimum de manipulations, afin de sécuriser l'analyse pour l'opérateur.

1.4.2 Analyse de 10 cytotoxiques dans des formulations par LC-MS/MS

Des méthodes multi-composés peuvent être utiles en contrôle qualité de formulations hospitalières, car la diversité de différentes préparations est très grande. Aux HUG, plus de 30 substances cytotoxiques sont utilisées pour la fabrication de chimiothérapies et si un contrôle qualité de ces formulations devait être instauré, une unique méthode pour plusieurs substances serait très attractive. L'**article VIII** décrit le développement d'une telle méthode générique pour 10 composés qui peut être utilisée pour le contrôle qualité.

1.4.2.1 Développement de méthode

La méthode LC-MS/MS a été initialement développée pour le contrôle environnemental et le développement est décrit plus en détail sous 2.1. La méthode permet de déterminer 10 cytotoxiques simultanément avec un temps d'analyse de 21 minutes (équilibrage de la colonne inclu). Dans le cadre du contrôle qualité de formulations pharmaceutiques, la méthode a montré de bonnes performances quantitatives à une concentration donnée (200 $ng \cdot mL^{-1}$) avec une erreur totale de moins de 5% pour tous les composés excepté l'irinotecan (erreur totale de 10%). Une dilution des formulations dans de l'acide formique 0.1% était effectuée avant l'analyse LC-MS/MS afin d'obtenir une concentration finale en agents cytotoxiques de 200 $ng \cdot mL^{-1}$. Ainsi, la méthode a pu être appliquée avec succès pour le contrôle qualité de quelques formulations produites à la pharmacie des HUG.

1.4.2.2 Analyse de préparations fabriquées aux HUG

Toutes les préparations cytotoxiques analysées ont donné un résultat conforme à la prescription (Tableau 7). Selon les règles usuelles, une concentration entre 90 et 110% est tolérée pour ces préparations.

Tableau 7: Contrôle qualité de préparations cytotoxiques par LC-MS/MS

Préparation cytotoxique	**Concentration calculée par rapport à la prescription**
CYT/10-115058: Gemcitabine 1800 mg dans 340 mL NaCl 0.9%	97% ± 2%
CYT/10-114026: Methotrexate 30 mg dans 62 mL glucose 5%	97% ± 2%
CYT/10-113521: Ifosfamide 2316 mg dans 1080 mL glucose 5%	97% ± 2%
CYT/10-116360: Cyclophosphamide 860 mg dans 293 mL glucose 5%	100 ± 2%
CYT/10-116778: Doxorubicine 44 mg in 72 mL dans NaCl 0.9%	106 ± 4%
CYT/10-115322: Epirubicine 190 mg dans 145 mL NaCl 0.9%	98% ± 4%
CYT/10-116578: Vincristine 1 mg 51 mL dans glucose 5%	93% ± 2%

La plupart des préparations cytotoxiques ne contiennent qu'un seul principe actif. Si cette méthode est appliquée en contrôle qualité de routine des formulations produites avant administration au patient, le temps d'analyse peut poser des problèmes et le développement doit alors être orienté vers des méthodes plus rapides. Par contre, la méthode trouve une raison d'être en assurance qualité avec des contrôles à postériori, pour vérifier la qualité des processus de fabrication de chimiothérapies. Etant donné que la concentration cible de l'échantillon injecté est faible, seuls quelques microlitres de la formulation sont nécessaires pour la préparation d'échantillon. La grande sélectivité offerte par la spectrométrie de masse, permet aussi d'utiliser la méthode pour des essais de stabilité, en étudiant d'abord les produits de dégradation de chaque substance.

1.4.2.3 Conclusion

La méthode LC-MS/MS a été utilisée avec succès pour le contrôle qualité de différentes formulations cytotoxiques. Il s'agit d'une méthode ''générique'', capable de déterminer simultanément 10 composés, qui peut être adaptée à d'autres applications, comme le contrôle environnemental (Chapitre 2 : 1.2 Toxicologie).

2. Toxicologie : analyse de traces de cytotoxiques sur des surfaces

L'analyse de composés anticancéreux dans l'environnement suscite un grand intérêt depuis quelques années (**article I**). Des traces de cytotoxiques ont été trouvées dans les locaux de préparation et d'administration de chimiothérapie [62-72] et dans les urines du personnel manipulant des composés cytotoxiques [73-79], montrant ainsi que le risque d'exposition à ces substances est réel. Un suivi biologique ou environnemental peut être mis en place, afin d'étudier les conditions de travail et de réduire l'exposition du personnel à ces substances [80]. Dans ce but, une méthode de prélèvement couplée à une analyse par LC-MS/MS a été développée dans ce travail de thèse permettant la détermination de 10 substances cytotoxiques sur différentes surfaces (**articles VIII, IX**). Finalement, cette méthode a été utilisée pour évaluer la contamination de surface au sein de l'unité cytotoxique de la pharmacie des HUG (**article IX**).

2.1 Analyse simultanée de 10 cytotoxiques par LC-MS/MS

Peu de méthodes validées et capables de déterminer simultanément plusieurs substances cytotoxiques ont été publiées. Ces méthodes multi-composés peuvent se révéler utiles dans le contrôle qualité (Chapitre 2 ; 1.4.2) ainsi que dans l'établissement d'un aperçu de la contamination par différents cytotoxiques dans le cadre d'un contrôle environnemental. L'**article VIII** expose le développement et la validation d'une telle méthode LC-MS/MS pour la quantification simultanée de 10 cytotoxiques.

2.1.1 Développement d'une méthode LC-MS/MS

Sélection de 10 substances cytotoxiques et du standard interne

Parmi les 20 composés cytotoxiques les plus utilisés à la pharmacie des HUG, 10 composés ont été choisis, i.e. cytarabine, gemcitabine, méthotrexate, étoposide phosphate, cyclophosphamide, ifosfamide, irinotecan, doxorubicine, épirubicine et vincristine (en noir dans la Figure 29).

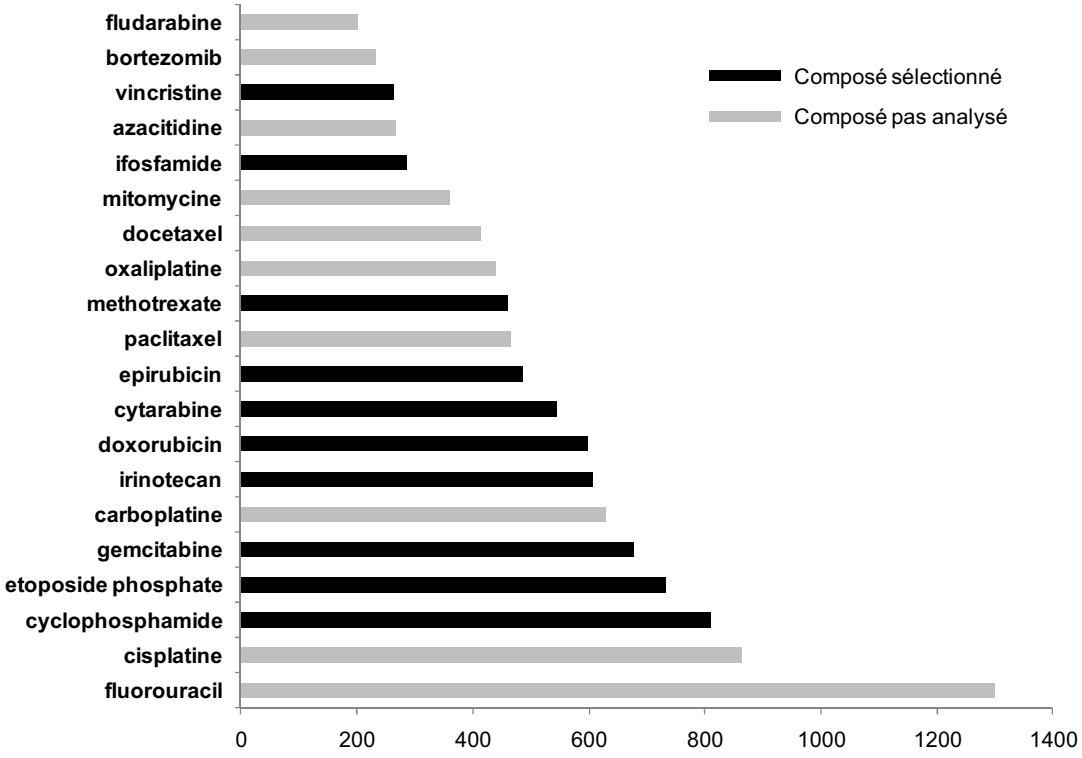

Figure 29: Quantité de préparations cytotoxiques fabriquées en 2009 par composé

La sélection était un compromis entre la fréquence de prescription, la toxicité de ces composés et les considérations analytiques liées à de tels composés. Les substances sélectionnées appartiennent à plusieurs familles cytotoxiques avec des structures chimiques et toxicités différentes, ce qui permet d'obtenir un excellent aperçu d'une éventuelle contamination. Les structures chimiques des candidats choisis sont représentées sur la Figure 30. Les complexes de platine et le 5-FU qui sont souvent préparés aux HUG n'ont pas été sélectionnés, suites à des aspects analytiques. Le 5-FU est un composé très polaire et il n'était pas retenu par la colonne dans nos conditions analytiques. Les complexes de platine (cisplatine, carboplatine et oxaliplatine) nécessitent d'autres techniques analytiques telles que l'ICP-MS ou la voltamétrie pour atteindre des LOQs intéressantes pour l'analyse de traces de cytotoxique.

Le [^{13}C, $^{2}H_3$]-méthotrexate a été choisi comme SI, afin d'exclure une contamination croisée, car il présente une forte réponse en ESI-MS/MS et est élue au milieu de la fenêtre de détection des 10 composés étudiés.

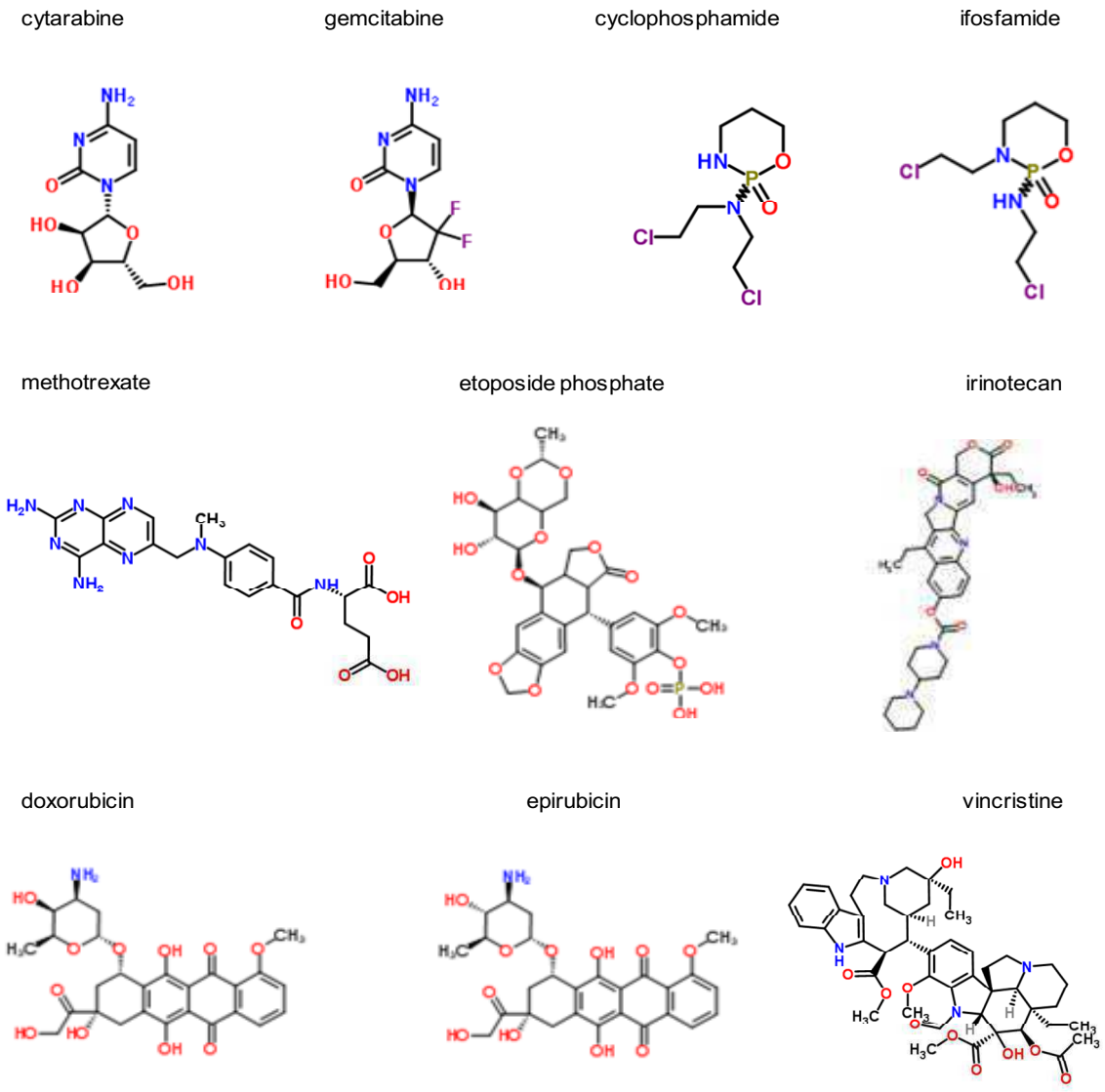

Figure 30: Structures chimiques des médicaments cytotoxiques sélectionnés

Optimisation des conditions LC-ESI-MS/MS

La séparation chromatographique a été réalisée en phase inverse, en utilisant un gradient eau/ACN en présence d'acide formique (FA) à 0,1%. La détection par spectrométrie de masse en mode tandem a été réalisée sur un triple quadripôle en mode SRM. Les conditions chromatographiques ont été optimisées afin d'obtenir une bonne séparation entre tous les composés dans un temps d'analyse raisonnable. Le gradient choisi est présenté dans le Tableau 8.

Tableau 8: Gradient de la méthode LC-MS/MS appliqué à un débit de 200 µL·min^{-1}

Temps [min]	Solvent A: Eau (%)	Solvent B: ACN (%)	Solvent C: Acide formique 1% [%]
0.0	88	2	10
2.0	88	2	10
2.5	69	21	10
10.0	69	21	10
13.0	60	30	10
13.5	40	50	10
15.5	40	50	10
16.0	88	2	10
21.0	88	2	10

Pour tous les composés, les paramètres ESI et MS/MS ont été étudiés. Un voltage de 4 kV a été appliqué à une température de 325°C, avec une pression du gaz de nébulisation de 45 psi et un gaz auxiliaire de 2 psi. Les conditions MS/MS sélectionnées pour chaque composé sont présentées dans le Tableau 9.

Tableau 9: Paramètres MS/MS pour l'analyse de 10 cytotoxiques avec le [^{13}C, $^{2}H_3$]-methotrexate comme SI

Segment [min]	Substance	Précurseur (*m*/*z*)	Fragment (*m*/*z*)	Energie de collision (eV)	Temps de rétention (min)
0 - 2.2	cytarabine	244.0	112.3	15	1.9
2.2 - 4	gemcitabine	264.7	112.3	20	2.6
4 - 7	méthotrexate	455.2	308.0	20	6.0
	[^{13}C, $^{2}H_3$] méthotrexate	459.2	312.2	20	6.0
7 - 10	étoposide 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	doxorubicine	544.6	379.2, 397.1	15	14.8
	epirubicine	544.6	379.2, 397.1	15	15.3
	vincristine	413.3	353.2	30	15.2

Tous les composés ont été détectés en moins de 16 minutes (21 minutes, avec le rééquilibrage de la colonne) et ils sont distingués soit par la chromatographie soit par la masse. Les chromatogrammes obtenus lors de l'analyse d'un échantillon contenant les 10 cytotoxiques et le SI à 50 ng·mL^{-1} dans FA 0,1%, est montré dans la Figure 31.

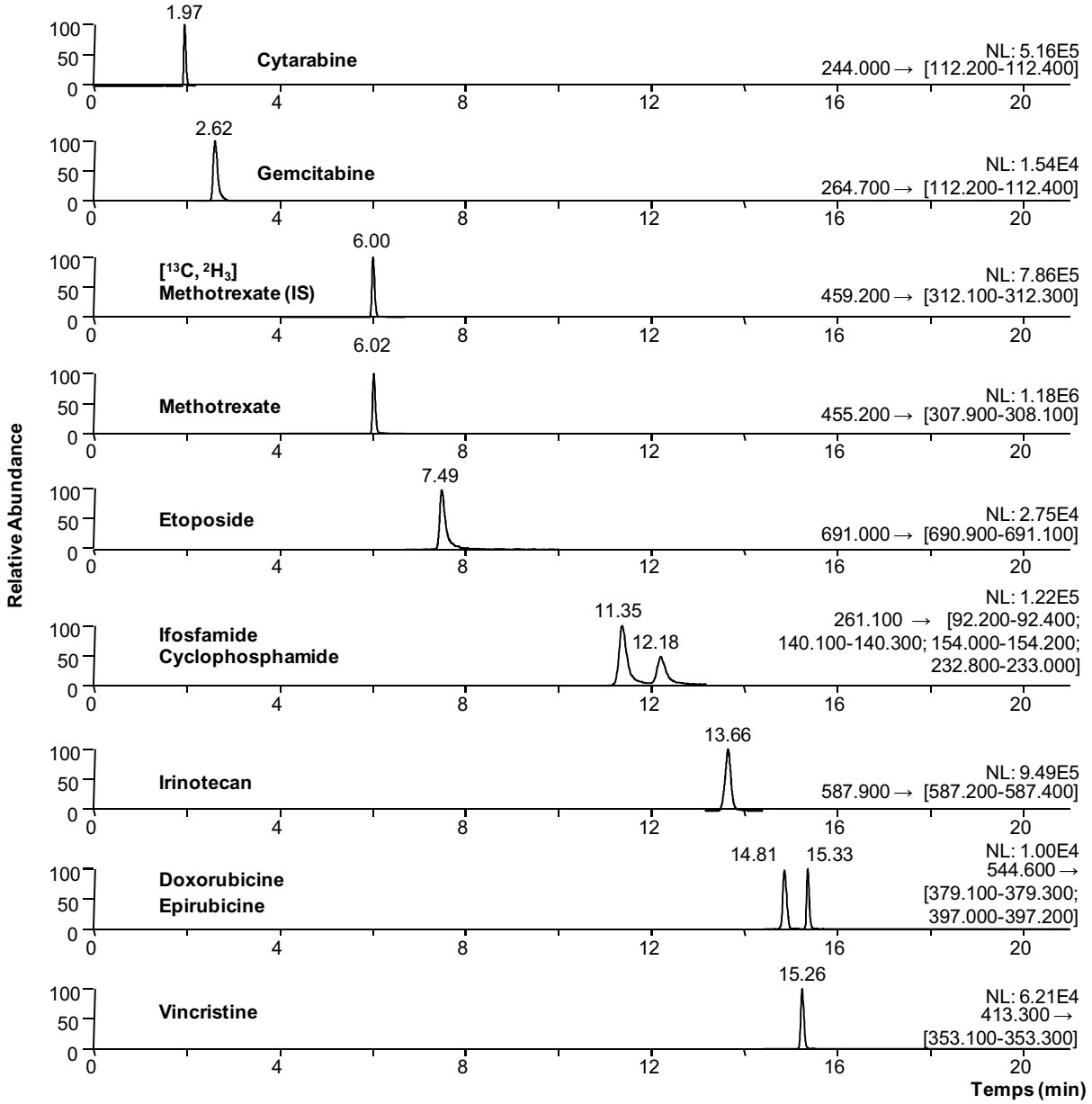

Figure 31: Chromatogramme obtenu lors de l'analyse LC-MS/MS d'un échantillon contenant les 10 composés cytotoxiques et le standard interne à 50 ng·mL^{-1}. Colonne: ZORBAX SB-C18 RR 2.1 x 100 mm 3.5 µm; débit: 200 µL min^{-1}; gradient et paramètres MS/MS sont décrits dans les tableaux 8 et 9.

2.1.2 Validation

Une validation a été réalisée pour estimer les performances quantitatives de la méthode analytique. Comme pour l'analyse des complexes de platine (1.4.1.4), la préparation des solutions a été faite dans des conditions sécurisées pour la manipulation de toxiques et des spécialités pharmaceutiques ont été utilisées à la place des poudres cytotoxiques. Les

échantillons de calibration et de validation ont été préparés dans l'acide formique 0.1% afin d'éviter une adsorption de quelques substances cytotoxiques (ex. anthracyclines) à la verrerie. La validation a été effectuée sur trois séries indépendantes: chaque série est constituée (i) de standards de calibration et de validation fraîchement préparés ainsi que de nouveaux solvants, (ii) du lavage de la colonne et du système LC, (iii) d'un arrêt de l'appareillage, (iv) du nettoyage du capillaire et du cône de la MS avec de l'eau et du méthanol et (v) d'un réglage et calibration du système MS. Les calculs ont été faits en utilisant les rapports d'aires des 10 cytotoxiques sur l'aire du SI. Deux préparations d'échantillons indépendantes (échantillons de calibration et de validation) à 12 niveaux de concentration (de 0.25 à 200 $ng \cdot mL^{-1}$) ont été effectuées avec des injections en triplica.

Un modèle de régression linéaire pondéré (1/x) a permis d'obtenir les meilleures performances quantitatives dans l'intervalle de dosage étudié avec un coefficient de détermination (r^2) supérieur à 0.996 pour tous les composés (Tableau 10). Pour visualiser la variabilité de la méthode globale, le profil d'exactitude de chaque substance a été construit (Figure 32). L'erreur totale ne dépasse pas ± 30% pour presque tous les composés entre 1 et 200 $ng \cdot mL^{-1}$. Seules la doxorubicine et l'épirubicine ont présenté des valeurs en dehors de cette limite de 30% à 1 $ng \cdot mL^{-1}$. Les LOD (rapport signal/bruit 3:1) sont inférieures à 1 $ng \cdot mL^{-1}$ pour tous les cytotoxiques et les LOQ inférieures (fidélité intermédiaire avec un CV < 15 % ou erreur totale < 30%) ont été déterminées entre 0,25 et 2 $ng \cdot mL^{-1}$. La LOQ supérieure a été fixée à 200 $ng \cdot mL^{-1}$ pour éviter de surcharger inutilement le système avec des agents cytotoxiques. Pour des raisons pratiques, l'intervalle de dosages retenu pour tous les médicaments cytotoxiques est compris entre 1 et 200 $ng \cdot mL^{-1}$. Par conséquent, la méthode développée LC-MS/MS présente des performances quantitatives entièrement compatibles avec un suivi environnemental de médicaments cytotoxiques sur les surfaces. De plus, la méthode développée peut être utilisée pour analyser des formulations produites par la pharmacie des HUG dans un contexte d'assurance qualité (1.4.2).

Tableau 10: Résultats de validation de l'analyse simultanée de 10 substances cytotoxiques par LC-MS/MS.

	Justesse	Répétabilité	Fidélité intermédiaire	LOD [ng·mL^{-1}]	LOQ [ng·mL^{-1}]	Intervalle de dosage [ng·mL^{-1}]	Coefficient de détermination (r^2)
Cytarabine	99-102%	1-3%	1-4%	0.025	0.25	1-200	0.9996
Gemcitabine	96-109%	1-5%	2-9%	0.25	0.5	1-200	0.9994
Methotrexate	97-104%	1-2%	1-2%	0.01	0.25	1-200	0.9997
Etoposide phosphate	99-106%	1-3%	1-7%	0.5	1	1-200	0.9994
Ifosfamide	98-101%	1-8%	2-8%	0.25	1	1-200	0.9993
Cyclophosphamide	99-104%	1-4%	2-5%	0.25	0.5	1-200	0.999
Irinotecan	90-106%	1-4%	3-10%	0.025	1	1-200	0.9992
Doxorubicine	86-103%	2-10%	3-10%	0.5	2	2-200	0.9962
Epirubicine	89-103%	2-9%	2-13%	0.5	2	2-200	0.9976
Vincristine	91-100%	1-7%	1-10%	0.25	1	1-200	0.9988

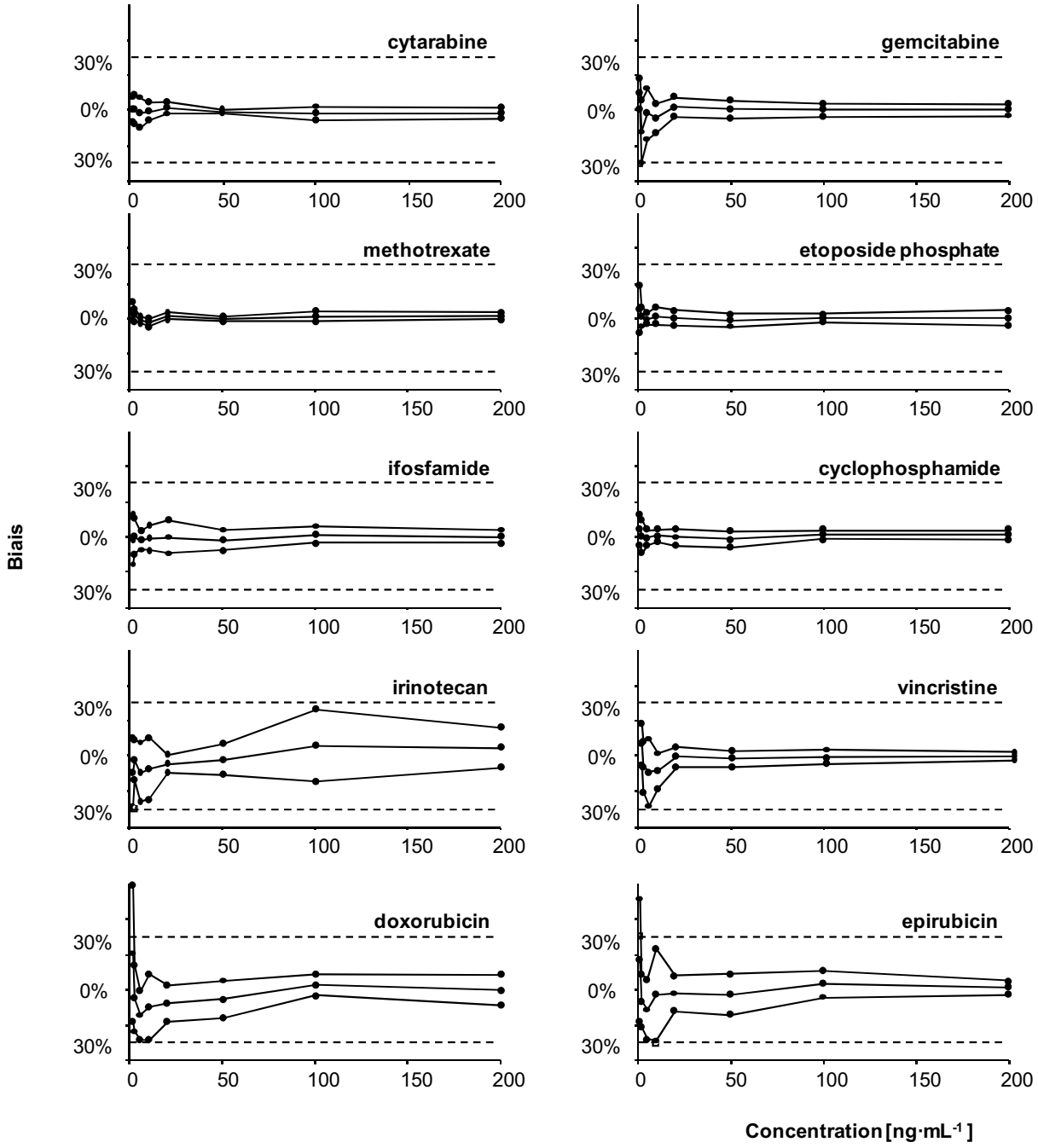

Figure 32: Profils d'exactitude pour la détermination de 10 cytotoxiques par LC-MS/MS de 1 à 200 ng·mL^{-1}. Les lignes en pointillé représentent la limite d'acceptation de ± 30%.

2.1.3 Conclusion

Une méthode simple LC-ESI-MS/MS a été développée pour la quantification simultanée de 10 cytotoxiques (cytarabine, gemcitabine, méthotrexate, étoposide phosphate, cyclophosphamide, ifosfamide, irinotecan, doxorubicine, épirubicine et vincristine) avec un temps d'analyse de 21 minutes (temps de rééquilibrage de la colonne inclu). Cette méthode a été validée et a démontré des performances quantitatives satisfaisantes en termes de LOQ, justesse et précision. Les profils d'exactitude présentent des erreurs totales inférieures à ± 30% pour tous les composés dans leur intervalle de dosages de 1 ou 2 $ng \cdot mL^{-1}$ à 200 $ng \cdot mL^{-1}$ et des erreurs totales inférieures à ± 5% à 200 $ng \cdot mL^{-1}$. La méthode peut donc être utilisée pour différentes applications, en combinant cette méthode avec un traitement d'échantillon adapté. Pour la détermination de la contamination cytotoxique de surfaces, une procédure de prélèvement et d'extraction a été développée (**article IX**).

2.2 Prélèvement et extraction de cytotoxiques sur des surfaces contaminées

Pour mesurer la contamination de cytotoxiques sur des surfaces, trois étapes peuvent être distinguées: i) le prélèvement de composés cytotoxiques de la surface déterminée sur un papier filtre; ii) la désorption de ces substances du papier filtre dans une solution; iii) l'analyse LC-MS/MS.

2.2.1 Désorption

Tout d'abord, plusieurs procédures de désorption (mélange doux, ultrasons pendant 5 et 20 min) ont été comparées en dopant une solution avec les 10 substances sur un papier buvard. Après désorption, l'échantillon est analysé par LC-MS/MS. Chaque procédure a été répétée 3 fois et le rendement ainsi que l'intervalle de confiance ont été calculés (Figure 33). Pour une description détaillée de ces essais, le lecteur peut se référer directement à l'**article IX**.

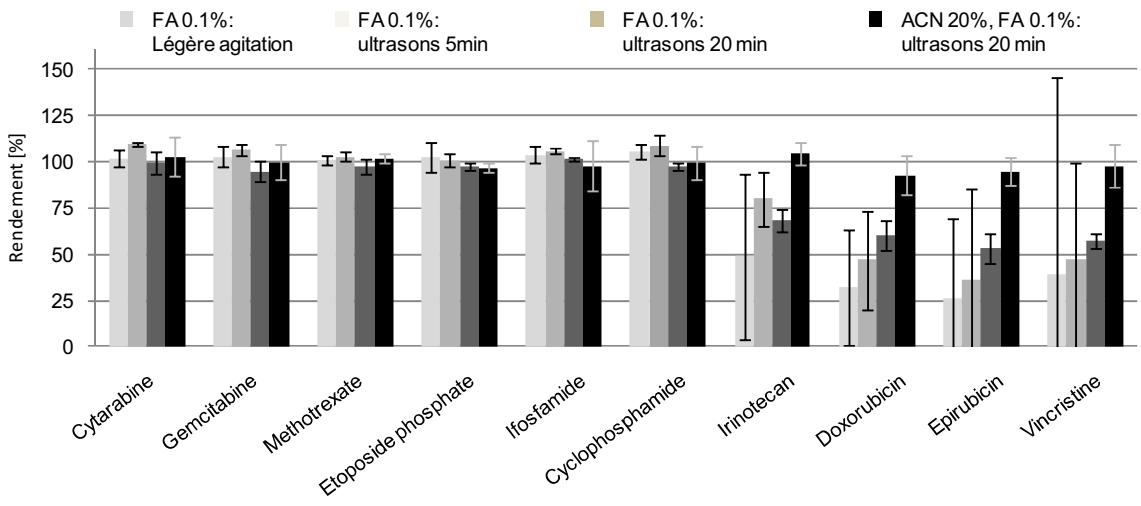

Figure 33: Evaluation de différentes procédures de désorption de papier Whatman (article IX)

Pour 6 composés (cytarabine, gemcitabine, méthotrexate, étoposide phosphate, ifosfamide et cyclophosphamide), des rendements de près de 100% ont été obtenus dans toutes les conditions testées. Pour les autres composés, des ultrasons pendant 20 minutes ont amélioré le taux de désorption par rapport aux autres procédures de désorption. De plus, avec les 20 min d'ultrasons, l'intervalle de confiance a diminué et était inférieur à 10% pour tous les composés. Dans un premier temps, la solution de désorption testée était du FA 0,1% pour être

entièrement compatible avec la méthode LC-MS/MS [81]. Mais des rendements de 50 à 70% ont été obtenus pour l'irinotecan, la doxorubicine, l'épirubicine et la vincristine. Afin d'améliorer la désorption de ces substances, 20% d'ACN ont été ajouté. Ainsi, des rendements proches de 100% sont obtenus pour tous les composés. La méthode LC-MS/MS [81] a été légèrement modifiée, car suite au solvant de désorption contenant 20% d'ACN, la cytarabine et la gemcitabine n'ont plus été retenues par la colonne chromatographique. Ainsi les standards de calibration ont été également préparés dans une solution de 20% ACN avec FA 0,1%. Le manque de séparation chromatographique de ces deux composés n'a pas posé de problème, étant donné l'utilisation d'une détection MS/MS.

La procédure de désorption sélectionnée a été évaluée via 3 séries à 3 niveaux de concentration différents (10, 50 et 200 $ng \cdot mL^{-1}$) avec 4 répétitions. La désorption des cytotoxiques, préalablement dopés sur un papier buvard de Whatman, est réalisée avec 1 mL d'ACN 20% et FA 0.1% dans des tubes en polyéthylène (PE) de 1.5 mL. Après 20 minutes sous ultrasons, le tube est centrifugé à 4000 tours par minutes pendant 5 minutes et 0.5 mL de la solution ont été récupérés et injectés dans le système LC-MS/MS. Le petit volume utilisé pour la désorption permet de réduire les déchets cytotoxiques et d'atteindre des LOQ satisfaisantes. Les performances quantitatives obtenues étaient très satisfaisantes. Le rendement est compris entre 93 et 102%. La précision et la fidélité intermédiaire sont inférieures à 8% à 10, 50 et 200 $ng \cdot mL^{-1}$ pour tous les composés. Dans le Tableau 11, le rendement et la fidélité intermédiaire à 50 $ng \cdot mL^{-1}$ sont présentés pour chaque composé.

Tableau 11: Performances quantitatives pour la désorption des 10 substances cytotoxiques d'un papier buvard de Whatman (50 ng par échantillon) avec 1 mL ACN 20%, FA 0.1%.

	Rendement [%]	Fidélité intermédiaire [%]
Cytarabine	102	5.7
Gemcitabine	99	5.2
Méthotrexate	99	1.6
Etoposide phosphate	95	1.9
Ifosfamide	102	7.6
Cyclophosphamide	100	4.9
Irinotecan	102	3.7
Doxorubicine	93	3.9
Epirubicine	94	3.6
Vincristine	98	6.4

La stabilité des médicaments cytotoxiques sur le papier filtre a été étudiée pendant 3 mois à 3 températures de stockage (-22°C, 4°C et 25°C) et protégée de la lumière. Les meilleures conditions de stockage pour tous les médicaments ont été obtenues à -22 ° C sur une période de 2 mois avec des rendements acceptables compris entre 90 et 110% (Figure 34). Après 3 mois, des valeurs supérieures à 50% ont été obtenues. Par conséquent, les prélèvements peuvent être conservés à -22 ° C pendant 2 mois jusqu'à la procédure de désorption et l'analyse LC-MS/MS.

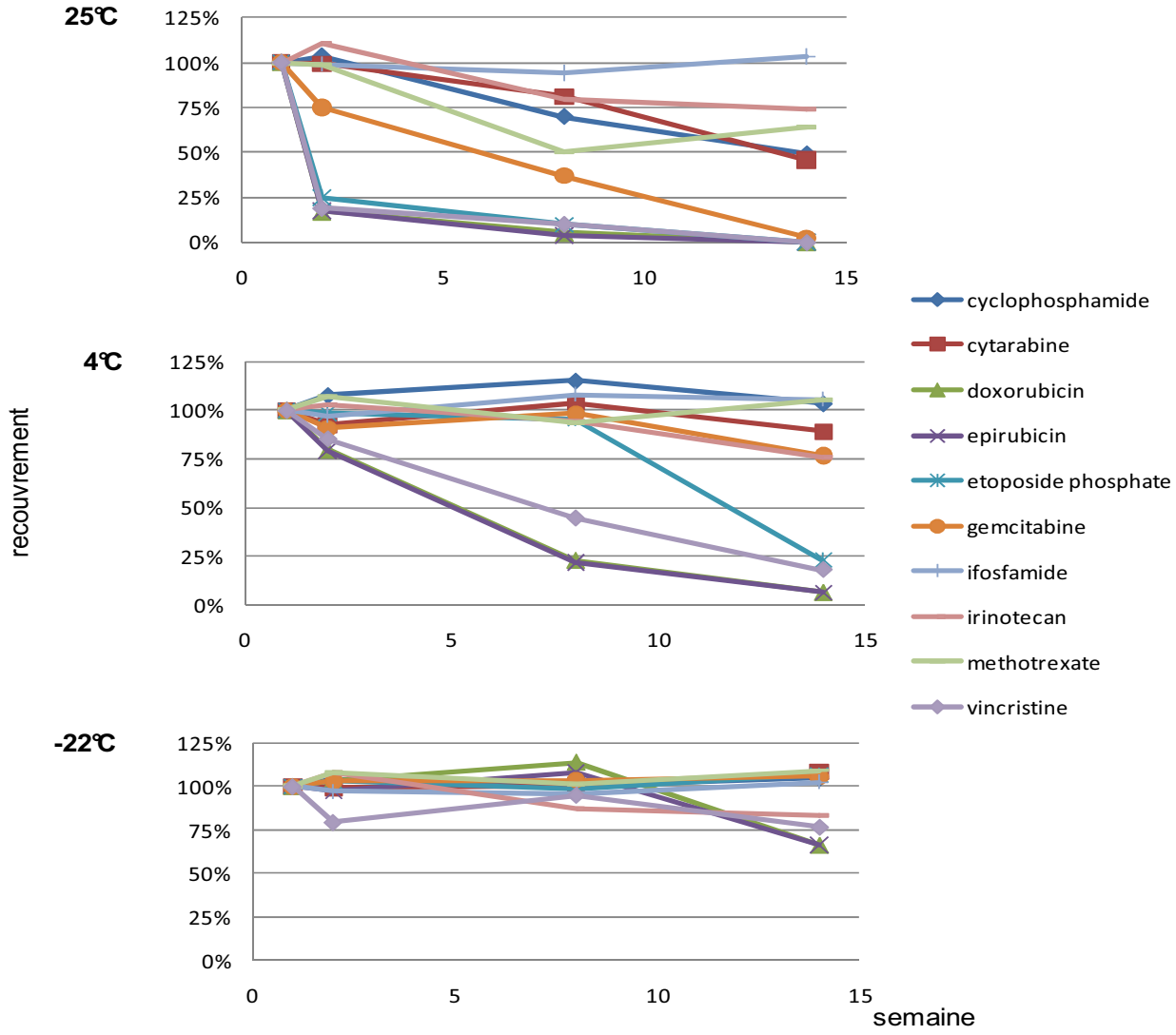

Figure 34: Stabilité des 10 cytotoxiques sur du papier Whatman à -22°C, 4°C and 25°C sur une période de 3 mois

2.2.2 Prélèvement

Après avoir étudié l'étape de désorption, plusieurs procédés de prélèvement ont été évalués. Les prélèvements ont été effectués sur des surfaces en acier inoxydable (10 x 10 cm) afin de comparer les différentes procédures dans les mêmes conditions. Les rendements et intervalles de confiance ont été calculés pour chaque procédure de prélèvement.

Différents supports de prélèvements ont été comparés (lingettes de Texwipe, papier filtre de Ahlstrom et de Whatman) et l'outil le plus adapté correspondait au papier filtre de Whatman® en termes de rendement, intervalle de confiance et utilisation pratique.

Les papiers filtres ont été imbibés avec 0.1 mL de différentes solutions : eau, NaOH 0,01 M, FA 0,1%, ACN 20% avec 0,1%, ACN 50% avec 0,1% de FA, ACN avec 0,1% FA (Figure 35). Le NaOH 0,01 M a du être écarté, car l'étoposide phosphate n'a pas été détecté dans les prélèvements et une dégradation de ce dernier est supposée en milieu alcalin. L'ajout de FA 0,1% à l'eau a permis d'augmenter le rendement pour la gemcitabine, l'étoposide phosphate, l'irinotécan et la vincristine et d'améliorer la précision pour la plupart des cytotoxiques. L'ajout de 20% d'ACN à la solution de FA 0.1% a été utile pour réduire l'adsorption des composés hydrophobes sur la surface et augmenter leurs rendements et la précision (intervalle de confiance inférieur à 20%).

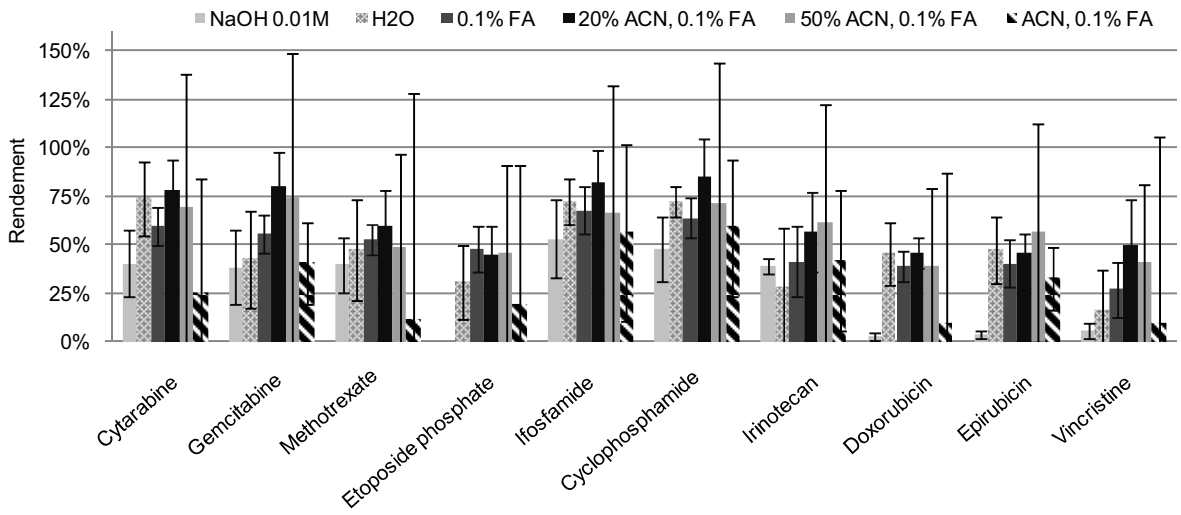

Figure 35: Rendements des cytotoxiques obtenus lors de prélèvements effectués sur une surface en INOX en fonction de la solution utilisée pour mouiller les papiers filtres de Whatman

Pour améliorer le rendement, un deuxième prélèvement avec un nouveau papier filtre et une étape de désorption séparée a été réalisée sur la même surface. Des rendements entre 10 et 15% de la concentration initiale ont été trouvés. En additionnant les deux prélèvements, des rendements totaux inférieurs à 75% ont été obtenus. Cette perte de cytotoxiques peut être due à des problèmes d'adsorption ou de dégradation. Seuls la cytarabine, la gemcitabine, l'ifosfamide et le cyclophosphamide ont présenté des rendements totaux compris entre 90 et 100%. Compte tenu de la faible amélioration des rendements avec un deuxième prélèvement, un seul prélèvement a été effectué dans la procédure finale et un facteur de correction par composé et par surface a été déterminé.

Les performances quantitatives de la méthode pour un prélèvement sur une surface en INOX et sur des poches de perfusion en polypropylène (les deux surfaces les plus souvent en contact avec les cytotoxiques) ont été étudiées via 3 séries indépendantes à 3 niveaux de concentration avec 3 répétitions. Des rendements constants ont été trouvés à 0.1, 0.5 et 2.0 $ng \cdot cm^{-2}$ (correspondant à 10, 50 et 200 $ng \cdot mL^{-1}$) pour tous les composés. Par conséquent, le rendement est considéré comme indépendant de la concentration entre 0.1 et 2.0 $ng \cdot cm^{-2}$. Les performances quantitatives pour le prélèvement sur d'autres surfaces (polystyrène, verre, gants en latex, souris d'ordinateur et cartons filmés) ont été déterminées à une seule concentration, soit 0.5 $ng \cdot cm^{-2}$ (50 $ng \cdot mL^{-1}$). Des rendements de 5 à 98% ont été obtenus en fonction du composé et de la surface (Tableau 12). En général, le prélèvement sur des surfaces lisses, comme l'INOX, le polypropylène, le polystyrène et le verre, a abouti à de plus grands rendements avec de meilleures précisions que les prélèvements sur des surfaces irrégulières telles que les gants en latex ou la souris d'ordinateur. La récupération des cytotoxiques d'un carton n'est pas satisfaisante (rendement entre 5 et 15%), probablement en raison de l'absorption des substances à l'intérieur du carton. Pour les autres surfaces, des rendements supérieurs à 50% avec une fidélité intermédiaire inférieure à 20% ont été déterminés. Certains composés (irinotecan, doxorubicine, épirubicine et vincristine) ont présenté des performances plus faibles, probablement dues à des problèmes d'adsorption ou de dégradation. Etant donné la bonne fidélité des mesures (< 20%), des facteurs de correction ont été calculés pour chaque composé par rapport à la surface en se basant sur les rendements moyens. Ce facteur peut être utilisé pour calculer la concentration de substances cytotoxiques sur les surfaces lors de prélèvements réels.

Les LOQ (basées sur la validation de la méthode LC-MS/MS [81] et les facteurs de correction par rapport à la surface) sont arrondis à 10 ng par prélèvement, ou 0.1 ng·cm^{-2} pour les 10 composés.

Tableau 12: Performances quantitatives de la méthode de prélèvement de 10 composés cytotoxiques sur différentes surfaces à 0.5 ng·cm^{-2}

		INOX	Polypropylène	Polystyrène	Verre	Gant en latex	Souris d'ordinateur	Carton filmé
Cytarabine	Rendement [%]	81	79	76	74	58	69	5
	Fidélité intermédiaire [%]	8.3	7.8	5.8	7.3	18.0	8.8	2.9
Gemcitabine	Rendement [%]	82	79	76	74	59	81	5
	Fidélité intermédiaire [%]	9.5	8.8	6.0	7.7	15.2	6.4	2.5
Methotrexate	Rendement [%]	63	85	75	72	50	64	5
	Fidélité intermédiaire [%]	9.8	5.1	5.2	6.5	12.8	9.8	3.2
Etoposide	Rendement [%]	45	82	73	68	58	81	5
phosphate	Fidélité intermédiaire [%]	7.8	8.2	10.4	11.7	11.0	22.6	1.9
Ifosfamide	Rendement [%]	82	91	79	85	65	98	7
	Fidélité intermédiaire [%]	10.4	8.2	11.2	11.7	17.0	24.8	2.5
Cyclophosphamide	Rendement [%]	86	94	71	80	57	77	5
	Fidélité intermédiaire [%]	10.8	4.8	10.0	15.3	11.4	20.4	2.5
Irinotecan	Rendement [%]	57	84	67	65	27	45	15
	Fidélité intermédiaire [%]	11.8	11.9	14.5	7.7	11.8	12.0	5.3
Doxorubicine	Rendement [%]	46	54	47	53	20	35	9
	Fidélité intermédiaire [%]	5.1	6.1	10.2	10.6	6.8	12.2	3.7
Epirubicine	Rendement [%]	46	58	45	55	16	19	13
	Fidélité intermédiaire [%]	5.2	6.2	6.0	7.6	5.0	11.1	10.6
Vincristine	Rendement [%]	50	55	35	56	27	22	13
	Fidélité intermédiaire [%]	12.0	11.9	13.3	7.6	13.0	12.3	7.1

2.2.3 Conclusion

Une méthode de prélèvement suivie d'une analyse LC-MS/MS (Figure 36) a été développée permettant la quantification simultanée de 10 médicaments cytotoxiques, appartenant à différentes familles thérapeutiques. Des performances quantitatives bien établies en termes de rendement et précision ont été démontrées. Sept surfaces, les plus couramment rencontrées dans les unités de production, ont été examinées et les rendements ont été clairement définis en fonction des types de surfaces et des composés. Ainsi, cette approche est particulièrement adaptée pour le suivi de l'environnement de travail et peut être utilisée pour identifier des voies d'exposition des opérateurs à ces substances et pour étudier des procédures de travail et de décontamination.

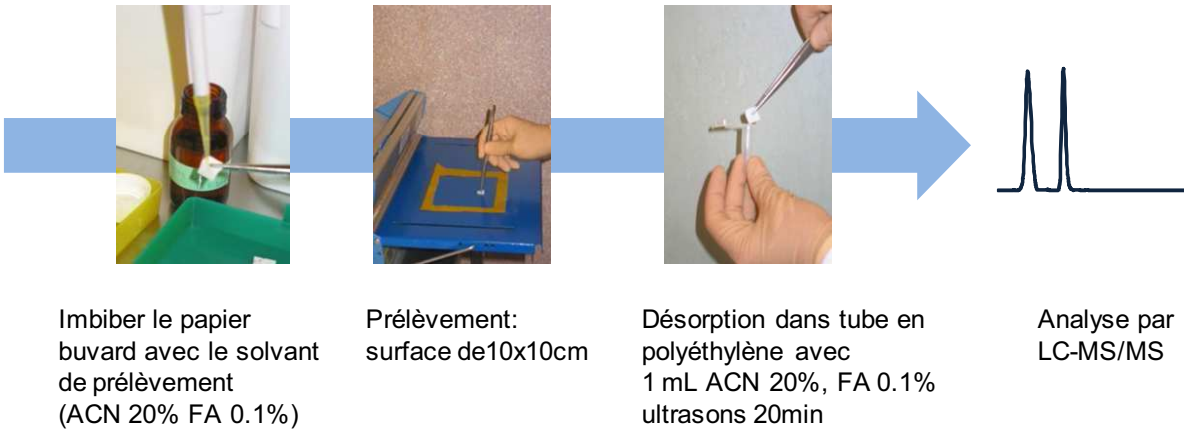

Figure 36: Méthode de prélèvement couplée à la LC-MS/MS

2.3 Contamination cytotoxique à la pharmacie des HUG

La méthode de prélèvement suivie d'une analyse LC-MS/MS a été utilisée pour mesurer la contamination de surfaces au sein de l'unité cytotoxique de la pharmacie des HUG. Les prélèvements ont été effectués dans la zone de production des cytotoxiques sur les surfaces de travail (Figure 37), le sol, les portes des postes à sécurité biologique, dans les lieux de stockage et de la logistique des cytotoxiques (par exemple le téléphone, le réfrigérateur, la souris d'ordinateur (Figure 38). En outre, les flacons des cytotoxiques en provenance des fournisseurs ont été examinés.

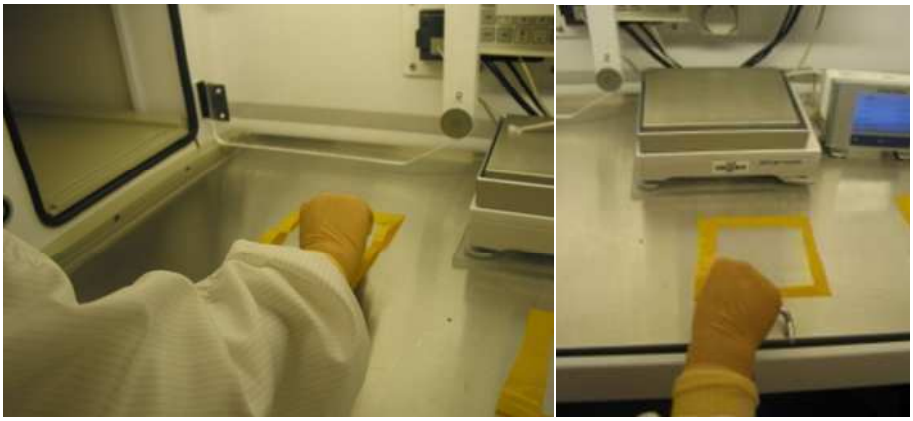

Figure 37: Prélèvements de surface dans un poste de sécurité de type 3, poste de préparation de formulations cytotoxiques à la pharmacie des HUG

Figure 38: Prélèvements dans la salle logistique des cytotoxiques de la pharmacie des HUG

2.3.1 Résultats aux HUG

Les résultats ont été comparés avec les taux de contamination trouvés par d'autres études, mais il était très difficile d'établir un lien direct, car ce ne sont pas toujours les mêmes substances investiguées et les procédures analytiques ne sont pas toujours bien établies. Jusqu'à ce jour, il n'existe pas de limites d'exposition officiellement reconnues. Pour avoir tout de même une idée, la SUVA recommande une concentration inférieure à 0.1 ng·cm^2 par substance, en se basant sur les valeurs obtenues lors d'une étude de contamination réalisée en Suisse alémanique. Cette valeur correspond à 10 ng avec la procédure utilisée.

Les endroits les plus contaminés se trouvaient à l'intérieur des isolateurs (Figure 39). La gemcitabine, l'ifosfamide et le cyclophosphamide sont les composés les plus souvent détectés. Ceci pourrait s'expliquer par la nécessité de reconstituer ces médicaments lyophilisés avant dilution, par les concentrations élevées prescrites et/ou par le grand nombre de produits fabriqués pendant les jours précédent le prélèvement.

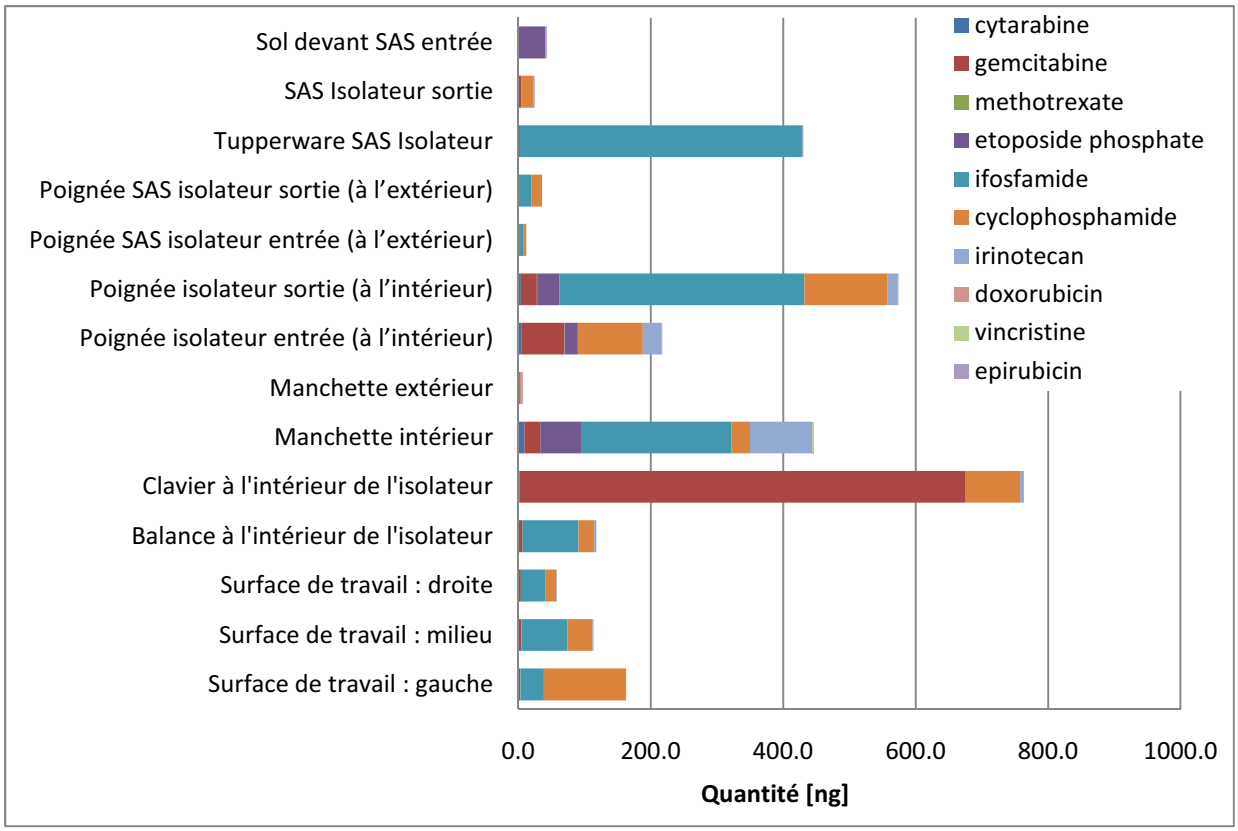

Figure 39: Contamination à l'intérieur et extérieur du poste à sécurité biologique n°2 de la pharmacie des HUG

Les prélèvements ont été faits avant et après nettoyage de l'isolateur, mais aucune diminution de la contamination n'a été observée. Ceci est probablement dû à une mauvaise procédure de décontamination qui est surtout orientée vers une décontamination microbienne et non chimique. Pour réduire la contamination, certaines actions doivent être entreprises, telles qu'une procédure de nettoyage plus efficace, en changeant les lingettes de nettoyage plus souvent et en allant de l'endroit le plus propre au plus sale. Jusqu'à présent, l'isopropanol 70% a été utilisé comme unique produit de décontamination. L'emploi d'un produit de type détergent pourrait aussi améliorer la décontamination chimique.

L'endroit où sont stockés des produits cytotoxiques entamés (reliquats) à l'intérieur de la salle de préparation a aussi montré une forte contamination, surtout de gemcitabine. Pour éviter une telle contamination, les flacons sont désormais emballés individuellement dans des sachets en plastique après la sortie de l'isolateur. Les produits cytotoxiques en provenance de l'industrie ont aussi montré des contaminations à l'extérieur des flacons, par le principe actif du produit, mais aussi par d'autres substances cytotoxiques, probablement lors du rangement de la marchandise.

Dans la salle logistique et les lieux de stockage des produits non entamés, seules quelques traces de substances cytotoxiques ont été détectées (valeurs inférieures à la LOQ de 10 ng).

2.3.2 Conclusion

Cette première application de la méthode de prélèvement et de dosage à un environnement de travail a confirmé sa faisabilité et sa bonne performance.

La quantité de contamination a diminuée fortement en allant de l'intérieur des isolateurs à l'extérieur jusqu'à la salle de logistique. Ceci est très rassurant, car la protection des opérateurs (double gants, manchette, masque,..) est beaucoup plus élevée lors de la fabrication qu'à l'extérieur. Même si les traces de cytotoxiques étaient très rares, (moins de 10 ng par prélèvement), l'importance de porter des gants aussi en logistique, pour toucher les flacons cytotoxiques et les préparations sortantes des lieux de fabrication, a été mise en évidence. Quelques actions ont été prises après cet état des lieux, comme l'emballage des flacons entamé dans des sachets individuellement. D'autres mesures sont en cours, comme l'évaluation puis la mise en place d'une procédure de nettoyage plus efficace.

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Chapitre 3: Articles

Article I

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, multicompound 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]

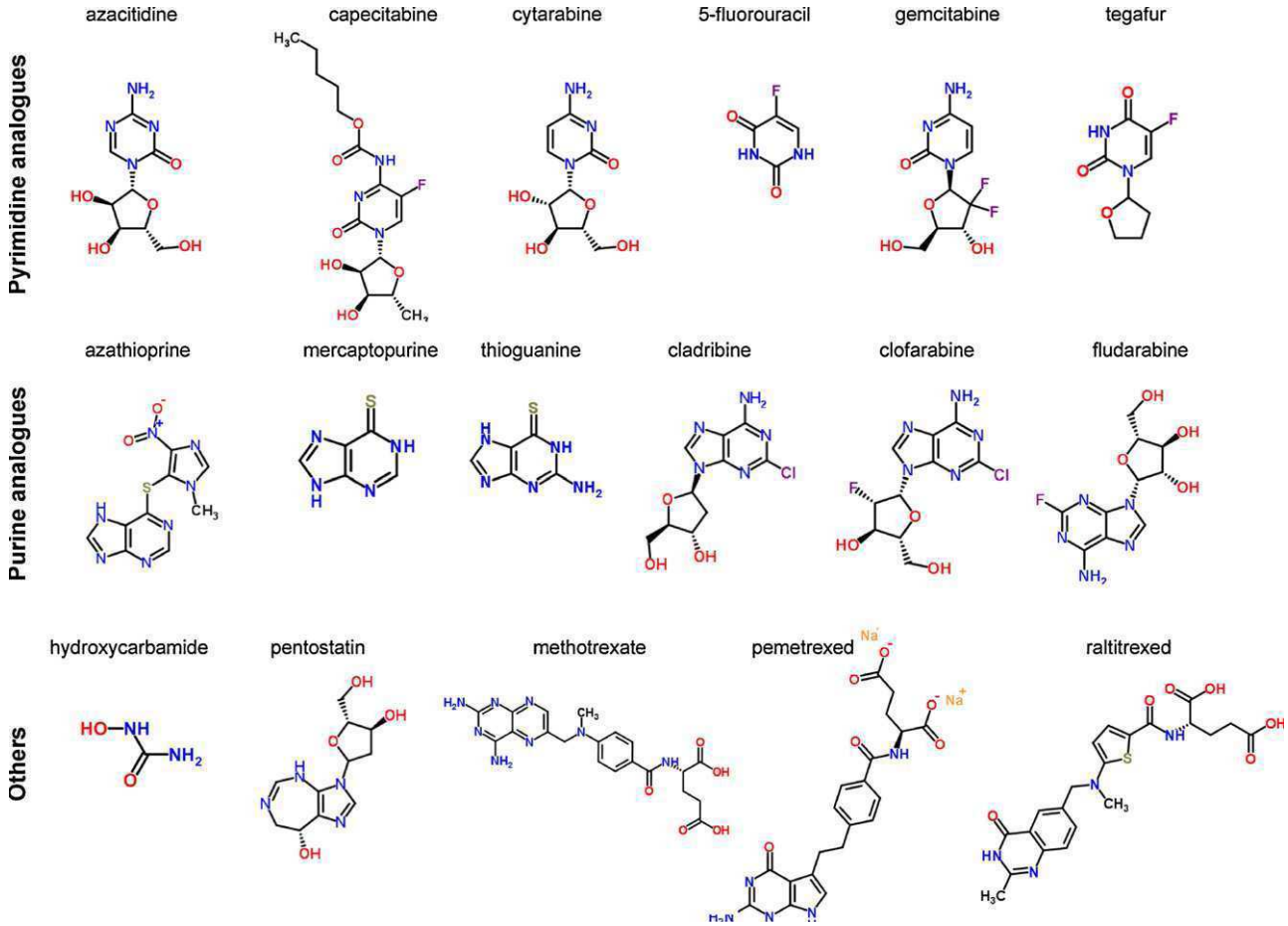

Fig. 1. Chemical structures of antimetabolites.

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-

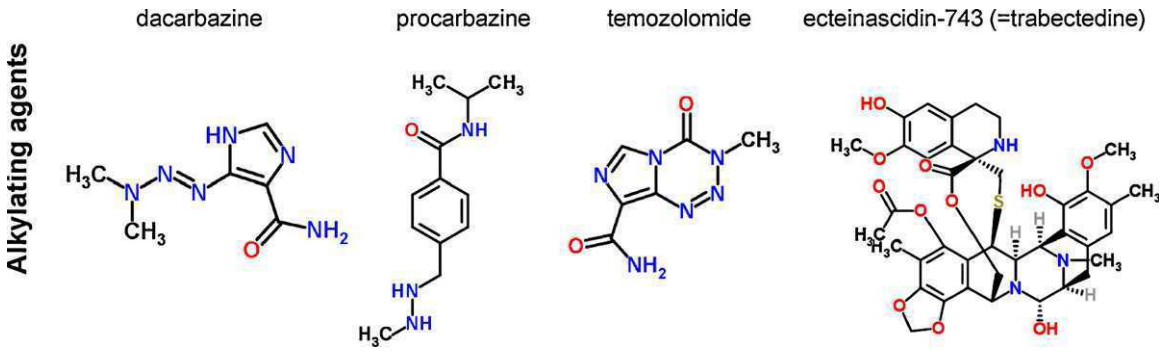

Fig. 2. Chemical structures of DNA-interactive agents: alkylating agents.

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

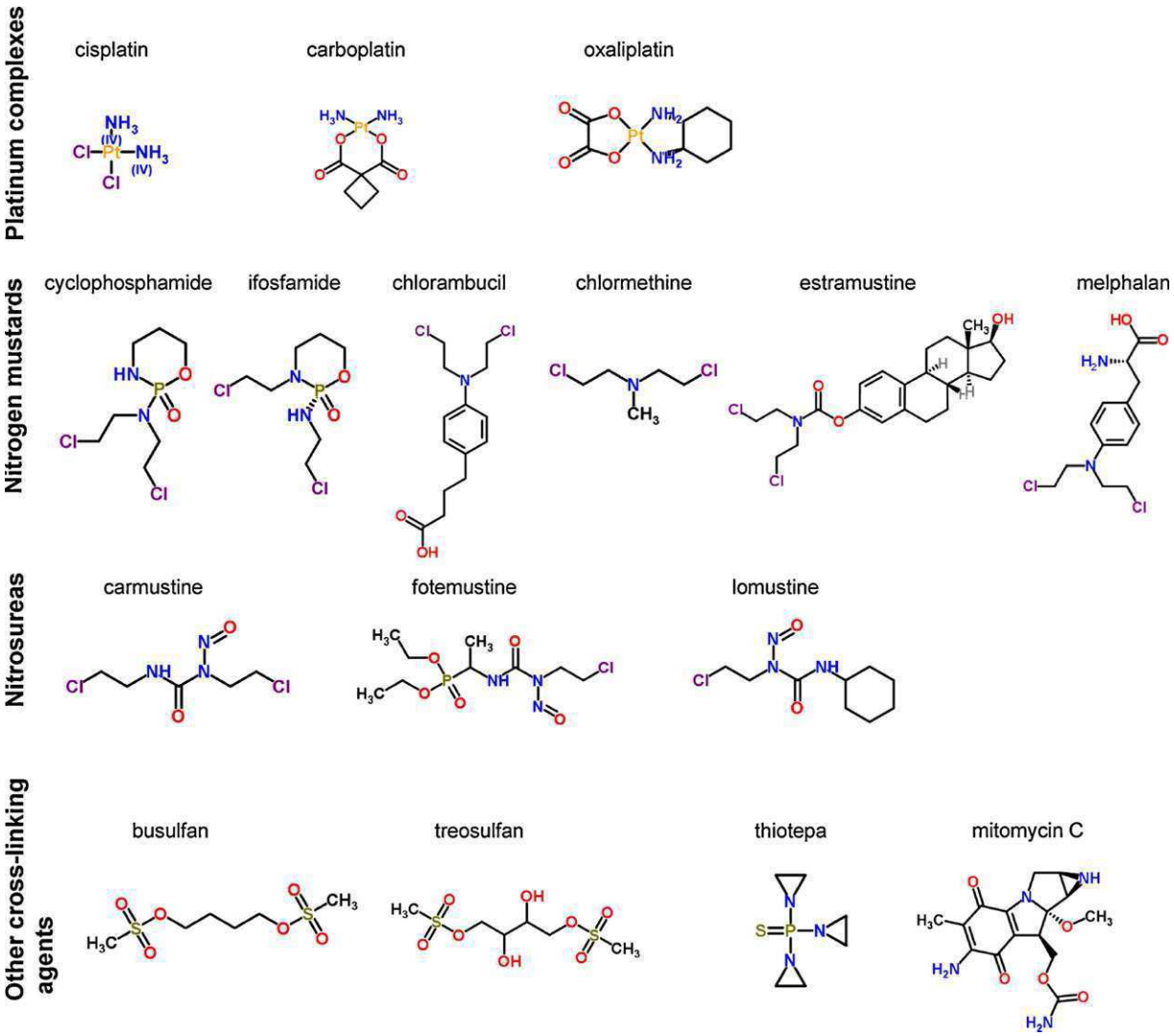

Fig. 3. Chemical structures of DNA-interactive agents: cross-linking agents.

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

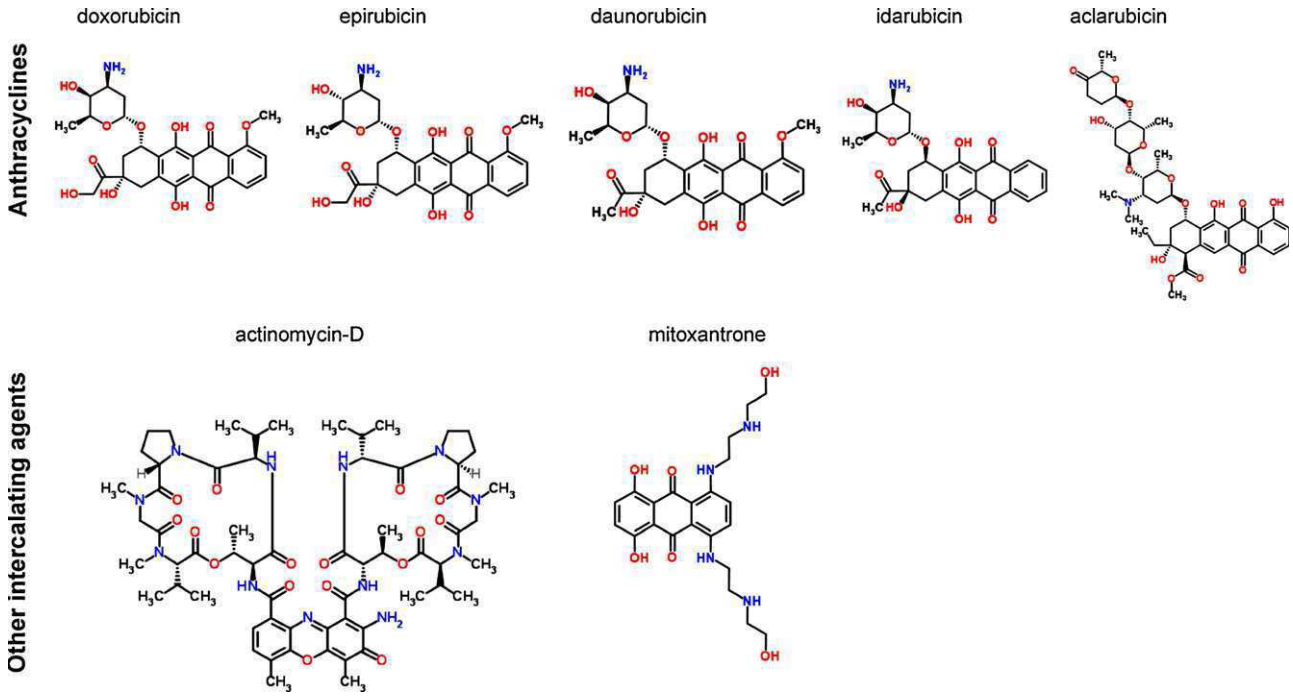

Fig. 4. Chemical structures of DNA-interactive agents: intercalating agents.

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

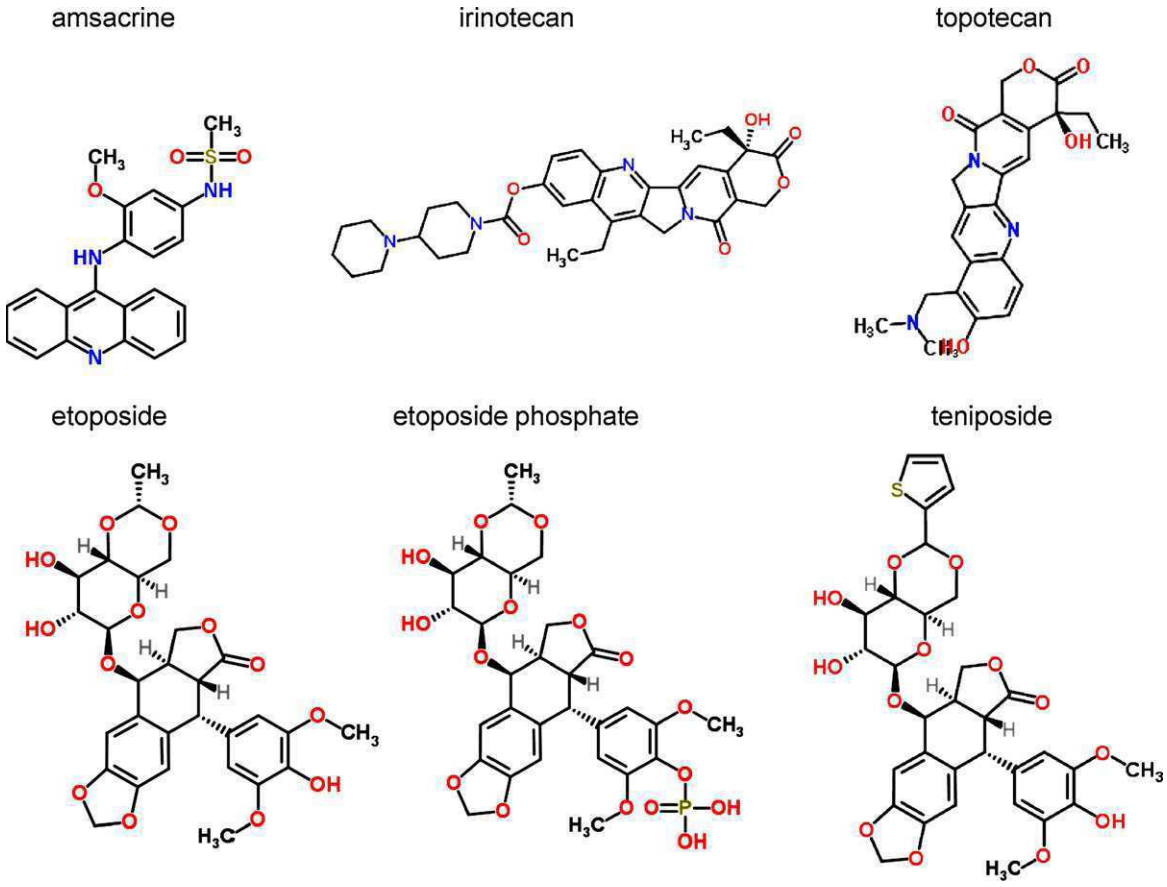

Fig. 5. Chemical structures of DNA-interactive agents: topoisomerase inhibitors.

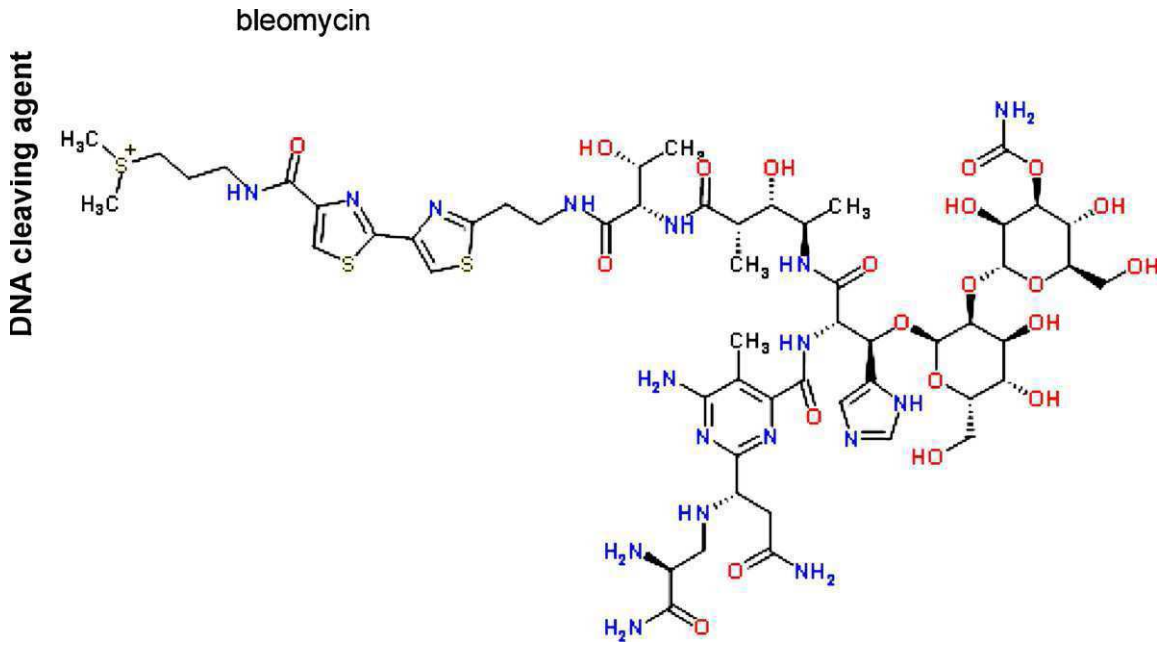

Fig. 6. Chemical structures of DNA-interactive agents: DNA-cleaving agent.

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} (~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} (~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

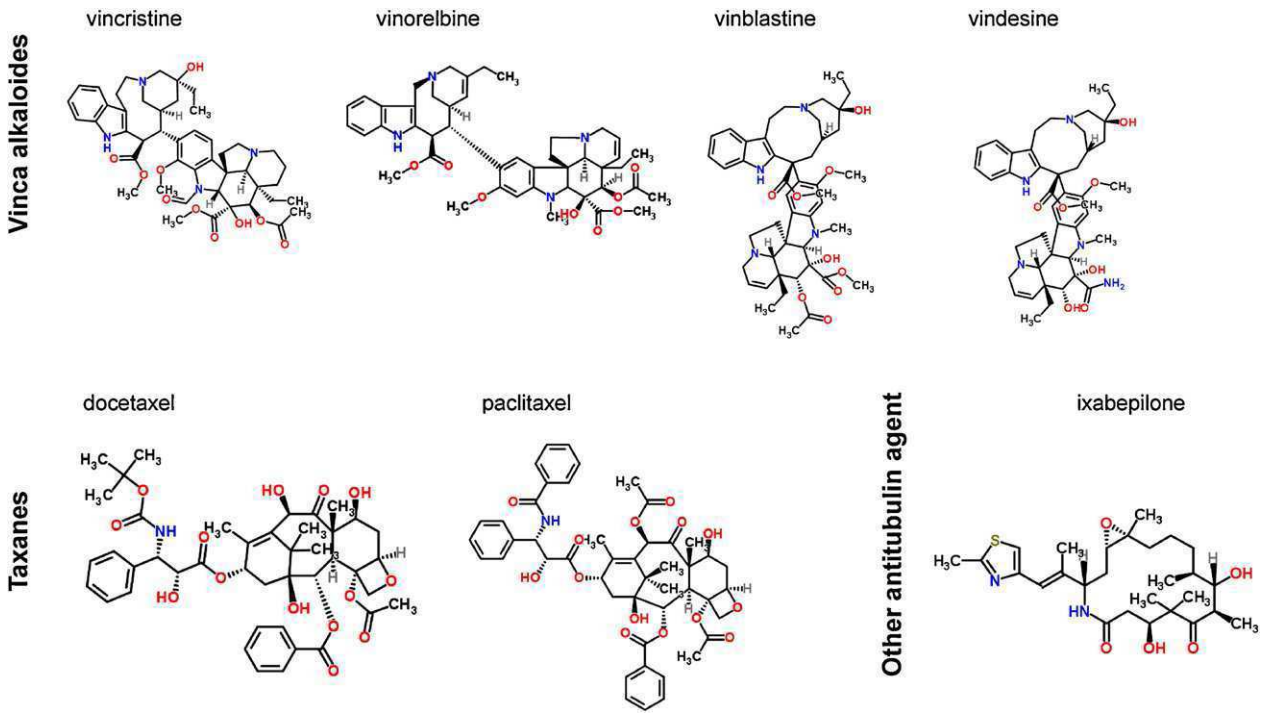

Fig. 7. Chemical structures of antitubulin agents.

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 II

Interest of capillary electrophoresis for the quality control of pharmaceutical formulations produced in hospital pharmacy

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Interest of capillary electrophoresis for the quality control of pharmaceutical formulations produced in hospital pharmacy

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Abstract

Study objectives: The aim of this paper is to share the experience of the Geneva University Hospitals' pharmacy regarding the establishment of capillary electrophoresis in the quality control laboratory.
Methods: Six capillary electrophoresis methods have been developed to quantify pharmaceuticals contained in more than 20 different formulations produced by the hospital pharmacy.
Results: All developed capillary electrophoresis methods exhibited good performance with the following main advantages: low organic solvent consumption, low cost (of capillary) and rapid development.
Conclusion: Capillary electrophoresis, a complementary technique to liquid chromatography, appears as a very attractive alternative for quality control of formulation produced in hospital pharmacy.

Keywords

Capillary electrophoresis, hospital pharmacy, quality control

Introduction

With 2,000 beds and more than 50,000 hospitalisations each year, Geneva University Hospitals (HUG) are among Switzerland's larger hospitals. One of the main objectives of the HUG pharmacy is to supply medicines corresponding to the clinical needs of all medical specialities. Most drugs used in HUG are commercially available on the pharmaceutical market. However, in some situations, there is an interest to produce suitable drugs in the hospital pharmacy, such as when they are not available on the market, not commercialised in an appropriate form, e.g. paediatrics, or when the safety can be improved, e.g. ready-to-use syringes.

According to the Good Manufacturing Practice, all pharmaceutical formulations such as injectable, ophthalmic, oral, topic solutions, suspensions, emulsions, and suppositories produced in batches by a hospital pharmacy are submitted to a quality control before administration to the patient. In addition to physico-chemical tests, e.g. pH, osmolarity, particulate matter, identification of drug(s) and/or excipients, the quantification of the active ingredient(s) in the formulation is an analysis of utmost importance. Generally, liquid chromatography (LC) is used for this purpose and its supremacy in the pharmaceutical field is illustrated by the recommendations of international pharmacopoeias, such as the *European Pharmacopoeia*.

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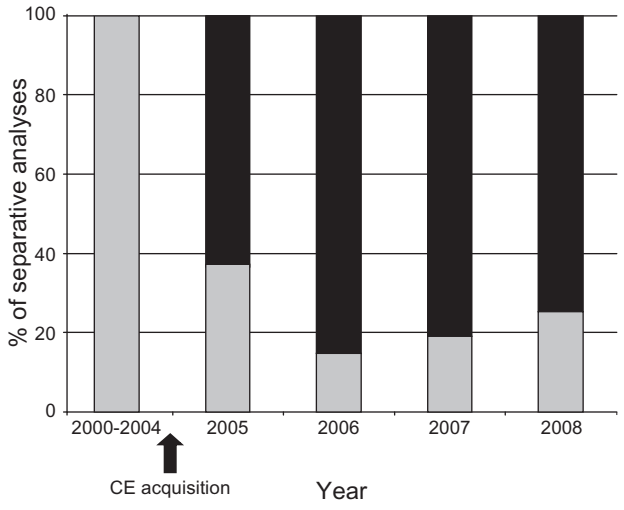
The Use Of Liquid Chromatography

Until 2005, LC-ultraviolet (UV) constituted the only separative technique used in the quality control laboratory of the HUG pharmacy for the quantification of drugs in pharmaceutical formulations, see Figure 1. The mean characteristics of a complete quantitative LC-UV analysis, including column equilibration and washing, formulations and standard sample preparations, separation and data handling, are reported in Table 1. In summary, the use of LC-UV represented a mean consumption of more than four litres of organic solvents, 200 hours of work and Euros 1,400 per year. The solvent consumption can be doubled, and even tripled, if method development and validation are considered.

The Use Of Capillary Electrophoresis

In 2004, the HUG pharmacy's laboratory had the opportunity to purchase new analytical instrumentation. The acquisition of a complementary analytical technique

Figure 1: The proportion of analyses achieved by liquid chromatography (LC; in grey) and Capillary Electrophoresis (CE; in black)*



*In the quality control laboratory of the Geneva University Hospitals' pharmacy.

to LC, based on different mechanisms of separation, appeared to be an attractive strategy. Therefore, capillary electrophoresis (CE) was selected which possesses several advantages such as high efficiency, low solvent consumption, low cost and rapid method development. It is noteworthy that one metre of fused silica capillary is 40-fold less expensive than a conventional reversed phase LC column. Moreover, CE is a versatile analytical technique which can be used for the analysis of a wide range of compounds from small inorganic ions to large proteins. Indeed, different separation modes with different selectivity can be applied at low cost because selectivity mainly depends on the nature and the pH of the background electrolyte, as well as the presence of additives.

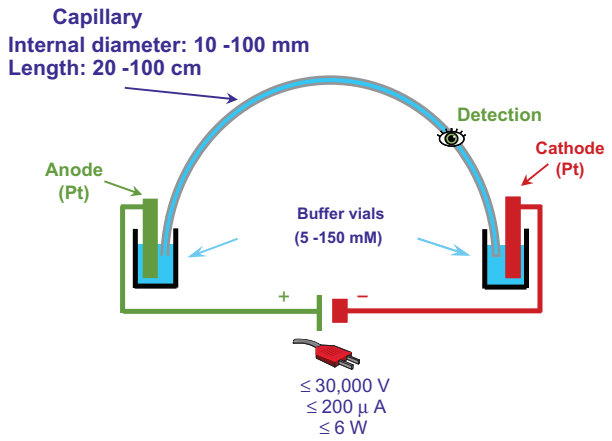
Figure 2 illustrates the main components of a typical CE instrument. Like LC, CE can also be coupled to

Table 1: Mean characteristics of the quantitative analysis of a pharmaceutical formulation batch produced by the Geneva University Hospitals' pharmacy

Mean criteria	LC	CE
Organic solvent consumption	≥ 100 mL	≤ 5 mL
Cost (capillaries or columns, consumables and products)*	Euros 30	Euros 7

*Instrumentation and human work are not considered because they are the same for both analytical techniques in our case.
LC: liquid chromatography; CE: capillary electrophoresis.

Figure 2: Main components of a typical capillary electrophoresis instrument

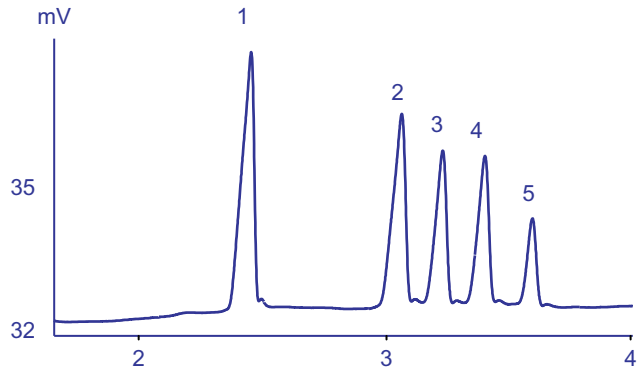


UV or alternative detection techniques such as mass spectrometry, laser-induced fluorescence or capacitively coupled contactless conductivity detection (C^4D). It should be noted that the lack of sensitivity of CE-UV, due to the low amount of sample injection to the capillary and the short detection pathway of the light, does not constitute a limitation for the analysis of pharmaceutical formulations, which generally possess active drug concentration(s) in the order of magnitude of $mg.mL^{-1}$. Thus, CE-UV is particularly adapted to the quality control of pharmaceuticals and is in harmony with the environmental protection policies and the budget limitation applied by the institution.

Since 2004, several CE-UV methods have been developed and validated in the laboratory. Some of these methods have substituted complex and relatively expensive LC-UV methods, such as for the analysis of adrenaline and isoprenaline (ion-pairing LC) or inorganic ions (ionic chromatography). CE-UV methods have also been developed for the analysis of new pharmaceutical formulations produced by the pharmacy and for the analysis of previous formulations, for which a purification step was required due to a lack of selectivity, for example: codeine syrup analysed by single UV spectrophotometry [1]. Besides low cost and increased selectivity, CE-UV features generic conditions. For instance, only one method was developed and could be used for the quantification of nine pharmaceutical compounds while LC needed at least four different columns. The different formulations analysed by the quality control laboratory and the characteristics of CE-UV methods used are reported elsewhere [1].

In order to analyse pharmaceutical compounds without chromophore groups, such as suxamethonium [2] or

Figure 3: Electropherogram of the analysis of a parenteral nutrition by capillary electrophoresis-capacitively coupled contactless conductivity detection



1: potassium; 2: sodium; 3: calcium; 4: magnesium; 5: lithium (internal standard). Based on reference [3].

inorganic ions contained in parenteral nutrition (PN) [3], a C^4D was acquired in 2007 and was easily coupled to CE. A typical electropherogram of the analysis of PN by CE-C^4D is reported in Figure 3.

All CE methods used in the laboratory have been validated and exhibit good performance. With CE, solvent consumption, the cost of analysis (only capillaries, consumables and products are considered) and the time needed for analysis are reduced by a factor of 20, 5-fold, and 2-fold, respectively, see Table 1. As previously mentioned, CE is a complementary technique to LC and its role is not to fully substitute conventional methods. LC is always successfully used in the laboratory to perform analyses of pharmaceutical formulations with compounds present at low concentration, with a low molar extinction co-efficient and/or not detectable by conductimetry. However, 80% of drug quantification is actually performed by CE in our laboratory today.

Conclusion

The establishment of CE in the quality control laboratory reveals a powerful analytical technique in terms of time saving and environmental impact and has a performance similar to the conventional LC for the analysis of pharmaceutical formulations. Its use in quality control laboratories should be strongly encouraged.

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Article III

Compounding of parenteral nutrition: usefulness of quality control methods

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Compounding of parenteral nutrition: usefulness of quality control methods

The implementation of the quality control of parenteral nutrition before patient administration

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At the Geneva University Hospitals (HUG), individualised parenteral nutrition (PN) are prepared daily using a MM12 MicroMacroR compounder (Baxa UK). An error in the concentration of electrolytes or glucose, due for example to an inversion of ingredients during the connection to the automatic filler, can induce an increased risk for the patient, especially for neonates. The objective of this study was to develop and validate quality control methods to analyse each PN before patient administration. The first method involved capillary electrophoresis with a capacitively coupled contactless conductivity detector (CE-C4D) for the quantification of potassium, sodium, calcium and magnesium in each PN. The second method, based on an enzymatic reaction, allowed the determination of glucose. The developed methods were found appropriate for controlling electrolytes and glucose in PN formulations and they were successfully applied in our daily quality control.

During the past few years, a lot of work has been carried out at HUG to improve the safety and the quality of drug use, from prescription to administration.[1–4] Particular attention was focused on drugs aseptically compounded in the hospital pharmacy, considered as a high-risk process, especially when individual formulations are prepared, such as PN. PN are complex mixtures of almost 50 components made from more than 10 different solutions, and a high risk of error and microbiological contamination exists during the production process. As shown by the proactive risk analysis of Bonnabry et al, the use of an electronic prescription and an automatic compounder to prepare PN reduced the criticality of the process.[5] This analysis also emphasised that new risks can appear during the re-engineering of a process, such as the inversion of compounds on the automatic filler, leading to a preparation error. Therefore, additional actions, such as quality control of PN before release, help to indicate the non-conforming PN before delivery, whatever the production process (automatic filling or not).

Choice and development of methods

Several points have to be taken into account during the development of quality control of PN before patient administration:

1. How much time has the laboratory to perform PN control?
2. Which critical parameters should be controlled?
3. Which methods are available in the laboratory?

How much time has the laboratory to perform PN control?

The analysis of PN has to be inserted into the process, from prescription to administration. In our hospital, electronic prescriptions are performed by physicians until 1pm and qualified operators produce PN under a laminar airflow hood (GMP Class A/ISO 4.8) in a cleanroom (GMP Class B/ISO 5) until 3pm with an automatic compounder (MM12 MicroMacroR compounder, Baxa UK). At 5pm, the PN should be transported to the wards, to allow their administration to the patient at 6pm. Consequently, the quality control laboratory has a two-hour period to analyse all PN (see Figure 1).

Which critical parameters should be controlled?

The concentration of the main electrolytes (potassium, sodium, calcium and magnesium) and glucose in PN are undoubtedly the most critical parameters. An error in the concentration of these compounds can induce serious clinical problems, especially in neonates. For these reasons, we decided to quantify the main four cations and glucose in order to ensure the adequacy of the production with the prescription.

The control of the sterility of PN also appears important because microbiological contamination of PN during compounding can have dramatic consequences for the patient. However, it cannot be performed in the time available before administration and other quality assurance methods (a posteriori sterility testing, media-fill tests) have to be implemented.

Which methods are available in the laboratory?

Microbiological quality

Nowadays, no sterility test, recognised by different pharmacopeias, on pharmaceutical fomulations can give a result in two hours. To bring confidence to the system, a full validation of the aseptic filling process

Concerning the chemical analysis of PN, it should be noted that the determination of four electrolytes in PN formulation is an extremely difficult task, given the multi-composition nature of these solutions

has been performed during the implementation process. Moreover, a sterility test, carried out according to the European pharmacopoiea, is performed on 'control' PN formulations produced over the weekend, to ensure the maintenance of sterility during the aseptic compounding process.[6] These PN contain standard concentrations of glucose, sodium chloride, potassium chloride, calcium chloride and water for injectables and are produced in the same way as PN for patients. Until now, results have shown no contaminated samples.

Quantification of the four main electrolytes

Concerning the chemical analysis of PN, it should be noted that the determination of four electrolytes in PN formulation is an extremely difficult task, given the multi-composition nature of these solutions. Usually, a rapid quantification of inorganic ions in aqueous solution is performed by atomic absorption. However, in the case of PN, an obstruction of the injection system is rapidly observed and a complete wash of this instrument part has to be achieved after a few injections even if diluted solutions of PN are used. Capillary electrophoresis (CE) coupled to a conductivity detection (capacitively coupled contactless conductivity detection, namely C4D), already and currently used in our laboratory in routine analysis, appeared to be an attractive strategy to perform the rapid analysis of inorganic ions in PN. Indeed, CE is particularly suitable for analysing small inorganic ions because the separation mechanism depends on the charge and the size of compounds. Moreover, the main advantages of CE are a high efficiency, low organic solvent consumption, low cost of capillaries, rapid method development and high versatility. The CE-C4D method developed for the rapid analysis of sodium, potassium, calcium and magnesium in PN is described elsewhere.[7] In summary, the CE-C4D method is considered as:

- Rapid, with a complete separation of the four ions in less than four minutes. A typical electropherogram obtained for the analysis of a PN is reported in Figure 2. This short analysis time is compatible with the two-hour period available to achieve the analysis of c.a. 10–15 produced PN bags.
- Easy to operate by the laboratory staff. Indeed, with the CE-C4D method, a diluted sample of PN is directly injected in the CE instrumentation.
- Reliable, as demonstrated by the complete validation of the developed method. Accuracy values between 99.7 and 101.9% were obtained with repeatability and intermediate precision values less than 2% for all cations.

Nowadays, more than 700 individualised PNs, produced by the HUG pharmacy, have been analysed with the CE-C4D method. Only one PN was considered as non-conforming because the potassium level was more than 110% (120%) of the prescribed concentration. This formulation was destroyed and a new one was produced and released after analysis. A calibration of the compounding system corrected this problem.

Quantification of glucose

The hexokinase method developed for the analysis of glucose in human blood is used for the determination of glucose in PN. This approach is based on the formation of NADPH proportional to the glucose concentration and can be measured photometrically at 340nm. A complete description of the reaction is reported in the guidelines of the reagent Gluco-quant Glucose/HK commercialised by Roche.[8] Like the CE-C4D method, the hexokinase method is:

- Rapid: the analysis of glucose in PN is performed in less than 10 minutes (including a reaction time of five minutes).
- Easy to operate: 100µL diluted sample of PN was added to 900µL of reagent. The solution is mixed and analysed by spectrophotometry after 5 minutes.
- Reliable: as demonstrated by the validation of the hexokinase method. Accuracy values between 99.4 and 102.1% were obtained with repeatability and intermediate precision values less than 2%.

A single non-conformity with glucose concentration has been revealed on 700 produced PN and human error was detected. After investigation, it appeared that the empty bottle of glucose G70% was substituted by an identical bottle of aminoacids during the production process. The PN with the wrong glucose

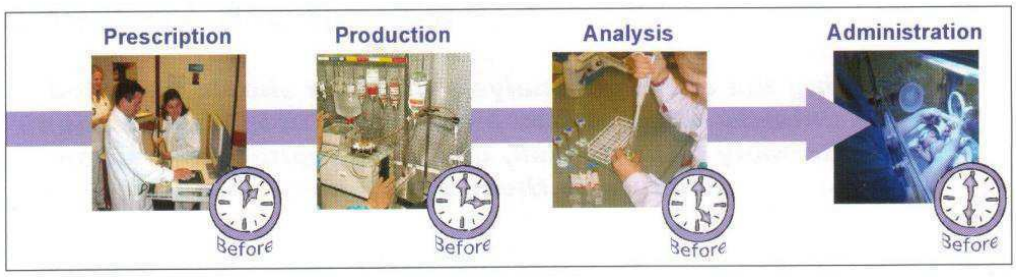

Figure 1. The parenteral nutrition process at the Geneva University Hospitals

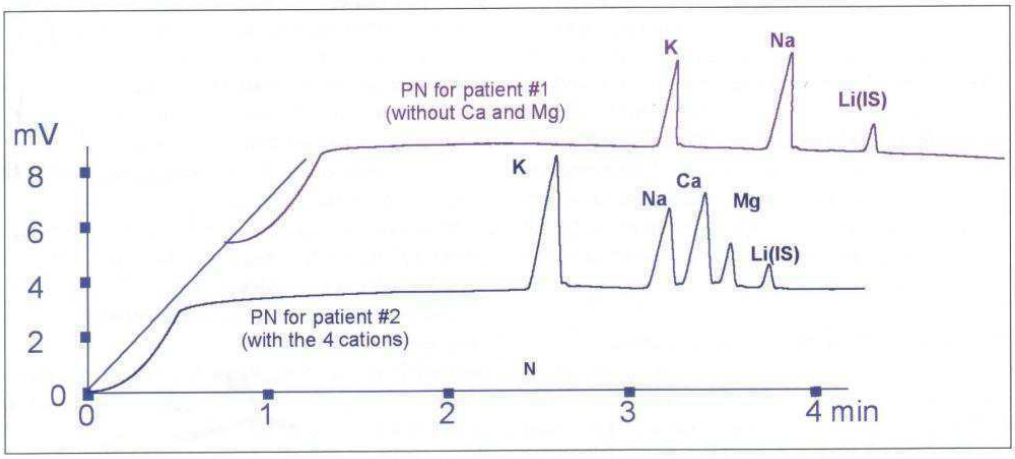

Figure 2. Electropherograms obtained for the analysis of two parenteral nutritions by the CE-C4D method

concentration was destroyed, and new PN was produced and released after analysis.

Data and non-conformity handling

All results obtained for the analysis of PN (cations and glucose determination and sterility test) are reported in a 'home-made computerised system' designed in collaboration with the medical informatics department of HUG. Production and analysis protocols for all prepared PN can be continually accessed by the pharmacy personnel. Thus, the traceability and the safety of PN flow are improved, as described in previously published work.[5]

Non-conforming PN are eliminated and new ones are produced and released after analysis. In this case, the physicians are informed about the delay and appropriate actions can be taken.

Conclusion

Quality control of PN formulations has been implemented at the HUG pharmacy. This included the quantification of sodium, potassium, calcium, magnesium and glucose for each PN before patient administration. The microbiological quality of the production process is checked by a complementary sterility test. Two simple and rapid analytical methods have been developed and validated. Both methods have been successfully applied in routine analysis for one year at the laboratory of the HUG pharmacy and permitted the detection of non-conforming PN. The implementation of a quality control of PN, in addition to an electronic prescription and an automatic compounding system, has contributed to improvements in the safety of the PN process in the hospital. ■

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Article IV

Determination of potassium, sodium, calcium and magnesium in total parenteral nutrition formulations by capillary electrophoresis with contactless conductivity detection

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Determination of potassium, sodium, calcium and magnesium in total parenteral nutrition formulations by capillary electrophoresis with contactless conductivity detection

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ABSTRACT

A simple method based on capillary electrophoresis with a capacitively coupled contactless conductivity detector (CE-C^4D) was developed for the determination of potassium, sodium, calcium and magnesium in parenteral nutrition formulations. A hydro-organic mixture, consisting of 100 mM Tris-acetate buffer at pH 4.5 and acetonitrile (80:20, v/v), was selected as the background electrolyte. The applied voltage was 30 kV, and sample injection was performed in 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. Under these conditions, complete separation between all cations was achieved in less than 4 min. The CE-C^4D method was validated, and trueness values between 98.6% and 101.8% were obtained with repeatability and intermediate precision values of 0.4–1.3% and 0.8–1.8%, respectively. Therefore, this method was found to be appropriate for controlling potassium, sodium, calcium and magnesium in parenteral nutrition formulations and successfully applied in daily quality control at the Geneva University Hospitals.

1. Introduction

Total parenteral nutrition (TPN) is the practice of feeding a person intravenously using nutritional formulas containing essential nutrients such as electrolytes, glucose, amino acids, trace elements and vitamins (see Table 1). These nutritional solutions are prepared daily at the pharmacy of the Geneva University Hospitals (HUG) for paediatric patients. Errors in the concentrations of electrolytes present increased risks for patients, especially for neonates. Therefore, TPN preparations are submitted to quality control before patient administration. Currently, sodium, potassium and calcium are checked at the HUG pharmacy using flame photometry or absorption spectrometry methods in control solutions without amino acids or vitamins. The constituents of real TPN samples (with increased concentrations of glucose, amino acids and vitamins) can interfere with the analysis of ions and contaminate the analytical system. Therefore, other analytical techniques are required.

Capillary electrophoresis (CE) coupled with indirect UV detection was developed for the analysis of inorganic cations [1–7], particularly sodium, potassium, calcium and magnesium, in TPN preparations [8,9]. These methods have been compared with flame atomic spectrometry and ion chromatography [1,9] and were found to be an acceptable alternative. However, UV-absorbing buffer additives and more complex buffer systems were needed to facilitate indirect absorbance detection [10], and weaker quantitative performance was achieved [1,9]. During the past few years, contactless conductivity detection has been recognized as an attractive alternative to optical detection techniques in CE because of its low cost, lack of maintenance requirements, easy handling and simple method development. Among the developed capacitively coupled contactless conductivity detectors (C^4D), we only consider in this paper the instrument used by Zemann [11,12]. The latter presents two metal tube electrodes, placed around the capillary. An oscillation frequency between 75 and 300 kHz is applied to one of the electrodes, and a signal is produced when an analyte zone with a different conductivity passes through the retention gap [2].

Numerous papers have described the analysis of inorganic cations (*e.g.*, sodium, potassium, calcium, magnesium) by CE-C^4D [2–4,10–23]. A buffer based on 2-(N-morpholino)ethanesulfonic

Abbreviations: BGE, background electrolyte; CS, calibration standard; C^4D, capacitively coupled contactless conductivity detector; EOF, electroosmotic flow; HIBA, α-hydroxyisobutyric acid; His, Histidine; HUG, Geneva University Hospitals; IS, internal standard; MES, 2-(N-morpholino)ethanesulfonic acid; SFSTP, Société Française des Sciences et Techniques Pharmaceutiques; TPN, total parenteral nutrition; Tris, tris(hydroxymethyl)-aminoethane; VS, validation standard.

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Table 1
Composition of TPN at HUG.

Solution	Composition	Manufacturer
NaCl 11.7%	Sodium: 2 mmol mL^{-1}	Bichsel (Interlaken, CH)
Calcium gluco-bionate 10%	Calcium: 0.16 mmol mL^{-1}, lactobionate: 0.16 mmol mL^{-1}, glucobionate: 0.16 mmol mL^{-1}	Bichsel (Interlaken, CH)
Phocytan®	Phosphate: 0.33 mmol mL^{-1}, glucose: 0.33 mmol mL^{-1}, sodium: 0.66 mmol mL^{-1}	Aguettant (Lyon, F)
KCl 7.5%	Potassium: 1 mmol mL^{-1}	Sintetica-Bioren (Couvet, CH)
$MgSO_4$ 5%	Magnesium: 0.2 mmol mL^{-1}	Pharmacy HUG
Sodium acetate 16.4%	Acetate: 2 mmol mL^{-1} Sodium: 2 mmol mL^{-1}	Pharmacy HUG
Vitamins	Vitamin A: 0.4 mg mL^{-1}, D3 1.1 μg mL^{-1}, E 2.04 mg mL^{-1}, C 25 mg mL^{-1}, B1 0.7 mg mL^{-1}, B2 0.83 mg mL^{-1}, B6 0.91 mg mL^{-1}, B12 1.2 μg mL^{-1}, B9 82.5 μg mL^{-1}, B5 3.45 mg mL^{-1}, B8 13.8 μg mL^{-1}, PP 9.2 mg mL^{-1}	Baxter (Volketswil, CH)
Cernevit®		
Trace elements	Fe^{2+}: 0.1 mg mL^{-1}, Zn^{2+}: 0.16 mg mL^{-1}, Mn^{2+}: 27.2 μg mL^{-1}, Cu^{2+}: 38 μg mL^{-1}, Cr^{3+}: 0.5 μg mL^{-1}, Mo(VI): 0.5 μg mL^{-1}, Se(IV): 1 μg mL^{-1}, F^-: 28.5 μg mL^{-1}, I^-: 6.5 μg mL^{-1}, Na^+: 1.9 μmol mL^{-1}, K^+: 0.05 μmol mL^{-1}, Cl^-: 17 μmol mL^{-1}	BBraun (Sempach, CH)
Tracutil® diluted		Pharmacy HUG
Heparin	50 UI mL^{-1}	Pharmacy HUG
Amino acids	Alanine: 6.3 g L^{-1}, arginine: 4.1 g L^{-1}, asparagine acid: 4.1 g L^{-1}, cysteine: 1 g L^{-1}, glutamic acid: 7.1 g L^{-1}, glycine: 2.1 g L^{-1}, histidine: 2.1 g L^{-1}, isoleucine: 3.1 g, leucin: 7.0 g L^{-1}, lysine: 5.6 g L^{-1}, methionine: 1.3 g L^{-1}, phenylalanine: 2.7 g L^{-1}, proline: 5.6 g L^{-1}, serine: 3,8 g L^{-1}, taurine 0,3 g L^{-1}, threonine: 3.6 g L^{-1}, tryptophan: 1.4 g, tyrosine: 0.5 g L^{-1}, valine: 3.6 g L^{-1}	Fresenius Kabi (Stans, CH)
Vaminolact®		
Glucosteril	Glucose 70%	Fresenius (Stans, CH)
Injection water	Water ppi	Bichsel (Interlaken, CH)

acid and histidine (MES/His) has been widely used for the determination of alkali and alkaline earth metals and ammonium ions [2–4,11–20]. Other background electrolytes (BGE) composed of citric, lactic or acetic acids and His or maleic acid/arginine have also been successfully used for the separation of these cations [15,20].

Weak complexing agents have been added to the BGE to modify the separation of inorganic cations, such as α-hydroxyisobutyric acid (HIBA) [4,7,17]. An organic solvent was added (10% methanol) to modify the selectivity and to obtain a complete separation of sodium, calcium and magnesium in blood samples [20]. However, to our knowledge, a validated CE-C^4D for TPN has not yet been reported.

In this study, a simple CE-C^4D method was developed and validated to determine sodium, potassium, calcium and magnesium in TPN and was applied to the quantitation of these cations in daily quality control.

2. Experimental

2.1. Chemicals

Sodium chloride, potassium chloride, calcium chloride, magnesium chloride, lithium chloride and tris(hydroxymethyl)-aminoethane (Tris) were purchased from Fluka (Buchs, Switzerland). Water and NaCl (0.9%) used for pharmaceutical preparations were obtained from Bichsel Laboratories (Interlaken, Switzerland). Acetic acid (glacial, 100%), methanol and acetonitrile were obtained from Merck (Darmstadt, Germany).

Parenteral nutrition solutions were prepared at the HUG pharmacy using the automated compounding system BAXA MM12 (Baxa corporation, Englewood, CO, USA) with the following solutions: Calcium glucobionate (10%) and NaCl (11.7%) obtained from Bichsel Laboratories (Interlaken, Switzerland), KCl (7.5%) from Sintetica-Bioren SA (Couvet, Switzerland), Phocytan from Aguettant (Lyon, France), Aminosteril Hépa (8%), Glucosteril (70%), and Vaminolact from Fresenius Kabi (Stans, Switzerland; Bad Homburg, Germany). Tracutil was diluted in a 1:2 ratio (BBraun, Sempach, Switzerland) and Cernevit was obtained from Baxter (Volketswil, Switzerland). Sodium acetate (16.4%), heparin (50 UI/mL) and magnesium sulfate (5%) were produced by the HUG pharmacy.

2.2. BGE preparation

Different BGEs (phosphate pH 2 and 7, borate pH 9, MES/His pH 6.1, citrate pH 3.1 and pH 4.8, lactate and acetate/Tris pH 4.5) were prepared for the method development. The final BGE was composed of a hydro-organic buffer corresponding to a mixture of an aqueous BGE (100 mM Tris-acetate buffer at pH 4.5) and acetonitrile (80:20, v/v). The aqueous BGE was prepared by an adequate dilution of the concentrated acid solution, and a solution of Tris at 1 M was added to adjust the solution to pH 4.5. The solution was then diluted to the final volume with distilled water. The BGE was degassed in an ultrasonic bath for 10 min before use.

2.3. Instrumentation and capillaries

CE experiments were carried out with an $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a power supply able to deliver up to 30 kV. $HP^{3D}CE$ was coupled to a TraceDec detector (Innovative Sensor Technologies GmbH, Strasshof, Austria). The conductivity sensor consisted of two electrodes separated by a detection gap of 1 mm, positioned along the capillary by sliding it into the desired position (14.5 cm from the cathode). A CE ChemStation (Agilent) was used for CE control and data handling, and a C^4D Tracemon (Innovative Sensor Technologies, Austria) was used for conductivity detector control and data acquisition. Analyses were performed in uncoated fused silica capillaries from BGB Analytik AG (Böckten, Switzerland) with an internal diameter of 50 μm, an external diameter of 375 μm and a total length of 64.5 cm (effective length of 50 cm). All experiments were performed in the normal mode (cathode at the outlet end of the capillary). The capillary was thermostated at 25 °C in a high velocity air stream, and a voltage of 30 kV was applied. The generated current was between 5 and 50 μA depending on the buffer solution. 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 final configuration of the C^4D was set at an output frequency of 150 kHz, an output voltage of 40 Vpp, 50% gain and an offset of ~30. The detector acquisition corresponded to the CE mode of 19.8 Hz. Before first use, capillaries were sequentially rinsed with methanol, 0.1 M NaOH, water, methanol, 0.1 M HCl, water and BGE for 5 min. A voltage of 30 kV was then applied for 60 min with the BGE. The

Table 2
Composition of the validation matrix.

Concentration	Nutriment	Composition
42 g/L	Amino acid	160.8 mL of Vaminolact
218.8 g/L	Glucose	78.2 mL of Glucosteril
500 U/L	Heparin	2.5 mL of Heparin
20 ml/L	Oligoelement	5 mL of diluted Tracutil

TraceDec was set to run for 1 h before the first analysis in order to obtain a constant signal. Prior to each sample injection, the capillary was rinsed by pressure (940 mbar) for 1 min with fresh BGE. When not in use, the capillary was rinsed with water and methanol. As the electrophoresis process altered the running buffer pH by electrolysis and subsequently changed the migration times, the separation buffer was refreshed every six runs.

2.4. Method validation

A validation was performed to estimate the quantitative parameters of the method for the analysis of potassium, sodium, calcium and magnesium in parenteral nutritional formulations. The validation was based on ICH recommendations following the guidelines of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [24] and carried out over three series. Each series involved the injection of a freshly prepared BGE, two calibration standards (CS) at 4 mM for K^+, Na^+ and 2 mM for Ca^{2+}, Mg^{2+}, four validation standards (VS) at 1, 2 and 4 mM for K^+, Na^+ and 0.5, 1 and 2 mM for Ca^{2+}, Mg^{2+}, complete washing of the capillary with water and methanol, and instrument shut-off. Lithium chloride was used as the internal standard (IS). The calculations were performed using normalised area (area/migration time) ratios of the cations on the IS.

2.5. Sample preparation

CS and VS were independently prepared. The IS stock solution was prepared by dissolving lithium chloride in water at a concentration of 50 mM.

2.5.1. Calibration standard

A standard stock solution was prepared by dissolving KCl, NaCl, $CaCl_2{\cdot}2H_2O$ and $MgCl_2{\cdot}6H_2O$ in water to obtain a concentration of 100 mM for K^+ and Na^+, and 50 mM for Ca^{2+} and Mg^{2+}, which were stored at 4 °C until use. Sample solutions were stable for more than 1 week at 4 °C, and no degradation was observed for the tested analytes during analysis. One concentration level sample was prepared by diluting the appropriate volume of standard stock solution in distilled water at a final concentration of 4 mM of K^+ and Na^+ and 2 mM of Ca^{2+} and Mg^{2+}. Lithium chloride was added as an internal standard to obtain a final concentration of 1.25 mM.

2.5.2. Validation standard

A TPN solution with 40 mM of K^+ and Na^+ and 20 mM Ca^{2+} and Mg^{2+} was prepared by diluting NaCl (11.7%), calcium glucobionate (10%), KCl (7.5%) and magnesium sulfate (5%) in a condensed TPN matrix consisting of glucose (Glucosteril, 70%), amino acids (Vaminolact), heparin and trace elements (Tracutil) as shown in Table 2. For VS, three concentration level samples were prepared at 25%, 50% and 100% of the highest value (4 mM K^+ and Na^+, 2 mM Ca^{2+} and Mg^{2+}) by diluting the appropriate volume of the TPN solution in water.

2.6. Application to TPN solutions

The four cations were determined in TPN solutions prepared at the pharmacy of HUG. Therefore, the formulations were diluted in distilled water to obtain a final concentration between 1 and 4 mM for K^+ and Na^+, and 0.5 and 2 mM for Ca^{2+} and Mg^{2+}. The quantitative analysis was repeated twice ($N = 2$) for each formulation.

3. Results and discussion

Paediatric TPN are produced daily in the HUG pharmacy and submitted to a quality control before patient administration. A CE-C^4D method was developed and validated for determining potassium, sodium, calcium and magnesium in these preparations.

3.1. Method development

3.1.1. Buffer selection

The selection of the BGE was based on conductivity detection of the four cations and selectivity toward other compounds of the TPN, such as amino acids, glucose or vitamins. In C^4D, the response arises from the difference in conductivity between analytes and BGE co-ions. For obtaining the highest signal-to-noise ratio, a large difference between the conductance of the analytes and the electrolyte is needed. Moreover, CE requires BGEs with a higher ionic strength compared to the sample zone to take advantage of the stacking effect. The compromise consists of using an amphoteric or low conductance buffer at high ionic strength [12]. Among the different BGEs tested, a good separation of the four cations was achieved as expected with the commonly used MES/His BGE [2–4,11–20], but also with the acetate/Tris buffer system (pH 4.5). With both BGEs, the resolution between sodium, calcium and magnesium had to be improved to determine magnesium and calcium in presence of sodium at high concentration, as it is generally the case in TPN. The acetate/Tris BGE was chosen for further development because it gave satisfactory results for the analysis of suxamethonium by CE-C^4D [25] and it possesses a low conductivity and can be used at a concentration of 100 mM without generating a high current (~20 μA). Lithium chloride was chosen as IS because it is not a constituent of TPN and it presents a much lower mobility than the four cations tested.

3.1.2. Influence of acetate concentration in the BGE

The first analyses were performed with an acetate/Tris buffer at 20 mM to reduce the background conductivity. Nevertheless, BGEs with different concentrations were tested (10, 20, 30, 50, 75 and 100 mM) to improve the resolution between sodium, calcium and magnesium. As shown in the literature, interactions of the analytes with BGE components could enhance selectivity in CE [4,5,7,8,17,21,26]. In these studies, a weak complexing agent (for example HIBA) was added to the BGE to modify the separation of the cations. The mobility of Mg^{2+} and Ca^{2+} was found to decrease due to a stronger interaction with HIBA [8]. In this work, acetate is a weak complexing agent that can interact with the studied cations [5]. Indeed, calcium and magnesium have higher complexation constants with acetate than potassium or sodium [27]. Increasing the acetate concentration therefore changed the migration order to potassium, sodium, calcium and magnesium (see Fig. 1). Furthermore, as expected, the electroosmotic flow (EOF) decreased with increasing acetate concentration, resulting in a net increase of the migration times of all cations.

The pH of the BGE modifies the EOF and the proportion of acetate, which can influence the separation of the cations. Therefore, acetate/Tris solutions with different pH were tested in the buffer region. In this work, the migration order of the cations changed with the pH value (pH 4.1: calcium–sodium–magnesium;

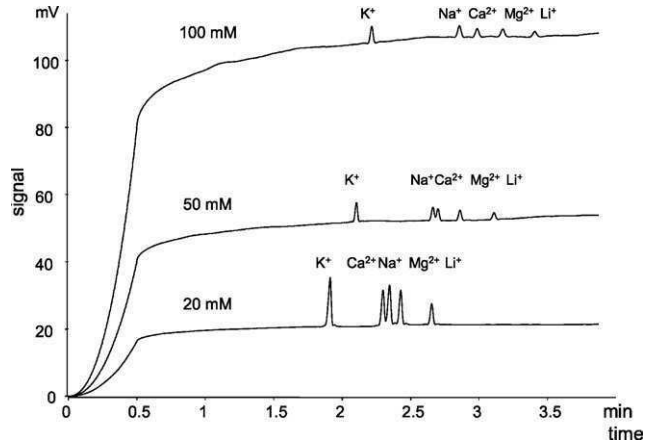

Fig. 1. Influence of BGE concentration: electropherograms of a sample containing sodium (1 mM), potassium (1 mM), calcium (0.5 mM), magnesium (0.5 mM), and lithium (1.25 mM) in an aqueous solution. BGE: 20, 50 and 100 mM Tris-acetate at pH 4.9. All other experimental conditions are described in Section 2.3.

pH 4.9: sodium–calcium–magnesium). This change of selectivity can also be explained by interactions of the cations with acetate. The best separation was obtained with a buffer pH of 4.9, but the signal-to-noise ratio was lower due to the higher conductance of the BGE (shown in Fig. 2).

Finally, a 100 mM Tris acetate buffer at pH 4.5 was selected since the signal-to-noise ratio of the cations was significantly enhanced compared to a BGE at pH 4.9 and the current generated was still inferior to 30 μA. Under these conditions, the complete separation of the four cations was achieved and the mobilities of the compounds were in the following decreasing order: potassium, sodium, calcium, magnesium and lithium.

3.1.3. Addition of an organic solvent

Ion-pair formation can be favoured by non-aqueous solvents due to their lower permittivity constant [28]. Organic solvents change the solvation radii of ions, which contributes to a modification of their mobilities [29–31]. They also alter the viscosity of BGE and directly affect the mobility of the analytes. Therefore, resolution can be enhanced by the addition of organic solvents. Separation of cations in purely non-aqueous buffers was achieved by Salimi-Moosavi and Cassidy and the effect of acetonitrile in methanol was demonstrated to be useful [31]. The addition of organic solvents

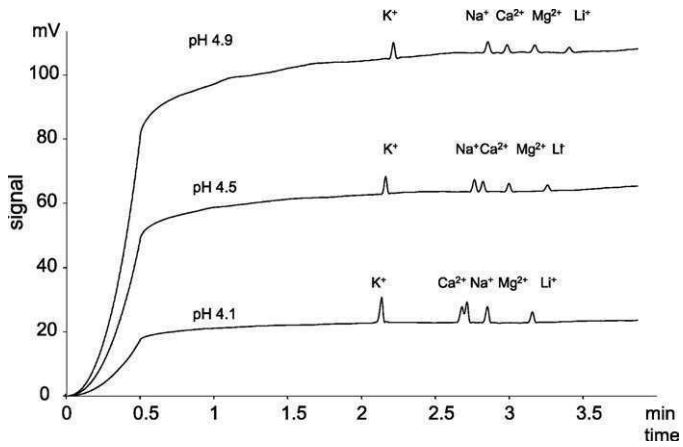

Fig. 2. Influence of BGE pH on the conductivity detection: electropherograms of a sample containing sodium (1 mM), potassium (1 mM), calcium (0.5 mM), magnesium (0.5 mM), and lithium (1.25 mM) in an aqueous solution. BGE: 100 mM Tris-acetate at pH 4.1, 4.5 and 4.9. All other experimental conditions are described in Section 2.3.

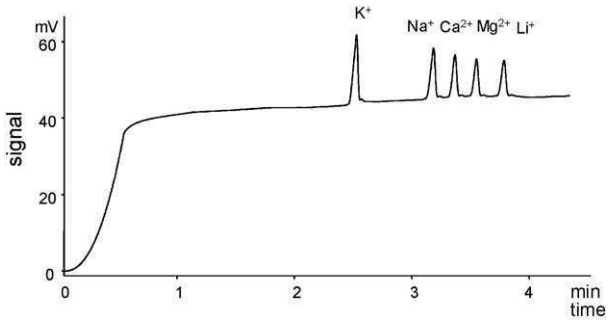

Fig. 3. Electropherogram obtained for the CE-C4D analysis of a sample containing sodium (1 mM), potassium (1 mM), calcium (0.5 mM), magnesium (0.5 mM) and lithium (1.25 mM) in an aqueous solution. BGE: 100 mM Tris-acetate at pH 4.5, acetonitrile (80:20, v/v). All other experimental conditions are described in Section 2.3.

to the electrophoretic medium can modify the selectivity through changes in the solvent pH and analyte pK_a. The increase of the pK_a values of aromatic acids with increasing concentration of acetonitrile was studied by Sarmini and Kenndler [32]. This is most easily understood in terms of a solvent-induced change of the analyte charge [33].

The addition of 10–30% methanol did not change the separation in the presented work, while it has been shown useful for changing the selectivity in other studies [29]. However, the addition of acetonitrile enhanced the separation of sodium, calcium and magnesium. Different concentrations of acetonitrile were added to the BGE (data not shown). The addition of 20% acetonitrile to the acetate/Tris BGE improved the separation significantly, without modifying the migration order.

Therefore, 100 mM acetate/Tris BGE, pH 4.5, 20% acetonitrile (v/v) was selected for the separation of the four cations (Rs > 1.5) (see Fig. 3).

3.1.4. C^4D parameters

Different oscillation voltages and oscillation frequencies of the C^4D were tested (data not shown). An oscillation voltage of 40 Vpp and a frequency of 150 kHz gave the best results with the selected BGE. The response of potassium, sodium, calcium and magnesium and lithium as a function of the excitation frequency was studied by Pavel and Hauser, where a maximal output voltage was observed at a frequency of 250 or 400 kHz with a BGE of His and acetic acid at pH 2.75 [14]. The difference of BGE did not allow a direct comparison of the optimal set-up parameters, but, in both cases, the detector response was enhanced with increasing output frequency.

3.2. Method validation

TPN is produced daily on prescription and the concentration of the different constituents varies in each case. The validation of the method could not include all dilutions and compositions possible, but was based on a worst case situation according to 4 years of TPN prescription at the HUG (internal unpublished data). In general, sodium is the most abundant cation in the TPN, while magnesium is the less concentrated. Calcium and magnesium are present at much lower concentrations than sodium or potassium and, therefore, the CS of Ca^{2+} and Mg^{2+} were chosen to be half of the concentration of Na^+ and K^+. First, the response function in the concentration range of 0.2–4 mM for Na^+ and K^+ and 0.1–2 mM for Ca^{2+} and Mg^{2+} was evaluated with ordinary linear regression using five concentration levels (5%, 10%, 25%, 50% and 100%). A linear response function ($r^2 > 0.999$) was achieved for all cations in the tested concentration range. Therefore, a 1-level calibration at 4 mM Na^+ and K^+ and 2 mM Ca^{2+} and Mg^{2+} (100%) was chosen for the validation in order

to shorten the analysis sequence time. The LOD of the method was estimated at 0.02 mM for all cations, while lowest quantification level obtained after dilution of the TPN was at 1 mM for Na^+ and K^+ and 0.5 mM for Ca^{2+} and Mg^{2+}.

For the VS, reconstituted dosage forms were obtained by a blank matrix built of glucose, amino acids, heparin and trace elements in the highest possible concentration, to mimic the highest concentrated samples, spiked with sodium, potassium, calcium and magnesium at usual TPN concentrations. The blank matrix composition is shown in Table 2.

The developed method was validated according to ICH guidelines following the SFSTP recommendations [24]. Quantitative performance was estimated in three separate series ($j = 3$) with the V1 protocol. This protocol involves one level ($k = 1$) at the upper end of the investigated range with two repetitions ($n = 2$) for CS and three concentration levels ($k = 3$) with four repetitions ($n = 4$) for the VS.

The concentrations of VS (25%, 50% and 100% of the target value) were computed from the analytical response to obtain trueness, repeatability and intermediate precision. Trueness was expressed in percent as the ratio between the theoretical and average measured values at each concentration level. Repeatability and intermediate precision were expressed as the coefficient of variation (CV %) of the ratio of the intra-day standard deviation (s_r) and between-day standard deviation (s_R), respectively, on the theoretical concentrations as described in [34]. The s_r and s_R values were obtained using ANOVA analysis. As reported in Table 3, the trueness and precision values were in accordance with regular recommendations for the analysis of pharmaceutical formulations over the tested concentration range. The CV (repeatability and intermediate precision) was lower than 2%, with trueness between 98.6 and 101.8% for all cations. To visualise the overall method variability, the accuracy profile of each cation was built combining trueness and intermediate precision as the confidence interval [35]. As presented in Fig. 4, the total error did not exceed the acceptance limits (±5%) for all concentration levels. Consequently, the developed CE-C^4D method is validated for determining the four cations over the tested concentration range.

Table 3
Validation results: trueness, repeatability and intermediate precision of the developed CE-C^4D method for the determination of the four cations in a pharmaceutical formulation.

	Trueness	Repeatability (CV)	Intermediate precision (CV)
Theoretical concentration of potassium [mM]			
1	100.6%	1.0%	1.3%
2	101.8%	1.2%	1.4%
4	101.6%	1.1%	1.1%
Theoretical concentration of sodium [mM]			
1	100.9%	1.2%	1.5%
2	100.9%	1.1%	1.5%
4	99.7%	0.9%	1.2%
Theoretical concentration of calcium [mM]			
0.5	100.5%	1.1%	1.1%
1	100.4%	1.3%	1.8%
2	99.0%	0.4%	1.1%
Theoretical concentration of magnesium [mM]			
0.5	99.1%	1.0%	1.2%
1	99.2%	0.8%	1.1%
2	98.6%	0.8%	0.8%

3.3. Application in the quality control laboratory of the HUG pharmacy

In order to demonstrate the applicability of the CE-C^4D method to real samples with different concentrations, quantitation of the four cations was achieved on several formulations prepared at the pharmacy of HUG. The concentrations of sodium, potassium, calcium and magnesium were calculated with reference to a central point, which was replicated twice. All concentrations of the four cations were found to be in the tolerated concentration of ±15% of the target value (internal fixed limits) by CE-C^4D. The results for

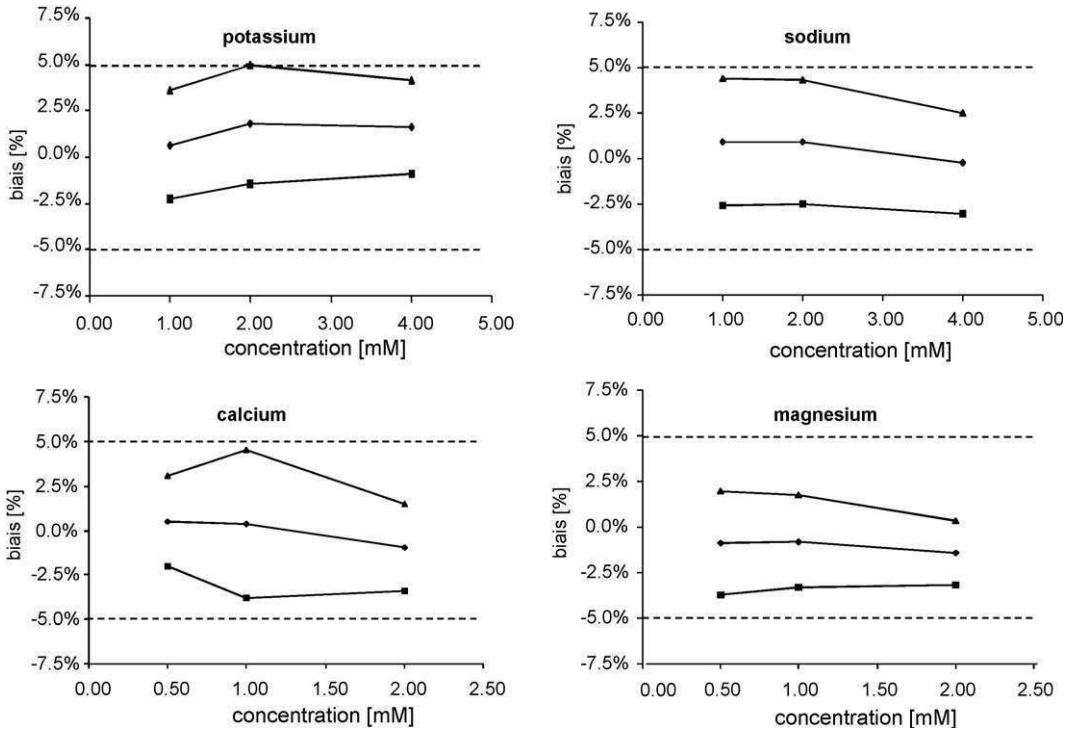

Fig. 4. Accuracy profile of the developed CE-C^4D method for the determination of sodium, potassium, calcium and magnesium in total parenteral nutrition. The dashed lines represent the acceptance limits of ±5%.

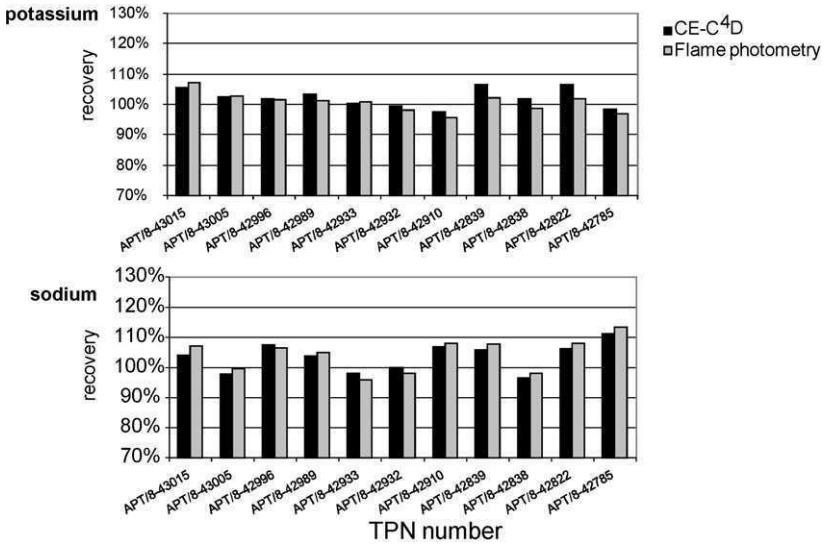

Fig. 5. Comparison of the results obtained by the developed CE-C^{4}D method and the determination by flame photometry for potassium and sodium.

sodium and potassium were confirmed by flame photometry (IL 243 flame photometer, Instrumentation Laboratory (Italy)) used as a reference method at the pharmacy of HUG. The results are shown in Fig. 5. The two methods were compared with the *t*-test for paired samples and were statistically identical for the determination of sodium and potassium (data not shown).

4. Conclusions

A simple method was developed for the quantitative determination of potassium, sodium, calcium and magnesium in TPN solutions by CE-C^{4}D. Under these conditions, even if the tested compounds did not possess chromophore groups, the developed method exhibited very good quantitative performance in terms of accuracy and precision with an analysis time of less than 4 min for all cations. The results demonstrated that CE-C^{4}D analysis is very useful for the determination of cations in parenteral nutrition formulations and the method was successfully applied in daily quality control.

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Article V

Determination of suxamethonium in a pharmaceutical formulation by capillary electrophoresis with contactless conductivity detection (CE-C^4D)

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Determination of suxamethonium in a pharmaceutical formulation by capillary electrophoresis with contactless conductivity detection (CE-C^4D)

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ABSTRACT

A simple method based on capillary electrophoresis with a capacitively coupled contactless conductivity detector (CE-C^4D) was developed for the determination of suxamethonium (SUX) in a pharmaceutical formulation. A hydro-organic mixture, consisting of 100 mM Tris–acetate buffer at pH 4.2 and acetonitrile (90:10, v/v), was selected as background electrolyte (BGE). 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. Under these conditions, a complete separation between SUX, sodium ions and the main degradation products (choline) was achieved in less than 4 min. The presence of acetonitrile in the BGE allowed a reduction of SUX adsorption on the capillary wall. The CE-C^4D method was validated, and trueness values between 98.8% and 101.1% were obtained with repeatability and intermediate precision values of 0.7–1.3% and 1.2–1.6%, respectively. Therefore, this method was found appropriate for controlling pharmaceutical formulations containing suxamethonium and degradation products.

1. Introduction

Suxamethonium (SUX) chloride (also known as succinylcholine) is a medication widely used in emergency medicine and anesthesia to induce muscle relaxation. It is used to make endotracheal intubation possible and acts as a depolarizing neuromuscular blocker [1]. SUX has two quaternary ammonium groups contributing to the very high polarity of the compound (Fig. 1). The chemical instability of SUX is well known [2]; it is rapidly hydrolyzed in aqueous alkaline solution to succinylmonocholine (SMC) and choline, and further hydrolyzed to choline and succinic acid. The chemical structures of these compounds are shown in Fig. 1. The adsorption on different surfaces, as well as the stability of SUX and its major hydrolysis product (SMC), was investigated by Tsutsumi et al. [2]. An adsorption of SUX to glassware (not to plasticware) occurred, and the sufficient stability of the samples was demonstrated in acidic conditions and in distilled water.

Several analytical methods were previously reported for the determination of suxamethonium [1–6]. The lack of a chromophore required other detection techniques in place of direct UV absorbance. Most of them used HPLC coupled with mass spectrometry (MS) [2,4] or electrochemical detection [1,3]. The separation of SUX and its degradation products by HPLC was often insufficient, and analysis times of more than 20 min were needed. Because these studies did not demonstrate a complete separation between SUX and its degradation products, a highly selective detector like MS was needed to counterbalance the low resolution of the analytical separation. Capillary electrophoresis (CE) with indirect UV detection is an alternative to analyze quaternary ammonium compounds [7–10]. SUX can be analyzed by CE, since it is a small molecule with a high polarity. With the CE-indirect UV analysis, it is well known that peak shapes and sensitivity depend on the relative mobilities of the analyte and the background electrolyte (BGE) [10]. Thus, in order to obtain a good detection signal, a BGE with mobility matching that of the counter-ions is required. The search for a suitable BGE in indirect optical detection remains a compromise between matching electrophoretic mobilities, concentrations, maximum absorption wavelength, molar absorptivity, and charge of the analyte [11]. Generally, indirect UV can provide an acceptable means of detection, however, with strongly reduced sensitivity [12]. Another approach to determine SUX was achieved by CE coupled with attenuated total internal reflectance infrared microspectroscopy (FT-IR) [6]. However, no simple method that sufficiently separated SUX and its degradation products in a pharmaceutical formulation has been described in the literature.

SUX possesses high conductivity due to the presence of quaternary ammonium groups allowing a conductimetric detection. During the past few years, contactless conductivity detection (CCD)

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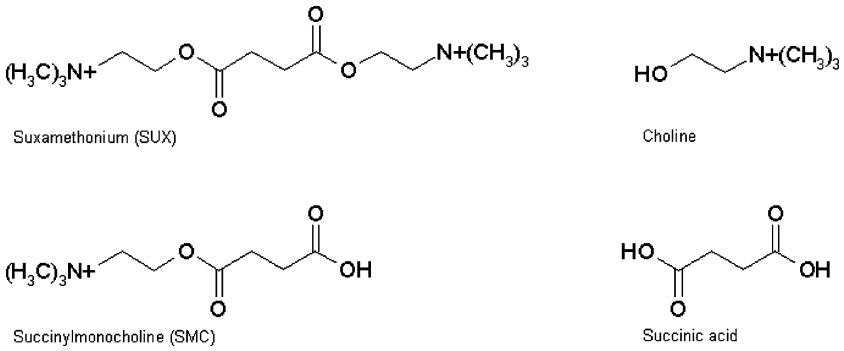

Fig. 1. Structures of suxamethonium and degradation products.

has become a good alternative to optical detection techniques in CE [12], and a capacitively coupled contactless conductivity detector (C^4D) was developed by Zemann [11,13]. The detector presents two metal tube electrodes, placed around the capillary. An oscillation frequency between 75 and 300 kHz is applied to one of the electrodes, and a signal is produced when an analyte zone with a different conductivity passes through the retention gap [14]. Inorganic cations and anions have been successfully analyzed by CE-C^4D, but the method is also suitable for organic ions such as alkylammonium cations [15]. In comparison with MS, C^4D can be considered a simple and inexpensive detection technique for routine analysis.

In this study, a CE-C^4D method was developed and validated to determine suxamethonium in a formulation and was applied to the quantitation of SUX in commercially available pharmaceutical products (Lysthenon and Succinolin).

2. Experimental

2.1. Chemicals

Succinylcholine chloride dihydrate, choline chloride, potassium chloride, and Tris(hydroxymethyl)–aminoethane (Tris) were purchased from Fluka (Buchs, Switzerland). Succinic acid was purchased from Sigma–Aldrich (Steinheim, Germany). Water and NaCl (0.9%) used for pharmaceutical preparations were obtained by Bichsel Laboratories (Interlaken, Switzerland). Acetic acid (glacial, 100%), methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). Succinylmonocholine was obtained by the degradation of a succinylcholine solution at 10 mg mL^{-1} in an alkaline solution [2]. Lysthenon (2% and 5%) was purchased from Nycomed Pharma SA (Dübendorf, Germany) and Succinolin was obtained from Amino AG (Neuenhof, Switzerland).

2.2. BGE preparation

Different BGEs (phosphate, citrate, 2-(N-morpholino) ethanesulfonic acid/histidine, lactate, acetate, at several pH and ionic strengths) were used for the development of the method. The final BGE was a hydro-organic buffer corresponding to a mixture of an aqueous BGE (100 mM Tris–acetate buffer at pH 4.2) and acetonitrile (90:10, v/v). The aqueous BGE was prepared by an adequate dilution of the concentrated acid solution, and a solution of Tris at 1 M was added to adjust the solution to pH 4.2. The solution was then diluted to the final volume with distilled water. The BGE was degassed in an ultrasonic bath for 10 min before use.

2.3. Instrumentation and capillaries

CE experiments were carried out with an $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a power supply able to deliver up to 30 kV. $HP^{3D}CE$ was coupled to a TraceDec detector (Innovative Sensor Technologies GmbH, Strasshof, Austria). The conductivity sensor consisted of two electrodes separated by a detection gap of 1 mm, positioned along the capillary by sliding it into the desired position (14.5 cm from the cathode). A CE ChemStation (Agilent) was used for CE control and data handling, and a C^4D Tracemon (Innovative Sensor Technologies) was used for conductivity detector control and data acquisition. 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 50 cm). Capillaries coated with poly(vinyl alcohol) (PVA) from Agilent (Waldbronn, Germany) with 50 μm i.d. and 32.5 cm total length were also tested. All experiments were performed in the cathodic mode. The capillary was thermostated at 25 °C in a high velocity air stream, and a voltage of 30 kV was applied. The generated current was between 5 and 50 μA depending on the buffer solution. 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 final configuration of the C^4D was set at an output frequency of 75 kHz, an output voltage of 80 Vpp, 50% of gain and an offset of ~50. The detector acquisition corresponded to the CE mode of 19.8 Hz. 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. A voltage of 30 kV was then applied for 60 min with the BGE. The TraceDec was set to run for 1 h before the first analysis in order to obtain a constant signal. Prior to each sample injection, the capillary was rinsed by pressure (940 mbar) for 3 min with fresh BGE. When not in use, the capillary was rinsed with water and methanol. As the electrophoresis process altered the running buffer pH by electrolysis and subsequently changed the migration times, the separation buffer was refreshed every six runs.

2.4. Method validation

A validation was performed to estimate the potential of the method for the quantitative analysis of suxamethonium in a pharmaceutical formulation. The validation was based on the guidelines of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [16] and carried out over three series. Each series involved the injection of a freshly prepared BGE, two calibration standards (CS), four validation standards or quality control (QC) samples at 80%, 100% and 120% each, water and methanol rinsing of

the capillary, and instrument shut-off. Potassium chloride was used as the internal standard (IS). The calculations were performed using normalized area (area/migration time) ratios of suxamethonium on the internal standard.

2.5. Sample preparation

A stock standard solution was prepared by dissolving suxamethonium in water for CS and in 0.9% NaCl for QC in order to obtain a concentration of 10 mg mL^{-1}, and was stored at 4 °C until use. The stock standard solution for QC corresponds to a commonly presented dilution of SUX in the hospital. The IS stock solution was prepared by dissolving potassium chloride in water at a concentration of 10 mM. For CS and QC, three concentration levels were prepared at 80%, 100% and 120% of the target value by diluting the appropriate volume of SUX stock solution in distilled water. Potassium chloride was added as an internal standard to obtain a final concentration of 0.4 mM. Sample solutions were stable for more than 2 days at 4 °C, and no degradation was observed for the tested analytes during analysis. To study the separation of SUX and its degradation products, samples with 10 mg mL^{-1} of SUX, choline, succinic acid and SMC were prepared in 0.9% NaCl.

2.6. Application to pharmaceutical products

Suxamethonium was determined in the commercially available pharmaceutical products Lysthenon® (2% and 5%) from Nycomed Pharma SA (Dübendorf, Germany) and Succinolin® (5%) from Amino AG (Neuenhof, Switzerland). Therefore, the formulations were diluted in distilled water to obtain a final concentration of 0.4 mg mL^{-1} of SUX with 0.4 mM of the IS corresponding to the 100% STD. The quantitative analysis was repeated five times ($N = 5$) for each formulation.

3. Results and discussion

SUX used in emergency medicine and anesthesia is administrated as an isotonic formulation. For determining this compound, a CE-C^{4}D method was developed and validated.

3.1. Method development

3.1.1. Buffer selection

The selection of the BGE was based on conductivity detection of suxamethonium and sufficient selectivity toward degradation products and sodium. In C^{4}D, the response arises from the difference in conductivity between the analytes and BGE co-ions. For obtaining the highest signal-to-noise ratio, the largest possible difference of the conductance of the analyte and electrolyte is required. Nevertheless, CE requires the use of electrolytes with a higher ionic strength compared to the sample zone in order to take advantage of the stacking effect. The compromise consists of using an amphoteric or low conductance buffer at high ionic strength [13]. Furthermore, the BGE requires a pH inferior to 7, in order to avoid the hydrolysis of suxamethonium during the analysis [2]. Among the different tested BGEs, the Tris–acetate buffer presented the best compromise for performing the complete separation of suxamethonium and its degradation products. This BGE possesses a low conductivity and can be used at an ionic strength of 100 mM without generating a high current (~20 μA). The pH range of this system was between 4 and 5, where SUX was stable.

Potassium chloride was chosen as the internal standard because it presents a much higher mobility than the other compounds (SUX, SMC, choline and sodium).

As expected with the Tris–acetate buffer, the effective mobilities of the compounds were in the following decreasing order: potassium, sodium, SUX, choline and SMC. Choline was detected close to SUX and SMC migrated afterwards, at 6 min. In these conditions, succinic acid did not interfere with the cation analysis. The other compounds were detected in less than 4 min as presented in Fig. 2.

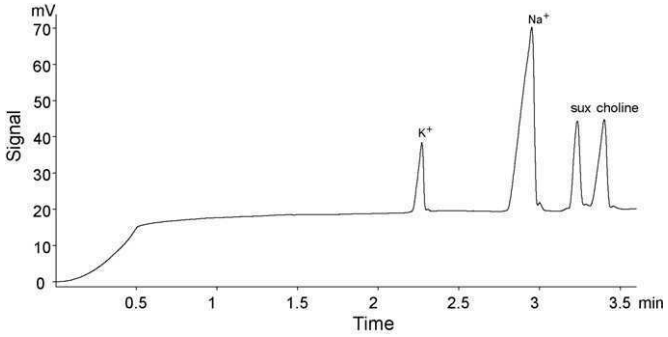

Fig. 2. Electropherogram obtained for the CE-C^{4}D analysis of a sample containing SUX (0.2 mg mL^{-1}), choline (0.2 mg mL^{-1}) and K$^+$ (0.4 mM) in an aqueous solution (presence of Na$^+$ at 3 mM). BGE: 100 mM Tris–acetate at pH 4.2, acetonitrile (90:10, v/v). All other experimental conditions are described in Section 2.3.

3.1.2. Influence of pH

The BGE conductivity depends mainly on the pH of the solution. Initial experiments were performed at pH 4.8 in order to work at the highest buffer capacity. However, the BGE conductivity decreased at lower pH values, while the detection of suxamethonium was improved. Acetate/Tris buffer solutions with different pH were tested in the buffer region, and pH 4.2 was selected since the signal to noise ratio of SUX was significantly enhanced (data not shown).

3.1.3. Influence of the buffer concentration

The first analyses were performed with a buffer concentration of 20 mM to reduce the background conductivity. Nevertheless, to improve the resolution between sodium and suxamethonium, buffer solutions with higher molarities were tested. Investigations of the effect of Tris–acetate ionic strength on the electrophoretic mobilities of organic anions showed that ion association and/or complexation equilibria could occur with this buffer system [17]. The electrophoretic mobility of ions was influenced by interactions with buffer components, which can enhance the selectivity in CE [18,19]. Acetic acid is a weak complexing agent that could interact with cations present in the system such as suxamethonium, choline, sodium or potassium. In this work, the resolution between sodium and SUX was improved by increasing the buffer concentration. Therefore, a 100 mM Tris acetate buffer at pH 4.2 was selected and, under these conditions, the generated current was still acceptable (inferior to 30 μA).

Different oscillation voltages and oscillation frequencies of the C^{4}D were tested (data not shown). An oscillation voltage of 80 Vpp and a frequency of 75 kHz gave the best results with the selected BGE.

The LOD of the method was estimated (*ca.* 10 μg mL^{-1}) and was much lower than the target value (*i.e.* 200 μg mL^{-1}) obtained after dilution of the pharmaceutical formulation.

3.1.4. Adsorption on the capillary wall

As described in the literature, quaternary ammonium groups can interact with the capillary walls [7,8,20], and a significant adsorption of SUX to glassware has been reported [2].

With the 100 mM Tris–acetate buffer at pH 4.2, deterioration of the SUX peak shape occurred after several runs (Fig. 3A). The capillary was flushed with the buffer solution for 5 min, and then a voltage of 30 kV was applied for 60 min before starting the

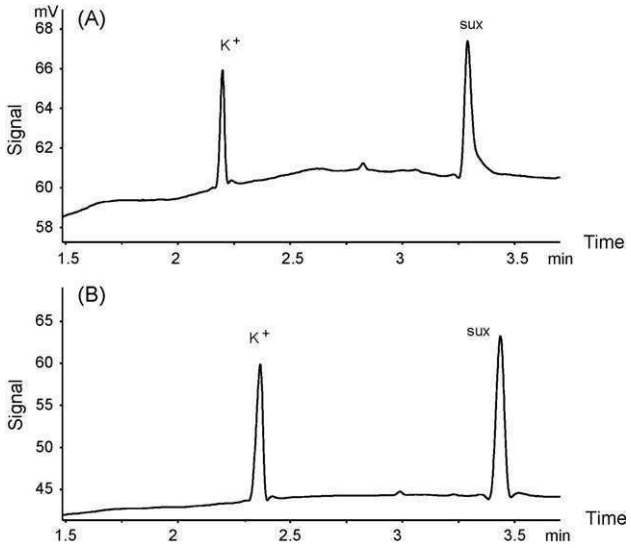

Fig. 3. Electropherograms obtained for the CE-C^{4}D analysis of a sample containing K^+ (0.4 mM) and SUX (0.2 mg mL^{-1}) in water using the BGE (A) 100 mM Tris–acetate at pH 4.2 and BGE (B) 100 mM Tris–acetate at pH 4.2, acetonitrile (90:10, v/v). All other experimental conditions are described in Section 2.3.

analysis. This procedure contributed to a constant peak shape, but a tailing was observed and the symmetry was insufficient (0.54).

To avoid the adsorption of the compound onto the capillary wall, different strategies could be applied. The use of higher temperatures or extreme pH values could help to decrease adsorption [21], but due to the low stability of suxamethonium, this approach was not investigated. In the literature, the use of organic modifiers in the BGE is often recommended [7,8,20] since they change the viscosity and the solvation ability of the carrier electrolyte, thus inducing better peak shapes. As an example, methanol, acetonitrile and tetrahydrofuran were tested as buffer additives in order to disrupt micelle formation within the sample of cationic surfactants (quaternary ammoniums) and to reduce the ability of surfactants to strongly adsorb onto the capillary walls [20]. In our case, the peak shape of suxamethonium was not influenced by methanol, introduced in the BGE at 10% and 20% (data not shown). However, 10% acetonitrile in the BGE gave a better peak shape (symmetry of 1.16), compatible with a quantitative determination of suxamethonium. Electropherograms with and without acetonitrile in the BGE are shown in Fig. 3.

Another approach to reduce adsorption on the capillary wall was the use of PVA-coated capillaries. These have been used to overcome adsorption problems of proteins [21] and quaternary ammoniums [22]. The peak shape of SUX was improved by using PVA capillaries and the aqueous acetate/Tris buffer (data not shown). Nevertheless, PVA capillaries are more expensive than ordinary uncoated capillaries. Therefore, the hydro-organic solution was preferred for further routine analyses of suxamethonium.

3.2. Method validation

The developed method was validated according to the SFSTP recommendations. Quantitative performance was estimated in three separate series ($j = 3$) with the V2 protocol [16]. This protocol involves three concentration levels ($k = 3$) with two repetitions ($n = 2$) for calibration standards and three concentration levels ($k = 3$) with four repetitions ($n = 4$) for validation or quality control samples.

Table 1
Validation results: trueness, repeatability and intermediate precision of the developed CE-C^{4}D method for the analysis of suxamethonium in a pharmaceutical formulation.

Theoretical concentration of suxamethonium	Trueness	Repeatability (CV)	Intermediate precision (CV)
80%	98.8%	1.1%	1.2%
100%	100.2%	1.3%	1.3%
120%	101.1%	0.6%	1.6%

The calibration curve was obtained for each series with conventional least-squared linear regression using the three concentration levels (80%, 100% and 120% of the target value). After establishing the calibration curves for each series, concentrations of the QC were computed from the analytical response to obtain trueness, repeatability and intermediate precision. Trueness was expressed as the ratio between the theoretical and average measured values at each concentration level. Repeatability and intermediate precision were expressed as the coefficient of variation (CV%) of the ratio of the intra-day standard deviation (s_r) and between-day standard deviation (s_R), respectively, on the theoretical concentrations as described in [23]. The s_r and s_R values were obtained thanks to ANOVA analysis. As reported in Table 1, the trueness and precision values were in accordance with regular recommendations for the analysis of pharmaceutical formulations over the tested concentration range. The CV (repeatability and intermediate precision) was lower than 2%, with trueness between 98.8% and 101.1%. To visualize the overall method variability, the accuracy profile was built combining trueness and intermediate precision as the confidence interval [24]. As presented in Fig. 4, the total error did not exceed the acceptance limits (±5%) for all concentration levels. Consequently, the developed CE-C^{4}D method could be considered accurate for SUX over the tested range.

3.3. Application to pharmaceutical products

In order to demonstrate the applicability of the CE-C^{4}D method to real samples, quantitation of SUX was achieved on three commercially available pharmaceutical products: Lysthenon® (2% and 5%) from Nycomed Pharma SA (Dübendorf, Germany) and Succinolin® (5%) from Amino AG (Neuenhof, Switzerland). The concentration of SUX was calculated with reference to a calibration curve constructed the same day. CS at three concentration levels were replicated twice, and conventional least-squared linear regres-

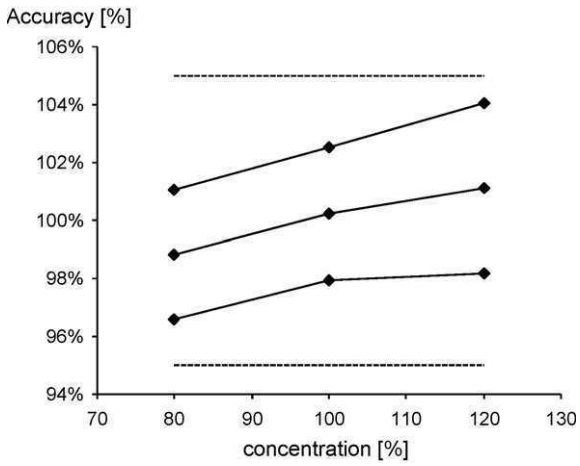

Fig. 4. Accuracy profile of the developed CE-C^{4}D method for the determination of suxamethonium in a pharmaceutical formulation using a linear regression model. The dashed lines represent the acceptance limits of 95% and 105%.

sion was applied. Since five independent analyses ($N = 5$) were performed on each pharmaceutical formulation, the result of the analysis could be expressed as

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

where N is the number of analyses performed during the routine analysis and $\bar{x}$ is the mean result. The $t_{\text{d.f.},\alpha}$ (student constant depending on d.f. and α set at 5%), s_r^2 and s_g^2 variance values were determined during validation with the regular ANOVA-based variance decomposition [24]. The analysis repetition was useful to obtain a smaller confidence interval, since most of the variability came from repeatability (s_r^2). In Lysthenon (2%), a SUX concentration of $20.2 \pm 0.2\ \text{mg mL}^{-1}$ was determined. Lysthenon (5%) contained $50.0 \pm 0.5\ \text{mg mL}^{-1}$ and Succinolin contained $51.3 \pm 0.5\ \text{mg mL}^{-1}$ of SUX. The indicated concentrations of the pharmaceutical products were confirmed to be in the authorized specifications of ±5% of the target value (19–21 mg mL^{-1} and 47.5–52.5 mg mL^{-1}, respectively) by the developed CE-C^{4}D method.

4. Conclusions

A simple method was developed for the quantitative determination of suxamethonium in pharmaceutical formulations by capillary electrophoresis with a capacitively coupled contactless conductivity detector. The developed method exhibited very good quantitative performance in terms of accuracy and precision with an analysis time of less than 4 min for SUX and its main degradation product (choline). The problem of adsorption onto the capillary wall could be reduced by the addition of acetonitrile. The results demonstrate that the CE-C^{4}D analysis is very useful for the determination of SUX in commercial products and can be used as a routine technique in quality control for compounds possessing quaternary ammonium groups.

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Article VI

Development of ready-to-use succinylcholine syringes for safe use in general anesthesia

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Development of ready-to-use succinylcholine syringes for safe use in general anesthesia

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KEYWORDS

Suxamethonium, Succinylcholine chloride, drug stability, centralised intravenous additive service (CIVAS), ready-to-use syringe, anaesthesia, risk management

ABSTRACT

Study objectives: To increase safety in the use of succinylcholine chloride (SUC) during anesthesia, by providing a ready-to-use (RTU) intravenous syringe. This preparation should be developed and produced according to good manufacturing practices.

Methods: The chemical stability of SUC solution (10mg/mL) in 0.9% sodium chloride was determined at 4°C, 25°C, and 40°C by a capillary electrophoresis method. pH and nonvisible particulate matter were measured throughout the study. Sterility testing including endotoxin analysis was also performed.

Results: The solution stored at 40°C was stable during 40 days. The loss in potency was less than 10% after 9 months at 4°C and 25°C. At 9 months, a degradation product (choline) appeared in the 25°C samples, and its concentration reached 8.6%.of the total peak area. After 1 year, losses in potency at 4°C, 25°C, and 40°C were 19%, 66%, and 79%, respectively. During the study, the pH diminished from 4.0–4.3 at the beginning to 3.6, 3.2, and 2.9 after 1 year at 4°C, 25°C, and 40°C, respectively. The sterility and endotoxin tests were negative in all cases and *Pharmacopeia* criteria in terms of nonvisible particles were always fulfilled.

Conclusion: RTU syringes of SUC with a 9 month period can be manufactured. Due to a pH decrease and the appearance of degradation products, we recommend the storage at 4°C. However, periods of storage at 25°C can be supported without any reduction of the expiry date. This product will reduce the risk of dilution errors and lead to significant economical advantages.

INTRODUCTION

Succinylcholine chloride is an ultrashort acting depolarizing skeletal muscle relaxant indicated as an adjunct to general anesthesia, to facilitate endotracheal intubation, and to provide skeletal muscle relaxation during surgery. Succinylcholine is also used as the sole muscle relaxant during electroconvulsive therapy.

The mechanism of action of succinylcholine involves a "persistent" depolarization of the neuromuscular junction. This substance combines with the cholinergic receptors of the motor end plate mimicking the effect of acetylcholine but without being rapidly hydrolyzed by acetylcholinesterase. Binding to the nicotinic acetylcholine receptor results in opening of the receptor's nicotinic sodium channel and leads to sodium movements into the cell. This results in a disorganized depolarization of the motor end plate. Succinylcholine causes profound muscle relaxation resulting in respiratory depression to the point of apnea.

The optimum dose varies among individuals ranging from 0.3 to 1.1 mg/kg for adults. Onset of flaccid paralysis is rapid (less than 1 minute after IV administration), and with single administration lasts approximately 4 to 6 minutes, a duration sufficient to allow endotracheal intubation. However, very large doses may result in more prolonged blockade.

Succinylcholine is a quaternary amine usually used in its bromide, chloride, or iodide form. The parenteral formulations are acidic (pH ≈ 3.5) and should not be mixed with alkaline solutions having a pH greater than 8.5.

In a retrospective survey of 896 incidents relating to drug error in anaesthetic practice reported to the Australian Incident Monitoring Study, 50.4% were related to the drug preparation step [1]. The drugs most commonly involved were neuromuscular blocking agents and among them succinylcholine was in the first position. Furthermore, to be prepared for the potential need of an emergency intubation, succinylcholine is prepared daily in our anaesthesiology department before each general anaesthesia. Most of the time, this preparation is not used and is discarded implying a consequent financial loss for the hospital (approximately 60 syringes representing 83€ each day). These safety and economic considerations have stimulated us to think of an improvement solution.

The aim of this study was to develop a ready-to-use (RTU) succinylcholine syringe for use in general anaesthesia, with a long shelf life and prepared under aseptic and good manufacturing practice (GMP) conditions to improve the safe administration of this drug.

MATERIAL AND METHODS

Material and chemical products

Polypropylene syringes: KlerpackTM BD/ 3325382 Ref KSY15010 (Shield Medicure, United Kingdom) Tamper-evident caps: TEC 1000 (BBraun, Germany)

Succinylchloride chloride**:** Ph. Eur. (Fagron, France)

Potassium chloride: Ph. Eur. (Hänseler, Switzerland)

Sodium chloride 0.9% solution : (Bichsel, Switzerland)

Tris (trishydroxymethylaminomethane): (Fluka AG, Switzerland)

Acetic acid concentrated: (Fluka AG, Switzerland)

Preparation of syringes for the stability study

An injection solution was prepared containing 0.9% sodium chloride in water and 10 mg/mL of succinylcholine chloride. The solution was sterilized by filtration (Millipore® 20 Millipack Gamma Gold 0.22μm) and 10 mL was transferred into 10 mL polypropylene syringes under a horizontal laminar-airflow hood in a GMP class B cleanroom. The syringes were closed using tamper-evident caps and stored at either 4 ± 2°C, 25 ± 2°C, or 40 ± 2°C. The solutions were analyzed at 8 different time points: immediately after preparation (day 1), after 4, 10, 40, 65, 180, 270, and 365 days.

Capillary electrophoresis (CE) analysis

CE instrumentation and conditions

We used a method developed in our laboratory; the article on this method has been previously published by Nussbaumer et al. [2]. The CE system was a CE apparatus (Agilent, Waldbronn, Germany) equipped with a contactless conductivity detector (CE-C4D) (Innovative Sensor Technologies GmbH, Strasshof, Austria). A CE ChemStation (Agilent) was used for CE control and data handling, and a C4DTracemon (Innovative Sensor Technologies) was used for conductivity detector control and data acquisition.

Separations were performed in a fused silica capillary from BGB Analytik AG (Böckten, Switzerland) with an inner diameter of 50 μm and a total length of 64.5 cm (distance to C4D detector: 50 cm). Experiments were carried out in cationic mode (i.e. anode at the inlet and cathode at the outlet) by applying a constant voltage of 30 kV with an initial ramping of

1 kV/s. The capillary was maintained at 25°C, samples were injected by pressure (40 mbar for 10 seconds), and conductivity detection was recorded. The background electrolyte solution was a 100 mM Tris-acetate buffer set at pH 4.2 and acetonitrile (90:10, v/v). The presence of acetonitrile in the background electrolyte (BGE) allowed a reduction of succinylcholine adsorption on the capillary wall. Before initial use, the capillary was sequentially washed with methanol, 0.1 M NaOH, water, methanol, 0.1 M HCl, water and BGE for 5 minutes each. Between analyses, the capillary was flushed with BGE for 3 minutes. Under these conditions, a complete separation between succinylcholine, sodium ions, and the main degradation product (choline) was achieved in less than 4 minutes. The CE-C^4D method was validated, and trueness values between 98.8% and 101.1% were obtained with repeatability and intermediate precision values of 0.7–1.3% and 1.2–1.6%, respectively. Therefore, this method was found appropriate for pharmaceutical formulations containing suxamethonium and degradation products.

Validation of the CE method

The CE method was validated according to the French Society of Pharmaceutical Science and Technology (SFSTP) guidelines [3]. Details about the validation and statistical analysis were described in the publication of Nussbaumer et al. [2].

Stability-indicating method

To study the separation of succinylcholine and its degradation products, samples with succinylcholine and the main degradation products (choline, succinylmonocholine, succinic acid) were prepared in NaCl 0.9%. With the used method, these compounds did not interfere with the succinylcholine analysis [2].

Sample preparation during the stability study

Stock solutions containing 10 mg/mL of succinylcholine hydrochloride and 10 mg/mL of potassium chloride (internal standard (IS)) were prepared using distilled water. Standard samples were prepared by diluting the stock solutions in distilled water to obtain a succinylcholine concentration ranging from 160 to 240 µg/mL and a potassium chloride concentration of 400 µg/mL. The 10 mg/mL succinylcholine hydrochloride assay solution (syringe) was diluted in distilled water to obtain a final concentration of 200 µg/mL. As with

the calibration samples, potassium chloride was added to the assay solution as an internal standard (final concentration of IS = 400 µg/mL).

Sterility testing

The tests were performed using a method developed and validated by our quality control laboratory [4]. Three syringes stored at each of the three temperatures (i.e. 4 ± 2ºC, 25 ± 2ºC, and 40 ± 2ºC) were tested for sterility on days 1, 4, 10, 40, 65, 180, 270, and 365 days.

Endotoxin (LAL) detection

The tests were performed using the method provided in the *US Pharmacopeia* for endotoxin detection [5]. Three syringes at each of the three storage temperatures were tested for endotoxin content at the eight different time intervals (see Sterility testing).

pH determination

The pH of three syringes stored at each of the three different temperatures was measured at each time interval with a glass electrode pH meter (Metrohm model 691, Herisau, Switzerland). Before each measurement, the pH meter was calibrated at pH 4.01 and 7.00.

Nonvisible particulate matter

A HIAC Royco counter (SKAN, Allschwil, Switzerland) with a HRLD-50 sensor module (serial no. 95080045) was used for the particle count determination. Three runs were carried out, and particle counts were performed at each time interval on 10 mL samples obtained from syringes maintained at the three storage temperatures. *US Pharmacopeia* standards on particulate matter contamination were applied to assess tested samples [6].

RESULTS

Figure 1 depicts a typical electropherogram of RTU succinylcholine chloride solution made of 10mg/mL in 0.9% NaCl after 12 months at 4°C with the three main peaks: succinylcholine chloride, potassium chloride (IS), and natrium chloride.

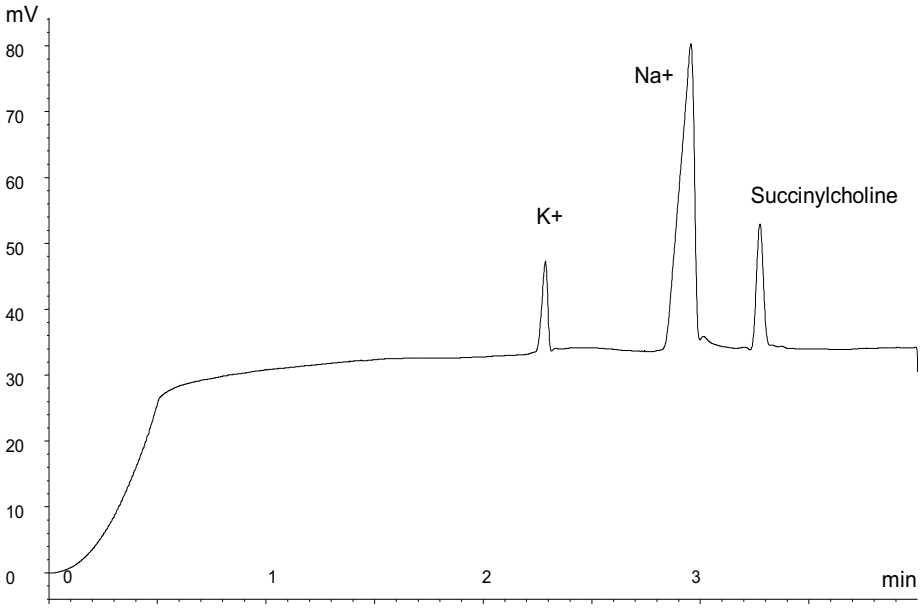

Figure 1. Typical electropherogram of succinylcholine chloride (succinylcholine chloride solution 10mg/mL in 0.9% NaCl after 12 months at 4°C)

The solutions were considered stable if the drug levels remained higher than 90% of the original concentration at the time of preparation. The solution stored at 40°C was stable for 40 days, whereas the concentration of those kept at 4°C and 25°C remained above 90% for 9 months (Figure 2).

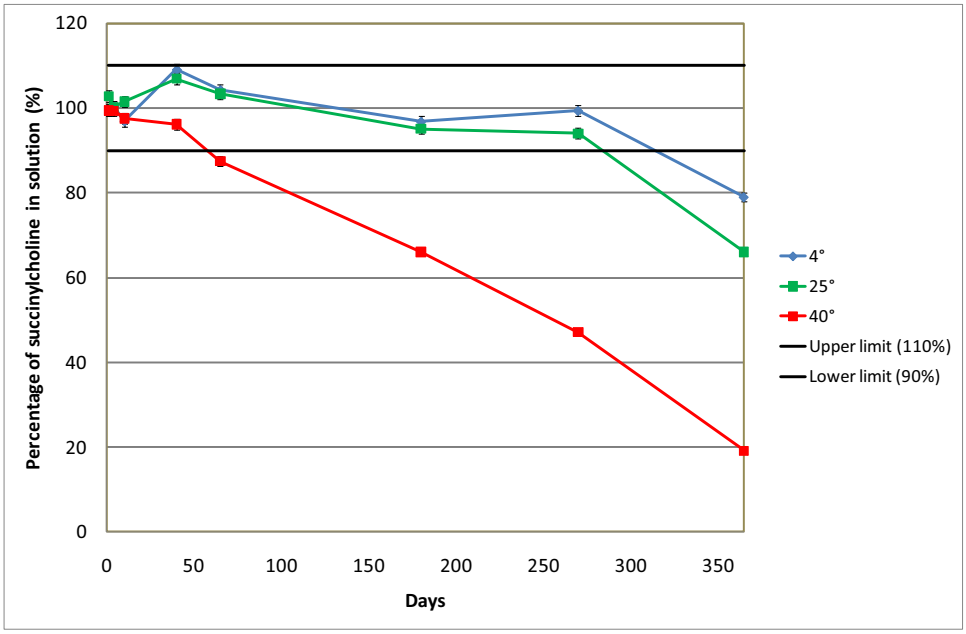

Figure 2. Stability of succinylcholine chloride 10mg/mL in NaCl 0.9% in polypropylene syringes for a period of 12 months at three temperatures.

During the first 6 months, only the succinylcholine chloride peak was detectable on the electropherograms of the syringes stored at 25°C and 4°C. At 9 months, a degradation product (choline) appeared in the 25°C samples (Figure 3), and its concentration reached 8.6% of the total peak area. After 1 year, losses in potency at 4°C, 25°C, and 40°C were 21%, 34%, and 81%, respectively.

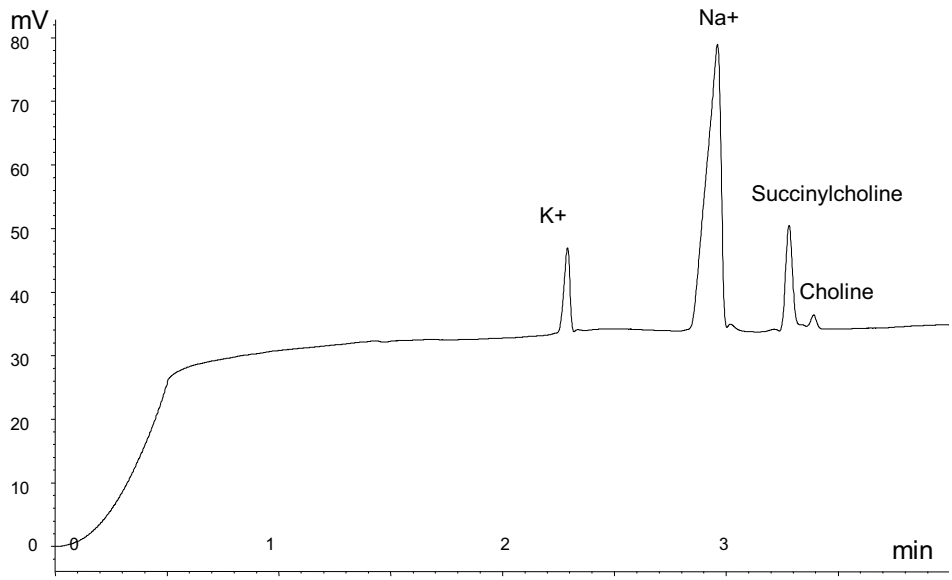

Figure 3. Succinylcholine chloride electropherogram with degradation product Choline peak (succinylcholine chloride solution 10mg/mL in 0.9% NaCl after 9 months at 25°C)

The pH diminished slightly during the study from 4.0–4.3 at the beginning to 3.6, 3.2, and 2.9 after 1 year at 4°C, 25°C, and 40°C, respectively (Figure 4). The sterility and endotoxin tests were found to be negative in all cases. The admixture remained clear without visible particulate matter throughout the study period. Furthermore, all tested syringes fulfilled *US Pharmacopeia* criteria in terms of nonvisible particles.

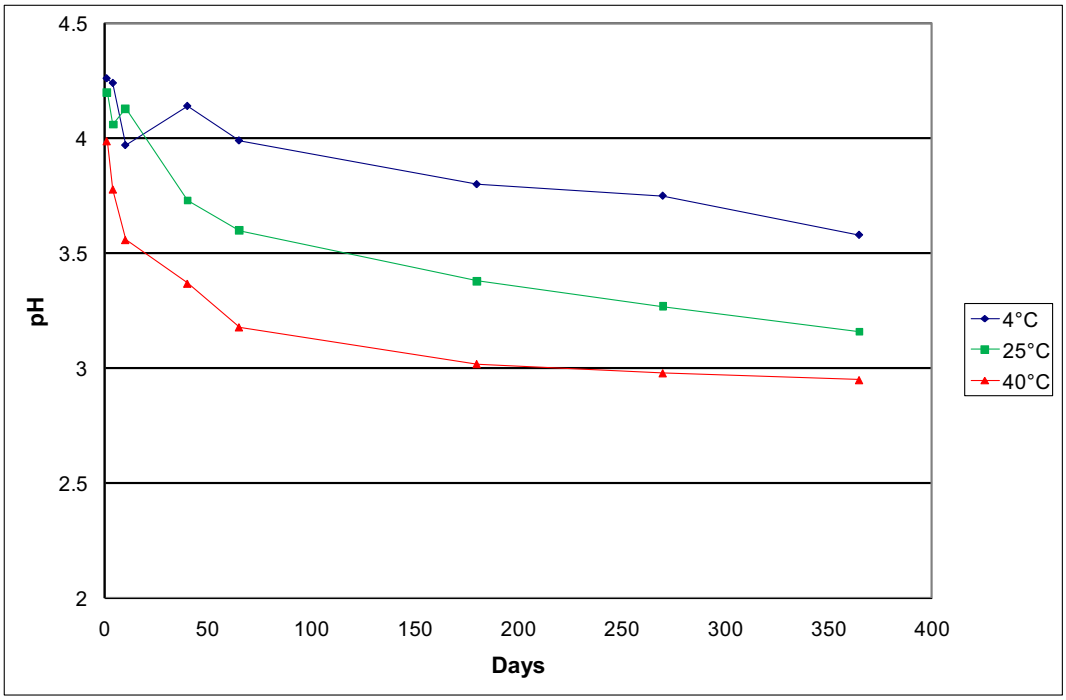

Figure 2. pH of succinylcholine chloride 10mg/mL in NaCl 0.9% in polypropylene syringes for a period of 12 months at three temperatures

DISCUSSION

The objective in developing a RTU succinylcholine syringe was to ensure the best possible safety for the patient. As already emphasized in a previous article published on a RTU ketamine syringes [7], the RTU succinylcholine syringe contributes to the safety in two ways. Firstly, because of manufacturing in aseptic conditions according to the GMP, the risk of microbiological contamination is reduced. Secondly, RTU succinylcholine syringes prevent dilution errors during preparation, which was our primary motivation for designing this new preparation. This is of significant importance, because the global error rate for drug preparation in anaesthesiology was measured to be 5% [8].

Our development contributes to an improvement of patient safety by reducing errors in succinylcholine preparation. This industrial production method has proven to be very safe and no incidents have been declared after the use of nearly 72, 000 RTU syringes (containing atropine, phenylephrine, ephedrine, insuline, isoprenaline, ketamine or vancomycine) over the last 6 years in our hospital. Furthermore, as RTU succinylcholine syringes are prepared by the hospital pharmacy, this allows for the production of standardized labels and packaging. A standardized coloured labelling scheme (in agreement with international

standards) and a standardized packaging of sealed boxes may both increase the safety and quality of this drug.

As for other RTU injectable products, it is of interest to have the longest possible shelf life, especially for succinylcholine, to optimize the production, the distribution, and the storage processes. With this objective, we carried out a long-term stability study and the results showed that the succinylcholine chloride solution remained chemically stable for 9 months, under refrigerated conditions (4°C) and at ambient temperature (25°C). Tests with methylene blue were carried out on the syringes used in this study, and the results show that these devices are totally impervious. This validates that the syringe dosage is not influenced by water evaporation and thus the chemical analysis during the 1 year period are accurate and exact.

Previously published stability studies involving succinylcholine chloride in solution have been carried out. For 20 and 50 mg/mL succinylcholine solutions at room temperature, a recent study has found a stability duration of up to 8.3 and 4.8 months, respectively [9–10]. A stability study of a 10mg/mL succinylcholine preparation compounded in vials with preservative [9] showed a stability for up to 5 months at room temperature and at least for 2 years at 4°C. In this study, succinylcholine solution protected from light or exposure to light had no significant differences in stability. Succinylcholine RTU solution prepared in this hospital pharmacy is however protected from light in a sealed cardboard box.

All these data are coherent with our results that bring new information on the stability of this active ingredient in syringes at three different temperatures. Our study provides actualized data, using capillary electrophoresis, a method suitable to simply and rapidly analyze pharmaceutical formulations in hospitals [11]. With this technique, the analysis time could be reduced by a factor of two to three in comparison with conventional techniques as HPLC. Moreover, it could represent a significant improvement not only for economical reasons (expensive LC columns are substituted by cheaper fused-silica capillaries) but also for ecological reasons (low consumption of organic solvents).

According to the Boehm et al. study [12], the pH range at which a maximal stability of succinylcholine is obtained is between 3.75 and 4.5 for unbuffered solution. At this pH range, succinylcholine degradation is independent of hydrolysis rate (zero order kinetics). In our study, the pH are maintained (Figure 4) in the range of 3.75–4.5 up to 30 days at 25°C and up to 9 months at 4°C. As a precaution, we recommend storing succinylcholine RTU at 4°C to

minimize the degradation occurring during long-term storage of solutions. This decision is reinforced by the appearance of a degradation product in significant amounts at 9 months at 25°C. However, succinylcholine concentrations remained over 90% after 9 months at 25°C and we concluded that short storage at ambient temperature during operation will not affect the shelf-life of the 9-month syringes. We recommend storing again the unused syringes in the refrigerator.

From an economic standpoint, RTU succinylcholine syringes are profitable for three main reasons. First, succinylcholine is always prepared before a general anaesthesia and most of the syringes are discarded at the end of the operation. Unused RTU syringes in their original unopened carton boxes can be returned to stock and made available for other patients. This reduces costs because of wastage and we estimated the saving to 20'000 syringes (27'700 €) per year in our hospital.

Second, as RTU succinylcholine syringes decrease the risk of error, we could hypothesize indirect savings consecutive to a reduction of mistakes. This may especially be important for drugs similar to succinylcholine, because a potential overdose may strongly depress vital organs, which would require expensive procedures, such as resuscitation in an intensive care unit.

Third, it is obvious that the time the anaesthetist staff previously spends in succinylcholine syringe preparation before anaesthesia can be saved for other activities allowing indirect cost savings.

CONCLUSION

Ready-to-use succinylcholine 10 mg/mL syringes supplied by the hospital pharmacy and stored in the anaesthesiology unit were found to be stable at 4°C and at room temperature for 9 months with no significant loss in potency. Due to a more marked pH decrease in solutions stored at higher temperature and the appearance of degradation products, we recommend by precaution to store the syringes at 4°C. However, periods of storage at ambient temperature can be supported without any reduction of the expiry date.

The long shelf life of these preparations are compatible with an efficient production of batches and the availability of pre-filled syringes of highly active injectable drugs should be encouraged in hospitals as part of a global strategy to improve the safety of drugs use.

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Article VII

Quality control of pharmaceutical 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).

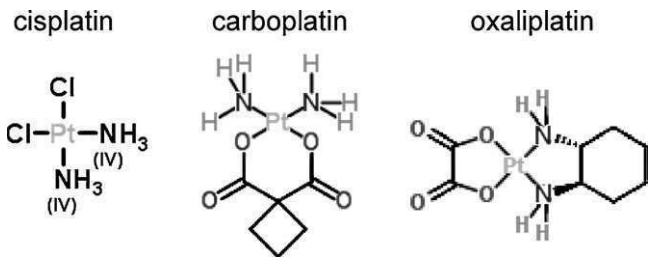

Fig. 1. Structures of cisplatin, carboplatin and oxaliplatin.

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-

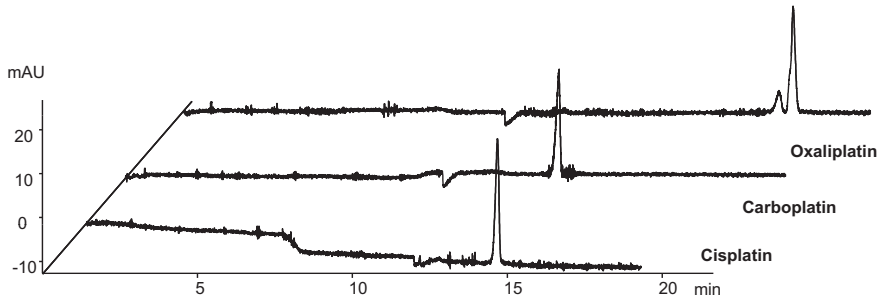

Fig. 2. Electropherogram obtained for the MEEKC–UV analysis of standard samples containing cisplatin, carboplatin, and oxaliplatin at 0.5 mg mL^{-1} in an aqueous solution. BGE: 20 mM phosphate at pH 7.0 with 6.6% (w/v) n-butanol, 3.3% (w/v) SDS, and 0.78% (w/v) n-heptane. Voltage: 20 kV. All other experimental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

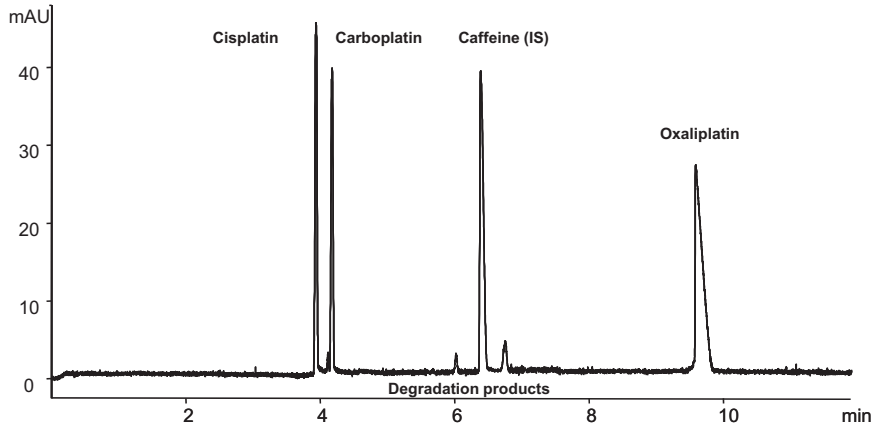

Fig. 3. Electropherograms obtained for the MEKC–UV analysis of a standard sample containing cisplatin, carboplatin, and oxaliplatin at 0.5 mg mL^{-1} with caffeine (IS) at 0.1 mg mL^{-1} in an aqueous solution. BGE: 25 mM phosphate at pH 7.0 with SDS 80 mM. All other experimental conditions are described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

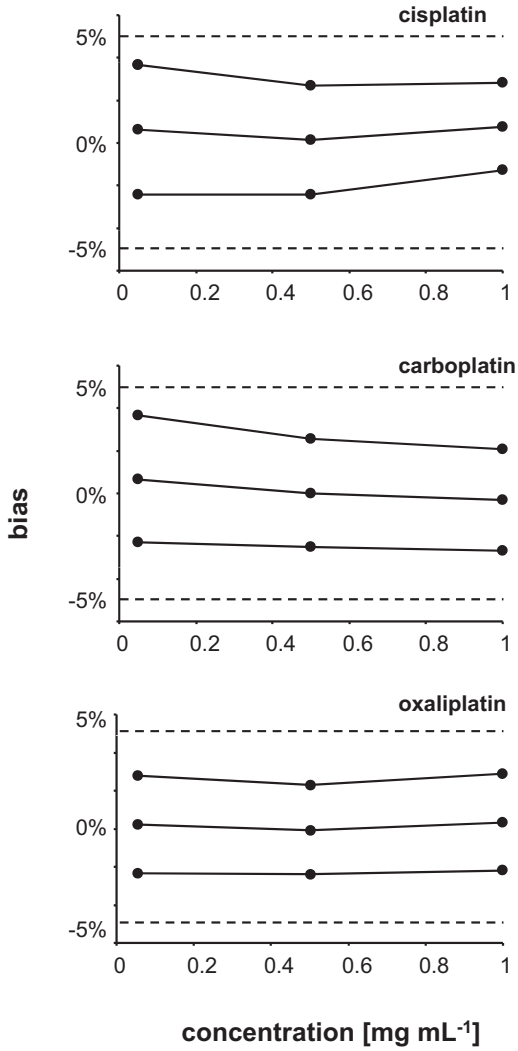

Fig. 4. Accuracy profiles of the developed MEKC–UV method for the determination of cisplatin, carboplatin, and oxaliplatin in a pharmaceutical formulation using a linear regression model. The dashed lines represent the acceptance limits of ±5%.

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 VIII

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

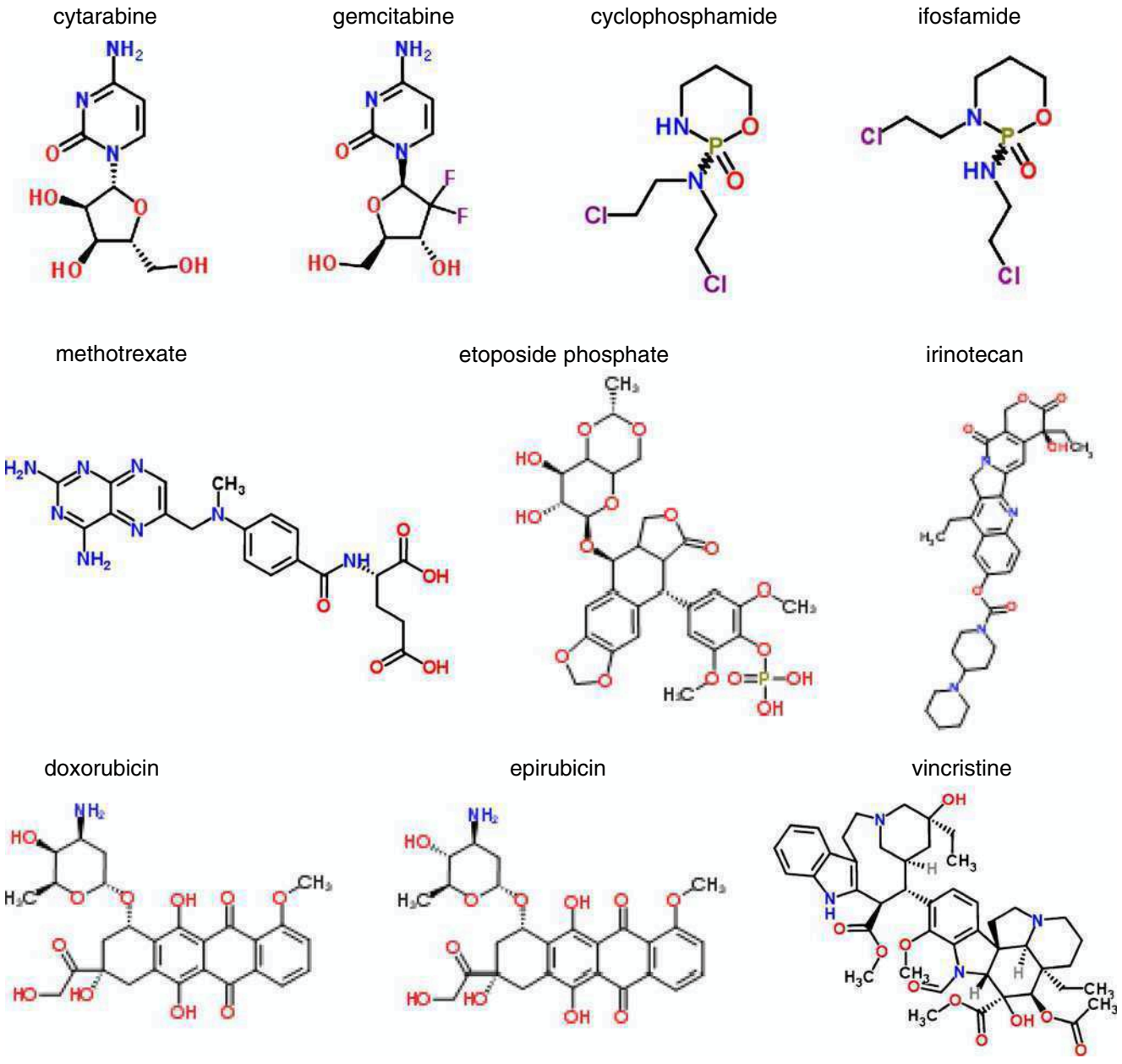

Fig. 1 Chemical structure of the ten studied cytotoxic drugs

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).

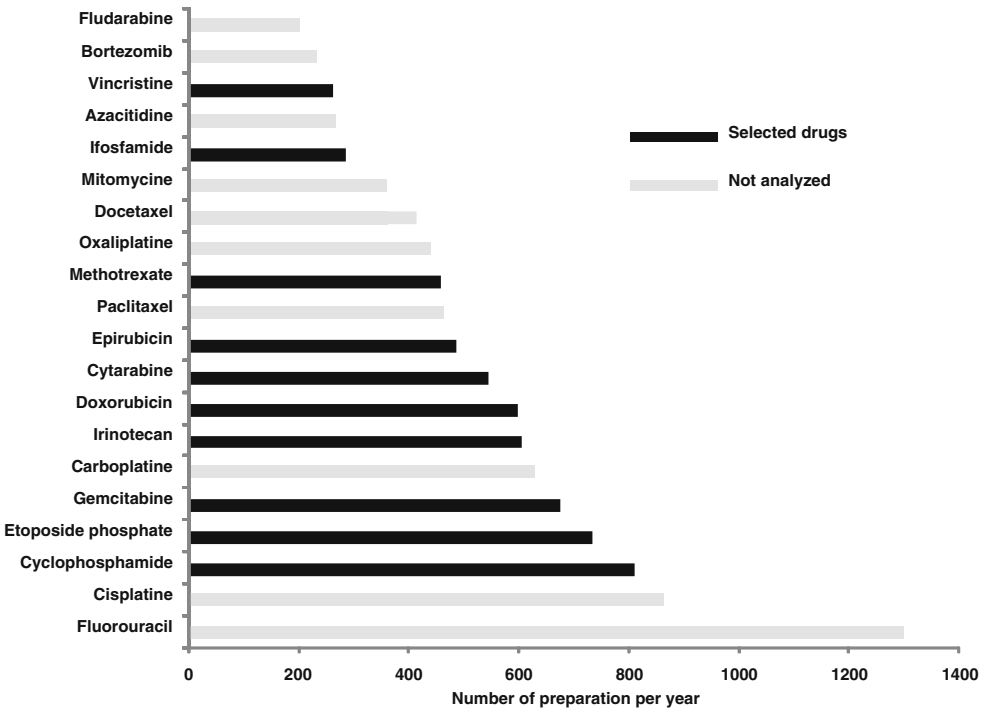

Fig. 2 Overview of the 20 most prepared cytotoxic drugs at the pharmacy of Geneva University Hospitals in 2009. *Black backgrounds* represent the selected compounds

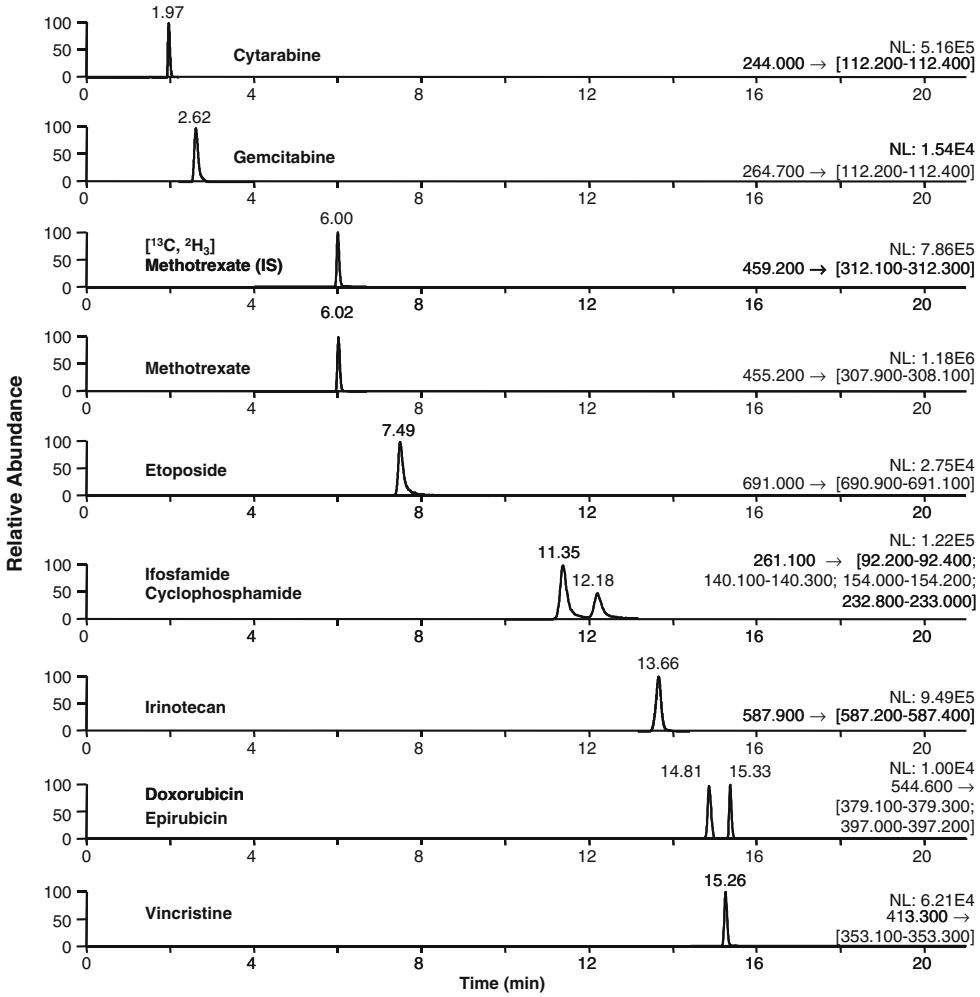

Fig. 3 LC–MS/MS chromatogram of a sample containing ten cytotoxic drugs and the internal standard at 50 ng mL^{-1}. Column: ZORBAX SB-C18 RR 2.1×100 mm 3.5 μm; flow rate 200 μL min^{-1}; gradient conditions and scan events are reported in Tables 2 and 3, respectively

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

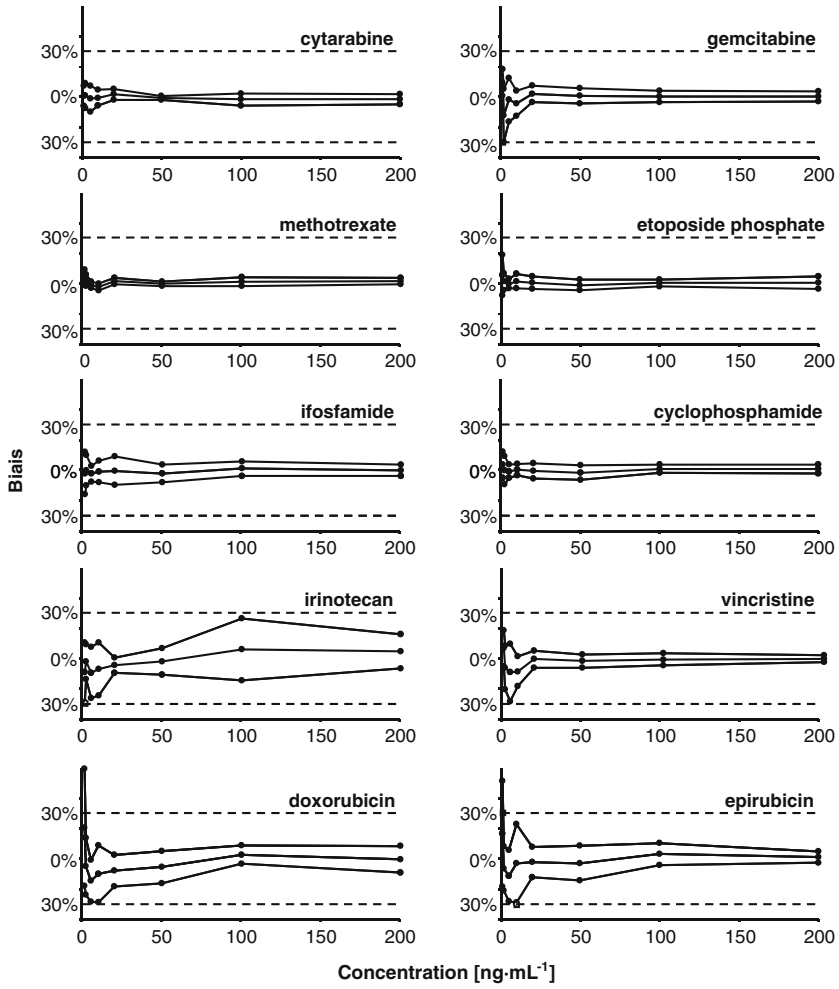

Fig. 4 Accuracy profile for the quantification of ten cytotoxic drugs by LC–MS/MS in the concentration range from 1 to 200 ng mL^{-1}, calculated from the trueness and intermediate precision reported in Table 4; *dashed lines* represent the acceptance limits of ±30%

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 IX

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.

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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,

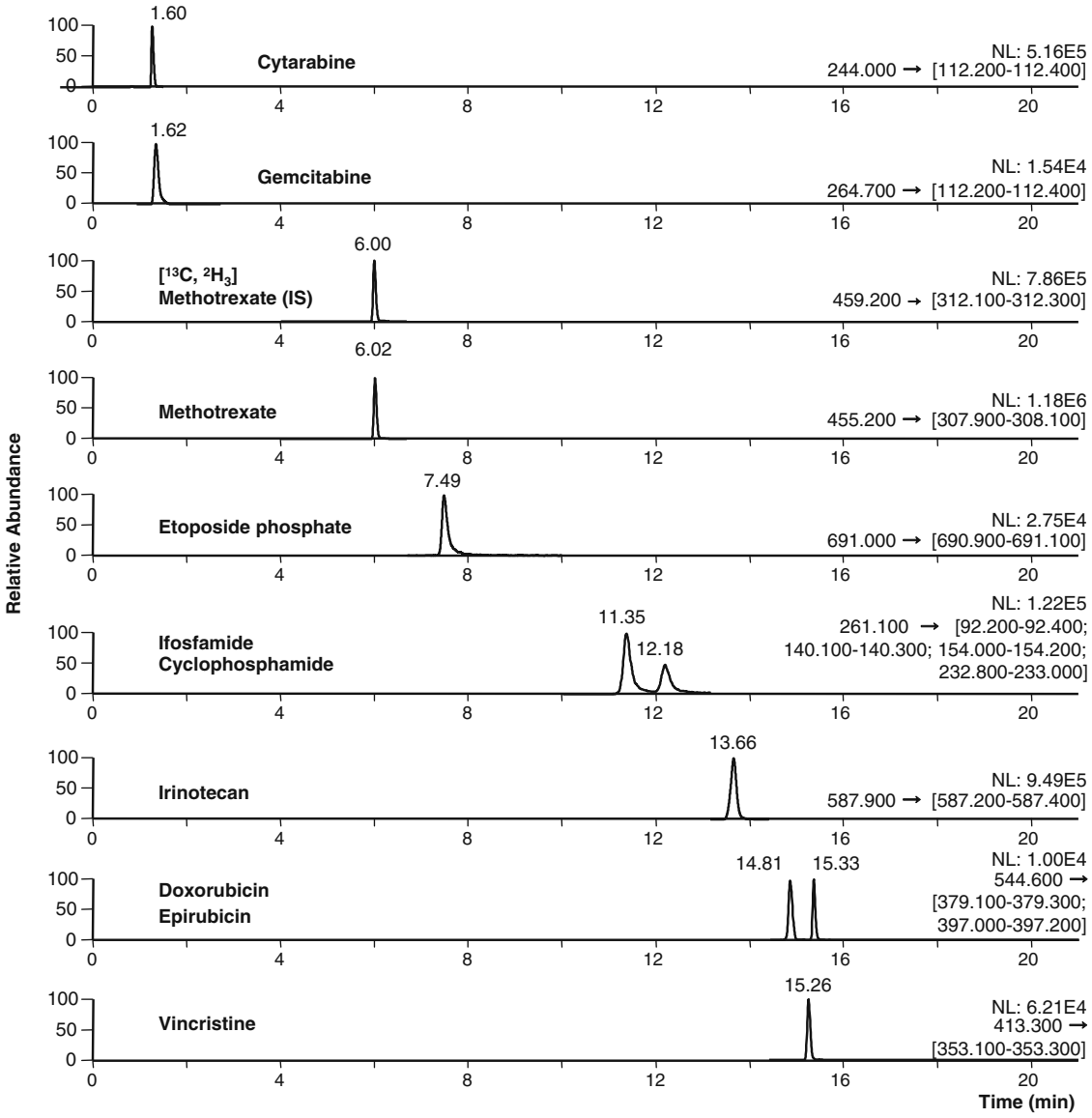

Fig. 1 LC–MS/MS chromatogram of a calibration sample containing the ten cytotoxic drugs and the internal standard at 50 ng mL^{-1} in ACN 20% with 0.1% FA. Column: ZORBAX SB-C18 RR 2.1 × 100 mm, 3.5 μm; flow rate, 200 μL min^{-1}; gradient conditions are reported in "Experimental" and scan events are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

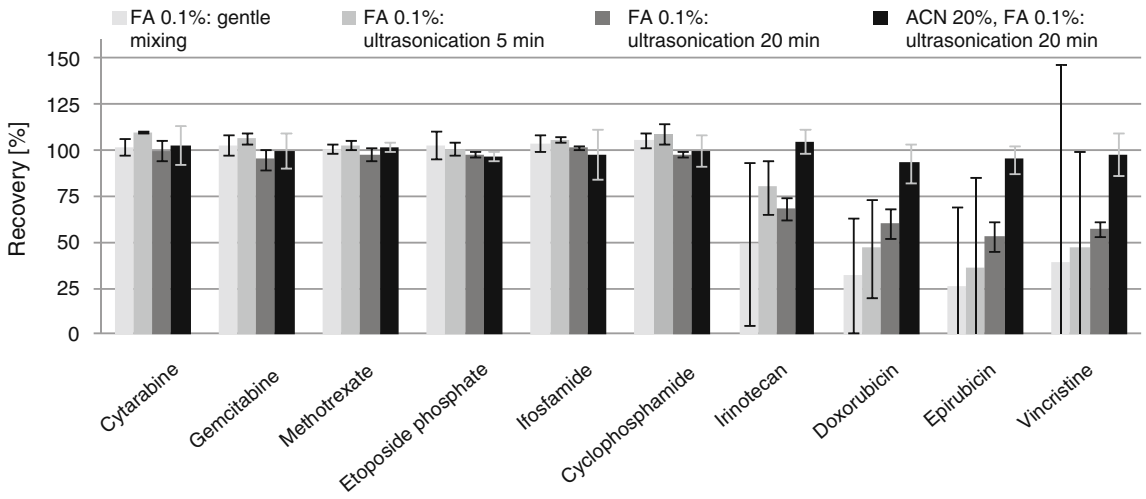

Fig 2 Desorption step development with Whatman papers: recovery of cytotoxic drugs as function of different procedures: FA 0.1% with gentle mixing, FA 0.1% with ultrasonication for 5 min, FA 0.1% with ultrasonication for 20 min, and 20% ACN, FA 0.1% with ultrasonication for 20 min

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.

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.

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

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

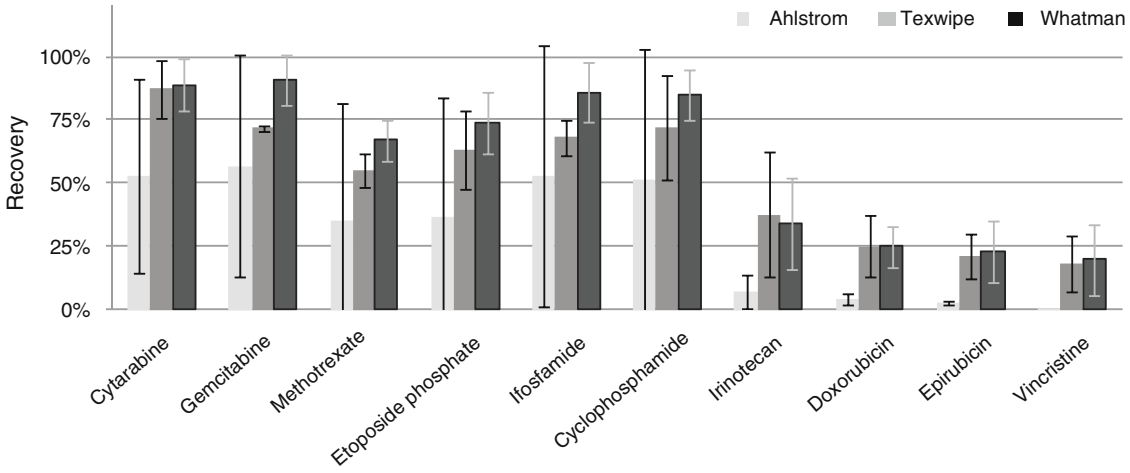

Fig 3 Recovery of ten cytotoxic drugs from a stainless steel surface obtained with different wiping papers (Ahlstrom, Texwipe, Whatman) previously wetted by water during wiping step development

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-

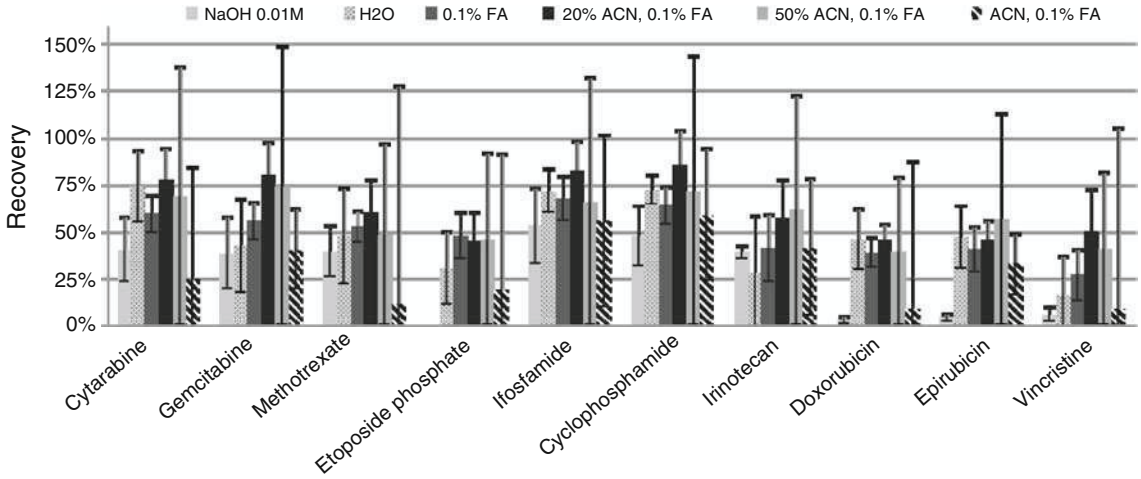

Fig 4 Wiping step development: recovery of cytotoxic drugs as function of different wiping solvents (water, FA 0.1%, NaOH 0.01 M, 20% ACN with 0.1% FA, 50% ACN with 0.1% FA and ACN with 0.1% FA). Wiping was performed with Whatman filter paper on stainless steel surface as described in "Experimental"

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

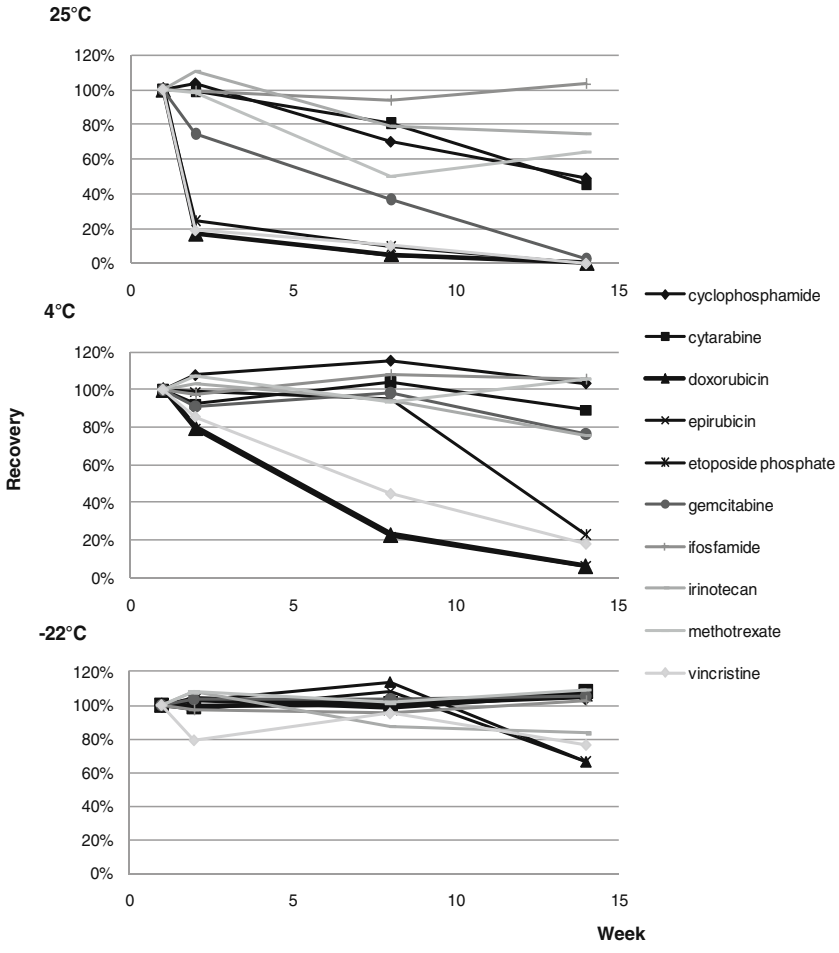

Fig 5 Stability data of the ten cytotoxic drugs on wiping papers stored at −22, 4 and 25°C for 3 months

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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Chapitre 4: Conclusions et perspectives

Ce travail de thèse a permis de démontrer l'importance de l'analyse de médicaments produits en milieu hospitalier, d'une part pour assurer la qualité du produit fabriqué et de l'autre pour sécuriser des processus de travail pour le personnel qui les manipule. Ceci a été illustré avec différentes applications concernant la détermination de composés non-UV absorbants et cytotoxiques contenus dans des formulations pharmaceutiques hospitalières.

Dans un premier temps, l'intérêt de la CE pour le contrôle qualité de médicaments en milieu hospitalier a été mis en évidence par les bonnes performances quantitatives, le gain de temps, d'argent, et par le respect de l'environnement. Deux méthodes CE-C^4D ont été développées et validées pour l'analyse de composés non-UV absorbants. La première méthode a permis la détermination des électrolytes dans les NP et la mise en place d'un contrôle qualité quotidien de ces formulations à haut risque avant administration au patient. Ce contrôle qualité est pratiqué aujourd'hui avec succès en routine et a permis la détection de NP non-conformes. Ainsi, il contribue à l'amélioration de la sécurité du processus des NP à l'hôpital. La deuxième méthode a permis la détermination du suxaméthonium dans des spécialités pharmaceutiques ainsi que la mise en œuvre d'une étude de stabilité concernant une nouvelle formulation prête à l'emploi. Cette seringue est maintenant à disposition des anesthésistes et présente une stabilité suffisante pour pouvoir répondre de manière optimale à leurs besoins, tout en garantissant une production efficiente par lots de grande taille. Pour le contrôle qualité de formulations cytotoxiques, la CE s'est montrée particulièrement adaptée, car la quantité de déchets contaminés est très faible et la séparation est réalisée en système clos. Ainsi, des méthodes basées sur la MEKC-UV et MEEKC-UV ont été développées pour la détermination de cisplatine, carboplatine et oxaliplatine dans des préparations de chimiothérapie. Une autre approche intéressante pour l'analyse des cytotoxiques est la LC-MS/MS, dont la sensibilité permet de détecter de très faibles concentrations de l'ordre du $ng \cdot mL^{-1}$. Ainsi, un échantillon de seulement quelques µL est nécessaire et l'exposition durant l'analyse est limitée. La méthode développée dans ce travail a pu être utilisée pour la détermination simultanée de 10 cytotoxiques, ce qui a facilité la mise en place d'un contrôle de ces formulations (avec une seule colonne, les mêmes solvants, pas de changement de méthode).

Parmi les préparations hospitalières étudiées dans ce travail, l'analyse d'une production de série, comme le suxaméthonium dans les seringues prêtes à l'emploi, est obligatoire. Par contre, les nutritions parentérales et les chimiothérapies, lorsqu'elles sont fabriquées sur prescription magistrale, ne requièrent aucune analyse quantitative. Toutefois, pour sécuriser ces traitements médicamenteux à haut risque pour le patient et/ou le personnel soignant, des analyses peuvent se révéler utiles. En outre, pour sécuriser le processus et pour simplifier la mise à disposition des nutritions parentérales, des formules standards ont été développées pour la néonatologie par une autre doctorante de la pharmacie (Lucie Bouchoud). Un contrôle qualité de ces préparations fabriquées en petites séries devient alors obligatoire du point de vue réglementaire. Pour les chimiothérapies, une situation similaire pourrait exister, puisqu'il est envisagé d'introduire le concept du ''dose-banding'', qui standardise les doses des cytotoxiques par paliers, afin de rationaliser la fabrication de traitements de chimiothérapie. Une fabrication en petite série permettrait d'augmenter la sécurité avec des préparations libérées par un contrôle qualité et d'économiser du temps et de l'argent pour le service qui prépare les chimiothérapies. Une production de chimiothérapies standardisées, nécessiterait aussi des méthodes indicatrices de stabilité, afin de déterminer la durée d'expiration et la conservation de ces préparations. L'analyse des préparations cytotoxiques pour le contrôle qualité présentera donc aussi dans le futur une grande importance, en permettant une fabrication sûre et rationnelle.

Dans la 2ème partie du travail, la problématique de l'exposition du personnel manipulant les cytotoxiques a été abordée avec la détermination de la contamination de traces de ces médicaments sur les surfaces. Une procédure de prélèvement de surface couplée à une méthode LC-MS/MS validée a été développée pour la détermination simultanée de 10 substances cytotoxiques. Les performances quantitatives ont été clairement définies en termes de rendement et précision en fonction du composé et de la surface testée. Finalement, cette procédure a permis de déterminer le degré de contamination au sein de l'unité cytotoxique de la pharmacie des HUG et a montré une importante contamination à l'intérieur des isolateurs où sont préparées les chimiothérapies, mais au contraire peu de traces dans la zone logistique. Ainsi, différentes actions pourront être entreprises :

- La réévaluation de la procédure de décontamination et de nettoyage des isolateurs. Des prélèvements avant et après nettoyage n'ont pas montré des différences significatives et le nettoyage n'était pas efficace du point de vue chimique. Différentes solutions de nettoyage peuvent être comparées via la méthode développée afin de déterminer le nettoyage chimique le plus efficace. Ce travail est repris par Thomas Queruau Lamerie, doctorant à l'Université de Lille et stagiaire au LCQ.

- La comparaison des taux de contamination entre les différents hôpitaux en Suisse, reposant sur le projet intitulé "Evaluation de la contamination chimique lors de la préparation des chimiothérapies: suivi de la contamination de surface et simulation à l'aide d'un marqueur non toxique" sélectionné par la GSASA, comme un projet de recherche sur la " Qualité et sécurité d'utilisation des médicaments à l'hôpital". Avec l'obtention de ce fonds de recherche, un pharmacien (Marc Mattiuzzo) a pu être engagé durant une année pour mener ce projet et faire des prélèvements dans les différents hôpitaux en Suisse. Ce projet permettra de comparer les taux de contamination des différents sites, de les mettre en relation avec les infrastructures et les modes d'organisation existantes, et d'apporter d'éventuelles actions correctives visant à sécuriser la manipulation de ces substances toxiques.

- La mesure des contaminations sur les flacons cytotoxiques en provenance de l'industrie sera également effectuée à l'aide de la méthode de prélèvement développée. Un premier essai, a montré des contaminations sur certains flacons, et une étude plus large, permettant de comparer les différents fabricants et leur qualité d'emballage, est en cours.

La méthode développée nous amène donc à poursuivre les travaux avec des études visant à améliorer et sécuriser la manipulation des cytotoxiques. Lorsque la mise en place de nouvelles recommandations sera effectuée, d'autres prélèvements pourront être effectués, afin de vérifier l'impact de ces mesures et d'avoir un suivi de la contamination non seulement au sein de la pharmacie des HUG mais également au sein des différents sites hospitaliers ayant participé à cette étude.