Analyse de protéines intactes par électrophorèse capillaire couplée à un spectromètre de masse à temps de vol

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THÈSE

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La Faculté des sciences, sur le préavis de Messieurs J.-L. VEUTHEY, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), S. RUDAZ, docteur et codirecteur de thèse (Section des sciences pharmaceutiques), L. SCAPOZZA, professeur ordinaire (Section des sciences pharmaceutiques), M. SEVE, professeur (Plateforme Protéomique Médicale, Centre d'Innovation en Biologie, Centre Hospitalier Universitaire de Grenoble, France), S. CHERKAOUI, docteur (Bracco Research S.A., Plan-les-Ouates, Genève, Suisse) et de Madame M. TAVERNA, professeure (Laboratoire de protéines et nanotechnologie en sciences séparatives, Université de Paris-Sud, France), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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Le Doyen, Jean-Marc TRISCONE

Seuls l'épreuve, l'expérience et l'échec provoquent un savoir nouveau et permettent de progresser pour, au final, être soi-même…

F.H.S

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Avant-propos

La présente thèse a été effectuée au sein de l'Ecole de Pharmacie de l'Université de Genève-Lausanne en Chimie Analytique Pharmaceutique. Elle a été réalisée sous la co-direction du Professeur Jean-Luc Veuthey et du Docteur Serge Rudaz.

Cette thèse s'inscrit dans la lignée des thèses déjà effectuées au sein du Laboratoire de Chimie Analytique Pharmaceutique sur la technique de l'électrophorèse capillaire (CE) couplée à la spectrométrie de masse (MS). Le Docteur Emmanuel Varesio avait débuté en montrant une première application du couplage de la CE avec la spectrométrie de masse (« Analyse des dérivés de l'amphétamine par électrophorèse capillaire », thèse n°3053 de l'Université de Genève, 1999). Des travaux montrant le potentiel du couplage CE-MS en milieux aqueux et non aqueux avaient ensuite été effectués par le Docteur Laurent Geiser (« 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 n°3442 de l'Universi té de Genève, 2003). Plus récemment, le Docteur Julie Schappler avait mis en évidence le couplage de la CE avec différents détecteurs et en particulier la MS pour l'analyse de composés pharmaceutiques dans les fluides biologiques (« Analyse de composés pharmaceutiques par électrophorèse capillaire couplée à des techniques de détection alternatives », thèse n°3937 de l'Université de Genève, 2008).

A la suite de ces travaux ayant clairement démontré le potentiel du couplage CE-MS pour l'analyse des composés pharmaceutiques de bas poids moléculaires, ma tâche a consisté à démontrer la place de ce couplage dans le cadre de l'analyse de protéines d'intérêt pharmaceutique. En effet, vu le nombre croissant de nouveaux médicaments protéiques, il était intéressant de voir quel rôle la CE-MS pouvait tenir. Dans cette optique, le laboratoire a fait l'acquisition d'un spectromètre de masse à temps de vol (TOF). Le travail de thèse a alors consisté en des études fondamentales (phénomènes d'adsorption et principes de quantification des protéines) et des applications dans divers domaines (forensique, dopage, contrôle qualité).

Structure de la thèse

Cette thèse se divise en quatre parties principales:

La première partie (**Chapitre I**) consiste en une présentation des sujets abordés. Elle comprend une introduction aux protéines d'intérêt pharmaceutique, à leur cycle de développement au niveau industriel, aux techniques analytiques employées et ceci avec un accent marqué sur l'électrophorèse capillaire couplée au spectromètre de masse à temps de vol.

La deuxième partie (**Chapitre II**) regroupe des travaux bibliographiques ainsi que l'ensemble des résultats obtenus durant cette thèse sous la forme de six articles rédigés en anglais et publiés dans des journaux scientifiques ou en cours de publication. Deux articles sont des revues tandis que les autres présentent des résultats expérimentaux. Les différents articles sont précédés par une partie introductive et explicative rédigée en français.

La troisième partie (**Chapitre III**) présente les conclusions et perspectives de ce travail de thèse.

La quatrième partie (**Chapitre IV**) contient les différentes annexes qui comprennent un article rédigé en amont de ce travail de thèse, un article invité, les résumés des différentes communications orales ainsi qu'une copie des posters présentés durant cette période.

Table des Matières

Abréviations

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

Techniques séparatives

AC	chromatographie d'affinité
ACE	électrophorèse capillaire d'affinité
BGE	solution tamponnée d'électrolytes
CE	électrophorèse capillaire
CEC	électrochromatographie capillaire
CGE	électrophorèse capillaire en présence de gel
CZE	électrophorèse capillaire de zone
d_p	diamètre des particules de phase stationnaire
E	champ électrique
FWHM	largeur à mi-hauteur
HILIC	chromatographie à interaction hydrophile
IEC	chromatographie à échange d'ions
IEF	focalisation isoélectrique
LC	chromatographie liquide
L_{eff}	longueur effective du capillaire
L_{tot}	longueur totale du capillaire
MEEKC	chromatographie électrocinétique en microémulsion
MEKC	chromatographie électrocinétique micellaire
N	efficacité
PAGE	électrophorèse sur gel de polyacrylamide
PLOT	tube ouvert à couche poreuse
r	rayon hydrodynamique
RPLC	chromatographie liquide en phase inverse
SDS	sodium dodécyl sulfate
SEC	chromatographie d'exclusion stérique
U	tension appliquée
UHPLC	chromatographie liquide à ultra-haute pression

Spectrométrie de masse

APCI	ionisation chimique à pression atmosphérique
e	charge élémentaire
E_c	énergie cinétique
E_p	énergie potentielle
ESI	ionisation par électrospray
FT-ICR	analyseur à résonnance cyclotronique d'ion à transformée de Fourier

ICP	ionisation par plasma induit
IT	trappe ionique
L	longueur du tube de vol
m	masse
Δm	largeur à mi-hauteur d'un pic sur l'échelle de masse
MALDI	désorption/ionisation laser assistée par matrice
MS	spectrométrie de masse
MS/MS	spectrométrie de masse en tandem
MS^n	spectrométrie de masse multidimensionnelle
m/z	rapport masse sur charge
oa	accélération orthogonale
PEEK	polyétheréthercétone
PMF	« peptide mass fingerprinting »
q	charge effective
Q-TOF	quadripôle-temps de vol
t	temps pour traverser le tube de vol
Δt	largeur à mi-hauteur d'un pic sur l'échelle de temps
TOF	analyseur à temps de vol
v	vitesse de l'ion
V_s	potentiel d'accélération

Divers

CD	dichroïsme circulaire
CSDD	« Centre for the Study of Drug Development"
DLS	diffusion dynamique de la lumière
EPO	érythropoïétine
FA	acide formique
FDA	« Food and Drug Administration"
IR	spectroscopie infrarouge
mAb	anticorps monoclonal
ppm	partie par million
PTM	modification post-traductionnelle
RMN	résonnance magnétique nucléaire
TFA	acide trifluoroacétique

Lettres grecques

η	viscosité
μ_{app}	mobilité apparente
μ_{eff}	mobilité effective

Communications scientifiques

Ce travail de thèse a fait l'objet d'articles publiés dans des revues scientifiques à comité de lecture ainsi que de présentations sous forme de conférences et de posters dans le cadre de congrès nationaux et internationaux.

Publications avec comité de lecture

I. Intact protein analysis in the biopharmaceutical field
A. Staub, D. Guillarme, J. Schappler, J.L. Veuthey, S. Rudaz, *J. Pharm. Biomed. Anal.* 2011, *55*, 810-822.

II. CE-TOF/MS: Fundamental concepts, instrumental considerations and applications
A. Staub, J. Schappler, S. Rudaz, J.L. Veuthey, *Electrophoresis* 2009, *30*, 1610-1623.

III. Use of organic solvent to prevent protein adsorption in CE-MS experiments
A. Staub, S. Comte, S. Rudaz, J.L. Veuthey, J. Schappler, *Electrophoresis* 2010, *31*, 3316-3333.

IV. CE-ESI-TOF/MS for human growth hormone analysis
A. Staub, S. Giraud, M. Saugy, S. Rudaz, J.L. Veuthey, J. Schappler, *Electrophoresis* 2010, *31*, 388-395.

V. Analysis of hemoglobin-based oxygen carriers (HBOCs) by CE-UV/Vis and CE-ESI-TOF/MS
A. Staub, S. Rudaz, M. Saugy, J.L. Veuthey, J. Schappler, *Electrophoresis* 2010, *31*, 1241-1247.

VI. Multiple injection technique for the determination and quantitation of insulin formulations by capillary electrophoresis and time-of-flight mass spectrometry
A. Staub, S. Rudaz, J.L. Veuthey, J. Schappler, *J. Chromatogr. A* 2010, *1217*, 8041-8047.

VII. Highly sensitive detection of pharmaceutical compounds in biological fluids using capillary electrophoresis coupled with laser-induced native fluorescence
J. Schappler, A. Staub, J.L. Veuthey, S. Rudaz, *J. Chromatogr. A* 2008, *1204*, 183-190.

Article invité

VIII. Blood doping detection – A new analytical approach with capillary electrophoresis A. Staub, S. Rudaz, M. Saugy, J.L. Veuthey, J. Schappler, *Chimia* 2010, *64*, 886.

Seuls les conférences et posters présentés en tant que premier auteur sont répertoriés ci-après:

Communications orales

- **New insights in protein analysis with CE-TOF/MS**
  A. Staub; *SCS Fall Meeting*, Septembre 2009, Lausanne (Suisse).

- **Analyse de protéines intactes par électrophorèse capillaire couplée à un spectromètre de masse à temps de vol (CE-TOF/MS)**
  A. Staub, J. Schappler, S. Rudaz, J.-L. Veuthey; *SEP 09*, Décembre 2009, Marseille (France).

- **Intact protein analysis by CE-ESI-TOF/MS, from method optimization to quantitation**
  A. Staub, J. Schappler, S. Rudaz, J.-L. Veuthey; *HPLC 2010*, Juin 2010, Boston (Etats-Unis).

- **Nouveaux développements dans l'analyse de protéines intactes par CE-TOF/MS**
  A. Staub, S. Rudaz, J.L. Veuthey. J. Schappler; *14èmes journées scientifiques du ccCTA*, Septembre 2010, Les Diablerets (Suisse).

- **Nouveaux développements dans l'analyse de protéines intactes par CE-TOF/MS**
  A. Staub, S. Rudaz, J.L. Veuthey, J. Schappler; *3ème Journée Scientifique du Club Jeunes de l'AfSep*, Octobre 2010, Paris (France).

  Prix "Jeune Chercheur" de la meilleure présentation orale.

Posters

- **CE-ESI-TOF/MS for human growth hormone analysis**
  A. Staub, J. Schappler, S. Rudaz, J.-L- Veuthey; *SCS Fall Meeting 2009*, Septembre 2009, Lausanne (Suisse).

- **Blood doping with hemoglobin-based oxygen carriers (HBOC): analysis by CE-UV/Vis and CE-ESI-TOF/MS**
  A. Staub, J. Schappler, S. Rudaz, J.-L- Veuthey; *SCS Fall Meeting 2009*, Septembre 2009, Lausanne (Suisse).

- **Blood doping with hemoglobin-based oxygen carriers (HBOC): analysis by CE-UV/Vis and CE-ESI-TOF/MS**
  A. Staub, J. Schappler, S. Rudaz, J.-L- Veuthey; *TIAFT 2009*, Août 2009, Genève (Suisse).

  1er prix pour “excellence in poster presentation” remis par the Youth Scientific Committee of TIAFT.

- **Intact protein analysis by CE-ESI-TOF/MS, from method optimization to quantitation**
  A. Staub, J. Schappler, S. Rudaz, J.L. Veuthey; *MSB 2010*, Mars 2010, Prague (République Tchèque).

Chapitre I | INTRODUCTION

I.1 LES PROTÉINES-MÉDICAMENTS

Depuis l'introduction de l'insuline en 1982, le nombre de protéines thérapeutiques recombinantes n'a cessé de croître. Cet important essor est principalement dû au développement des technologies basées sur l'ADN recombinant. En effet, si certaines protéines thérapeutiques continuent d'être obtenues par purification de matériel biologique (α-anti-1-trypsine ou α-1-protéinase par exemple), la grande majorité des protéines-médicaments sont actuellement obtenues de sources recombinantes et sont donc considérées comme des biomédicaments car issues des biotechnologies. Les sources recombinantes sont variées et comprennent les bactéries, les cellules d'insectes ou de mammifères ainsi que les animaux et les plantes transgéniques. Le choix du système dépend souvent des coûts de production ou des modifications post-traductionnelles (PTM) nécessaires à la fonction de la protéine (les bactéries sont par exemple incapables de produire des PTMs).
Les protéines mises sur le marché ont des domaines d'application très variés, comprenant par exemple l'oncologie, le traitement des inflammations ou encore de l'anémie. Un récent article de Leader *et al.* [1] propose une classification intéressante des protéines-médicaments, basée sur la pharmacologie. Quatre groupes principaux ont été constitués :

- **Groupe 1**: protéines thérapeutiques ayant une activité enzymatique ou de régulation. Trois sous-catégories sont ensuite proposées: la première comprend les protéines remplaçant une protéine déficiente ou anormale (exemples: l'insuline employée pour réguler le taux de glucose sanguin en cas de diabète et l'hormone de croissance utilisée en cas de problèmes de croissance); la seconde concerne celles augmentant une voie existante (exemples: l'érythropoïétine pour le traitement des anémies sévères et certains interférons utilisés dans le traitement de l'hépatite C ou de certains cancers); la troisième est constituée de protéines permettant une nouvelle activité ou fonction (exemple: la papaïne employée pour aider au débridement des tissus nécrotiques).

- **Groupe 2**: protéines thérapeutiques ayant une activité ciblée et spécifique. Ce groupe est composé de deux sous-catégories, les protéines interférant avec une molécule ou un organisme (exemples: l'etanercept utilisé dans le traitement de l'arthrite rhumatoïde et le ranibizumab employé pour ralentir la dégénérescence maculaire) et celles aidant au relâchement d'autres composés ou protéines (exemple: le tositumomab utilisé dans certaines chimiothérapies en seconde ligne de traitement).

- **Groupe 3**: protéines-vaccins. Ce groupe se décompose en trois sous-catégories. La première rassemble les vaccins protégeant contre un agent étranger néfaste (exemples: les vaccins contre l'hépatite B et contre la maladie de Lyme), la seconde concerne les vaccins traitant une maladie auto-immune (exemple: l'immunoglobuline G utilisée pour l'immunisation rhésus chez les femmes rhésus négatif) et la dernière

comprend les vaccins traitant les cancers, ces derniers étant encore en essais cliniques.

- **Groupe 4**: protéines-diagnostics. Ce dernier groupe comprend des protéines qui permettent d'aiguiller fortement sur un diagnostic (exemple: la sécrétine utilisée pour le diagnostic d'une dysfonction du pancréas exocrine).

De manière générale, les protéines présentent un certain nombre d'avantages sur les médicaments de bas poids moléculaires principalement issus de la synthèse chimique. Elles peuvent en effet accomplir un ensemble complexe de fonctions hautement spécifiques qui ne peuvent aucunement être imitées par de simples petits composés. De plus, la haute spécificité des mécanismes d'action complexes des protéines réduit les effets indésirables et les risques d'interférences avec les processus biologiques normaux. Il est à noter également que les protéines peuvent aussi être attractives financièrement parlant dans la mesure où elles sont uniques tant dans leur forme que dans leur fonction et par conséquent, les brevets sont souvent obtenus plus facilement par les compagnies pharmaceutiques.

Cependant, les protéines soulèvent aussi des problématiques souvent plus complexes que celles mises en évidence lors du développement de molécules thérapeutiques de bas poids moléculaires. La solubilité, la voie d'administration, la distribution et la stabilité des protéines sont des facteurs pouvant notablement empêcher le succès des thérapies associées. En effet, leurs propriétés tant hydrophiles qu'hydrophobes et leur temps de demi-vie peuvent être fortement influencés par l'action de protéases ou de mécanismes d'élimination. Le corps peut aussi déclencher de violentes réactions immunitaires à l'encontre des protéines thérapeutiques et ainsi neutraliser le médicament voire même provoquer d'importants effets secondaires pour le patient. Pour contrecarer ces problèmes, la PEGylation, ou greffage de sous-unités polyéthylène glycol aux protéines, est un domaine d'intérêt grandissant. Cette étape permet en effet de protéger la protéine contre la protéolyse, résultant en un temps de demi-vie augmenté dans le corps et améliorant ainsi l'efficacité, mais aussi de prolonger l'absorption, de diminuer la clérance rénale et de réduire l'immunogénicité des protéines [2]. Comme évoqué plus haut, la présence de PTMs est souvent nécessaire à l'activité physiologique de la protéine et par conséquent le choix de l'hôte producteur est crucial, selon qu'il soit capable ou non de les synthétiser. De plus, la quantité de protéines doit être suffisante pour permettre les tests sur les animaux et les essais cliniques. Par conséquent, le coût de production des protéines recombinantes est très important, pouvant même atteindre plus de 100'000$ par an et par patient.

La présence des protéines thérapeutiques au sein des très recherchés médicaments les plus vendus au monde est incontestable. Certaines prévisions optimistes indiquent qu'en 2014, cette classe de médicaments pourrait en dépasser les 50% [3]. La Figure I.1 présente le nombre de molécules approuvées par la Food and Drug Administration (FDA) en 2009, molécules de bas poids moléculaires et produits biologiques étant distingués.

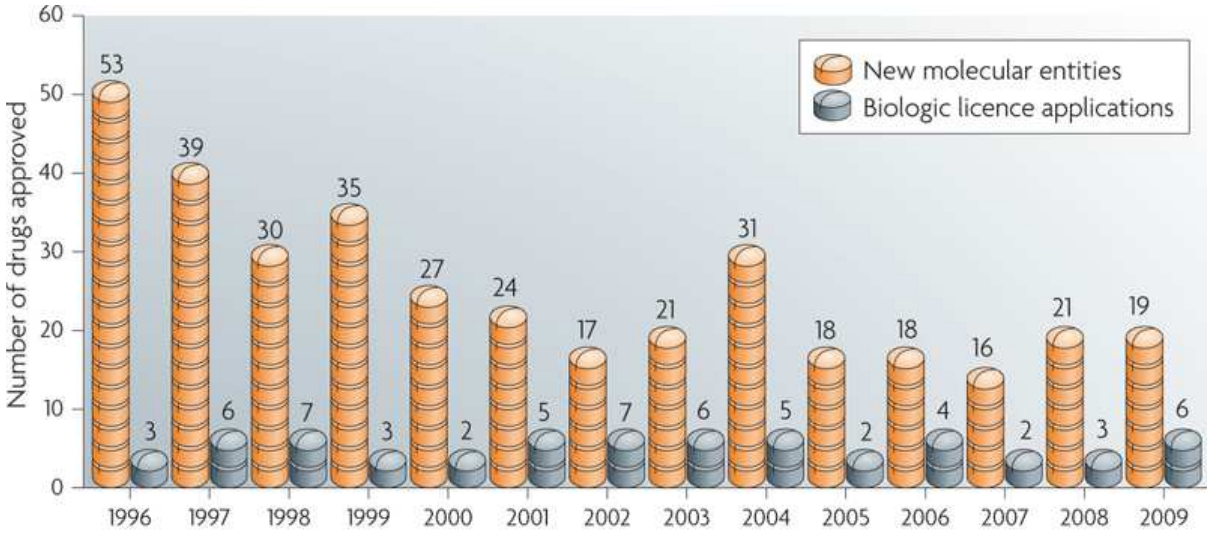


Figure I.1: Médicaments approuvés par la FDA en 2009 [4].

Six nouveaux produits biologiques ont été approuvés en 2009, dont quatre anticorps monoclonaux (mAb), ce qui amène à plus de 25 le nombre total de mAbs disponibles sur le marché américain en 2010. Ce nombre peut apparaître décevant au vu des sommes dépensées annuellement par les firmes pharmaceutiques pour développer leur secteur biopharmaceutique mais ces produits ne sont que la pointe de l'iceberg. En particulier, le marché des mAbs paraît des plus prometteurs avec plus de 240 molécules dans le pipeline, sans oublier également les autres protéines thérapeutiques qui sont plus de 120 [5]. Sur la base d'un taux de réussite de mise sur le marché d'environ 20% pour des molécules ayant déjà passé les premières phases cliniques, on peut espérer environ 70 molécules biologiques supplémentaires dans les dix prochaines années.

L'arrivée à échéance des brevets des premières protéines pharmaceutiques mises sur le marché complexifie encore le domaine des médicaments biologiques, avec l'arrivée des biosimilaires. En effet, l'activité de ces produits dépend totalement de leur structure primaire, secondaire, tertiaire, voire quaternaire et également de leurs PTMs. Par conséquent, cette complexité rend les spécifications utilisées pour les génériques de molécules chimiques de bas poids moléculaires inutilisables pour établir la qualité, l'efficacité et la tolérance des biosimilaires [6]. De ce fait, non seulement des études de qualité et de pharmacocinétique sont requises mais également des études pharmacodynamiques, toxicologiques, ainsi que des comparaisons d'efficacité et de tolérance. Le sénat américain n'a d'ailleurs légiféré sur ce sujet qu'en mars 2009 et proposé une voie de régulation avec des critères à remplir pour que la FDA reconnaisse le biosimilaire (très grande similarité du point de vue de la structure moléculaire avec la molécule innovatrice originale et pas de différences cliniques entre les deux produits) [7]. Ces deux critères paraissent évidents, pourtant dans le cas des protéines, prouver que la structure et l'action clinique sont équivalentes fait appel à d'innombrables techniques analytiques qui ne garantissent d'ailleurs souvent pas une image complète de la protéine étudiée. Le corolaire à cet état de fait est que le domaine des techniques analytiques des protéines est actuellement en plein développement.

I.2 Place des méthodes analytiques dans le cycle de vie d'une protéine-médicament

Si le développement d'une formulation pharmaceutique de protéine suit globalement les mêmes phases que celles empruntées pour un médicament classique issu de la synthèse chimique (Figure I.2), le déroulement et la durée de chaque phase est néanmoins différent tant par exemple dans le processus de sélection des candidats que dans la nature des tests, ceci principalement de par la plus grande complexité structurale des protéines.

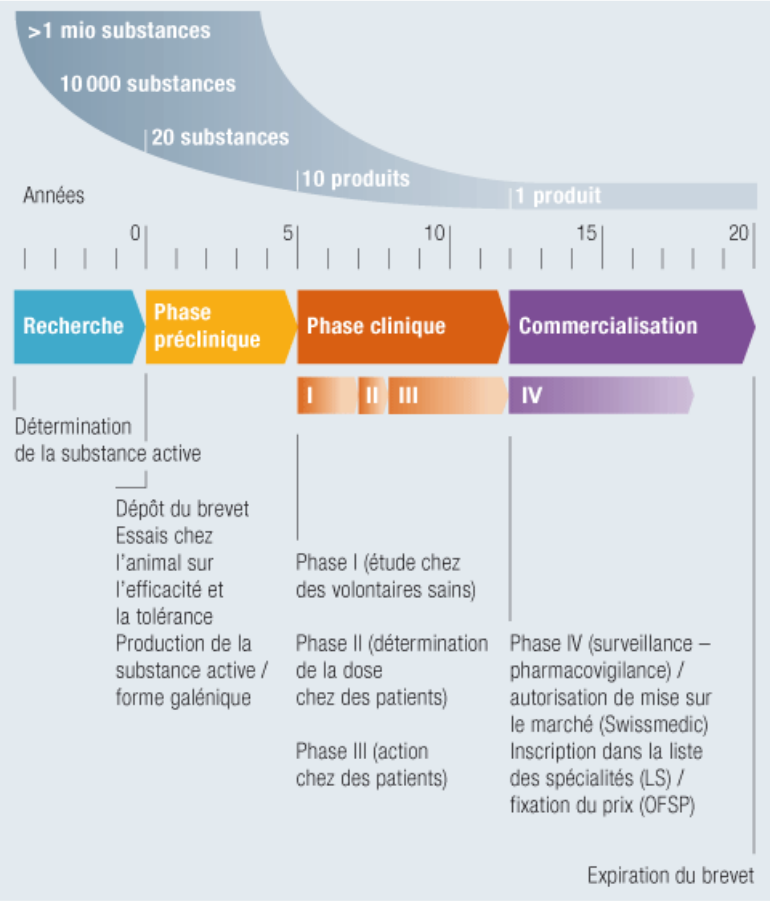


Figure I.2: Cycle de vie d'un médicament, de la détermination d'une substance active à sa mise sur le marché [8].

Il a été établi en 2007 que le coût de recherche et développement dans le domaine des biotechnologies était équivalent à celui rencontré dans les firmes pharmaceutiques. Le Tufts Centre for the Study of Drug Development (CSDD) a démontré qu'il fallait 1.2 milliards de dollars pour développer un médicament biopharmaceutique mais qu'il fallait en moyenne 8% de temps en plus pour y parvenir [9,10,11]. A noter aussi que le taux de succès de mise sur le marché est en faveur des produits biotechnologiques avec 30.2% d'acceptation contre 21.5% pour les voies pharmaceutiques traditionnelles.
Ces chiffres peuvent paraître dans un premier temps surprenants dans la mesure où on connaît la complexité intrinsèque plus importante des produits issus de la biotechnologie par rapport à ceux issus de la synthèse chimique. En effet, pour un temps de développement

plus long, les coûts engendrés restent semblables à ceux rencontrés dans les firmes pharmaceutiques. Ceci s'explique partiellement par le fait que les spécialités biotechnologiques s'inspirent de substances qui existent déjà naturellement dans le corps. Par conséquent, un principe déjà établi est utilisé, engendrant des coûts de recherche primaire moindres.
Quoi qu'il en soit, la production de produits biopharmaceutiques implique un nombre plus grand de tests de qualité (>2000 vs. <100), plus d'étapes critiques (>5000 vs. <100) et des informations beaucoup plus fournies (>60'000 vs. <4000) [12]. Passer d'une molécule de bas poids moléculaire à une protéine ne consiste pas uniquement à changer de domaine de masse, la complexité augmente aussi considérablement. En effet, les protéines possèdent non seulement une structure primaire, mais aussi secondaire, tertiaire voire même quaternaire selon le composé. Cette structure multidimensionnelle doit être élucidée et s'accompagne également très souvent de modifications post-traductionnelles (PTM) qui doivent aussi être caractérisées. Selon la complexité du mélange de protéines et leur degré de glycosylation par exemple, la caractérisation complète ne peut tout simplement pas être achevée.
Au vu de ces challenges, il devient alors évident que les méthodes analytiques employées pour parvenir à résoudre les écueils, tout du moins partiellement, sont nombreuses et possèdent des principes très différents les unes des autres. Elles interviennent à toutes les étapes de la vie de la protéine-médicament [13]:

- Phase préclinique: tests pour déterminer la pureté et identifier le produit.
- Phases I et II: établissement de méthodes pour analyser le produit et ses produits intermédiaires; l'accent est mis sur la caractérisation du produit et de ses impuretés, des études de stabilité sont aussi conduites.
- Phase III: comme pour les phases I et II, le produit continue d'être caractérisé ainsi que ses impuretés minoritaires et majoritaires.
- Avant la phase de validation: des études de stabilité continuent d'être menées et les méthodes analytiques sont en voie de validation.
- Avant la phase d'approbation finale: des études comparatives sont conduites avec d'autres produits.
- Après l'approbation finale: suivi de tous les processus de production et améliorations constantes des techniques analytiques concernées.

La classe de protéines thérapeutiques qui présente le plus fort taux de croissance et semble la plus prometteuse est celle des anticorps monoclonaux (mAb) [14]. En 2006, ils étaient déjà en tête des ventes au sein des biomédicaments, devant les vaccins, les érythropoïétines (EPO), les insulines et les interférons. Les domaines d'application sont nombreux et variés mais concernent des domaines où les progrès médicaux sont impatiemment attendus. Cela comprend le domaine de la lutte contre le cancer (exemples: le Trastuzumab et le Bévacizumab de Roche™ contre le cancer du sein et du côlon respectivement) ou encore le traitement de maladies dégénératives et invalidantes comme l'arthrite rhumatoïde (exemples: le Rituximab de Roche™ et l'Infliximab de Johnson & Johnson™) [15,16]. Les premières générations de mAbs ont déjà été rejointes par une

seconde génération composée de mAbs avec des structures plus stables (acides aminés mutés permettant de diminuer l'agrégation par exemple), voire même maintenant par une troisième génération promettant des temps de demi-vie plus longs et une action plus efficace (par exemple, modulation du domaine variable pour diminuer le point isoélectrique et ainsi ralentir leur élimination) [17]. Le fait que les mAbs connaissent le plus fort engouement au sein des firmes biotechnologiques augmente le challenge analytique sous-jacent vu la complexité de ces derniers. Mais la manne financière qu'ils représentent permet aussi de voir des progrès technologiques rapides au niveau des instrumentations analytiques et des kits d'analyse disponibles. Il n'est pas certain qu'il soit possible un jour de caractériser complètement certains mélanges complexes de mAbs mais la marge de progression est encore importante.

I.3 Les méthodes d'analyse des protéines

Deux approches fondamentalement différentes sont habituellement employées pour l'analyse des protéines. La première consiste en une première étape de digestion enzymatique des protéines puis de l'analyse des peptides ainsi obtenus. La technique analytique utilisée est alors principalement voire exclusivement de la chromatographie liquide (LC) couplée à un spectromètre de masse (MS) ou à un MS en mode tandem (MS/MS). L'ensemble est ensuite assisté par de puissants outils de bioinformatique. On parle alors de technique « Bottom-Up ». La seconde approche propose d'analyser les protéines sous leur forme intacte. Cette stratégie permet l'utilisation de techniques analytiques variées (spectroscopie, LC, électrophorèse, MS) qui chacune apporte des informations complémentaires. Lorsque la MS est employée pour les protéines intactes et qu'une fragmentation a lieu dans la source, le terme consacré est la technique dite « Top-Down » [18].
Ces deux approches d'analyse des protéines présentent des avantages et des inconvénients qui seront discutés dans les parties suivantes, avec l'accent mis sur l'analyse des protéines sous leur forme intacte vu que cette stratégie a été employée pour les travaux de cette thèse. Il est à noter aussi que seuls les aspects qualitatifs seront développés.

I.3.1 Analyse des protéines sous leur forme digérée

L'analyse des protéines sous leur forme digérée est particulièrement employée dans le domaine de la protéomique au sens strict du terme, c'est-à-dire l'étude de l'ensemble des protéines d'une cellule, d'une organelle, d'un tissu, d'un organe ou d'un organisme. Ceci nous éloigne quelque peu de l'objet de cette thèse vu que celle-ci s'est concentrée uniquement sur l'analyse des protéines-médicaments tant d'un point de vue caractérisation et contrôle qualité que leur analyse dans les fluides biologiques. Cependant, l'approche Bottom-Up peut aussi être employée pour l'analyse d'échantillons protéiques moins complexes et en cela, s'appliquer au domaine de l'analyse des protéines pharmaceutiques. Son principe est donc à expliquer ici.

Cette approche comprend schématiquement quatre étapes lorsqu'elle est appliquée à des échantillons biologiques comme le sérum, par exemple [19]:

- Une première étape consiste en la simplification de l'échantillon protéique (purification ou pré-fractionnement). Cette simplification peut se faire en utilisant des colonnes d'(immuno)affinité permettant d'éliminer les protéines les plus abondantes ou en utilisant des techniques séparatives des protéines sous leur forme intacte. Ces techniques seront abordées plus en détails dans la partie suivante mais comprennent les techniques électrophorétiques sur gel (à une ou deux dimension(s), 1D ou 2D), capillaires, ainsi que les techniques de LC à 1D ou 2D.

- La seconde étape consiste en la digestion des protéines avec une protéase (généralement la trypsine, peu chère et très sélective). Les peptides obtenus sont

composés de 10 à 12 acides aminés en moyenne, rendant leur analyse MS aisée. La digestion enzymatique peut être effectuée in-gel ou en solution [20]. Le choix est souvent effectué en fonction de la technique de pré-fractionnement utilisée précédemment.

- Les peptides générés sont ensuite séparés lors de la troisième étape, le plus souvent par LC suivie d'une détection MS à haute résolution [21]. Le couplage LC-MS est principalement assuré par une source électrospray (ESI). Les analyseurs de masse employés peuvent être de différentes natures. Le triple quadripôle et la trappe ionique sont couramment employés mais peuvent être insuffisants en termes de résolution selon la complexité de l'échantillon analysé et par conséquent, les MS à haute résolution et avec une haute exactitude de masse sont de plus en plus couramment utilisés (temps de vol ou TOF, quadripôle temps de vol ou Q-TOF, Orbitrap [22] et MS à résonance cyclotronique ionique à transformée de Fourier ou FT-ICR).

- La dernière étape consiste en l'identification des protéines présentes grâce à la consultation de bases de données protéomiques MS, à l'aide de logiciels de comparaison (Mascot par exemple). L'identification par « peptide mass fingerprinting » (PMF) est possible dans la mesure où chaque protéine possède un profil peptidique spécifique et que la masse de ces peptides déterminée avec une très haute exactitude de masse assure l'identification de la protéine. Le PMF est donc surtout utilisé après une analyse avec un MS à haute exactitude de masse. L'identification est aussi possible en générant des fragments peptidiques spécifiques par MS/MS. Il s'agit alors de « peptide mass sequence tag ». La Figure I.3 présente le schéma général de l'identification avec la stratégie Bottom-Up.

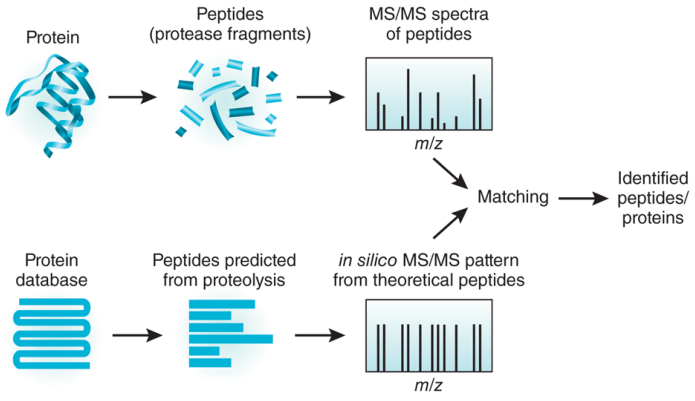


Figure I.3: Approche générale utilisée dans la stratégie Bottom-Up pour l'identification de protéines dans des échantillons complexes [23].

Les avantages de cette stratégie sont multiples [24]. Premièrement, l'approche Bottom-Up est celle qui est la plus employée et par conséquent la plus mature pour l'identification et la caractérisation de protéines. De plus, des instruments avec des outils élaborés de bioinformatique sont largement disponibles dans le commerce, rendant l'emploi de cette stratégie accessible à des laboratoires non-experts dans le domaine. La somme

d'informations obtenue avec cette stratégie est impressionnante et beaucoup d'applications rendant compte de la caractérisation et de l'identification de nombreuses protéines ont été publiées à ce jour. On peut citer par exemple la mise en évidence récente des modifications post-traductionnelles (PTM) d'une sous-unité du complexe C1, qui joue un rôle-clé dans les réactions immunitaires [25]. La déamidation de l'immunoglobuline gamma a aussi été mise en évidence récemment avec cette approche. Il est important de pouvoir détecter cette dégradation dans la mesure où elle facilite l'agrégation protéique et donc peut réduire l'efficacité et augmenter l'immunogénicité de la protéine [26].
A l'opposé, l'approche Bottom-Up présente certains inconvénients [24]. Le premier concerne la digestion tryptique elle-même. En effet, le postulat que l'enzyme produit effectivement et de manière répétable les peptides prédits est à la base de l'identification. Cependant, il a été mis en évidence que le nombre de produits protéolytiques est souvent supérieur à celui prédit (clivages inattendus, produits résiduels suite à des étapes d'alkylation et/ou de réduction). Ces artefacts sont des produits minoritaires et ne posent pas de problème lorsque peu de protéines sont analysées. Cependant, lorsque l'échantillon est complexe, les artefacts de protéines présentes en grandes quantités peuvent masquer les produits majoritaires des protéines présentes à basse concentration. Les algorithmes de reconnaissance peuvent aussi présenter des limitations (les peptides glycosylés sont par exemple souvent non détectés car ils s'ionisent moins bien). Le temps pour obtenir les résultats d'un échantillon est important et par conséquent plusieurs échantillons sont souvent assemblés pour gagner du temps, ceci masquant parfois des variations individuelles et moyennant les données protéomiques. Finalement, la couverture partielle de la séquence de la protéine (une fraction seulement de la population totale des peptides est identifiée) peut engendrer des pertes d'information, par exemple au niveau des PTMs.

I.3.2 Analyse des protéines sous leur forme intacte

Les concepts développés dans cette partie sont l'objet de l'article 1 qui consiste en une revue reprenant globalement la place de l'analyse des protéines pharmaceutiques sous leur forme intacte. Les points les plus importants sont repris ici, avec une mise en avant prononcée de la technique utilisée pour ces travaux de thèse, c'est-à-dire l'électrophorèse capillaire (CE) couplée à la spectrométrie de masse à temps de vol.

L'analyse des protéines sous leur forme intacte permet l'utilisation de techniques très variées, de la spectroscopie UV à la MS, en passant par des techniques séparatives aux principes orthogonaux telles que la LC et les méthodes électrophorétiques (sur gel et capillaires), incluant également la combinaison de ces techniques [27]. De façon globale, le fait de conserver les protéines intactes lors de leur analyse donne accès à des informations structurales qui peuvent être parcellaires dans le cas d'une approche Bottom-Up. En particulier, des informations quant à la structure tridimensionnelle de la protéine ainsi que la position de ses PTMs peuvent s'avérer plus faciles à acquérir *via* l'analyse de protéines intactes. En effet, comme évoqué ci-avant, l'approche Bottom-Up ne couvre qu'une partie de la séquence de la protéine ce qui peut provoquer une perte d'information, particulièrement

au sujet des PTMs. La grande force de cette approche demeure donc la grande diversité des techniques à disposition et la variété d'informations complémentaires qui en découlent.
Cette approche présente aussi certains inconvénients. Ainsi, l'analyse de protéines intactes s'avère rapidement plus complexe que celle des peptides issus de ces mêmes protéines (problèmes d'adsorption, de dégradation). Certaines techniques se montrent plus naturellement compatibles à l'analyse de ces composés (électrophorèse sur gel, chromatographie d'exclusion stérique) que d'autres (chromatographie liquide en phase inverse, électrophorèse capillaire de zone), et ceci en lien avec les problèmes évoqués ci-dessus. Des ajustements, tant technologiques que méthodologiques, sont alors à effectuer rendant par la suite de nombreuses techniques utilisables. De plus, les mélanges de protéines à analyser sont souvent complexes nécessitant dans certains cas des équipements de pointe et par conséquent coûteux. Par exemple, l'utilisation de la MS en mode Top-Down implique l'emploi de spectromètres de masse à haute résolution et à haute exactitude de masse (tels que les FT-ICR, Orbitrap et TOF) [28,29]. L'analyse des protéines sous leur forme intacte ne s'inscrit donc pas comme une solution de remplacement de l'approche après digestion, mais plutôt comme un gain d'informations, ce qui n'est pas négligeable au vu des difficultés rencontrées pour résoudre ces structures complexes.

Les sous-chapitres suivants décrivent les quatre grandes familles de techniques permettant d'analyser les protéines sous leur forme intacte. Comme dans l'article 1, l'accent a été mis sur les techniques séparatives que sont la chromatographie liquide et les méthodes électrophorétiques. De plus, les approches faisant appel au transport de masse hydrodynamique, comme la viscosité et l'ultra-centrifugation analytique ne sont pas abordées ici.

I.3.2.1 Techniques spectroscopiques

Les techniques spectroscopiques sont très largement utilisées au cours de toutes les étapes du développement d'une protéine thérapeutique. Elles sont principalement employées pour caractériser les structures secondaires et tertiaires de ces molécules et consistent en l'étude des interactions (absorption, émission et diffusion de photons) de radiations électromagnétiques avec les molécules. Différentes techniques existent: par exemple, les rayons-X [30], la résonance magnétique nucléaire (RMN) [31], l'absorption (dans le domaine de l'ultraviolet et du visible) [32], la fluorescence [33], le dichroïsme circulaire (CD) [34], la diffusion dynamique de la lumière (DLS) [35] et la spectroscopie infrarouge (IR) [36]. Ces techniques présentent l'avantage de pouvoir être employées dans des plateformes de criblage à haut débit, particulièrement utiles dans des étapes de caractérisation de la protéine et de ses produits de dégradation [37]. De plus, dans le cas de la fluorescence, de l'absorption UV-visible ou encore de la DLS, ce sont des approches non-destructives qui permettent par conséquent leur utilisation en série, tout en préservant le produit.
Globalement, ces techniques n'étant pas séparatives, elles sont souvent couplées à la chromatographie liquide ou à l'électrophorèse capillaire (cas de l'absorption UV-visible et de la fluorescence principalement), jouant alors le rôle de systèmes de détection. En effet, une séparation préalable permet souvent de mettre en évidence les produits minoritaires

potentiellement présents en les séparant des produits majoritaires. Les techniques spectroscopiques sont largement utilisées à toutes les étapes de développement d'une protéine-médicament, le plus souvent en combinaison, chacune apportant de nouveaux éléments pour caractériser le produit.

I.3.2.2 Spectrométrie de masse

Comment mentionné dans la partie I.3.2, l'approche de spectrométrie de masse proposant d'analyser les protéines sous leur forme intacte s'intitule technique Top-Down. Cette stratégie utilise la fragmentation des protéines directement dans la source du MS, permettant l'identification et la caractérisation de ces molécules, incluant aussi des informations précieuses sur les PTMs. La Figure I.4 présente le principe général de cette approche, avec en regard le principe général de l'approche Bottom Up.

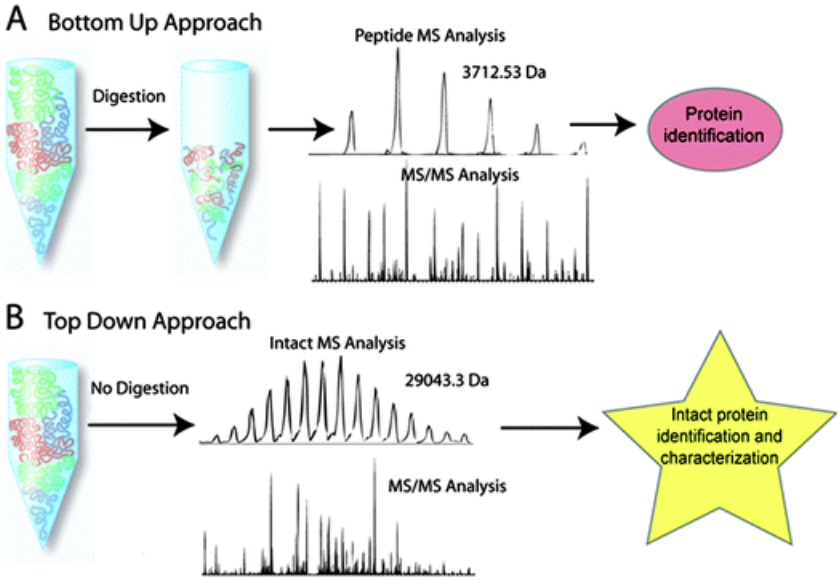


Figure I.4: Schémas des approches Bottom Up et Top Down pour l'analyse des protéines [38].

L'élément primordial du spectromètre de masse employé en mode Top Down est le type d'analyseur de masse employé. Les trappes ioniques (IT), les TOF, les Orbitraps et les FT-ICR sont couramment utilisés [28,29]. Les différences principales entre ces différents appareils résident dans leur pouvoir de résolution et leur exactitude en masse. Ces deux paramètres se calculent le plus souvent comme suit :

$$Résolution = \frac{masse\ mesurée}{FWHM} \qquad \text{Equation 1}$$

$$Exactitude\ en\ masse\ [ppm] = \frac{\Delta\ |masse\ exacte - masse\ mesurée|}{masse\ exacte} \cdot 10^6 \qquad \text{Equation 2}$$

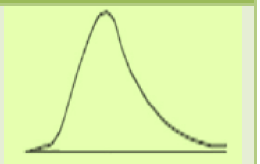
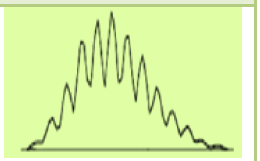
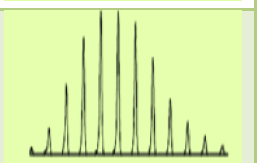
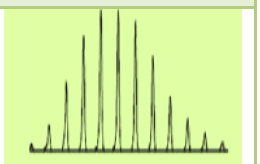
où FWHM équivaut à la largeur du pic à mi-hauteur ou *full-width at half-height maximum*. Par conséquent, pour un composé d'une masse exacte de 400.0000 Da, d'une masse mesurée

de 400.0020 et d'une largeur à mi-hauteur de 0.1, la résolution de l'appareil est alors de 5000 et son exactitude en masse de 5 ppm.

Ainsi, si les IT permettent une très bonne sensibilité et une très bonne détection de nombreux fragments, leurs limitations en termes de résolution (1000) et d'exactitude de masse (100-200 ppm) les rendent beaucoup moins fiables pour l'identification [38]. L'Orbitrap et le FT-ICR apparaissent comme étant les analyseurs de masse les plus adaptés à ce type d'approche, tant leurs performances en termes de résolution (60'000 et 200'000 respectivement) et d'exactitude en masse (2-5 et 0.5-3 ppm respectivement) sont impressionnantes. Le TOF ainsi que ses combinaisons, le TOF-TOF et le Q-TOF, sont des alternatives intermédiaires qui apportent des résolutions de l'ordre de 10'000 à 40'000 et des exactitudes de masse aux alentours de 2-10 ppm. Ils permettent donc une identification plus sûre et une caractérisation plus poussée que les IT. De plus, ils possèdent un large domaine de masse (virtuellement illimité) permettant l'emploi de sources d'ionisation ne produisant pas d'ions multichargés (par exemple, la désorption/ionisation laser assistée par matrice ou MALDI).

Le type d'analyseur de masse utilisé dépend bien évidemment aussi du budget à disposition. Si l'Orbitrap rend l'accès aux très hautes résolutions et exactitudes en masse possible pour un coût « moindre » par rapport au FT-ICR, le TOF demeure le plus attractif en termes de compromis coût-résultats. La Figure I.5 résume les caractéristiques de ces quatre types d'analyseurs de masse.

Table I.1: Caractéristiques des analyseurs de masse couramment utilisés dans l'analyse Top Down des protéines. Adapté de [38,39,40,41].

Analyseur de masse	Approprié pour l'approche Top Down	Temps d'acquisition du spectre/s	Résolution/Da	Exactitude en masse [ppm]	Performance à 8 kDa
IT	+	0.05-0.3	1'000	100-200	
TOF, TOF-TOF et Q-TOF	++	<0.01	10'000-40'000	2-10	
Orbitrap	+++	0.1-1	60'000-100'000	2-5	
FTICR	+++	0.1-1	200'000	0.5-3	

L'approche Top Down ne saurait remplacer la Bottom Up, ces deux techniques sont définitivement complémentaires. En effet, si la MS Bottom Up ne saurait être détrônée dans les cas où il s'agit de détecter la présence d'une protéine ou encore de l'identifier grâce à une base de données, la MS Top Down a apporté ces dernières années des informations que la MS Bottom Up ne permet pas d'obtenir. Par exemple, la caractérisation complète de la structure primaire d'une protéine (composition en acides aminés et modifications) par une approche Top Down ciblée donne des résultats très intéressants. L'approche Top Down ciblée consiste en la comparaison des masses des ions précurseurs et fragments avec une base de données. Cela permet une localisation et une caractérisation complètes des PTMs. Les bases de données Top Down n'atteignent pas encore la qualité de celles consacrées à l'approche Bottom Up mais les progrès sont constants [42].

Il existe deux approches mixtes qui sont nommées Middle Up et Middle Down. La première consiste comme la Bottom Up en une digestion enzymatique de la protéine, mais cette digestion résulte en des peptides plus grands qui sont analysés tels quels dans le MS. La deuxième approche utilise les mêmes peptides que la Middle Up mais ceux-ci sont encore fragmentés dans le MS (MS/MS). Afin de démontrer la grande complémentarité de ces quatre approches, la Figure I.5 présente leur emploi au cours de la caractérisation la plus complète possible de mAbs.

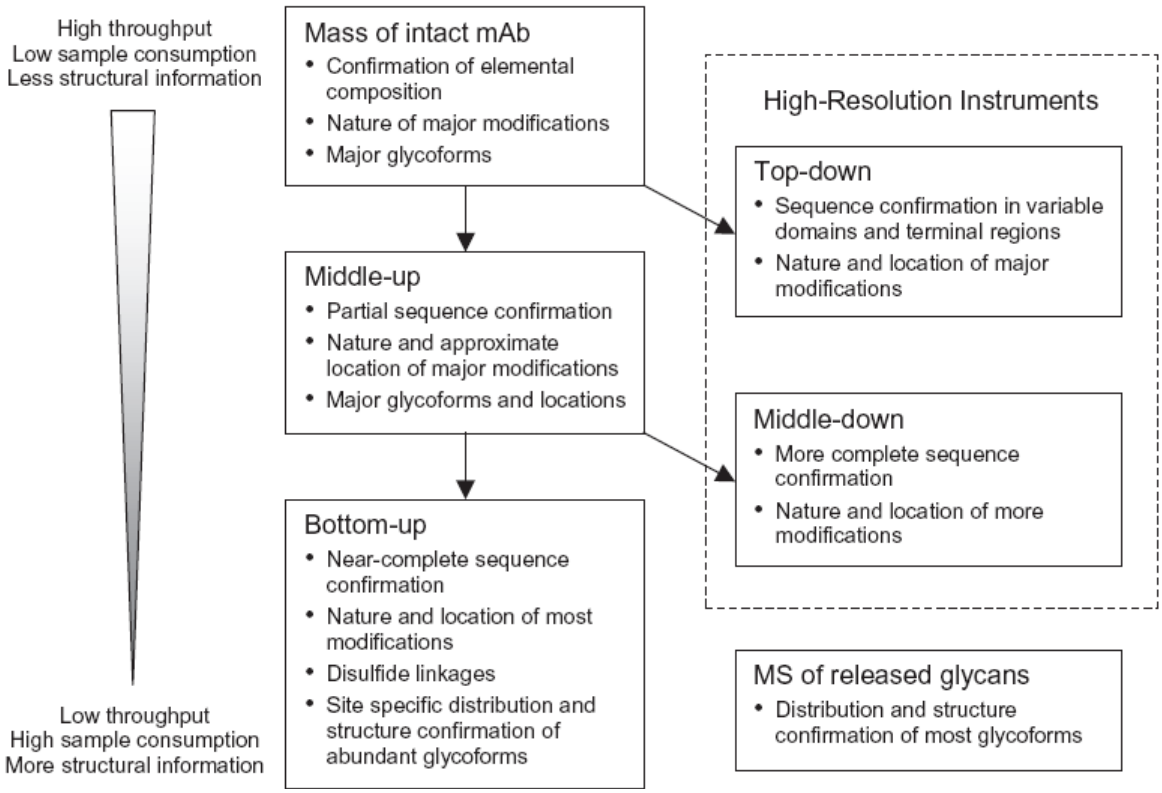


Figure I.5: Résumé des différentes techniques MS employées pour la caractérisation structurale des mAbs [43].

Même si les mAbs sont dans l'extrême de la complexité au niveau structural, cette approche multi-modes peut s'appliquer à d'autres classes de protéines et démontre une fois de plus la nécessité d'avoir une palette de techniques pour pouvoir tenter d'approcher la structure véritable de la protéine.

I.3.2.3 Chromatographie liquide

La chromatographie liquide (LC) est une technique séparative très employée pour l'analyse des protéines intactes de par son grand pouvoir résolutif et sa haute reproductibilité ainsi que sa compatibilité avec la spectrométrie de masse. Les modes utilisés pour la séparation de protéines intactes sont la LC en phase inverse (reversed-phase LC ou RPLC), la chromatographie d'exclusion stérique (SEC), la chromatographie d'échanges d'ions (IEC), la chromatographie à interaction hydrophile (HILIC) et la chromatographie d'affinité (AC). D'une manière générale, les systèmes de LC sont similaires quel que soit le mode employé (injecteur, pompe, colonne,…). Par conséquent, de manière globale, l'emploi du polyétheréthercétone (PEEK) pour les tubes de connexion ainsi que pour les aiguilles est déconseillé lors de l'analyse de protéines de par sa grande hydrophobicité et donc sa grande propension à l'adsorption des protéines hydrophobes. Les matériaux inertes tels que le titane, l'acier inoxydable ou le PEEK-Sil (PEEK à l'extérieur et silice fondue à l'intérieur) sont préférables, même s'ils n'annulent pas totalement les phénomènes d'adsorption. Les constructeurs de systèmes LC commencent par ailleurs à mettre sur le marché des appareils dédiés à l'analyse des protéines intactes. Ces derniers sont faits dans des matériaux biocompatibles et inertes.
L'accent a été mis dans cette partie consacrée à la LC sur la RPLC car ce mode connaît actuellement les plus grands développements technologiques.

I.3.2.3.1 Chromatographie liquide en phase inverse

Les protéines intactes ne s'analysent pas par chromatographie liquide en phase inverse (RPLC) de la même manière que les molécules de bas poids moléculaires ou les peptides car elles posent des problèmes d'adsorption, de carryover, de rétention insuffisante (exclusion des pores), de formation de pics multiples ou encore de traînée et d'élargissement de pics. Ces aspects sont attribuables à une diffusion lente au sein des pores et à des interactions secondaires avec la phase stationnaire. De plus, la très forte dépendance de leur rétention avec de petits changements dans la force éluante de la phase mobile a pour conséquence la nécessité d'employer des méthodes gradients car le changement de seulement 0.1% d'acétonitrile peut drastiquement modifier la rétention des protéines [44]. La Figure I.6 montre la grande augmentation de rétention de l'insuline alors que la teneur en acétonitrile n'a été modifiée que de 3% entre les deux pics extrêmes.

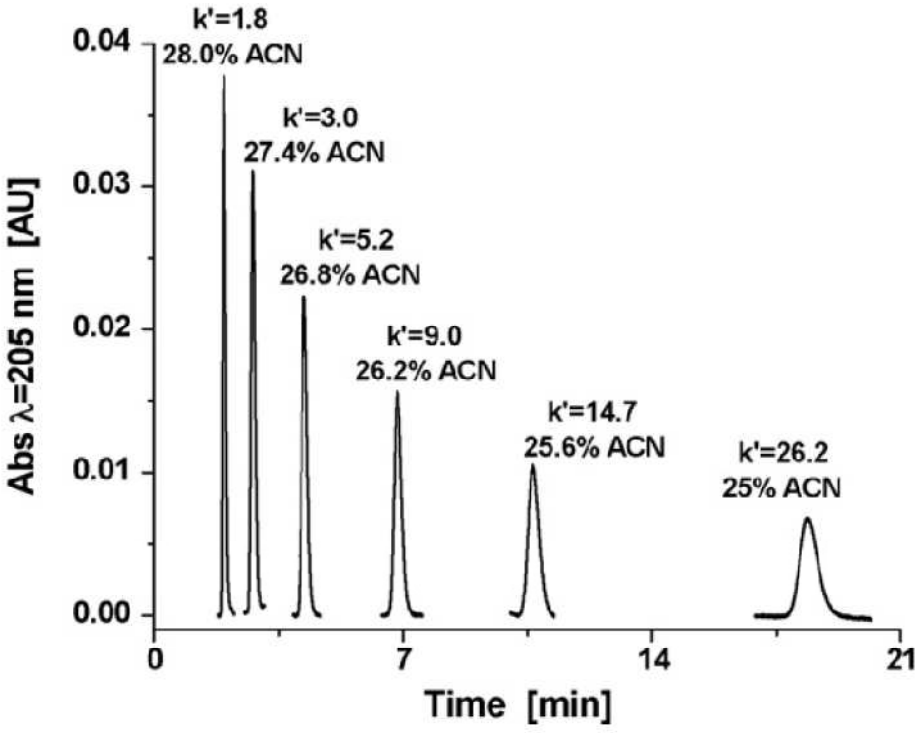


Figure I.6: Profils d'élution du pic de l'insuline en mode isocratique en faisant varier la teneur en acétonitrile de 28 à 25% [44].

Pour améliorer la qualité des chromatogrammes obtenus, trois stratégies différentes sont envisageables:

- **Réduction de l'adsorption**: l'adsorption d'analyte au niveau de la phase stationnaire ou du système chromatographique lui-même provoque des pertes de sensibilité. Les recommandations en termes de limitation de l'adsorption au niveau du système LC ont déjà été évoquées dans la section précédente. Pour minimiser l'adsorption au niveau des colonnes RPLC, l'utilisation de chaînes carbonées plus courtes (C_4 vs. C_8 ou C_{18}) s'avère intéressante. En effet, les chaînes C_4 sont moins hydrophobes et par conséquent génèrent moins d'interactions avec les protéines [45].

- **Réduction de la traînée des pics**: des interactions secondaires se produisent entre les silanols résiduels chargés négativement de la phase stationnaire et les protéines chargées positivement. Ces interactions ioniques étant d'une cinétique plus lente que celle des interactions hydrophobes, les performances chromatographiques sont réduites (traînée et élargissement des pics). Quatre aspects sont à considérer. Premièrement, les phases stationnaires peuvent être composées de silices à accès restreint aux silanols résiduels (silice hybride, à greffage dense, endcappées). Ensuite, l'emploi de hautes températures de séparation (attention toutefois à ne pas générer une dégradation des protéines par la chaleur) permet d'accélérer les cinétiques d'interactions secondaires et donc de minimiser les asymétries [46]. Il est aussi possible d'utiliser un agent de paire d'ions dans la phase mobile. L'acide trifluoroacétique (TFA) et l'acide formique (FA) sont couramment utilisés, avec la

détection UV et MS, respectivement [47]. Une dernière approche consiste en l'utilisation de conditions très basiques au niveau de la phase mobile (rendues possible avec certaines silices hybrides) pour réduire les interactions ioniques. Ainsi, comme les peptides, les protéines présentent une majorité de groupes ionisables basiques et sont sensibles à un changement de pH de la phase mobile, tant au niveau de leur rétention que de leur forme de pic. Pour l'instant, cette approche n'a été appliquée qu'à l'analyse de peptides [48].

- **Réduction de l'élargissement des pics**: les protéines étant de grosses molécules avec de faibles coefficients de diffusion, leurs pics chromatographiques sont élargis lors de l'utilisation de particules poreuses conventionnelles. Trois approches sont employées pour améliorer la forme des pics des protéines intactes:

    - Matériaux à pores plus larges: pour assurer une diffusion satisfaisante dans les pores, ces derniers doivent être au moins dix fois plus grands que les analytes. Pour cela, des particules avec des pores de 300 à 1000 Å sont utilisées pour les protéines au lieu des 80 à 120 Å traditionnels [49,50].

    - Haute température de phase mobile: comme mentionné ci-dessus, une haute température évite les traînes de pic par augmentation des cinétiques de sorption. De plus, elle permet aussi d'augmenter les coefficients de diffusion (qui sont proportionnels au rapport température sur viscosité) et ainsi d'améliorer les performances cinétiques [46]. La température est un paramètre important à optimiser pour trouver le bon compromis entre amélioration des performances et non-dénaturation des protéines et des phases stationnaires.

    - Modification de la morphologie de remplissage des colonnes: plusieurs stratégies sont envisageables. Les supports non poreux constituent une solution car ils minimisent la résistance au transfert de masse, permettant une diffusion améliorée de l'analyte et une bonne efficacité [51]. La capacité de chargement est par contre faible et la rétention réduite due à la faible surface spécifique du support. Ce problème de rétention réduite est aussi rencontré avec les tubes ouverts à couche poreuse (porous layer open tubular ou PLOT) qui, s'ils permettent une capacité de chargement plus grande, posent aussi des problèmes de rétention et de sélectivité de par leur faible surface spécifique [52]. Les monolithes sont intéressants car ils possèdent une grande perméabilité et par conséquent génèrent de basses contre-pressions et des transferts de masse rapides. Les monolithes organiques sont particulièrement appropriés à l'analyse des protéines car ils sont totalement biocompatibles. Cependant, leurs performances chromatographiques restent limitées. Les deux dernières stratégies sont les plus prometteuses. La première consiste en l'utilisation de particules sub-2µm. La réduction du diamètre des particules apporte des améliorations en termes de performance cinétique. En effet, le

débit optimal de phase mobile est inversement proportionnel au diamètre des particules (d_p) tandis que la résistance au transfert de masse est directement proportionnelle au carré de d_p [49,53]. Le principal problème de cette approche est la contre-pression engendrée, mais les systèmes à ultra-haute pression (UHPLC) permettent de le contourner. Finalement, la dernière stratégie est l'emploi de la technologie fused-core (appelée aussi technologie à particules superficiellement poreuses ou poroshell). Cette dernière consiste à utiliser des particules de silice superficiellement poreuses, tandis que le cœur de ces particules est non poreux. Le chemin de diffusion est ainsi réduit par rapport aux particules poreuses de même taille et les transferts cinétiques ainsi que les performances sont améliorées [54]. Cette technologie est aussi disponible en format sub-2µm améliorant encore les performances cinétiques. La Figure I.7 présente la structure des particules fused-core en comparaison avec les particules poreuses traditionnelles.

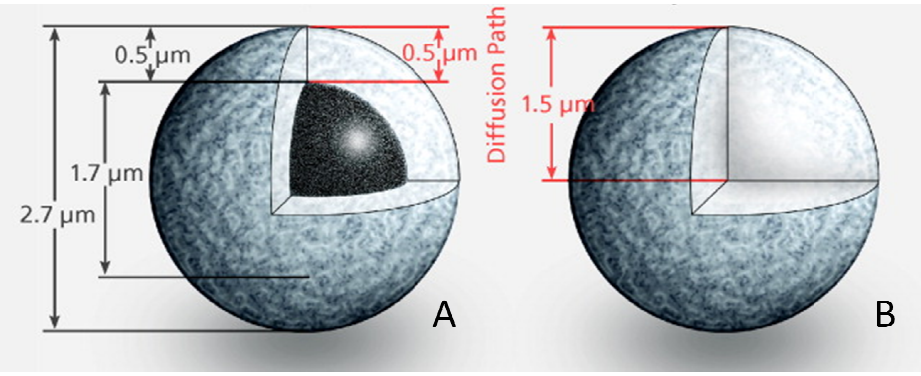


Figure I.7: Structure des particules A. fused-core et B. poreuses traditionnelles [55].

Globalement, les meilleures conditions en RPLC consistent certainement en une combinaison de stratégies permettant le meilleur compromis entre efficacité/capacité de pics, sélectivité/rétention, capacité de chargement et adsorption. Pour cela, l'emploi de l'UHPLC ou de la technologie fused-core avec des chaînes alkyles courtes et de larges pores paraît adéquat. La phase mobile devra elle contenir du TFA ou du FA et être chauffée.
La Figure I.8 présente une séparation rapide de quatre protéines combinant plusieurs des stratégies évoquées.

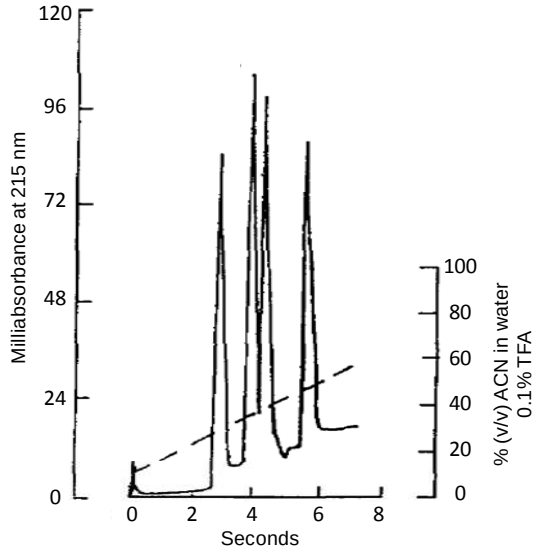


Figure I.8: Séparation rapide de quatre protéines par RPLC (phase stationnaire pelliculaire) à haute température (120°); 1, ribonucléase A; 2, cyt ochrome; 3, lysozyme; 4, β-lactoglobuline [46].

La RPLC est déjà couramment utilisée pour le contrôle de la pureté des lots, la mise en évidence des produits de dégradation et également des PEGylations. Les récents progrès technologiques devraient permettre d'asseoir plus encore cette technique séparative au sein des différentes étapes du développement des protéines pharmaceutiques.

I.3.2.3.2 Chromatographie d'exclusion stérique

La chromatographie d'exclusion stérique (SEC) est une des techniques chromatographiques les plus utilisées dans le domaine de l'analyse des protéines pharmaceutiques intactes de par sa simplicité, sa robustesse et le grand choix de colonnes disponibles. De plus, par rapport à l'électrophorèse sur gel qui partage un principe de séparation similaire (ségrégation par la taille), les débits d'analyse peuvent être plus importants. Les protéines sont séparées selon leur taille après un passage au travers d'une colonne remplie de matériaux aux pores de taille définie. La composition de la phase mobile est primordiale pour éviter la dénaturation des protéines et les colonnes doivent être aussi inertes que possible pour éviter les interactions parasites [56]. Cette technique est principalement employée pour la caractérisation de la taille des protéines ainsi que celle des agrégats.

I.3.2.3.3 Chromatographie d'échange d'ions

La chromatographie d'échange d'ions (IEC) est très employée pour la séparation de protéines car ces dernières possèdent de très nombreuses charges positives et négatives. Le processus d'IEC dépend fortement de la concentration en sels et du pH de la phase mobile [57]. L'échange de cations est un mode très utilisé car en dessous de pH 3, les charges négatives des groupes carboxyliques des protéines sont neutralisées au contraire

des groupes N-terminaux qui sont alors positivement chargés. L'IEC reste la méthode de choix pour l'analyse des hétérogénéités de charge des protéines pharmaceutiques.

I.3.2.3.4 Chromatographie à interaction hydrophile

La chromatographie à interaction hydrophile (HILIC) est une variante de la LC en phase normale avec une phase stationnaire hydrophile et une phase mobile consistant en un mélange d'eau et de plus de 70% de solvant organique. La rétention est due à une adsorption de surface par des ponts hydrogène, un partage hydrophile entre la phase stationnaire et la phase mobile moins polaire et /ou des interactions électrostatiques avec des groupements chargés à la surface de la phase stationnaire [58]. Cette technique est encore peu utilisée pour l'analyse de protéines intactes (il est fait mention de l'analyse d'histones avec détection UV [59] et de protéines solubles [60]) mais la disponibilité récente de colonnes avec des particules sub-2µm ou fused-core pourrait faire évoluer cette situation. Il est à noter que la nature du solvant de dissolution est primordiale en HILIC et qu'il doit être composé le plus souvent de solvant organique. Le comportement des protéines dans le solvant organique choisi devra être évalué car des dénaturations sont possibles.

I.3.2.3.5 Chromatographie d'affinité

La chromatographie d'affinité (AC) se base sur les interactions entre les protéines-cibles et des ligands spécifiques immobilisés. Les ligands employés peuvent être des immunoglobulines, des fragments d'anticorps, des protéines bactériennes, des lectines ou des peptides [61]. Cette approche est très utile pour la préparation d'échantillon afin d'éliminer certains types de protéines comme par exemple les protéines très abondantes du plasma. Elle peut aussi être employée comme technique d'extraction très spécifique, permettant par exemple de sélectionner la forme active d'une protéine thérapeutique. Elle est beaucoup utilisée dans l'étude des PTMs (phosphorylations, glycosylations).

I.3.2.4 Techniques électrophorétiques

De manière générale, les techniques électrophorétiques permettent de séparer selon différents modes (par exemple, rapport charge sur taille des molécules, affinité pour des micelles, combinaison avec des phénomènes de rétention) des molécules chargées par l'intermédiaire de l'application d'un champ électrique. De par le caractère multichargé des protéines, ces approches sont particulièrement adaptées à leur analyse. Plusieurs formats d'électrophorèse sont possibles pour l'analyse des protéines intactes, les analyses sur plaque d'une part et les analyses en format capillaire d'autre part. La première catégorie consiste principalement en la grande famille des électrophorèses sur gel tandis que la seconde regroupe plusieurs sous-groupes d'électrophorèse capillaire comprenant entre autres l'électrophorèse capillaire de zone et l'électrophorèse capillaire en présence de gel. Ces approches sont très largement utilisées tout au long du développement des protéines pharmaceutiques et certaines sont mêmes les méthodes de référence pour la détermination

de paramètres particuliers (exemple: l'électrophorèse sur gel bidimensionnelle pour établir le poids moléculaire de la protéine).
Les paragraphes suivants présentent les différents types d'électrophorèse et un développement particulier est fait pour le couplage électrophorèse capillaire de zone et spectromètre de masse à temps de vol.

I.3.2.4.1 Electrophorèse sur gel

Cette technique lorsqu'elle est effectuée en une dimension permet la séparation de macromolécules sur la base de leur taille, leur forme et leur charge nette. Le milieu de séparation consiste le plus souvent en un gel de polyacrylamide (électrophorèse sur gel de polyacrylamide ou PAGE), qui joue le rôle de tamis moléculaire. La combinaison de ce dernier et des forces électriques résulte en des vitesses de migration différentes pour les protéines contenues dans un mélange. La séparation des protéines natives par PAGE est néanmoins limitée car elle est le résultat des influences indistinctes des paramètres charge, taille et forme. Ainsi, des protéines avec des poids moléculaires différents pourraient ne pas être séparées [62].
Pour contrecarrer cela, l'électrophorèse sur gel de polyacrylamide en présence de sodium dodécyl sulfate (sodium dodécyl sulfate polyacrylamide gel electrophoresis ou SDS-PAGE) a été introduite. Cette technique impose à toutes les protéines du mélange des caractéristiques hydrodynamiques et de charge équivalentes. Les protéines sont traitées pendant la préparation d'échantillon avec du SDS chaud ce qui permet la formation de complexes protéine-SDS présentant tous une charge globale négative. Ces derniers possèdent alors une migration à l'anode dont la vitesse est inversement proportionnelle au logarithme de leur poids moléculaire. Ainsi, en établissant une droite de calibration avec des protéines de poids moléculaires connus, il est possible de déterminer celui d'une protéine inconnue. En plus de cette utilisation, la SDS-PAGE est aussi employée en technique préparative, les protéines d'intérêt étant excisées du gel pour être ensuite analysées par exemple par LC-MS.
L'électrophorèse sur gel est cependant le plus souvent effectuée en deux dimensions (2D). La première dimension consiste en une séparation basée sur la charge (focalisation isoélectrique ou IEF) et la seconde est une SDS-PAGE [63]. L'IEF permet la séparation des protéines selon leur charge nette qui dépend de l'ensemble des groupements ionisables de la molécule et donc de son point isoélectrique. Un gradient de pH est créé dans le gel à l'aide d'ampholytes ou plus récemment de gradients immobilisés directement sur le gel de polyacrylamide. Un produit chaotrope est ajouté à l'échantillon protéique pour éviter la formation d'agrégats et de complexes puis l'échantillon est déposé sur le gel et soumis à un champ électrique. Chaque protéine migre jusqu'à atteindre la région de pH correspondant à son point isoélectrique où elle s'immobilise, sa charge globale étant alors neutre. L'IEF est une technique présentant un pouvoir résolutif très élevé de par le fait que chaque protéine se concentre dans une région du gel bien précise. Le point isoélectrique des protéines peut être déterminé par cette technique en utilisant une calibration. Les gels sont ensuite incubés dans le tampon SDS et une SDS-PAGE est effectuée en seconde dimension. Les résolutions

obtenues sont excellentes et permettent par exemple de distinguer des PTMs qui altèrent la charge (phosphorylations, acétylations,…).

Le point faible de ces approches sur gel demeure la détection des protéines une fois séparées. En effet, si les colorations au bleu de coomassie ou à l'argent (dix fois plus sensible) sont couramment utilisées, elles impliquent des manipulations supplémentaires et n'apportent pas toujours une bonne répétabilité ni une relation exploitable entre l'intensité observée et la concentration. La détection grâce à des agents de dérivation fluorescents permet cependant d'améliorer cette situation, apportant de plus une gamme dynamique plus grande (jusqu'à cinq ordre de magnitude). La Figure I.9 montre une représentation d'un gel d'électrophorèse 2D d'une fraction mitochondriale après une révélation avec un agent de dérivation fluorescent. La technique a permis de mettre en évidence plus de 200 protéines dont 170 ont ensuite été identifiées par désorption/ionisation laser assistée par matrice (MALDI) et MS/MS.

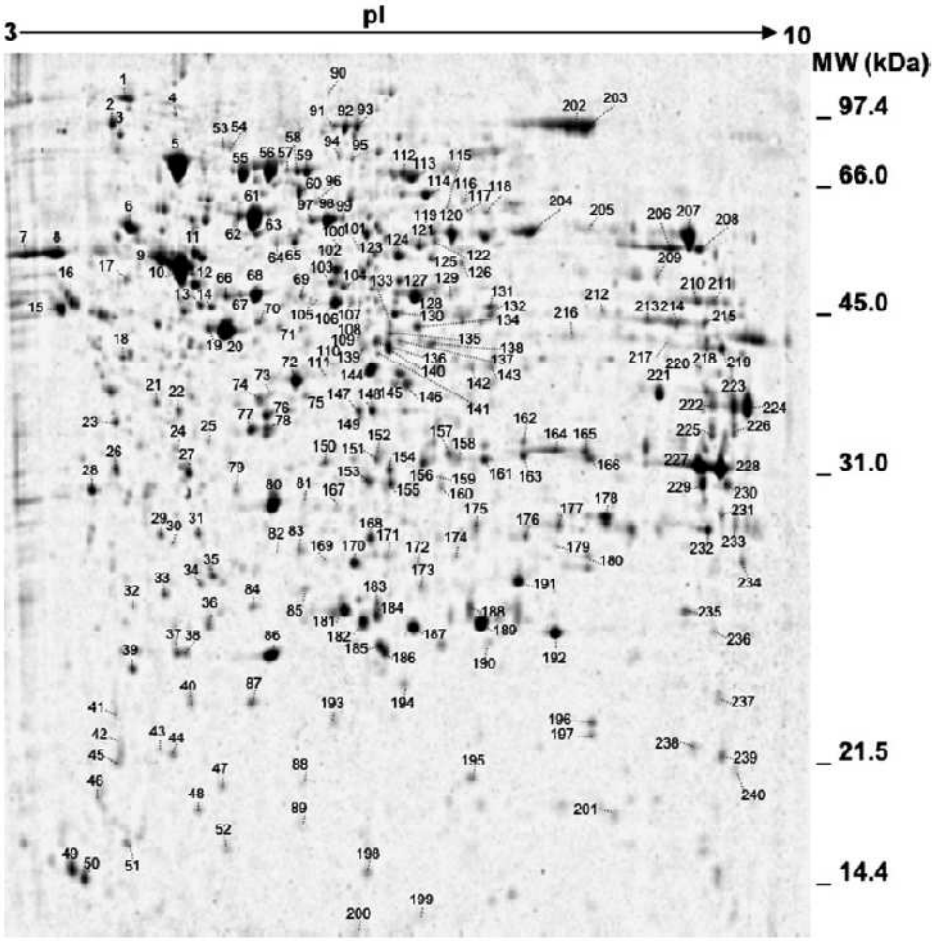


Figure I.9: Séparation de plus de 200 protéines d'une fraction mitochondriale de cellules neuronales par électrophorèse sur gel à 2D (révélation avec un agent de dérivation fluorescent) [64].

Malgré le savoir-faire technique nécessaire, les longs temps d'analyse et les problèmes de détection, les techniques d'électrophorèse sur gel demeurent les techniques de référence pour la séparation de protéines dans des échantillons complexes (préparation d'échantillon),

la détermination des poids moléculaires et points isoélectriques ainsi que la mise en évidence de PTMs impliquant des modifications de l'état de charge.

I.3.2.4.2 Electrophorèse capillaire

L'électrophorèse capillaire (CE) s'avère être une technique séparative très intéressante car elle permet des efficacités très importantes, une rapidité de mise en œuvre, de faibles volumes d'échantillon et une complète automatisation [65]. Les faibles volumes d'échantillons nécessaires (de l'ordre de 1 à 20 nanolitres) sont à mettre en comparaison des microlitres nécessaires en électrophorèse sur gel et en LC. Le format capillaire permet d'effectuer plus d'analyses ce qui peut se révéler intéressant lors du développement d'une protéine-médicament dont peu de quantité est disponible. De plus, le principe de séparation étant très différent de la RPLC, la CE peut être considérée comme une technique orthogonale de séparation. Par rapport à l'électrophorèse sur gel, la CE apporte une meilleure résolution, des temps de séparation plus courts, la possibilité d'automatisation et une détection en temps réel [66]. Comme pour la LC, des problèmes d'adsorption des protéines sur les parois du capillaire habituellement en silice fondue se posent. En effet, même si l'appareil de CE contient moins de tubes et autres connectiques qu'en LC, les phénomènes d'adsorption restent importants, en particulier directement au niveau du capillaire. Ces aspects seront plus largement discutés au paragraphe suivant.
Plusieurs modes de CE sont communément utilisés pour l'analyse des protéines intactes: l'électrophorèse capillaire en présence de gel (CGE), la focalisation isoélectrique capillaire (CIEF), l'électrochromatographie capillaire (CEC) et l'électrophorèse capillaire de zone (CZE). La chromatographie électrocinétique micellaire (MEKC) peut aussi être utilisée mais de par les faibles efficacités obtenues pour les protéines, elle est peu employée. La MEKC et la chromatographie électrocinétique en microémulsion (MEEKC) sont par contre parfois utilisées en format miniaturisé lors de séparations de protéines en 2D. Il est enfin à noter que l'électrophorèse capillaire d'affinité (ACE) permet surtout d'étudier les interactions non covalentes entre biomolécules et de déterminer les constantes de liaison et de dissociation des complexes formés [67]. Dans les sections suivantes, l'accent a été mis sur la CGE, la CIEF, la CEC et plus particulièrement encore, la CZE.

I.3.2.4.2.1 Les phénomènes d'adsorption

Les séparations de protéines intactes par CE sont souvent détériorées par la tendance de ces dernières à s'adsorber aux parois du capillaire de silice fondue. Le phénomène d'adsorption peut être divisé en deux catégories: l'adsorption réversible d'une part et irréversible de l'autre. La Figure I.10 présente les phénomènes impliqués pour les deux types d'adsorption.

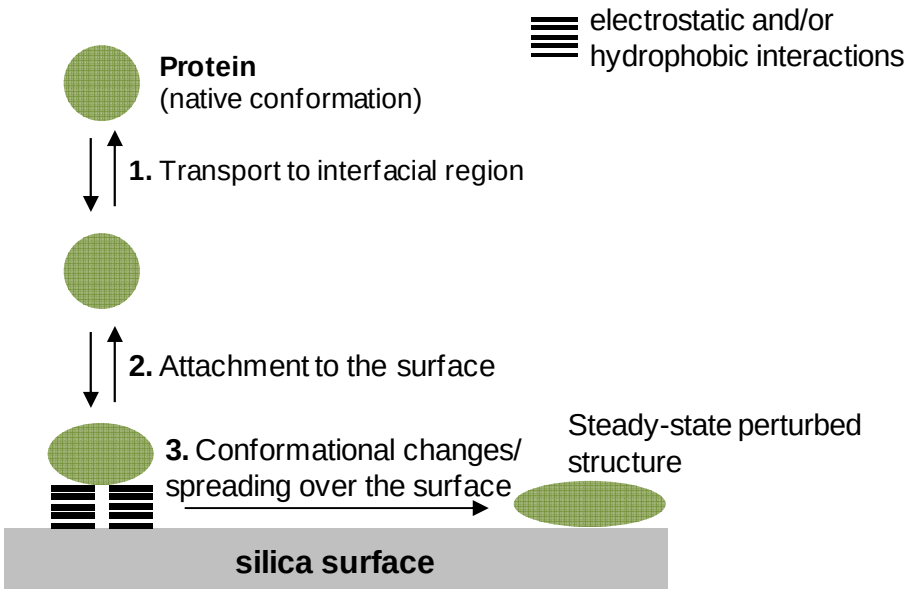


Figure I.10: Schéma mettant en évidence les différentes étapes du phénomène d'adsorption des protéines aux parois d'un capillaire; adapté de [68,69].

La première étape consiste en le transport de la protéine en direction de la surface du capillaire. La seconde étape fait intervenir des interactions entre la protéine et la surface qui peuvent être de natures électrostatique ou hydrophobe principalement. Les interactions électrostatiques dépendent à la fois des charges à la surface du capillaire et de la protéine, les deux étant sous l'influence du pH de l'électrolyte. Les interactions hydrophobes résultent des liaisons entre les parties hydrophobes de la protéine et les sections de même nature de la paroi du capillaire. A ce point du processus, la protéine est attachée à la paroi mais une désorption est possible, il s'agit alors d'adsorption réversible. La troisième étape consiste en des réarrangements structuraux de la protéine qui mène à une structure protéique perturbée mais en situation d'équilibre. La protéine est alors irréversiblement adsorbée. De manière générale, les deux types d'adsorption sont délétères pour la qualité de la séparation en termes de résolution, d'efficacité et de répétabilité. Plus précisément, l'adsorption réversible provoque des irrépétabilités au niveau des temps de migration [70] et une perte en efficacité [71], les deux phénomènes étant dus à des évènements d'adsorption et de désorption. L'adsorption irréversible induit une perte d'échantillon protéique le long du capillaire (se traduisant par un recouvrement d'aire inférieur à 100%) [72,73] et une altération du flux électrosomotique attribuable aux irrégularités de la paroi du capillaire provoquées par le dépôt de protéines [74]. Ces paramètres (répétabilité du temps de migration, efficacité, recouvrement d'aire et conservation du flux électroosmotique) sont donc des descripteurs utilisés pour caractériser et déterminer les phénomènes d'adsorption.

Il est par conséquent nécessaire d'employer des moyens pour diminuer voire supprimer l'adsorption, ce qui fait entièrement partie du processus de développement de méthode. En effet, si l'ampleur de l'adsorption dépend du type de protéine (flexible, rigide, point isoélectrique), du pH et de la composition du tampon, de la température de séparation ou encore de la nature de la surface du capillaire, elle n'est cependant pas prédictible en

étudiant la structure primaire de la protéine. Plusieurs approches sont envisageables, seules ou en combinaison, pour minimiser le phénomène d'adsorption:

- **Désorption par rinçage**: les stratégies qui apparaissent comme étant les plus efficaces consistent en l'utilisation de rinçages basiques (hydroxyde de soude le plus souvent) et/ou acides (acide chlorhydrique ou phosphorique). Ces rinçages sont d'autant plus efficaces s'ils sont faits immédiatement après l'analyse, mais il a été établi que des résidus d'échantillon demeuraient attachés à la paroi du capillaire [75]. L'utilisation de SDS est aussi parfois prônée. Il n'existe pas de consensus quant au type de rinçage à effectuer et cette approche est souvent utilisée en combinaison avec une autre stratégie visant à diminuer l'adsorption.

- **Sélection du tampon**: les tampons phosphate sont reconnus comme étant favorables *via* la formation de complexes entre le tampon et les silanols, diminuant ainsi la charge nette de la paroi du capillaire, particulièrement à bas pH. De plus, les ions phosphate peuvent interagir et former des paires d'ions avec les zones de la protéine chargée positivement et ainsi augmenter la répulsion entre la surface de silice et la protéine [76]. Cette option est par contre inutilisable dans le cas d'un couplage avec la MS, ce tampon étant non volatil. L'ajout de solvant organique en proportions variables dans le tampon peut aussi se montrer efficace et présente de plus l'avantage d'être totalement MS-compatible.

- **Revêtements de capillaire**: il s'agit de la méthode la plus utilisée. Ils peuvent consister en une approche dynamique (le revêtement est généré par l'addition de polymères ou d'amines directement dans le tampon) ou statique (les polymères sont fixés sur la surface du capillaire). Le choix du type et de la nature du revêtement dépendent de plusieurs paramètres comme le système de détection [77] (si la MS est choisie, les revêtements statiques sont préférés pour éviter une suppression d'ionisation, un important bruit de fond et/ou une contamination de la source d'ionisation et du spectromètre de masse) et le mode CE employé (en CIEF par exemple, un faible flux électroosmotique doit être garanti donc des polymères neutres et hydrophiles sont le plus souvent utilisés).

L'étape de caractérisation et de minimisation des phénomènes d'adsorption est donc cruciale dans le développement des méthodes d'analyse de protéines intactes par électrophorèse capillaire.

I.3.2.4.2.2 Electrophorèse capillaire en présence de gel

L'électrophorèse capillaire en présence de gel (CGE) utilise le même principe fondamental que la SDS-PAGE. En effet, l'échantillon protéique est chauffé en présence de SDS pour dénaturer la protéine et lui conférer une charge négative uniforme. La protéine peut être analysée sous sa forme réduite ou non réduite. L'échantillon est ensuite injecté dans un capillaire rempli d'un gel comprenant un polymère linéaire ou ramifié (polyacrylamide, PEG

ou dextrane). Le flux électroosmotique doit être supprimé pour permettre une séparation basée uniquement sur le rayon hydrodynamique, la protéine migrant à travers le gel qui joue le rôle de tamis moléculaire [78]. Pour diminuer le flux, il est possible d'employer un tampon à haute concentration ou de revêtir dynamiquement ou statiquement le capillaire. La CGE possède des avantages intéressants comme une possible automatisation, une grande vitesse d'analyse, une bonne résolution et une détection en ligne [79]. La haute résolution en particulier est un avantage sur la SDS-PAGE mais aussi sur la SEC qui constitue l'autre technique majeure pour la détermination des variants en taille. La CGE se positionne comme la méthode pouvant apporter la caractérisation de la taille des protéines en évitant certains inconvénients de la méthode de référence, la SDS-PAGE, comme la reproductibilité souvent insuffisante ou encore les longs temps d'analyse. Elle connaît d'ailleurs un essor important dans le domaine du contrôle qualité des mAbs [80].

I.3.2.4.2.3 Focalisation isoélectrique capillaire

La focalisation isoélectrique capillaire (CIEF) combine la haute résolution et la capacité de concentration des analytes de l'IEF et les avantages du format capillaire [81]. Comme avec l'IEF, les protéines sont séparées selon leur point isoélectrique dans un gradient de pH formé par des ampholytes, suite à l'application d'un champ électrique. La CIEF présente de très hautes résolutions permettant de distinguer deux protéines ayant un point isoélectrique qui ne diffère que de 0.005 unités de pH. Comme en CGE, le flux électroosmotique est minimisé voire supprimé par l'utilisation de revêtements de capillaire [82]. La CIEF est employée au sein des industries biopharmaceutiques principalement pour la détermination des points isoélectriques, pour caractériser les impuretés et monitorer les hétérogénéités de charges des protéines. La CIEF présente l'avantage de pouvoir être couplée avec la MS, ceci étant néanmoins possible moyennant d'importantes optimisations au niveau de la composition du gradient de pH. Cette association est une alternative à la 2D-PAGE dans la mesure où la première dimension est aussi basée sur les points isoélectriques (IEF) et que la seconde permet une séparation selon le rapport masse sur charge. Le couplage hors ligne avec une source MALDI a par exemple été effectué via une interface de collecte de fractions [83]. La source ESI est aussi employée pour un couplage en ligne mais des ajustements sont alors nécessaires, comme l'utilisation de concentrations d'ampholytes moins élevées car les ampholytes employés pour le gradient de pH provoquent souvent de la suppression ionique [84].

I.3.2.4.2.4 Electrochromatographie capillaire

L'électrochromatographie capillaire (CEC) est une méthode hybride qui sépare les protéines par une combinaison complexe de rétention chromatographique et de migration électrophorétique. Ainsi, les avantages de la CE tels que la faible consommation de solvant, la haute efficacité et le profil de flux plat sont combinés avec ceux de la LC comme la sélectivité élevée et la possible séparation de molécules neutres. De plus la combinaison avec la MS est possible *via* des interfaces comme l'ESI et l'ionisation chimique à pression atmosphérique (APCI) [85]. Cependant, la CEC-MS n'a pas encore été appliquée à l'analyse

de protéines, au contraire d'applications à des acides aminés ou des peptides, certainement du fait du cumul des inconvénients de la CE et de la LC, comme les phénomènes d'adsorption.
Trois types de CEC peuvent être distingués selon le format de colonne utilisé: les colonnes (capillaires) remplies avec des particules, les monolithes ou les systèmes tubulaires ouverts [45,86]. Les approches les plus employées pour l'analyse de protéines intactes sont les colonnes remplies en mode RPLC ou échange d'ions. Cependant, le potentiel de la CEC dans ce domaine reste peu exploité de par le manque de phases stationnaires fabriquées pour les protéines [87,88].

I.3.2.4.2.5 Electrophorèse capillaire de zone

L'électrophorèse capillaire de zone (CZE) permet la séparation de molécules chargées dans un capillaire dont les extrémités plongent dans des récipients contenant une solution liquide appelée électrolyte. Ce dernier remplit le capillaire et sa composition est très variable: totalement aqueux, aqueux avec un/des modificateur(s) organique(s), non aqueux, mais toujours avec un ou plusieurs sels qui lui confèrent une force ionique et permettent le contrôle du pH. Ce mode assure des séparations rapides avec de bonnes sélectivités et des efficacités très importantes [89]. La rapidité de séparation est une conséquence de la relation directe entre la vitesse du ion et le champ électrique appliqué:

$$v = \mu_{eff} E \qquad \text{Equation 3}$$

où v est la vitesse du ion [cm·s^{-1}], μ_{eff} est la mobilité électrophorétique effective du composé [cm^2·V^{-1}·s^{-1}] et E le champ électrique appliqué [V·cm^{-1}]. Ainsi, comme une tension jusqu'à 30 kV peut être appliquée, des séparations rapides sont obtenues.
Les sélectivités atteintes sont très intéressantes, particulièrement dans le cadre de l'analyse des protéines intactes. En effet, la mobilité électrophorétique d'un composé est définie comme suit:

$$\mu_{eff} = \frac{q}{6\pi\eta r} \qquad \text{Equation 4}$$

où μ_{eff} est la mobilité électrophorétique effective du composé [cm^2·V^{-1}·s^{-1}], q sa charge effective [Cb], η la viscosité de l'électrolyte [Cp] et r le rayon hydrodynamique du composé [cm]. La sélectivité en CZE est donc gouvernée par le rapport charge sur taille des composés. Les protéines étant des macromolécules pouvant présenter des charges et des tailles très différentes, une grande sélectivité peut ainsi être atteinte.

Plusieurs facteurs influencent la largeur des pics et par conséquent l'efficacité. Les facteurs principaux sont la diffusion de l'analyte, la longueur de la zone d'injection, l'hétérogénéité de la température de l'électrolyte et des interactions entre l'analyte et les parois du capillaire.

Les trois derniers pouvant être contrôlés et minimisés, l'efficacité est principalement gouvernée par la diffusion de l'analyte et peut être définie comme suit:

$$N = \frac{\mu_{app} U}{2D} \frac{L_{eff}}{L_{tot}} \qquad \text{Equation 5}$$

où N est l'efficacité, μ_{app} la mobilité électrophorétique apparente [$cm^2 \cdot V^{-1} \cdot s^{-1}$], U la tension appliquée [V], D le coefficient de diffusion [$cm^2 \cdot s^{-1}$], L_{eff} la longueur effective du capillaire (du site d'injection au détecteur) [cm] et L_{tot} la longueur totale du capillaire [cm]. Les protéines ayant des tailles importantes, elles possèdent des coefficients de diffusion très faibles (de l'ordre de 10^{-10} $cm^2 \cdot s^{-1}$) et leur analyse par CZE peut par conséquent permettre d'atteindre de très hautes efficacités (jusqu'à un million de plateaux). Ceci est d'autant plus vrai que de hautes tensions sont appliquées.
Les détecteurs classiquement utilisés avec la CZE demeurent les détecteurs UV/visible, de fluorescence et conductimétrique. Ces détecteurs permettent de déterminer de nombreuses molécules et sont faciles d'utilisation. Cependant, dans l'optique de gagner en sensibilité et en sélectivité, la MS s'est affirmée comme le mode de détection de choix. Or, en plus des performances en termes de sélectivité et d'efficacité déjà évoquées, la CZE présente également le grand avantage d'être naturellement couplable avec la MS [90]. En effet, contrairement à d'autres modes de CE qui impliquent l'emploi de gels ou d'ampholytes dans le milieu de séparation, la CZE emploie un milieu de séparation fait le plus souvent uniquement de tampon. Ainsi, en privilégiant des composants volatils (acide formique ou acétique, ammoniaque), la détection MS en ligne est possible.
Les chapitres suivants vont décrire les sources d'ionisation, les interfaces et les analyseurs de masse couramment utilisés dans le cadre de l'analyse des protéines intactes par CZE-MS.

I.3.2.4.2.5.1 Electrophorèse capillaire de zone et spectrométrie de masse

Les aspects généraux du couplage CE-MS ont été décrits dans l'article 2. Sous la forme d'une revue, cet article présente plus particulièrement le couplage de la CE avec le spectromètre de masse à temps de vol.

I.3.2.4.2.5.1.1 Sources d'ionisation

Le type de source d'ionisation à utiliser est lié aux caractéristiques physicochimiques de celle-ci. Par exemple, l'emploi d'une source APCI est difficilement envisageable de par les hautes températures de l'évaporateur et le caractère thermosensible de bon nombre de protéines. Ainsi, les sources les plus employées sont l'électrospray (ESI) et la désorption/ionisation laser assistée par matrice (MALDI). L'emploi d'une source d'ionisation par plasma induit (ICP) est aussi possible mais son utilisation est restreinte aux protéines contenant ou liant un métal et ne sera pas détaillée ici.

- **Désorption/ionisation laser assistée par matrice (MALDI)**: la source MALDI est très largement répandue pour l'analyse des protéines intactes [91]. Son principe consiste tout d'abord en la co-crystallisation de la protéine avec une matrice UV-absorbante. L'échantillon ainsi obtenu est ensuite frappé par un faisceau laser qui excite et vaporise partiellement la matrice, celle-ci entraînant également les protéines en phase vapeur. Un échange de protons se produit alors entre celles-ci et les molécules de matrice induisant une voire deux charge(s) sur la protéine. Les spectres de masse obtenus sont donc relativement simples. Le MALDI présente aussi les avantages de n'être que peu sensible à la présence de sels ou de tampons dans l'échantillon et de permettre l'analyse de protéines de grandes tailles. Cependant, ce dernier point n'est possible que par l'emploi d'analyseurs de masse à grand domaine de masses comme le quadripôle-TOF. De plus, le processus MALDI provoque souvent la formation d'adduits qui rendent parfois la détermination de la masse exacte de la protéine difficile et les informations obtenues via ce mode d'ionisation se limitent souvent à la masse moléculaire de la protéine. Le couplage entre la CZE et le MALDI est souvent effectué hors ligne car la co-crystallisation doit être obtenue avant l'analyse. Plusieurs techniques de collecte de fractions existent mais elles ne diffèrent que par la manière dont l'effluent CZE est déposé sur la plaque MALDI. Quelques interfaces de couplage CZE-MALDI en ligne ont été développées (avec une boule de dépôt rotative ou avec des interfaces de dépôt sous vide) mais elles sont relativement complexes et restent encore très peu utilisées.

- **Ionisation par électrospray (ESI)**: Un puissant champ électrique est appliqué à pression atmosphérique sur un liquide traversant une aiguille à faible débit. Le champ est produit par une différence de potentiel entre l'aiguille et une contre-électrode. Suite à l'accumulation de charges à la surface du liquide situé à l'extrémité de l'aiguille (cône de Taylor), la surface finit par rompre et générer un spray de gouttelettes fortement chargées. La Figure I.11 montre la désorption d'ions positifs suite à l'application d'une tension positive au niveau de l'aiguille.

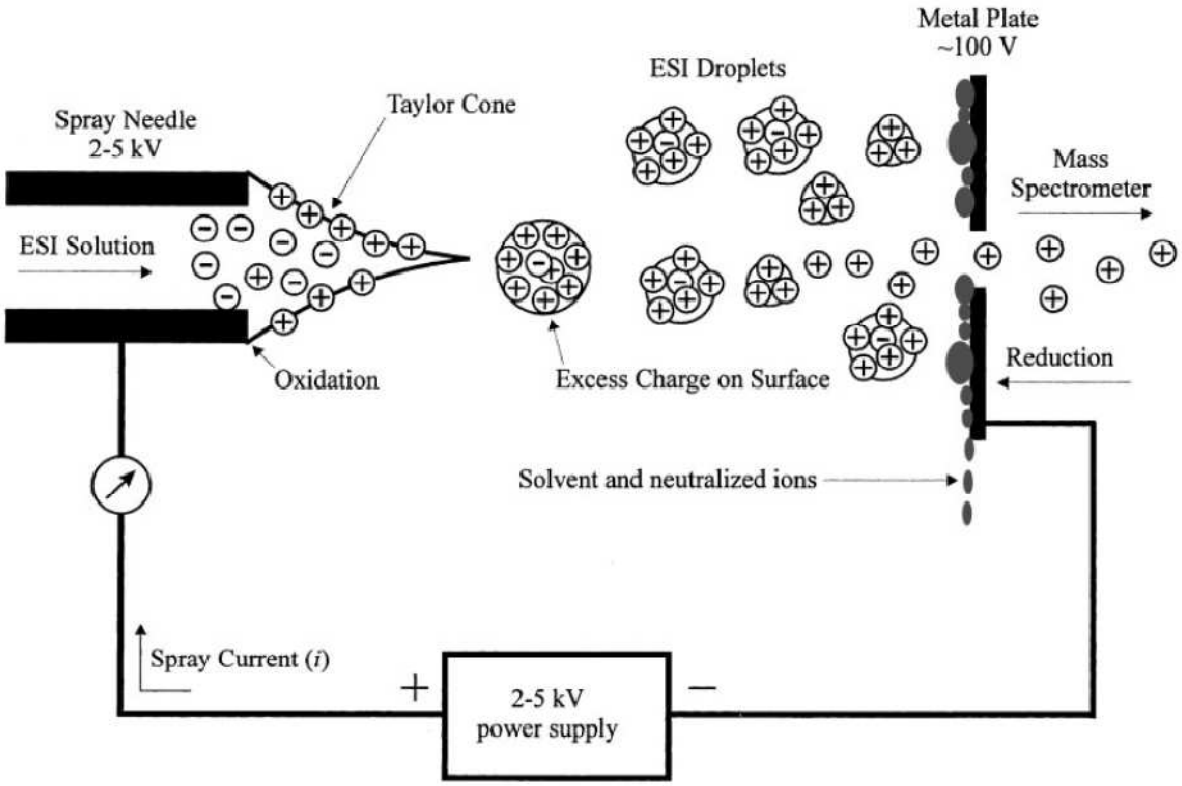


Figure I.11: Représentation schématique du processus de formation des ions dans une source électrospray [92].

La stabilité du processus ESI dépend tout autant de paramètres de l'interface (débit, tension de surface) que de la source elle-même (géométrie, voltage appliqué). Dans le cas du couplage avec la CZE, le circuit CE doit de plus être fermé au niveau de l'aiguille ESI et la compatibilité de la source avec les très faibles débits de la CE doit être rendue possible. Pour cela, deux types d'interface sont utilisés, avec et sans liquide additionnel [93].

- L'interface avec liquide additionnel est la plus utilisée car elle permet des conditions robustes et faciles à mettre en oeuvre. Dans cette configuration, le voltage est appliqué au tampon CE *via* un liquide de contact. Ce liquide permet à la fois de fermer le circuit électrique CE et de rendre les débits CE compatibles avec la source. Ce dernier point s'avère nécessaire car les sources ESI sont prévues pour une utilisation avec de la LC et donc ses débits beaucoup plus importants. Deux types d'interfaces avec liquide additionnel sont possibles, les deux étant facilement mises en place mais impliquant des réglages fins (débit et composition du liquide additionnel, position du capillaire CE dans le nébuliseur) pour obtenir un spray stable. La première est l'interface coaxiale qui implique la perfusion à un débit de quelques microlitres par minute du liquide additionnel à travers le nébuliseur autour du capillaire CE. Le mélange avec le tampon CE a lieu à l'extrémité du sprayeur ce qui assure la connexion électrique. La composition du liquide peut être optimisée, ce qui rend cette méthode très versatile. La seconde approche

est l'interface à jonction liquide. La connexion électrique est ici établie à la jonction entre le capillaire CE et un second capillaire inséré dans l'aiguille du sprayeur. Cette jonction est reliée à un réservoir de liquide additionnel. Le liquide entraîne l'effluent CE à travers le second capillaire par effet siphon (dû au champ électrique de l'ionisation et au gaz de nébulisation).

- Dans l'interface sans liquide additionnel, le voltage est directement appliqué sur le tampon CE. La plus grande difficulté demeure la fermeture du circuit CE. Pour cela, une aiguille constituée d'un matériau conducteur peut être employée, celle-ci étant de faible diamètre. Une autre option consiste en l'utilisation de l'extrémité du capillaire CE lui-même comme aiguille de nébulisation. Pour assurer la connexion électrique, l'extrémité du capillaire est revêtue d'un matériau conducteur où une électrode est directement insérée dans le capillaire. L'extrémité du capillaire peut aussi être rendu poreuse, permettant le contact entre l'intérieur du capillaire et un liquide conducteur infusé dans l'aiguille ESI [94]. Ce type d'interface présente l'avantage de ne pas diluer l'échantillon en sortie de capillaire et de permettre à une quantité maximale d'ions d'atteindre le MS, l'orifice d'entrée étant très proche du capillaire. Cependant, la robustesse du système reste faible, la détérioration des revêtements conducteurs étant par exemple assez rapide.

Les protéines intactes analysées par ESI-MS tendent à donner des ions multichargés de par la haute densité de charges dans les gouttelettes. Les espèces multichargées donnent naissance à une enveloppe de charges dans le spectre de masse de la protéine. Cette enveloppe consiste en un ensemble de pics avec des valeurs de rapports masse sur charge de l'ordre de 500 à 3000. Ainsi, les protéines sont détectées dans un domaine de masse beaucoup plus bas que leur poids moléculaire réel ce qui permet l'utilisation d'analyseurs de masse à moins grand domaine de masses comme le quadripôle ou la trappe ionique. Les masses moléculaires des protéines sont ensuite obtenues par déconvolution du spectre de masse ESI.
La Figure I.12 montre le résultat de l'analyse de l'hormone de croissance humaine par CZE-ESI-TOF/MS, comprenant l'électrophérogramme, le spectre de masse de la protéine ainsi que son spectre de masse déconvolué.

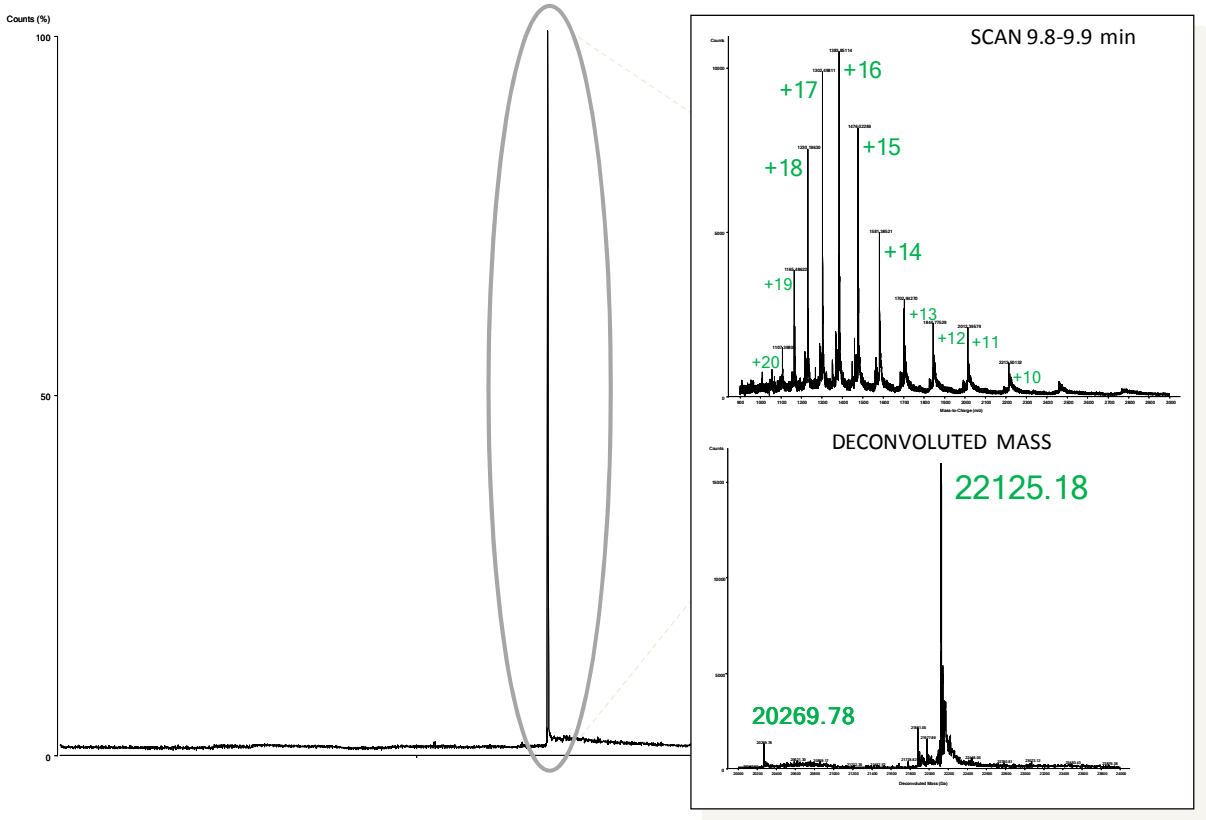


Figure I.12: Electrophérogramme, spectre de masse et spectre de masse déconvolué de l'hormone de croissance humaine analysée par CZE-ESI-TOF/MS [données obtenues lors de ce travail de thèse].

De manière générale, l'emploi de l'une ou l'autre des sources en couplage avec la CZE dépend des résultats souhaités. Le fait de pouvoir aisément coupler en ligne la CZE et la MS *via* l'ESI rend cette source particulièrement populaire et des applications sont publiées dans de nombreux domaines comme la recherche de biomarqueurs et la caractérisation de glycosylations. La source MALDI est plutôt utilisée hors ligne et plus particulièrement pour l'obtention des poids moléculaires des protéines, préalablement séparées par CZE et collectées sur une plaque MALDI. Cependant, le nombre de publications reste limité en comparaison avec celles impliquant l'ESI.

I.3.2.4.2.5.1.2 Analyseurs de masse

Comme déjà évoqué précédemment, le choix de l'analyseur de masse est primordial selon le type d'information désiré. Le fait de pouvoir employer une source ESI pour l'analyse des protéines et d'ainsi générer des ions multichargés permet l'utilisation des quadripôles et trappes ioniques, malgré leur domaine de masses limité. Le quadripôle présente une résolution en masse faible (de l'ordre de l'unité de masse) ce qui limite les identifications. En effet, l'exactitude de masse du spectre déconvolué est alors insuffisante pour par exemple distinguer entre deux espèces de protéines de masses proches [90]. Le spectromètre de masse à temps de vol (TOF) se présente donc comme un bon compromis entre coût et efficacité vu qu'il permet des exactitudes de masse de l'ordre de 2 à 10 ppm et des

résolutions de 10'000 à 40'000 pour les modèles les plus performants. Le MS à résonance cyclotronique ionique à transformée de Fourier (FT-ICR) et l'Orbitrap sont beaucoup plus performants encore en termes de résolution et de masse exacte mais les coûts sont aussi plus importants.
Les paragraphes suivants concernent le TOF, ses principes fondamentaux et quelques aspects techniques, dans la mesure où le couplage CZE-ESI-TOF/MS a été utilisé tout au long de ce travail de thèse.

Le principe fondamental du TOF est relativement simple. Le TOF sépare les ions produits dans la source du spectromètre de masse selon leur vitesse alors qu'ils se déplacent dans le tube de vol, zone libre de tout champ. Une différence de potentiel a été préalablement appliquée entre la source et l'analyseur pour accélérer les ions. Ces derniers possédant la même énergie cinétique (E_c), une distribution de vitesses est obtenue en cas de distribution de masses. Les rapports *m/z* (où *m* est la masse du ion et *z* sa charge) sont déterminés en mesurant le temps nécessaire aux ions pour parcourir la distance de vol *L* qui équivaut à la longueur du tube de vol [95].

Ainsi, pour un ion de masse *m* et de charge totale *q=ze* (où *e* est la charge élémentaire = 1.6×10^{-19} C) accéléré par un potentiel V_s, son énergie potentielle (E_p) équivaut à:

$$E_p = qV_s \qquad \text{Equation 6}$$

Vu que l'ion est accéléré, son E_p est convertie en E_c qui est égale à:

$$E_c = \frac{mv^2}{2} \qquad \text{Equation 7}$$

où *v* est la vitesse de l'ion. Les deux équations peuvent être combinées pour obtenir:

$$E_p = E_c = qV_s = \frac{mv^2}{2} \qquad \text{Equation 8}$$

Le tube de vol étant libre de champ et sous haut vide, *v* n'est pas affectée et reste constante. Elle peut donc être définie par le temps *t* mis pour traverser le tube de longueur *L*:

$$t = \frac{l}{v} \qquad \text{Equation 9}$$

Finalement, en substituant *v* dans l'équation 9 par sa valeur obtenue dans l'équation 8, l'équation suivante est obtenue:

$$t^2 = \frac{m}{z}\left[\frac{L^2}{2eV_s}\right] \qquad \text{Equation 10}$$

Par conséquent, la mesure du t^2 permet directement le calcul du rapport *m/z*, les termes inclus dans la parenthèse étant constants. Plus ce rapport est petit et plus l'ion atteindra le détecteur rapidement.

En MS, la résolution est habituellement mesurée par le rapport $m/\Delta m$, où Δm est la largeur à mi-hauteur d'un pic sur l'échelle de masse. Dans le cas de la spectrométrie de masse à temps de vol, il est préférable de travailler en termes de temps: $t/\Delta t$, où Δt est la largeur à mi-hauteur d'un pic sur l'échelle de temps. La résolution est donc limitée par les petites différences dans les temps mesurés pour des ions de même masse [96].
Ces différences sont dues à la combinaison des dispersions temporelle, spatiale et énergétique des ions qui conduisent à une dispersion en temps et en énergie cinétique. La dispersion temporelle se produit lorsque des ions de même masse formés à des temps différents avec la même énergie cinétique parviennent à des temps différents dans le tube de vol. La dispersion spatiale concerne des ions de même masse formés au même temps et avec la même énergie cinétique initiale mais dont le lieu de formation est différent. Ainsi, les ions se trouvant à l'arrière de la source vont subir un plus grand gradient de potentiel et être accélérés à une énergie cinétique plus grande que ceux se trouvant plus près de la zone d'extraction. La dispersion énergétique est induite par la variation de la vitesse initiale des ions lors de leur formation. La conséquence est une distribution des temps de vol.

Il existe plusieurs approches pour améliorer la résolution du TOF [97,98]:

- allongement du tube de vol, la résolution étant proportionnelle au temps de vol, mais la sensibilité est alors affectée (cas des TOF permettant l'usage du mode en « W »).

- abaissement de la tension d'accélération (avec une diminution concomitante de la sensibilité).

- emploi de l'extraction retardée: les ions sont accélérés après un délai par un champ électrique pulsé et pour un domaine de masses réduit. Cela est principalement utilisé avec les sources MALDI et permet d'améliorer sensiblement les résolutions pour les masses jusqu'à 20 kDa.

- utilisation du réflectron (ou ion mirror): ce dernier induit un ou plusieurs champs électrique(s) de sens opposé à celui de la zone d'accélération. Les ions arrivant dans le réflectron vont être ralentis jusqu'à s'arrêter et faire demi-tour en direction du tube de vol (Figure I.12). Ainsi, un ion d'énergie cinétique plus grande va pénétrer plus profondément dans le réflectron qu'un ion de même *m/z*, mais de moindre énergie cinétique qui va mettre moins de temps pour être réfléchi. Les deux ions sont donc focalisés sur un même plan et la résolution améliorée.

- emploi de l'accélération orthogonale (oa): la source d'ionisation, l'accélérateur et le détecteur décrivent un angle de plus de 90° dans un oa-TOF/MS. L'oa a été développée pour permettre le couplage d'une source continue comme l'ESI avec le

TOF qui demande la production d'ions par paquets successifs. Les ions sont injectés à intervalle régulier dans le tube à l'aide d'un champ électrique orthogonal à l'axe du faisceau d'ions incidents (Figure I.13). Cela permet une grande efficacité pour focaliser les ions provenant d'une source continue et de corriger simultanément les dispersions spatiales et temporelles. Une fois que le premier paquet d'ions a été dirigé vers le tube, un second paquet remplit l'accélérateur orthogonal, transformant une source continue en un flux pulsé compatible avec le TOF.

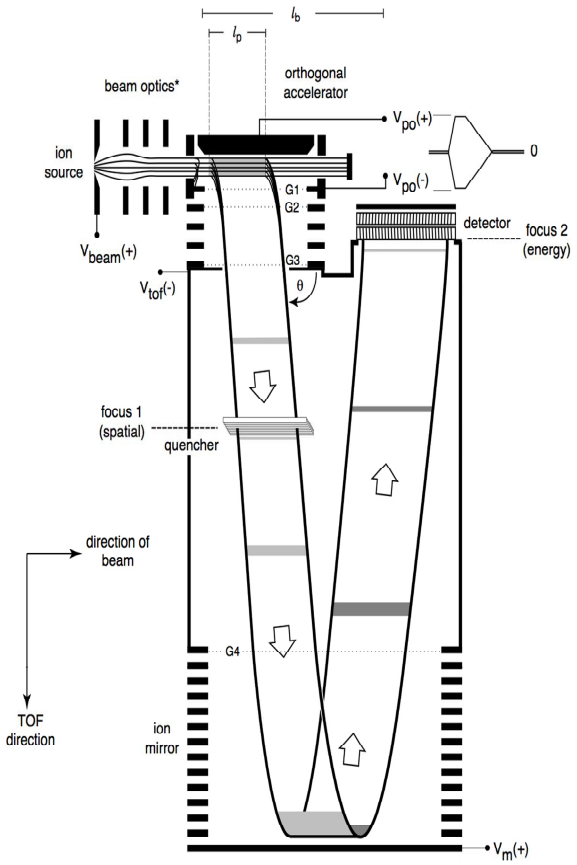


Figure I.13: Principe d'un système TOF/MS avec injection orthogonale et réflectron [97].

Même si les résolutions du TOF demeurent assez éloignées de celles obtenues avec un FT-ICR ou un Orbitrap, son couplage avec la CZE dans le cadre de l'analyse des protéines intactes permet néanmoins de combiner les avantages de la CZE (sélectivité et efficacité) avec ceux du TOF (résolution et exactitude de masse). Le couplage *via* une source ESI a déjà été largement employé dans le domaine des protéines avec par exemple l'identification des glycoformes de l'érythropoïétine (EPO) [99].

Il est à noter également que l'utilisation d'un TOF en mode hybride peut être intéressante. Ainsi, il est possible d'employer des instruments hybrides de type quadripôle (Q-TOF), à trappe d'ions (IT-TOF) et TOF-TOF [100]. Le Q-TOF peut être vu comme l'addition d'un quadripôle et d'une cellule de collision à un TOF ou au contraire le remplacement du troisième quadripôle par un TOF. Il en résulte quoi qu'il en soit une sensibilité, un pouvoir de résolution et une exactitude en masse améliorés en mode MS et MS/MS [101]. L'IT-TOF permet d'accomplir des analyses en mode MS^n et d'améliorer simultanément l'exactitude de

masse [102]. Les TOF-TOF consistent en deux TOF en série. Le premier sélectionne, isole et fragmente les ions en utilisant la collision à l'aide d'un gaz, tandis que le second ré-accélère les ions précurseurs et les fragments et mesure leurs masses et intensités [103].

I.4 REFERENCES

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Chapitre II | ARTICLES SCIENTIFIQUES

II.1 L'analyse des protéines intactes dans le domaine pharmaceutique

L'**article I** est un article de revue qui survole les principales techniques analytiques permettant l'analyse des protéines sous leur forme intacte et qui sont employées tout au long du cycle de vie d'une protéine pharmaceutique. La structure plus complexe des protéines en comparaison avec les molécules médicamenteuses de bas poids moléculaires demande des techniques analytiques adaptées et pose des problèmes nouveaux. Cette revue permet d'avoir une vue globale du challenge analytique que pose les protéines et de mieux saisir le rôle que peut jouer l'électrophorèse capillaire couplée à un spectromètre de masse à temps de vol dans ce cadre précis.
L'accent est tout d'abord mis sur les étapes-clés du développement de la protéine qui nécessitent des procédures d'analyse sous leur forme intacte, c'est-à-dire en particulier les étapes de caractérisation et d'études de stabilité. La caractérisation comprend un nombre important de tests pour évaluer entre autres les structures primaire, secondaire, tertiaire voire quaternaire de la protéine, son point isoélectrique, sa masse moléculaire et ses éventuelles modifications post-traductionnelles. Les instabilités peuvent elles être classées en deux catégories: chimiques (par exemple: oxydation, déamidation) et physiques (par exemple: agrégation, précipitation).

Au niveau des techniques analytiques elles-mêmes, quatre types de méthodes sont disponibles: la chromatographie, l'électrophorèse, la spectrométrie de masse et la spectroscopie. La revue décrit les quatre approches mais l'accent est mis sur les techniques séparatives, à savoir la chromatographie et l'électrophorèse.

La chromatographie liquide est beaucoup utilisée dans les étapes du développement des protéines, en particulier la chromatographie d'exclusion stérique qui demeure la méthode de référence pour déterminer la taille des protéines et celle des agrégats. Les modes les plus prometteurs de par les récents progrès technologiques (par exemple: diverses phases stationnaires disponibles en format sub-2 µm) et le potentiel couplage avec la MS sont la chromatographie en phase inverse (RPLC) et à interaction hydrophile (HILIC).
Au niveau des techniques électrophorétiques, le mode le plus employé demeure l'électrophorèse sur gel bidimensionnelle. Elle est largement utilisée au sein des industries pharmaceutiques pour la détermination de la taille des protéines et leur pureté. Les techniques électrophorétiques capillaires font également l'objet d'un intérêt croissant de par leur format miniaturisé. L'électrophorèse capillaire en présence de gel par exemple est déjà bien implantée dans le domaine de l'analyse des anticorps monoclonaux, permettant la mise en évidence entre autres des chaînes lourdes et légères de ceux-ci. L'électrophorèse capillaire de zone paraît être un mode prometteur de par la simplicité de son milieu de séparation et sa naturelle compatibilité avec la MS.

La revue conclut globalement sur la nécessité de détenir dans les laboratoires d'analyses de protéines pharmaceutiques de nombreuses techniques analytiques avec des principes

d’analyse différents. En effet, une seule approche ne permettra par exemple jamais de mettre en évidence la structure secondaire ou tertiaire d’une protéine de manière complète et sûre.

II.1.1 Article de revue I

A. Staub, D. Guillarme, J. Schappler, J.L. Veuthey, S. Rudaz. **Intact protein analysis in the biopharmaceutical field**, *J. Pharm. Biomed. Anal.*, 2011, *55*, 810-822.

INTACT PROTEIN ANALYSIS IN THE BIOPHARMACEUTICAL FIELD

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ABSTRACT

In recent years, a growing number of biopharmaceutical proteins has been produced and is already available, or will be soon available, on the market. These molecules are more complex to analyze than conventional low molecular weight drugs, and thus need performance analytical approaches for the entire development and delivery process. This review summarizes the analytical techniques available for intact protein determination and the main development steps in which they are applicable. A strong emphasis has been put on separation techniques, liquid chromatography and electrophoretic techniques, but mass spectrometry and spectroscopic approaches are mentioned. Overall, we highlight how several analytical strategies are necessary to obtain global information.

KEYWORDS

Intact protein, biopharmaceutical analysis, analytical methods, liquid chromatography, capillary electrophoresis

1. INTRODUCTION

During the last decade, the biotechnology sector has taken particular interest in pharmaceuticals. Insulin was the first product available from this area in 1982 [1,2], and the number of drugs originating from biotechnology is expected to reach 50% of all new chemical entities in a near future [3]. The development of these biomolecules is primarily related to the huge improvements in recombinant DNA technology. Indeed, only a small number of therapeutic proteins are purified from a native source, such as pancreatic enzymes from hog and pig pancreas [4]. In 2008, Leader *et al.* proposed a complete classification of all therapeutic proteins in current use, based on their pharmacological action [5]. Four groups were distinguished: therapeutic proteins with enzymatic or regulatory activity (*e.g.*, insulin, growth hormone, and erythropoietin), those with special targeting activity (*e.g.*, etanercept and abciximab), protein vaccines (*e.g.*, hepatitis B surface antigen), and protein diagnostics (*e.g.*, glucagon and growth hormone releasing hormone). The authors also called attention to the important potential of these proteins, considering that thousands of proteins are known to be produced by living organisms.

In terms of production, formulation, and quality control, therapeutic proteins pose many challenges compared to small molecules because of their inherent complexity [6]. For instance, manufacturing of biopharmaceuticals requires a greater number of batches (>250 *vs.* <10), a larger number of product quality tests (>2000 *vs.* <100), and larger process data entries (>60000 *vs.* <4000) [7]. The proteins' complexity is related to their numerous molecular weights, possible conformations, solubilities, stabilities, *in vivo* lifetimes, posttranslational modifications, and microheterogeneity [8]. Concerning their heterogeneity, protein modifications could occur during production, extraction, purification, formulation, and storage. Small differences in manufacturing processes can affect the efficacy and safety of recombinant proteins [9]. Some heterogeneity is natural and has no consequences, but some variants could have adverse biological and clinical effects [10]. For example, variations in glycosylation influence the biological effect of erythropoietin [11]. Taking into account the number of co- and post-translational modifications it takes to "fine-tune" the activity of proteins (*e.g.*, enzymatic cleavages, attachment of lipids, or glycans), it is consequently also complicated to produce a generic version of a biological drug [12]. Indeed, proteins require elaborate and sophisticated manufacturing processes, and their properties are highly dependent on the process employed. Legislation introduced in the U.S. Senate in March of 2009 describes a new regulatory pathway that would require manufacturers to demonstrate the following: (i) that generic versions of biological drugs are both very similar in molecular structure to the original one and share the same mechanism of action and (ii) that there are no significant clinical differences between the two products [13]. In this context, two distinct categories were defined: (i) "biosimilars", which include generic drugs that are merely similar to the brand-name drug and (ii) "biogenerics", which include generic drugs that are essentially identical to the brand-name drug, and thus can be substituted for the brand-name drug [13,14,15,16].

The specific characteristics of proteins compared to small molecules create the need for additional analytical methodologies. A variety of techniques including reversed-phase liquid chromatography (RPLC), size-exclusion chromatography (SEC), native gel electrophoresis

and other electrophoretic techniques, mass spectrometry (MS), and UV and fluorescence spectroscopy have been used to study proteins in research, development, production, and quality control [3]. Analytical chemistry plays an important role in supporting these activities by helping to understand the impact that changes in manufacturing processes and scale have on the quality and consistency of the drug's final form [18]. A single method is never sufficient to resolve and characterize a protein. Multidimensional separation techniques using orthogonal separation modes with MS are also often unable to fully resolve all of the variants present within complex protein products [8].

The aim of this review is first to summarize the key steps in biopharmaceutical development for intact protein analysis. Then, the most widely used analytical methods and applications are highlighted, with particular emphasis on the electrophoretic and chromatographic separation techniques widely used in the biopharmaceutical field today.

2. BIOPHARMACEUTICALS: KEY STEPS IN INTACT PROTEIN ANALYSIS

In this part of the review, the main stability issues and characteristics of therapeutic proteins as intact-molecule analysis are described. The corresponding analytical methodologies are further explained in related sections.

2.1 Characterization

Overall, proteins should be characterized in terms of identity, heterogeneity and impurity content. Proteins exhibit primary, secondary, tertiary, and, in some cases, quaternary structure. Characterization includes many parameters, such as molecular weight, size, isoelectric point, structure determination, purity assessment, charge state and charge microheterogeneity studies. Each property of the protein must be determined by at least two analytical strategies to ensure a coherent result. For example, the molecular weight can be calculated using size-exclusion chromatography, sodium dodecyl sulfate polyacrylamide gel electrophoresis, mass spectrometry (MS), light scattering and analytical ultracentrifugation. Since the underlying principles of these analytical methods are different, all results need to be corroborated to find a consensus value.

Proteins can also possess some posttranslational modifications, such as phosphorylation, *N*- and *C*- terminal amino acid heterogeneity and glycosylation. The latter is the most common form of posttranslational modification and consists of the enzyme-mediated process by which oligosaccharidic side chains are covalently attached to either the side chain of asparagine (N-linked) or serine/threonine (O-linked) [19]. The oligosaccharidic moieties of proteins are often essential for recognition, signaling and interaction events within and between cells and proteins, as well as for folding and defining protein conformation. Moreover, about 40% of approved therapeutics are glycoproteins [20]. Oligosaccharides are generally analyzed while still attached to the protein (peptide mapping and glycopeptide analysis), after intact releasing from the protein (oligosaccharide profiling) or after being broken down into their monosaccharidic constituent units (monosaccharide analysis). However, the analysis and characterization of intact proteins' glycoforms, such as recombinant erythropoietin [21], can also be achieved using mass analyzers with high or very high resolution (e.g., time-of-flight

mass spectrometers (TOF/MS), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS) and Orbitrap).

2.2 Stability

Protein instabilities can be classified into two categories, chemical and physical. A recent review from Manning *et al.* [22] describes these phenomena. The resulting degradations can be implicated in causing adverse reactions like anaphylactoid reactions [23] and renal failure [24]. The manufacturing process consists of several operational steps, referred to as “unit operations”, where the media is subjected to different stressors and conditions that could compromise quality and stability [25]. Instabilities can occur during processing, handling, storage or use; it is therefore important to understand stability issues in all of these stages to successfully apply proteins as pharmaceuticals [26].

Chemical instabilities are due to processes that make (*e.g.*, oxidation) or break (*e.g.*, deamidation) covalent bonds, potentially generating new chemical entities [27]. Manning presents chemical instabilities into the following classes: deamidation, aspartate interconversion/isomerization, racemization, proteolysis, beta-elimination, oxidation, disulfide exchange, diketopiperazine formation, condensation reactions, pyroglutamate formation, hinge region hydrolysis and tryptophan hydrolysis. All of these reactions are dependent on numerous parameters, including pH, nature/concentration of excipients and temperature. Consequently, operating production conditions, purification, formulation and storage should be optimized and controlled to minimize chemical instabilities. Characterization of chemical degradation products is often performed by analyzing proteins in their intact form using liquid chromatography (LC), electrophoretic techniques or direct infusion or hyphenation with MS [28]. The combination of several analytical methods is often necessary to obtain a complete view of the degraded protein.

Physical instability is rarely encountered for low molecular weight molecules. Proteins, because of their polymeric nature and ability to form superstructures (*e.g.*, secondary, tertiary and quaternary), can undergo a variety of structural changes independent of chemical modifications [29]. The principal types of physical instabilities are denaturation, surface adsorption, aggregation and precipitation. In particular, the control and analysis of protein aggregation is a growing challenge in pharmaceutical research and development [30]. Proteins may aggregate through several different mechanisms, classified as soluble/insoluble, covalent/noncovalent, reversible/irreversible, and native/denatured. Aggregates produced as a result of different stressors may have different size distributions, and their component proteins may contain different secondary and tertiary structures [31]. Protein aggregation can also lead to loss of activity, immunogenic reactions (*e.g.*, in small aggregates) or adverse effects during administration (*e.g.*, particulates) [32,33]. Several analytical methodologies for intact proteins are used for the determination of these physical degradations, such as multiangle light scattering, chromatographic and electrophoretic techniques, circular dichroism (CD), MS, and analytical ultracentrifugation [1]. As for chemical degradation, results obtained from several orthogonal analytical strategies are required to describe physical instabilities accurately.

3. ANALYTICAL METHODOLOGIES

Analytical methodologies for intact protein analysis include chromatography, electrophoresis, mass spectrometry (MS) and spectroscopy. Particular attention has been paid to separation techniques such as chromatography and electrophoresis. Mass spectrometry and spectroscopy are mentioned with relevant references, but not in details. Hydrodynamic mass transport methods (e.g. viscosity, analytical ultracentrifugation) can also be used during pharmaceutical development, but these approaches are not discussed here.

3.1 Spectroscopy

Spectroscopy is commonly used to assess protein secondary and tertiary structure. Numerous techniques are available, such as X-ray, nuclear magnetic resonance (NMR), absorption, fluorescence, CD, dynamic light scattering (DLS), and infrared spectroscopy (IR) [10]. X-ray and NMR are often used to determine the three-dimensional structures of proteins, but present some limitations in routine implementation. For X-ray experiments, the proteins must be crystallized, which is particularly difficult for glycosylated proteins [6]. NMR is restricted to structures up to 25 kDa in size and often required protein concentrations higher than those used in the formulations. Absorption and fluorescence spectroscopy are used to describe the secondary structure of intact proteins (*e.g.*, study of folding/unfolding). Absorption spectroscopy is also used to quantify proteins, according to the Bradford or Lowry methods. These approaches, which present an important lack of selectivity, are only capable of measuring an average signal change and cannot resolve contributions from individual amino acid residues. They provide indirect information on structure and conformation. CD spectroscopy is the technique of choice for studying chirality, particularly for monitoring and characterizing molecular recognition phenomena in solution [34]. It can also provide insights into the stereochemistry of a protein-bound drug or protein folding. Aggregates can also be analyzed by CD. Infrared spectroscopy can provide information about the protein secondary structure but involves complex spectra due to the number of atoms or groups of atoms in the protein. It is often used to complement CD methods, improving the estimation of secondary structure. DLS is primarily used to determine particle size and investigate protein aggregates [35].
Spectroscopy is widely employed in the pharmaceutical industry to elucidate structure and alteration during purification and formulation.

3.2 Mass spectrometry

Two fundamental strategies are used in the characterization and identification of proteins by MS. In the most commonly used “bottom-up approach”, the mixture of proteins of interest is usually first digested by trypsin, and peptides generated are analyzed by MS and MS/MS. In the “top-down approach”, intact protein molecular ions generated by electrospray (ESI) or matrix-assisted laser desorption (MALDI) are introduced into the mass analyzer [36]. The bottom-up strategy involves the analysis of peptides from the digested proteins, which is out of the scope of this review. The focus will thus be on the top-down approach only. The latter

has the advantage of providing access to the complete protein sequence and the ability to locate and characterize posttranslational modifications. However, in the case of ESI-MS, multiply charged ions are produced during ionization, generating complex mass spectra. Consequently, the method is often limited to isolated proteins or simple protein mixtures. In the context of protein development, this issue is often overcome since proteins are often isolated in pharmaceutical formulations. Thus, top-down MS is often preceded by a separation step (on-line or off-line), consisting of LC, capillary electrophoresis (CE) or other electrophoretic strategies [37]. In this context, mass spectrometers with high resolving power and accuracy should be used, such as TOF mass spectrometers, Orbitrap and FT-ICR instruments [38]. In biopharmaceutical development, intact protein analysis using the top-down approach should become more widely available, with Orbitrap instruments becoming less expensive to purchase and use compared to FT-ICR [39]. A milestone in protein characterization, MS provides precise and complementary structural information when used with other analytical techniques.

It has to be noted that two other MS approaches are also available: the Middle Up and Middle Down strategies. The Middle Up one consists, as the Bottom Up, in an enzymatic digestion but in bigger peptides, which are directly injected in the mass spectrometer. The Middle Down mode uses the same peptides than the Middle Up but they are fragmented in the mass spectrometer (MS/MS). The four approaches provide different information and are used in combination for the characterization of mAbs, for example.

3.3 Liquid chromatography

Liquid chromatography (LC) is recognized as an indispensable tool for intact protein analysis because of its high speed, high-resolving power, important reproducibility, and compatibility with MS [1]. Five different modes of LC have been employed for the analysis of intact proteins: reversed-phase LC (RPLC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), hydrophilic interaction liquid chromatography (HILIC), and affinity chromatography (AC). Each mode comes with its advantages and limitations, which will be discussed in the upcoming sections, with comments about applications in biopharmaceutical analysis. A common aspect that should be evaluated for all modes is the adsorption of proteins onto the HPLC device. It is important to avoid the use of polyether ether ketone (PEEK) in connection tubings and injection needles. PEEK is a very hydrophobic material that causes strong protein adsorption. Inert materials such as titanium, stainless steel or PEEK-Sil (fused silica inside, PEEK outside) are preferable, although stainless steel and fused silica do not completely eliminate adsorption [40]. Recently, Agilent Technologies (Palo Alto, USA) introduced a new LC system (1260 Infinity Bio-inert HPLC Solution) dedicated to biomolecules analysis. The system is iron- and steel-free in solvent delivery and the sample contacting surface is completely metal-free, minimizing unwanted surface interactions. Moreover, Waters Corporation (Milford, USA) proposed a new UHPLC system (ACQUITY UPLC® H-Class Bio System), which also features an inert flow path and permits to perform four chromatographic modes on a single system (RPLC, IEC, SEC, and HILIC).

3.3.1 Reversed-phase liquid chromatography

In comparison to low molecular weight molecules and peptides, reversed-phase liquid chromatography (RPLC) of proteins is more problematic due to adsorption, carryover, lack of retention (pore exclusion), multiple peak formation, and low chromatographic performance. These issues arise because of slow diffusion through and secondary interactions with the stationary phase [41]. In addition, retention of proteins is strongly dependent on small changes in solvent strength, as recently reported by Gritti *et al.* [42]. For this reason, isocratic conditions are usually impractical. Even a change of 0.1% acetonitrile (ACN) would lead to a strong modification of protein retention, and percentage windows are quite narrow for each protein. Gradient elution is thus mandatory.
To improve RPLC on intact proteins, several approaches can be applied to reduce adsorption, minimize secondary interactions and enhance diffusion coefficients.

3.3.1.1. Reduction of protein adsorption

Adsorption of proteins onto the solid phase largely decreases sensitivity, due to analyte loss. This issue is particularly problematic when quantitation is to be performed or when small amounts of protein have to be analyzed. The first variable to consider modifying is the use of less hydrophobic stationary phases instead of conventional C_{18} phases [1]. Various types of sorbents (*e.g.*, C_2, C_4, C_8, and C_{18}) are used in protein separation [43], but long-chain phases like C_8 or C_{18} can cause peak tailing or lower recovery of large protein due to their high hydrophobicity. Therefore, packing with shorter alkyl chain lengths like C_4 is preferable for proteins. However, this kind of chemistry is less resistant to hydrolysis in acidic pH conditions than C_8 or C_{18} phases. Adsorption issues with LC have been mentioned in section 3.3. It is also worth noting that hydrophobic adsorption phenomena are partially overcome in RPLC due to the use of gradients to analyze proteins by LC. The increase in organic solvent composition with time could counteract and reduce this type of adsorption with numerous analyzed proteins.

3.3.1.2 Reduction of protein peak tailing

Secondary interactions occur between the positively charged analytes and the remaining negatively charged silanol groups of the stationary phase [44,45]. Because the kinetics of secondary ionic interactions are slower than that of hydrophobic interactions, chromatographic performance is reduced, resulting in peak tailing and broadening. To minimize secondary interactions, it is possible to use silica-based stationary phases with restricted access to residual silanols (*e.g.*, endcapped, bidendate, hybrid silica, high density bonding, or embedded polar group stationary phase). Alternatively, the temperature of the mobile phase can be increased to enhance performance [46]. At elevated temperatures, mobile phase viscosity is reduced, analyte diffusivity is enhanced, and sorption kinetics are accelerated by improving mass transfer and kinetic rates [47]. Consequently, peak tailing and broadening are strongly reduced. However, temperature should be set with caution due to potential thermal degradation of proteins.

The third aspect to consider is the addition of an ion-pairing agent to the mobile phase. Trifluoroacetic acid (TFA) at a concentration of 0.1% is commonly used for protein analysis, as it possesses excellent ion pairing and solvating characteristics and inhibits peak tailing and broadening [48]. However, when coupled with MS detection, ion suppression can occur with TFA, both in negative and positive modes. In this scenario, formic acid (FA) may be preferred. Finally, since some hybrid silica-based stationary phases are stable within a wide pH range (*i.e.*, up to pH 12), it would be interesting to evaluate basic pH conditions to further reduce ionic interactions [49]. This option has only been reported for peptide analysis so far, but could certainly be extended to proteins [50,51]. Since peptides and proteins are multicharged molecules, comprised of mostly basic, ionizable functional groups, the change of mobile phase pH will have a pronounced effect on their retention behavior and peak shape.

3.3.1.3 Reduction of protein peak broadening

Conventional porous packing materials are well adapted for relatively small proteins and peptides obtained after proteolytic digestion. However, high-molecular-weight proteins have large radii and low diffusion coefficients that cause peak broadening when using conventional porous particles. Analyte diffusion in the pores significantly slows down as the pore size becomes smaller than approximately 10-fold the size of the analyzed compound. Therefore, larger porous packing materials with 300- or 1000-Å pore sizes have been introduced in place of the conventional 80–120 Å sizes. Numerous applications with these larger-pore materials can be found in the literature [52, 53, 54]. An alternative approach is the use of elevated mobile phase temperature. As previously mentioned, temperature can be optimized to avoid peak tailing by accelerating the sorption kinetics of proteins. In addition, because diffusion coefficients are drastically enhanced with increasing temperature (*i.e.*, Dm is proportional to the ratio of temperature/viscosity), chromatographic performance are improved. The use of high temperature (120°C) LC was reported for the first time by Chen and Horvath in 1995, and a separation of four proteins in less than 10 s was successfully performed (Figure 1) [46]. However, most stationary phases have limited thermal stability [55], and proteins are thermolabile molecules that can quickly be denaturated. For these reasons, high temperatures should be applied with caution.

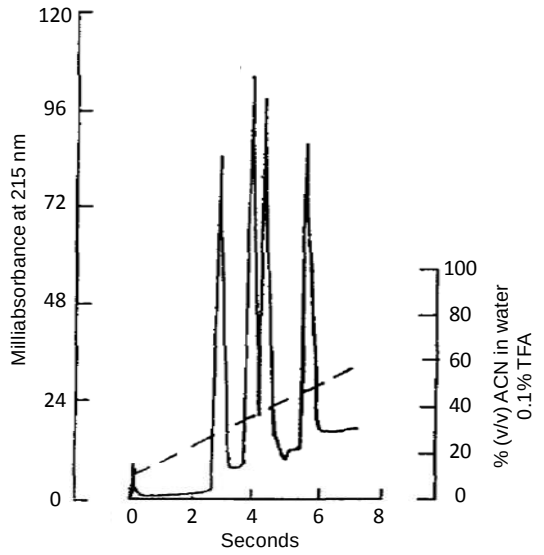

Figure 1: Rapid separation of four proteins by RPLC (pellicular stationary phase) at elevated temperature (120°); 1, ribonuclease A; 2, cytochrom e; 3, lysozyme; 4, β-lactoglobulin. Reprinted from [46] with permission from Elsevier B.V.

One of the best solutions to limit band broadening in the case of intact protein analysis is the optimization of packing morphology. For this purpose, various solutions have been proposed. In the 1980–90s, RPLC protein analysis was primarily carried out on non-porous sorbents [56]. The typical structure of non-porous stationary phases includes a fluid-impervious support physically or covalently attached to a layer of functional groups on its surface. A major advantage of this sorbent is that it decreases or avoids significant mass transfer resistances, resulting in improved analyte diffusion and high efficiency. However, the loading capacity is reduced proportional to the small specific surface area of the non-porous particles. Retention is also drastically decreased, which may hamper separation of most hydrophilic proteins. Porous layer open tubular (PLOT) columns that reduce mass transfer resistance have also been developed. They do not consist of a specific solid phase, but only a porous layer of stationary phase on the inner column wall, similar to a GC column. As non-porous supports, PLOT columns can provide high efficiencies for large molecules [57,58] with higher loading capacities. However, they may have some limitations in retention and selectivity, since the specific surface is not equivalent to that of sorbent particles.

Because of the inherent limitations of non-porous and PLOT materials, progress has also been made in optimizing the kinetic performance of packed columns with fully porous particles. Monolithic supports offer obvious kinetic advantages, including high permeability, low backpressure, and rapid mass transfer. These supports can be made with inorganic (*e.g.*, silica, carbon, zirconia and titania) or organic (*e.g.*, polymethacrylate and poly(styrene-divinylbenzene), polyacrylamide) materials. Organic monoliths are of limited interest for the separation of low molecular weight molecules, but useful for analyzing macromolecules because of their inert and biocompatible properties [59,60]. In the last two years, several applications have been published. Causon *et al.* studied the kinetic performance of a poly(styrene-divinylbenzene) monolithic column [61], and its use with elevated temperatures

and alternative solvents [62]. They demonstrated some obvious advantages in cost, time, and reduction in organic solvent consumption. Eeltink *et al.* studied the same column and showed the interplay of the primary chromatographic parameters for the separation of intact proteins [63]. They highlighted the importance of column length and macropore size in achieving maximum peak capacity. In a previous work, they also employed such monoliths for one- and two-dimensional LC separations of intact proteins, reaching the highest possible resolution [64].

An alternative strategy consists of using columns packed with sub-2µm particles. Reducing particle size leads to significant improvements in kinetic performance. This is particularly useful for large molecules, since optimal mobile phase flow-rate is inversely proportional to particle diameter and mass transfer resistance (*i.e.*, C-term of the Van Deemter equation) is directly proportional to the square of d_p [65]. However, sub-2µm supports generated high backpressure (>400 bar), which is not compatible with conventional LC instrumentation. Nevertheless, since the advent of commercial ultra-high pressure liquid chromatography systems (UHPLC) that withstand pressures up to 1300 bar, this issue can be counteracted. In 2008, Everley *et al.* used a combination of high temperatures (up to 65°C), a strong organic modifier (i.e., isopropanol) and columns packed with sub-2µm particles at very high pressure to yield enhanced resolution, sensitivity and a threefold increase in throughput for the analysis of ten proteins [41]. Figure 2 compares the original HPLC with the optimized UHPLC method, where enhanced resolution was obtained. However, when dealing with UHPLC conditions, it is important to keep in mind that pressure can have a strong influence on protein retention [66]. Indeed, when the linear velocity is increased between HPLC and UHPLC, the average column pressure is also significantly increased, and the retention factor could be strongly altered [42].

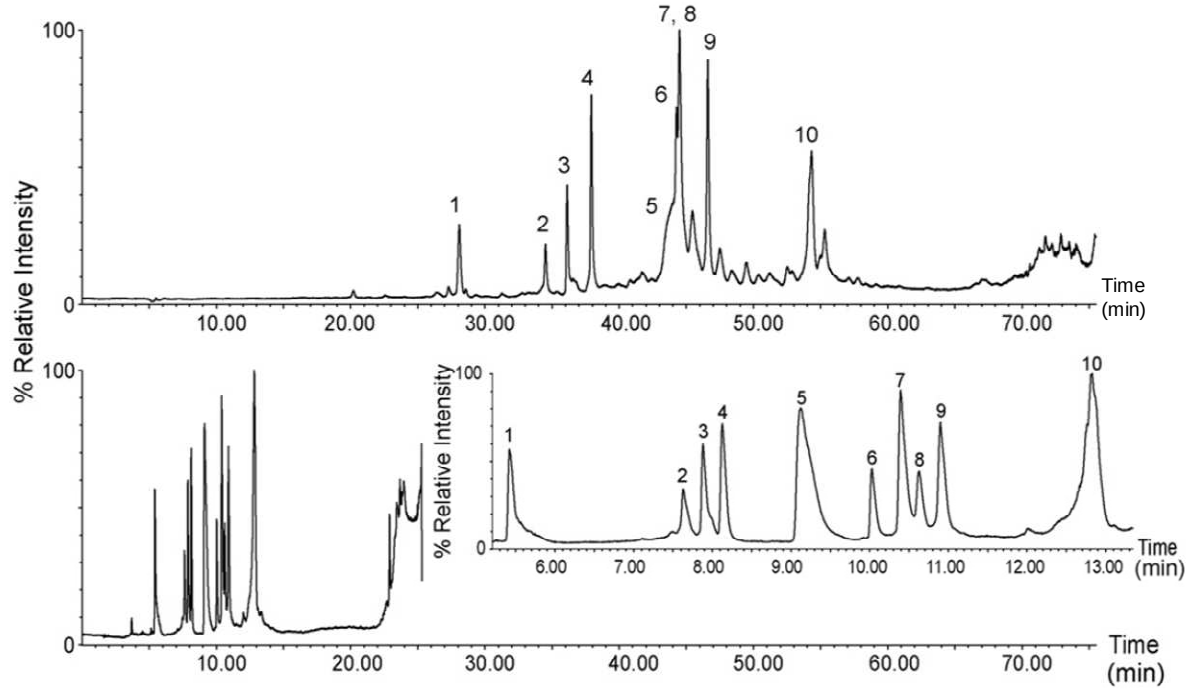

Figure 2: Comparison of the original HPLC method (upper trace) and the optimized UHPLC method (lower trace) for the separation of ten proteins. Reprinted from [41] with permission from Elsevier B.V.

To increase the separation performance for large molecules, fused-core (also called core-shell or superficially porous particle) technology with 5-µm particles was originally introduced by Kirkland in 1992 [67]. It is now commercially available from numerous providers as sub-3 µm particles, allowing a significant improvement in kinetic performance. Compared to completely porous particles of similar sizes, the diffusion path is much shorter in fused-core technology because the inner core is solid fused silica, which is poorly penetrable by analytes [59]. For small molecules, it provides superior mass transfer kinetics and better performance at high mobile phase velocities [68] and lower backpressure [69]. On the other hand, Gritti *et al.* showed that the minimization of peak broadening at elevated linear velocities can be very attractive for intact proteins analysis [70].

3.3.1.3 Generic RPLC conditions and applications

To summarize, optimal RPLC conditions for intact protein analysis should consist of a compromise between efficiency/peak capacity, selectivity/retention, loading capacity, and protein adsorption. In this context, it seems suitable to employ UHPLC or fused-core technologies with a short alkyl chain length (C_4 phase) and a large pore size of 300Å. The mobile phase temperature should be increased, and 0.1% TFA (with UV detection) or 0.1% FA (with MS detection) should be added to the mobile phase for their ion-pairing abilities.
In the biopharmaceutical field, RPLC appears to be promising for both the assessment of protein batch purity and to highlight any protein degradation (*e.g.*, truncation, glycosylation, and isomerization) misfolds or PEGylation [7]. Due to recent advances in RPLC and its straightforward coupling to MS, its usefulness in intact protein analysis will become increasingly important.

3.3.2 Size-exclusion chromatography

Size-exclusion chromatography (SEC) is widely used for protein analysis because it is simple, robust, relatively high-throughput (compared to slab-gel electrophoresis), and readily available. A recent review by Arakawa *et al.* highlighted the importance of mobile phase composition (salt content, buffer concentration and organic solvent addition) on retention and recovery of proteins during SEC analysis [71]. On the other hand, significant drawbacks of SEC are its limited dynamic range, low efficiency and loading capacity, complex MS-coupling (few reported applications [1]), limited throughput, and protein adsorption onto the column. Diol-coated SEC columns packed with 1.7-µm particles (Waters Corporation, Milford, USA) were recently proposed to minimize secondary interactions, reduce column/system clogging and requirements for high-salt-concentration mobile phases, and significantly improve throughput. In the biopharmaceutical field, SEC is the method of choice for the characterization of aggregate size and content [72], although large aggregates could not enter into the column due to clogging in the frits. Figure 3 presents dual-wavelength SEC chromatograms for the IgG1 monoclonal antibody, exhibiting several aggregates. Finally, SEC is also widely used for protein purity and PEGylation determinations, ensuring batch-to-batch consistency.

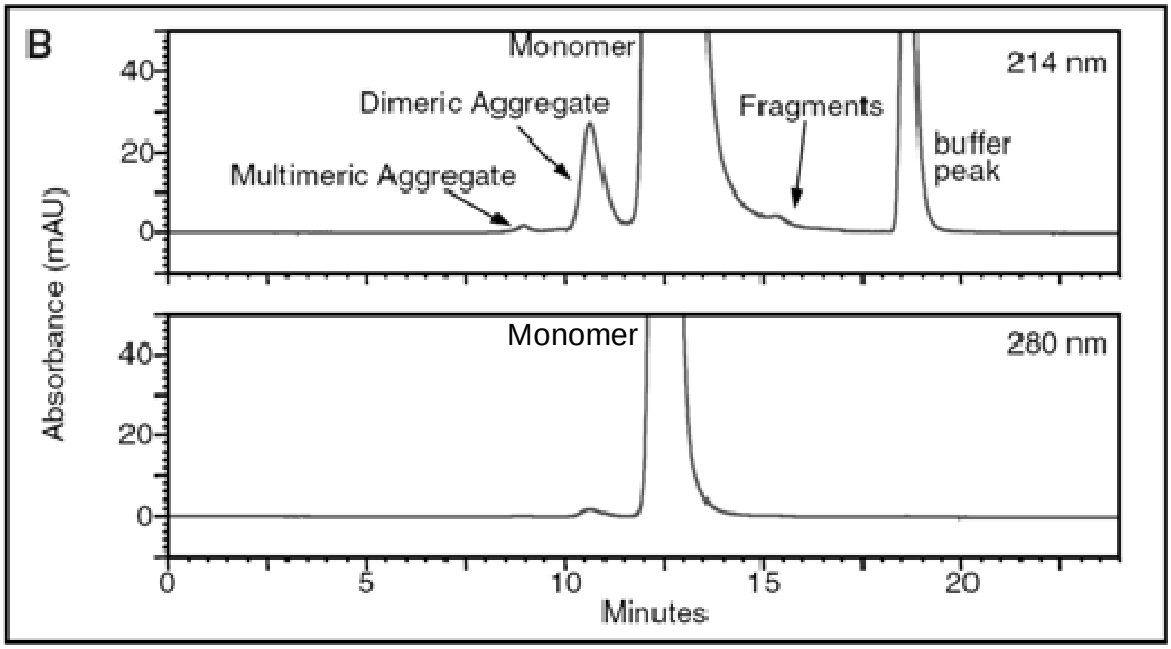

Figure 3: Dual-wavelength size exclusion chromatography for aggregate analysis of the IgG1 monoclonal antibody. Reprinted from [72] with permission from Wiley-VCH Verlag GmbH.
Finally, SEC is also widely used for protein purity and PEGylation determinations, ensuring batch-to-batch consistency.

3.3.3 Ion-exchange chromatography

Ion-exchange chromatography (IEC) is extensively used in protein separation, which usually involves many positive and negative charges. As with SEC, mobile phase composition is a critical because the IEC process strongly depends on salt concentration and pH. Cation-exchange chromatography is the gold standard for protein analysis because at a pH lower than 3, negative charges on carboxyl groups are neutralized, while N-terminus groups are protonated. Anion-exchange chromatography, which implies that carboxylic groups are negatively charged and N-terminus groups are neutralized, involves the use of high pH values (*i.e.*, greater than 12) and is often incompatible with silica columns. Finally, IEC is often used in two-dimensional LC with hydrophobic interaction chromatography for the separation of native proteins [73]. In biopharmaceutical development, IEC remains the method of choice to analyze the charge heterogeneity of proteins and can be useful to determine PEGylations.

3.3.4 Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a variant of normal-phase chromatography in which the stationary phase is hydrophilic (*i.e.*, bare silica or silica derivatized with various polar functional groups including amine, amide, cyano or diol), while the mobile phase consists of a mixture of water with more than 70% organic solvent (generally ACN). Retention is governed by different interactions including hydrogen bonding,

hydrophilic partitioning between the stationary phase and the less polar mobile phase, and electrostatic interactions with charged groups at the surface of the stationary phase [74]. This technique is often used to analyze glycopeptides or glycans from glycoproteins [75]. HILIC has not yet been widely applied for the analysis of intact protein, aside from applications in the analysis of histones [76,77,78] and soluble proteins [79]. Recently, a paper describing online coupling of RPLC and HILIC for protein and glycoprotein characterization has been published [80]. In this work, the authors validated the applicability of their setup for the analysis of very complex biological samples. HILIC could be a valuable alternative to ion exchange chromatography (*e.g.*, by selecting an appropriate HILIC column with positive or negative charges at the surface), since it is directly compatible with MS instrumentation. Because HILIC columns packed with sub-2µm or fused-core particles are now commercially available from several providers, interest in such chromatographic modes for intact protein analysis should quickly grow in the fields of protein characterization and stability studies. However, the possible irreversible adsorption of proteins onto the HILIC material still needs to be evaluated. Moreover, the dissolution solvent plays a major role in HILIC, and organic solvent solubility may be an issue for proteins, causing denaturation.

3.3.5 Affinity chromatography

Affinity chromatography (AC) is based on the interaction between target proteins and specific immobilized ligands. It creates either an enrichment or depletion of a specific class of proteins (*e.g.*, highly abundant proteins in serum). AC can also separate proteins based on their biological activity, where an active form can be separated from the inactive one or a form with different biological function [81]. Interactions can occur through several entities: immunoglobulin (immunoaffinity), antibody fragments, bacterial proteins (protein A or G), lectins, or peptides [82]. Particularly, AC is widely used in the study of post-translational modifications, such as phosphorylation, glycosylation or cysteine oxidation-reduction.

3.4. Electrophoresis

Electro-driven separations are often used in the analysis of charged macromolecules. The principle is similar for all modes and consists of the separation of molecules under an electric field. Gel electrophoresis remains dominant, but capillary electrophoresis (CE) possesses some attractive characteristics, which will be highlighted in the next sections.

3.4.1 Gel electrophoresis

Fractionation by gel electrophoresis (polyacrylamide gel electrophoresis or PAGE) is based on sizes, shapes, and net charges of macromolecules. Systems designed to fractionate native proteins cannot distinguish between the impacts of size, shape, and charge on electrophoretic mobility. Consequently, proteins with different molecular weights could have the same mobility in these systems. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an efficient strategy to overcome these limitations. SDS-PAGE imposes uniform hydrodynamic and charge characteristics on all proteins present in a

sample mixture. Interactions with SDS disrupt all noncovalent protein bonds, causing the macromolecules to unfold. Electrophoretic mobility of the detergent-protein complexes is then related to molecular weight [83]. Detection is generally accomplished by staining with either Coomassie Brilliant Blue, or more sensitive silver stain dyes. However, SDS-PAGE requires extensive skill in gel pouring, sampling, separation, and staining/destaining for the visualization and evaluation of separated bands [84]. Moreover, very hydrophobic, extremely large, and highly basic proteins are often missing, and, finally, the reagents are quite toxic [85]. This approach is well suited for proteins with molecular masses higher than 10'000 Da with no post-translational modifications, where SDS-PAGE is commonly used to determine apparent molecular weight, size heterogeneity, purity, and manufacturing consistency [86].
Gel isoelectric focusing (IEF) methods are also often used for the characterization of therapeutic proteins (e.g., monoclonal antibodies). IEF is an electrophoretic separation method that separates amphoteric molecules like proteins according to their charge. The sample is prepared and conditioned with a chaotropic agent, a zwitterionic detergent, a reducing thiol, and carrier ampholytes to avoid the formation of aggregates and complexes between proteins. After the separation, when the proteins have reached their isoelectric point, gels are often incubated in SDS buffer to be subjected to SDS-PAGE as a second dimension. IEF can provide very high resolving power, including the separation of protein post-translational modifications that alter their charge (*e.g.*, phosphorylation, acetylation) [87,88]. If desired, isoelectric points of proteins can be estimated with a calibration curve using marker proteins. The second dimension (SDS-PAGE) allows further separation based on the apparent molecular mass.
These methods are often used during the characterization step, due to their great resolving power and sensitivity. This is especially true for IEF because of the high concentration of proteins at their isoelectric point. Gel methods thus remain the gold standard among electro-driven techniques, although they are time-consuming and require good technical skills. They are also currently used as a preparative technique, where proteins of interest are excised from the gel prior to analysis by LC- or MALDI-MS [37].

3.4.2 Capillary electrophoresis

Capillary electrophoresis (CE) has several well established, attractive features, such as high speed and great efficiency. Moreover, CE presents advantages of the capillary format (*i.e.*, µL range): a smaller sample size, improved resolution, decreased separation time, full automation and real-time detection [89,90]. In the case of intact protein analysis, small differences between proteins may be sufficient for separation, since CE is a function of size, charge and shape. This method is promising for biopharmaceutical development because it is viewed as being orthogonal to RPLC and generally considered to be superior to classical electrophoresis.
Four modes of CE are commonly used for intact protein analysis: capillary gel electrophoresis (CGE), capillary isoelectric focusing (cIEF), capillary electrochromatography (CEC), and capillary zone electrophoresis (CZE). Micellar electrokinetic chromatography (MEKC) can also be used, but is less common due to its low efficiency for protein analysis [91]. MEKC and micro-emulsion electrokinetic chromatography (MEEKC) have been used in

microfluidic chips to perform two-dimensional separations [92]. Affinity capillary electrophoresis (ACE) can also be used in intact protein analysis. ACE is particularly useful for studying biomolecular, noncovalent interactions and determining binding and dissociation constants of formed complexes. The review from Liu *et al.* summarizes these findings [93]. In following sections, we will focus on CGE, CIEF, CEC and CZE.

3.4.2.1 Adsorption issue

The separation of intact proteins by CE is often hampered by their tendency to adsorb onto the negatively charged surface of conventional fused-silica-based capillaries. Adsorption can be reversible or irreversible, and both have a negative effect on CE separation performance. Reversible adsorption retards the migration time of proteins and decreases separation efficiency through adsorption/desorption events. Irreversible adsorption causes loss of proteins within the capillary and alteration of the electroosmotic flow (EOF) velocity. To monitor the reversible adsorption, peak efficiency and migration time relative standard deviation are recorded. EOF conservation and peak area recovery are likewise documented for irreversible adsorption [94]. Adsorption is influenced by the type of protein (*e.g.*, flexible, rigid, isoelectric point), the pH and composition of the background electrolyte (BGE), the separation temperature, the nature of the solid surface, and the coating modification of the silica surface [95]. Minimizing protein adsorption is an important part of any optimization procedure, especially as the degree of adsorption to the capillary surface is not predictable from protein primary structure. Several strategies are possible to decrease protein adsorption: desorption by rinsing (*e.g.*, NaOH, HCl or SDS solutions), electrolyte selection (*e.g.*, phosphate buffers, addition of organic solvent in the BGE [96]), and capillary coatings. The latter is the most often used. Two types of coatings are available, dynamic and static. Dynamic coatings are versatile and cost effective, made by adding amines, surfactants or some neutral polymers directly to the BGE. Static coatings are made by permanently modifying the fused-silica surface through chemical reactions or physical adsorption of coating agents [97]. Choosing the type and nature of coating depends on both the detection system (*e.g.*, static coatings are often preferred with MS detection to avoid serious background noise, suppression of analyte signal and/or contamination of ion source and MS optics) and the CE mode (*e.g.*, in cIEF, no or low EOF is preferred with neutral or hydrophilic polymers). In all cases, evaluation of adsorption is a crucial step to use CE in the analysis of proteins. However, the potential adsorption surface is drastically reduced in CE compared to LC with packed material.

3.4.2.2 CE modes

3.4.2.2.1 Capillary gel electrophoresis

Traditionally, SDS-PAGE has been used to monitor the size-based separation of proteins. As mentioned above, this technique presents some drawbacks including the use of toxic reagents and the low reproducibility associated with the staining/destaining steps [84,85]. The fundamental principle of capillary gel electrophoresis (CGE) is similar to SDS-PAGE—

samples are heated in the presence of SDS to denature the protein and impart a uniform negative charge. Proteins can be analyzed under their reduced or non-reduced form. The former is prepared by adding reducing agents such as β-mercaptoethanol, and the latter by adding an alkylating agent to prevent inter-chain disulfide bond shuffling during sample preparation. The sample is then injected into a capillary filled with a sieving gel comprised of a linear or branched polymer (*e.g.*, polyacrylamide, PEG or dextran). The EOF must be strongly reduced by either a high buffer concentration, a buffer with a dynamic coating, or coated capillaries to achieve separation that is based solely on hydrodynamic radii as analytes migrate through the gel-sieving matrix [98]. CGE possesses some inherent advantages, such as automation, enhanced precision, high-speed analysis, improved resolution for closely migrating species, and online quantitative detection [99]. The high resolution of CGE is an advantage over SDS-PAGE and SEC. Over the past decade, CGE has been considered the primary method for size-based protein analysis, and its use has consequently grown in analytical and quality control laboratories, particularly for monoclonal antibodies (mAb) [100].

Recently, purity analysis of reduced and non-reduced IgG2 by CGE was developed, validated and implemented by Pfizer (Chesterfield, MO, USA) as a replacement for SDS-PAGE [98]. Figure 4 shows the separation of a reduced IgG2 mAb using a mixed buffer matrix. The light and the heavy chains are perfectly separated.

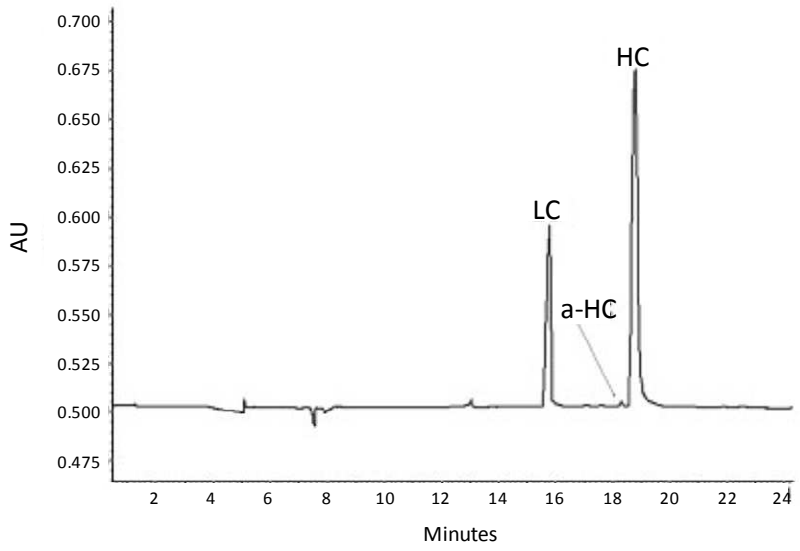

Figure 4: Separation of a reduced IgG2 mAb using a mixed buffer matrix, which contained a dynamic coating (LC, light chain; HC, heavy chain; a-HC, aglycosylated heavy chain). Reprinted from [98] with permission from Wiley-VCH Verlag GmbH.

Guo *et al.* highlighted the structural isoforms of IgG2 in a non-reduced CGE method and demonstrated that both isoforms were disulfide bond-related species. Bioactivity studies of both isoforms have shown that they both possess the same potency [101]. In the development of targeted ultrasound contrast agents (UCA), monoclonal antibodies are often used due to their high specificity and affinity for the target (*e.g.*, vascular molecular targets). The chemical coupling of UCA and mAb could have negative effects on mAb integrity. Cherkaoui *et al.* demonstrated that CGE-UV can be a powerful technique for monitoring IgG

structural integrity under various reduction conditions [102]. CGE can also be used in miniaturized systems, as recently described by Wenz *et al.* They detected protein impurities down to a level of 0.05% relative to the main component using a microchip CGE and fluorescent derivatization [103]. This sensitivity could permit the routine use of miniaturized systems for purity and integrity analyses of biopharmaceuticals.

3.4.2.2.2 Capillary isoelectric focusing

Capillary isoelectric focusing (CIEF) combines the high resolving power and analyte concentration capacity of classical IEF with the advantages of the capillary format [104,105]. As in IEF, proteins are separated according to their isoelectric point (pI) in a pH gradient formed by carrier ampholytes when an electric field is applied [86]. CIEF has proven to be one of the most powerful methods in the analysis of protein isoforms, as it can distinguish between two proteins whose pI differs by as few as 0.005 pH units [106]. Coated fused-silica capillaries are usually used in CIEF to decrease EOF and reduce protein adsorption onto the capillary wall [107]. CIEF is commonly used to determine the pI of proteins, characterize impurities, and monitor protein charge heterogeneity (*e.g.*, mAb). A great advantage of CIEF is its compatibility with MS detection. CIEF coupled online with MS is a promising alternative to 2D-PAGE, since it is also a 2D-separation [108]. Offline coupling with MALDI/MS was performed by Minarik *et al.* by using a fraction collection interface and analyzing a mix of standard proteins [109]. This set-up was also used in 2009 to characterize glucagon and its deamidation product [108]. The online coupling of CIEF with MS via an electrospray ionization (ESI) interface is also promising, but the presence of carrier ampholytes and polymeric additives could interfere with protein ionization (*i.e.*, ion suppression). To overcome this limitation, relatively low concentrations of carrier ampholytes should be used [110]. Mokaddem *et al.* proposed an online coupling of CIEF and ESI/MS in a glycerol-water media [111]. Glycerol was a good alternative to conventional aqueous gels for CIEF separations, since the presence of gel in the separation medium is a major constraint in CIEF-ESI-MS. Since glycerol strongly reduced EOF, no capillary coating was needed. The procedure may allow the characterization of both hydrophilic and hydrophobic proteins in samples of intermediate complexity. Figure 5 shows data for the scan-mode MS signal, single ion monitoring (SIM) signal and extracted ion current (EIC) chromatogram of six model proteins obtained with sufficient resolving power.

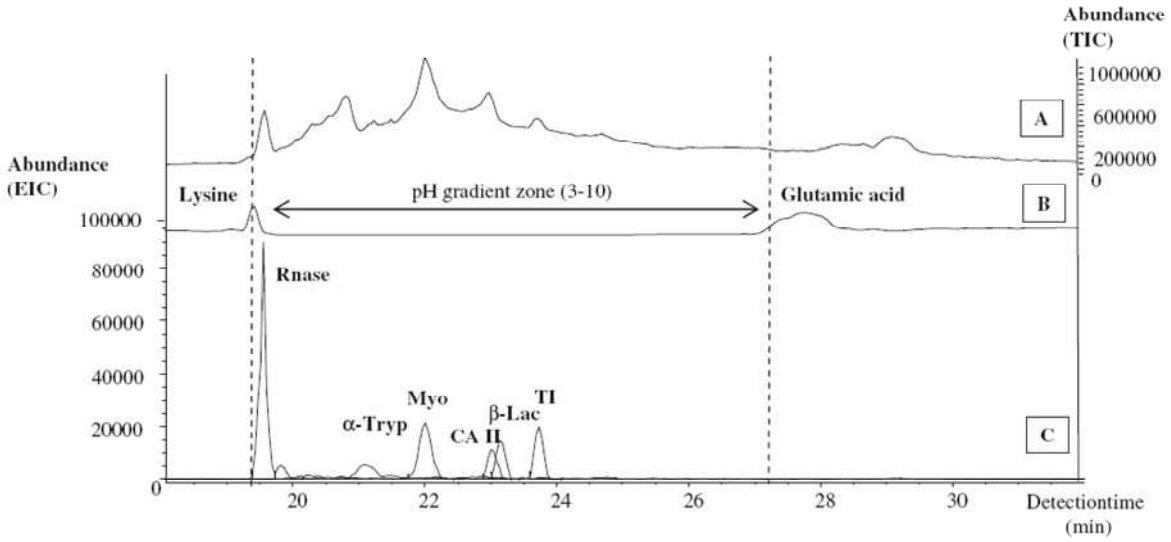

Figure 5: CIEF-ESI/MS and extracted MS spectra of six model proteins: (A) total ionic current electropherogram; (B) single ion monitoring electropherogram; (C) extracted ion chromatogram of Rnase (ribonuclease A), α-Tryp (α-chymotrypsinogen), Myo (myoglobin), CA II (carbonic anhydrase II), β-Lac (β-lactoglobulin), and TI (trypsin inhibitor). Reprinted from [111] with permission from Wiley-VCH Verlag GmbH.

A recent review by Shimura highlighted advances in CIEF with microchips [112]. Overall, automation and separation resolution should be improved before this technique can be widely used.

3.4.2.2.3 Capillary electrochromatography

Capillary electrochromatography (CEC) is a hybrid technique where the separation between analytes results from a combination of electrophoretic migration and chromatographic retention. Consequently, advantages of CE (*i.e.*, the capillary format, low consumption of solvents, high efficiency, and flat flow profile) are theoretically achieved along with those of LC (*i.e.*, elevated selectivity and possible separation of neutral molecules). However, some limitations are encountered in CEC such as poor robustness, low sample capacity, and insufficient reproducibility. There are three modes of CEC: columns (capillaries) packed with porous particles, columns with monolithic materials, and open-tubular systems. Reviews published by Oliva *et al.* [1] and Miksik *et al.* [113] extensively described these different strategies. The traditional approach uses columns packed with particular chromatographic materials. For protein separation, RP and ion-exchange materials are typically used. Zhang *et al.* proposed a CEC separation of four standard proteins with a strong anion-exchange column [114]. Columns made of a cationic acrylic monolith have been used by Zhang *et al.* to separate a mixture of standard proteins and peptides [115]. They highlighted the complex interplay between selective chromatographic retention and differential electrophoretic migration. The use of open-tubular systems requires a coating to avoid protein adsorption. Recently, Moore *et al.* developed a zwitterionic coating with both carboxylic acid and amine groups. It creates either an overall positively or negatively charged coating, depending on

BGE pH [116]. Microfluidic devices can also be used in CEC, although development has been limited due in part to the difficulty of packing microfluidic networks with stationary phase materials. In 2009, Jemere *et al.* presented a baseline separation of labeled insulin and immunoglobulin G with microchip-based bead-packed columns [117].
CEC can also be coupled to MS detection [118]. The most common interfaces are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). CEC-MS is commonly used for the analysis of amino acids, peptides, saccharides, and chiral compounds, among others. To the best of our knowledge, intact protein analysis by CEC-MS has not yet been reported.
The potential of CEC in protein analysis is still negligible due to the lack of stationary phases specially designed for protein separation [119]. Combined with the issues associated with CEC, it is clear why there are only a small number of applications in biopharmaceutical protein analysis [120].

3.4.2.2.4 Capillary zone electrophoresis

In capillary zone electrophoresis (CZE), the capillary is generally filled with a background electrolyte (BGE) and separation is accomplished by differences in the analytes' electrophoretic mobility [89]. High separation efficiencies can be obtained, with longitudinal diffusion being the only source of band broadening. In CZE, efficiency is inversely proportional to the diffusion coefficient of molecules. This is particularly attractive for intact protein analysis, since these compounds have low diffusion coefficients. CZE is the most frequently used mode that can be hyphenated with MS for intact protein analysis. Since the BGE can be made of only volatile components, it is directly compatible with MS, commonly through an ESI interface. MS compatibility provides useful structural information, particularly when coupling with high resolution mass spectrometers [89,121]. The study of adsorption is crucial in CZE, since fused-silica capillaries are generally used with a BGE containing only buffer. As explained previously, the use of capillary coatings (dynamic or static) is the best approach to counteract protein adsorption onto the capillary wall. Catai *et al.* proposed for example the use of noncovalently bilayer-coated capillaries for intact proteins analysis [122,123,124]. In biopharmaceutical protein research, CZE is now often used to identify the therapeutic mAb, and examine its charge heterogeneity. He *et al.* developed a CZE method in a 40-cm, uncoated capillary for the separation of IgG1 and IgG2 monoclonal antibodies [125]. Charge variants were separated with a short capillary (10 cm effective length). CZE can also be applied to the identification of protein isoforms. Bohoyo *et al.* used CZE in the analysis of different isoforms of unphosphorylated recombinant tau protein and for the separation of the phosphorylated and unphosphorylated protein forms [126]. A polybrene coating was used to reduce adsorption of the tau protein, which presents numerous cationic moieties that interact strongly with the capillary wall. Balaguer *et al.* characterized the glycoforms of erythropoietin by combining glycan and intact protein analysis using CE and time-of-flight MS [127]. The molecular masses and the quantitation of each intact glycoform were determined. Figure 6 presents the mass spectrum and corresponding deconvoluted mass spectrum of the analyzed recombinant human erythropoietin. The spectrum obtained is

relatively simple, and, as shown in Figure 6b, a charge envelope corresponding primarily to one glycoform is obtained.

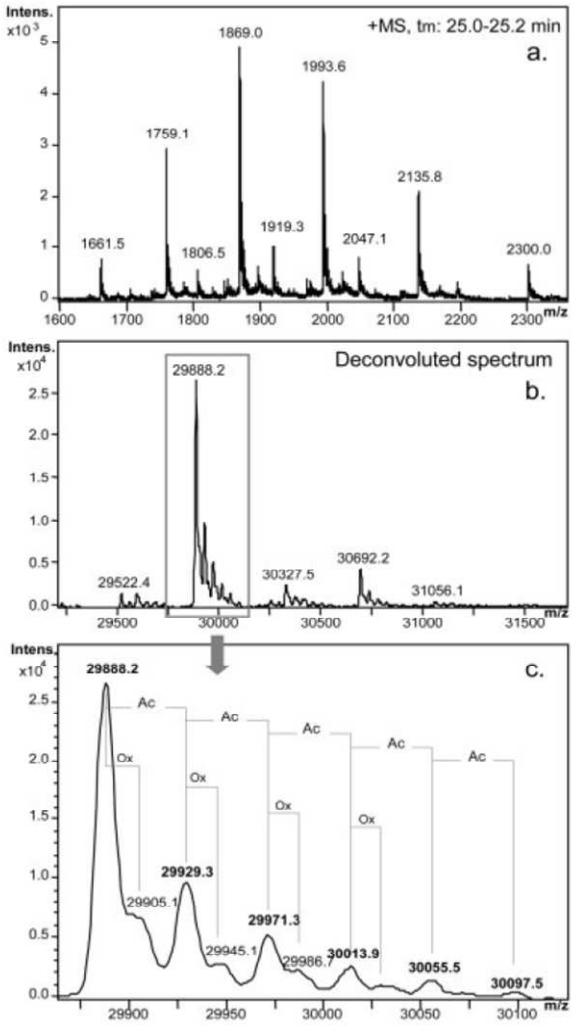

Figure 6: CE-TOF/MS mass spectrum a. from separation of intact recombinant human erythropoietin, b. the deconvoluted mass spectrum, and c. details of the deconvoluted spectra (Ac, acetylation; Ox, oxidation). Reprinted from [127] with permission from Wiley-VCH Verlag GmbH.

Berkowitz *et al.* developed a CZE method to detect various forms of oligosaccharides and the amount of deamidation on the glycoprotein [128].
CZE is particularly well adapted for intact protein analysis during development (*e.g.* for quality control), where simple and efficient analytical methods are required. In this context, Staub *et al.* presented the analysis of human growth hormone seized samples [129] and identification and quantification of insulin formulations using CE and a time-of-flight mass analyzer [130].

3.4.2.3 CE perspective

Every pharmaceutical protein either on the market or in development has been characterized by electro-driven approaches. CE is now recognized by pharmacopeias and extensively used for quality control by companies in the context of lot release, product development, recovery, process design, formulation and stability analyses [90]. Microfluidic CE devices for proteins show promises to increase the contribution of CE to this area. Development of miniaturized CE systems for protein analysis has advanced tremendously in recent years, and significant progress has been made in terms of EOF control and limits of detection [131]. Improvements in detection, reproducibility and ease of fabrication will provide solutions for the analysis of new biopharmaceutical drugs, particularly in the context of onsite analysis.

4. CONCLUSION

Over the last decade, the number of pharmaceutical proteins in development and on the market has become more significant. This is principally due to the advances made in the field of biotechnology. These new products are much more difficult to analyze than “classical” drugs resulting from chemical synthesis (*e.g.*, microheterogeneity, numerous molecular weights, possible conformations, and posttranslational modifications). Their complexity necessitates the development of new analytical strategies to characterize and ensure the safety of these biopharmaceuticals. Physical and chemical stabilities also have to be studied. If chemical instabilities are well known for low molecular weight chemical molecules, physical instabilities will be more specific for proteins. To obtain a comprehensive picture of a protein in terms of its structure, conformation, posttranslational modifications and stability, numerous analytical strategies with different principles are needed. In this review, emphasis has been put on intact protein analysis and separation techniques. Figure 7 summarizes the separation methods described in this review, highlighting the gold standards and promising techniques, as well as applications for each analytical technique.

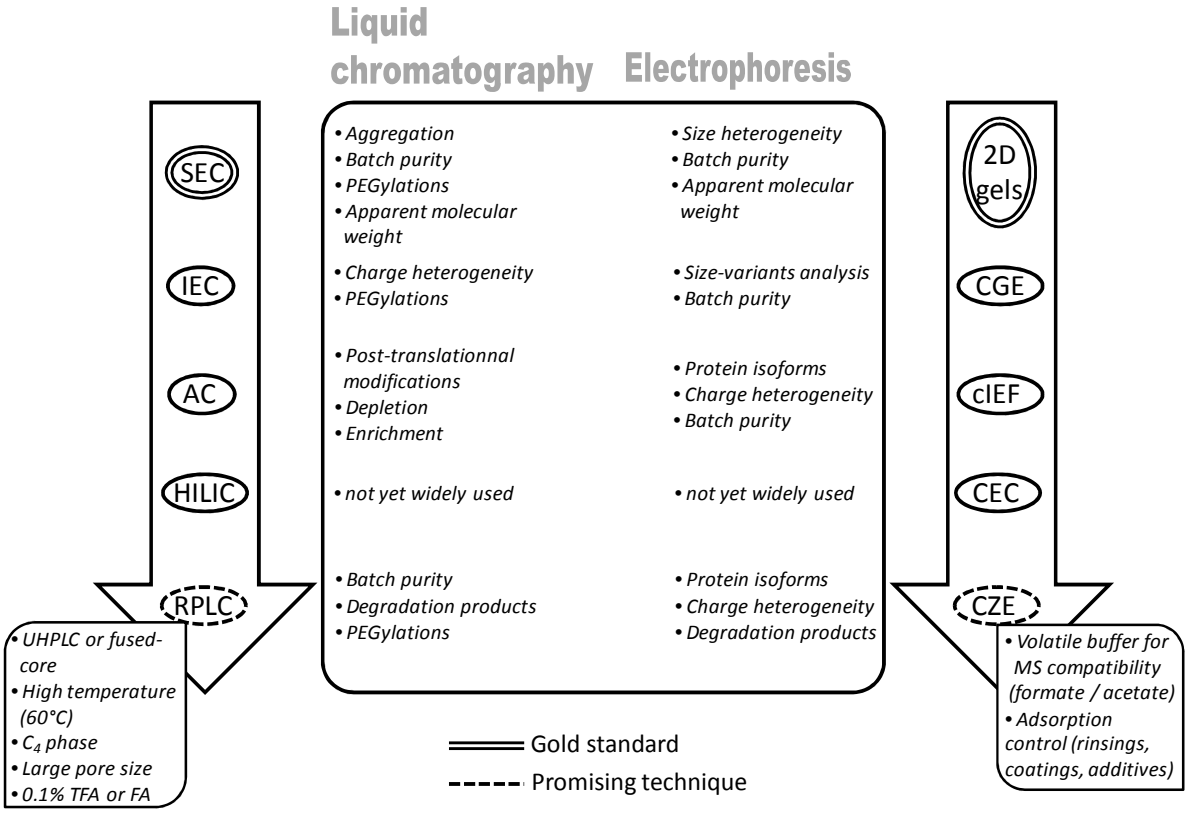

Figure 7: Separation techniques described in this review and their applications.

Liquid chromatography is already well established in industrial laboratories for intact protein analysis (*e.g.*, size-exclusion, ion-exchange chromatography). However, recent technological developments of RPLC stationary phases for the analysis of intact proteins (*i.e.*, sub-2µm or fused-core particles with short alkyl chain lengths, C_4, and large pore sizes of 300Å) make

this a promising technique. Gel electrophoretic approaches remain the gold standard for apparent molecular weight, size heterogeneity, purity, and manufacture consistency determinations, although they are time-consuming and need good technical skills. Capillary electrophoresis is also commonly used in the biopharmaceutical industry. Specifically, capillary gel electrophoresis and capillary isoelectric focusing modes permit the combination of the high resolution of gel techniques and the advantages of the microfluidic format of capillaries. In this context, capillary electrophoretic techniques could partially substitute future gel electrophoretic methods. Capillary zone electrophoresis appears to be a good candidate, since its easy coupling with time-of-flight mass spectrometry could provide important information with simple and efficient analytical methodology. Mass spectrometry (top-down approach) and spectroscopy are also widely used to collect complementary structural information regarding 2D and 3D protein conformation. Overall, several analytical approaches are always needed to cover all protein properties. Recent technological progress will contribute to a better knowledge of these parameters and help to understand the impact of changes in manufacturing on the quality and consistency of biopharmaceutical drugs.

5. ABBREVIATIONS

ACN	acetonitrile
BGE	background electrolyte
EIC	extracted ion chromatogramm
EOF	electrosomotic flow
SIM	single ion monitoring
UCA	ultrasound contrast agent

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II.2 La place du CE-TOF/MS dans le domaine analytique

L'**article II** est un article de revue qui discute plus particulièrement de l'intérêt du couplage de l'électrophorèse capillaire (CE) avec le spectromètre de masse à temps de vol (TOF). Cette revue est complémentaire à l'article I dans la mesure où elle permet une vue d'ensemble des aspects techniques de l'appareillage employé tout au long de ce travail de thèse, tout en ayant un regard plus global sur les applications possibles.

Tout d'abord, les caractéristiques instrumentales du TOF sont mises en évidence. L'accent est porté sur les améliorations technologiques qui sont intervenues pour permettre son couplage avec des sources continues telles que l'électrospray (ESI). Il s'agit principalement de l'injection orthogonale qui permet de modifier la source d'ionisation initialement continue en une source pulsée par un système de stockage d'ions. L'intérêt du réflectron est également abordé, ce dernier permettant d'améliorer considérablement la résolution du TOF.
Les aspects de couplage entre la CE et le TOF sont ensuite décrits. Deux types de sources d'ionisation sont communément utilisés, l'ESI et la désorption/ionisation laser assistée par matrice (MALDI). L'ESI est employée avec la CE *via* des interfaces (avec ou sans liquide additionnel) qui ont principalement le rôle d'assurer la fermeture du circuit électrique CE et d'adapter les faibles débits de la CE avec des sources d'ionisation pensées et construites pour les débits de la chromatographie liquide. Les couplages CE-ESI-TOF/MS sont donc principalement effectués en ligne. Au contraire, le MALDI est surtout employé hors ligne *via* des collecteurs de fractions. Il existe quelques interfaces permettant le couplage en ligne mais cela reste encore peu répandu.
La suite de la revue présente sous forme de tables et de textes les principales applications développées en CE-TOF/MS. Trois domaines sont distingués: les biomolécules, les produits naturels et toutes les autres applications. Le potentiel de la technique est particulièrement intéressant au niveau des protéines, principalement par l'approche Top Down.

La revue conclut globalement sur le grand potentiel de ce couplage qui mêle les grandes sélectivités et efficacités de la CE avec la haute masse exacte et la résolution satisfaisante du TOF. Si cet analyseur de masse reste pour l'instant principalement employé pour les macromolécules, un intérêt croissant est remarqué pour son utilisation à l'analyse de molécules de bas poids moléculaires, comme dans le domaine de la métabolomique.

II.2.1 Article de revue II

A. Staub, J. Schappler, S. Rudaz, J.L. Veuthey. **CE-TOF/MS: Fundamental concepts, instrumental considerations and applications**, *Electrophoresis* 2009, *30*, 1610-1623.

CE-TOF/MS: FUNDAMENTAL CONCEPTS, INSTRUMENTAL CONSIDERATIONS AND APPLICATIONS

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ABSTRACT

This review discusses the fundamental principles of TOF analyzers and covers the great progress that has been made in this area in recent years (*i.e.*, orthogonal acceleration, reflectron). This paper also gives an overview of applications performed by CE coupled to TOF/MS detection. The main domains of interest include the analysis of biomolecules and natural compounds.

KEYWORDS

CE, MS, TOF mass spectrometer

1. INTRODUCTION

Today, in several domains (*e.g.* pharmaceutics, environment, food) there is a strong need to have at disposal different separation techniques coupled to various detection systems. Aside from chromatography (*i.e.*, GC and LC) which remains the gold standard of separation techniques and is compatible with diverse modes of detection, CE has gained a large acceptance in analytical laboratories. UV-VIS spectrophotometry is probably the most widely used detection mode with CE because of the simplicity of the on-line configuration. However, its sensitivity, directly related to the optical pathlength afforded by the internal diameter of the capillary (in the µm range), is low, and this remains the major bottleneck of this technique. In this context, the on-line combination of CE and MS is an attractive option, and presents some major benefits. Among them, an enhancement of sensitivity and the enabling of the determination of co-migrating compounds with different mass to charge ratios (*m/z*). MS provides a high potential for the identification and confirmation of components in complex mixtures and potentially gives some information concerning the structure of the separated compounds. Therefore, CE-MS coupling provides a powerful combination for performing rapid, efficient and sensitive analysis [1]. Almost all mass analyzers, such as quadrupole, ion trap (IT), time-of-flight (TOF), and Fourier transform ion cyclotron (FTICR) can be on-line coupled with CE, generally using electrospray (ESI) as the ionization source. Quadrupoles offer limited value if structural information on the analytes is required, while IT analyzers allow MS^n experiments and provide additional information through the multi-stage fragmentation of analytes [2]. Because increased resolution and the highly accurate determination of molecular masses are often required, TOF mass analyzers are gaining large acceptance in several domains, especially for the analysis of macromolecules. Moreover, taking into account that CE can provide fast and efficient separations that often yield very small peak widths, the mass spectrometer must be able to produce a sufficient number of data points across the peak width. Again, TOF technology can fulfill this task due to its high data acquisition rate [3, 4]. Hence, the characteristics of TOF/MS, such as speed, unlimited mass range, high duty cycle, and sensitivity, meet CE requirements and qualify it as the analyzer of choice for CE-MS [5].

TOF/MS was first introduced by Stephens in 1946, but CE-TOF/MS was only investigated in the 1990s [6-8] thanks to two major technological developments: the use of a reflectron (or ion mirror) and orthogonal acceleration (oa) [9]. Reflectrons substantially increased the interest in TOF technology, attributable to the great resolution improvement and reduction of bench space and cost. Orthogonal acceleration is now considered to be preferred method for coupling continuous ion sources to a TOF mass analyzer, and its impact has been very significant with the ESI source [10]. This technological progress explains the increasing number of CE-ESI-TOF/MS publications over the last 16 years (Figure 1).

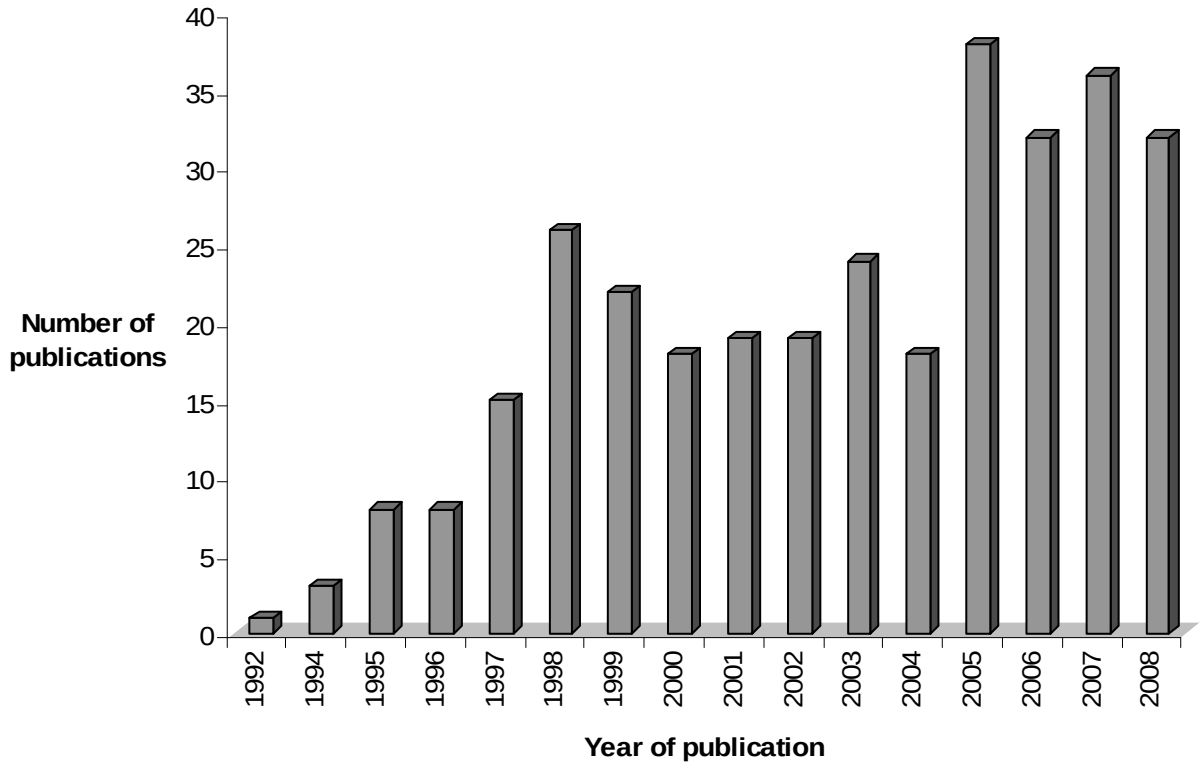

Figure 1: Publications on CE-ESI-TOF/MS over the last 16 years (Scifinder Scholar® 2007, search in the Chemical Abstracts database from 1992 to 2008. Date of information gathering: January 2009).

MALDI is also a very common MS ionization source for performing CE-TOF/MS. In contrast to ESI, approaches integrating MALDI are essentially carried out off-line [11], even though several strategies for the direct hyphenating of CE to MALDI-TOF/MS have been described [12]. An ICP (inductively coupled plasma) source can also be used to hyphenate CE with TOF/MS. Although this technique allows speciation analysis [13-16], major limitations prohibit its widespread use (*e.g.* complicated coupling of all components, discrepancy between CE typical liquid flow rates and ICP-MS, insufficient detection capability resulting from the use of extremely small sample volumes [17]).

The present paper reviews instrumental aspects of TOF/MS and the fundamental concepts for achieving successful coupling to CE, particularly considering MALDI and ESI coupling. A number of tables providing a comprehensive list of CE-TOF/MS applications are also included.

2. THEORY

2.1. TOF/MS

2.1.1. Principles, benefits, and limitations

The essential principle of TOF/MS is that a population of ions moving in the same direction and having a distribution of masses but a constant kinetic energy will have a corresponding distribution of velocities inversely proportional to the square root of *m/z*. Therefore, the ion arrival times at a target plane will be distributed according to the *m/z* ratios [18]. In MS, resolving power is usually measured by the ratio of $m/\Delta m$, where Δm is a discernable mass difference. In TOF/MS, it is convenient to work in the time domain. The resolving power $m/\Delta m$ can be measured in terms of $t/\Delta t$, and the finite time interval, Δt, is typically the full-width at half-height of the peak. Mass resolving power is thus limited by small differences in the measured flight times for ions of the same mass.

TOF technology presents numerous advantages over other analyzers, such as high mass resolution, high mass accuracy, theoretically unlimited mass range, and relatively low cost. Moreover, TOF/MS is ideal for pulsed or spatially confined ionization, and a complete mass spectrum for each ionization event can be obtained, as well as spectra from extremely small sample amounts [3, 9].

Nevertheless, several phenomena, known as temporal, spatial, and kinetic distributions, can degrade mass resolution and must be taken into account in TOF/MS. A temporal distribution occurs when ions of the same mass formed at different times with the same kinetic energy go through the field-free region maintaining a constant difference in time and space. A spatial distribution takes place when ions of the same mass are formed at the same time with the same initial kinetic energy, but are generated at different locations in the extraction field. The ions near the back of the source will experience a larger potential gradient and be accelerated to higher kinetic energy than those formed close to the extraction grid. A kinetic energy distribution occurs when ions formed with different initial kinetic energies have different final velocities after acceleration and arrive at the detector at different times. The kinetic energy distribution also includes ions with the same kinetic energy but velocities in different directions. It is also important to note that ions initially moving towards the detector arrive before ions initially moving away from it. Indeed, the latter ions are first decelerated to zero velocity before being re-accelerated and passing through their original position. This phenomenon is known as “turn-around time”.

Other significant physical and technical limitations must be overcome in TOF/MS. First, ions must be “gated”, *i.e.*, they have to be formed into temporally discrete packets. The MALDI process is particularly useful because the pulsed ionization process is shorter than the desired ion arrival-time spread. For continuous sources like ESI, an ion gating device must be used. Second, the ion arrival event at a focal plane detector is, in principle, instantaneous. This event must then be transduced to an electrical signal at the detector, and the associated electronics display their own characteristic temporal response. Lately, detectors have been developed to give pulse widths of 1-2 ns. Finally, another critical point relates to metastable decomposition. In TOF/MS, fragment ions can be generated in field-free regions and move

with nearly the same velocity. Therefore, the parent, fragment ion, and neutral species are detected at the same place in the spectrum. It is well known that some internal energy is converted into translational energy when an ion fragments. The effect of the energy release, and hence the increased range of velocities of the fragments relative to the parent must be considered as a factor that decreases the resolving power. However, metastable decomposition could be used in combination with collision-induced dissociation (CID) to greatly increase the amount of structural information in the TOF/MS of large molecules.

2.1.2. Instrumental considerations

The first TOF mass spectrometers were of a fairly simple construction and were known as linear time-of-flight mass spectrometers [19]. As mentioned above, some technical issues can degrade resolution and these limitations should be overcome. Two technological advances have thus been implemented to partially account for these limitations: the reflectron and orthogonal acceleration (oa) (Figure 2).

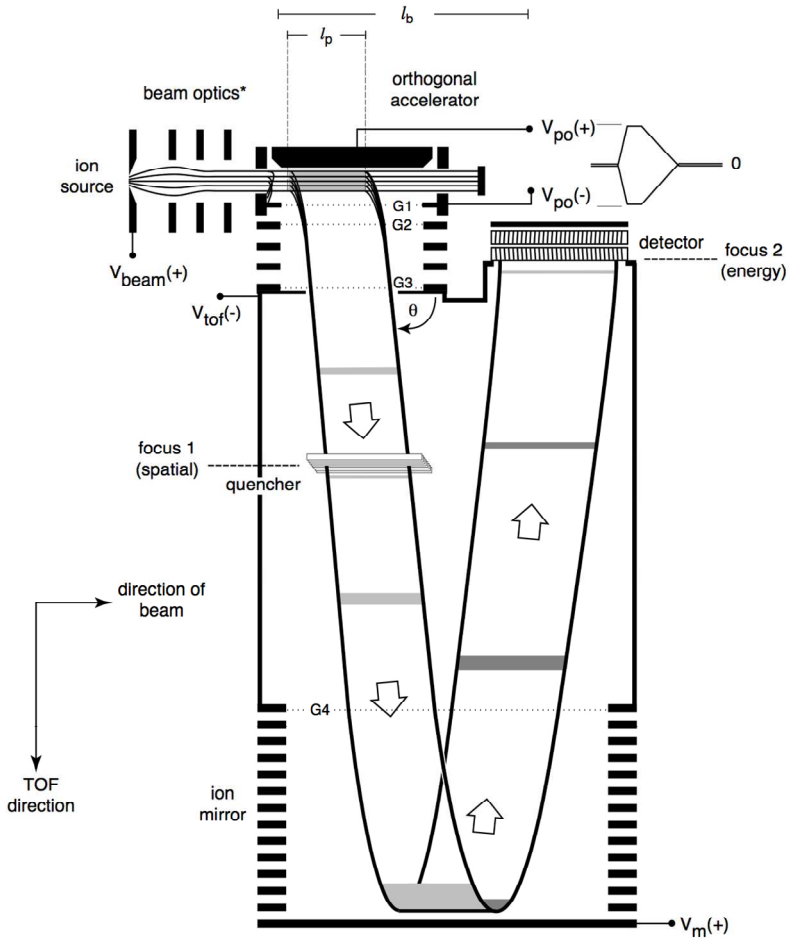

Figure 2: Main components and layout of typical oa-TOF systems with reflecting mass analyzer Reprinted from [10] with permission from Wiley-VCH Verlag GmbH.

The most notable advance has been the recent commercialization of hybrid TOF mass spectrometers that offer, in addition to high mass accuracy measurements, the possibility of MS/MS experiments, providing additional information by fragmentation of the analytes.

2.1.2.1. Reflectron

The reflectron (or ion mirror) creates one or more retarding fields (*i.e.*, decelerating and reflecting fields) after a drift region. These are oriented to oppose the accelerating field. For ions of the same *m/z* entering such a field, those with higher kinetic energy (and velocity) will penetrate the decelerating field further than ions with lower kinetic energy. An advantage of the mirror is an increased drift-length without increasing the size of the instrument. The problem of the “turn-around time” cannot be corrected by the reflectron. On the other hand, the former can be compensated by a strong extraction field. In this case, ions are brought to a sharp spatial focus located at a short distance along the drift region. Different masses will focus there at different times, but at the respective times, the ion packets will be sharply focused in the direction of the drift velocity. However, an initial spread in position will then occur at a short distance into the drift region and be converted into a substantial velocity spread [18]. The latter can be corrected by the reflectron. Therefore, the ion mirror is particularly adapted to compensate for (i) ion distributions decreasing resolution and (ii) devices that generate a strong accelerating field in order to minimize the turn-around effect.

2.1.2.2. Orthogonal acceleration

In oa-TOF/MS, the ionization source, the accelerator, and the detector describe an angle slightly greater than 90°. Oa-TOF presents numerous benefits: (i) high efficiency in gating ions from any external continuous source, (ii) simultaneous correction for velocity and spatial dispersions, (iii) capability without an ion mirror of mass resolving powers ten-times higher than with conventional TOF instruments fitted with gaseous ionization sources, (iv) maximum duty factor at the high mass end of the mass spectrum, and (v) minimal temporal dispersion from the gating method. Oa consists of different steps. Ions are first sampled from a nearly parallel ion beam from the continuous ionization source. A low energy beam is then allowed to fill an ion accelerator area where an electric field is propagated by a fast pulse at one or more electrodes that define this area. The field is designed to apply a force that is strictly and exclusively at a right angle to the axis of the ion beam. As the beam is nearly parallel, ions have zero average velocity and minimal velocity spread in the direction of this force prior to its application. The finite width of the beam (spatial dispersion) can readily be refocused without the complication of simultaneous and substantial velocity dispersion. Oa-acceleration TOF/MS is superior to in-line gating where the ion beam is trapped and analyzed along the same axis. Other gating approaches for continuous ion sources involve ion trapping [20, 21].

2.1.2.3. Hybrids TOF/MS

Many combinations of hybrid TOF/MS are possible, such as Q-TOF, IT-TOF, and TOF-TOF [22]. These instruments do not present the same characteristics. The configuration of Q-TOF

can be considered either as the addition of a mass-resolving quadrupole and collision-cell to a TOF, or as the replacement of the third quadrupole by a TOF mass spectrometer [23, 24]. The benefits are high sensitivity, mass resolution, and mass accuracy of the resulting tandem mass spectrometer in both precursor (MS) and product ion (MS/MS) modes. IT-TOF mass spectrometers have been developed to achieve high mass accuracy and MS^n analysis simultaneously [25]. IT is particularly adapted with the discontinuous work process of TOF/MS because the ion trap is used to focus ions before ejection into the time-of-flight, as well as supporting MS^n analysis with effective precursor ion selection capabilities. TOF-TOF consists of two successive TOF accelerations. The first one selects, isolates, and fragments (usually by collision with a neutral gas) a precursor ion of interest. The second reaccelerates the precursor ion and fragments, and then measures the masses and intensities of the fragment ions [26].

2.2. CE coupling

The most important sources used for CE-TOF/MS coupling are ESI and MALDI [12, 27, 28]. Approaches integrating MALDI are essentially off-line (since it requires a solid matrix), while ESI enables the direct transfer of molecules from the liquid to the gas phase [4-8, 29, 30]. On the one hand, MALDI is a pulsed source, and ions generated with pulsed energy sources are readily amenable to TOF analysis. On the other hand, ESI is a continuous source, and selecting packets of ions from a continuous stream is by no means straightforward. The most appropriate approach consists of continuous ionization followed by gating of the resulting ion beam, which can be efficiently achieved by orthogonal acceleration, as explained previously.

2.2.1. MALDI interfaces

MALDI is a method for generating small to very heavy gas phase ions *via* delivery of pulsed laser radiation to a highly absorbing matrix that contains the sample. The rapidly moving matrix molecules “sweep” along the slower high mass ions, which increase their kinetic energy (and kinetic energy spread as well). These translational effects depend on the laser power and the sample preparation technique. Therefore, internal standards are often required for a reasonable mass measurement.
A substantial amount of progress has been made in the domain of MALDI coupling to TOF/MS, such as delayed extraction MALDI and orthogonal acceleration applied to MALDI. The former exploits the correlation between ion position and velocity at an optimal delay after instantaneous desorption from a flat surface. Ions are accelerated after a delay by a pulsed electric field and for a limited mass range, and the acceleration conditions can be chosen to bring a very narrow arrival time spread. The delayed extraction allows for an improvement of the resolving power up to masses of about 20 kDa. Oa-TOF/MS could be hyphenated to pulsed sources such as MALDI even if they have been developed for continuous ionization processes. This requires a double-pulsed experiment in which the first pulse arises from a laser while the second, after a delay, consists of sampling the desorbed ions [31]. Overall, the resolving power of a MALDI-oa-TOF/MS configuration is about the same as that reported for a delayed extraction instrument of the same size [32].

A very comprehensive review written by Huck *et al.* [11] describes CE hyphenation with MALDI-TOF/MS. Two alternatives have emerged, the off-line and the on-line approaches.

2.2.1.1. Off-line approaches

Off-line strategies involve the deposition of separated analytes on a MALDI target plate. The latter is stable for an extended time; therefore, the analysis can be repeated and in special cases, even performed several weeks after sample acquisition.
The most common off-line approach consists of depositing the sample on a stainless steel target plate (*i.e.*, fraction collection). The matrix solution can be added to the fraction as a separate step or can be deposited onto the target before sample introduction. To collect the fractions, robotic x-y-z axis motion systems are available [33-41]. Another possibility for collecting fractions is to switch off the voltage, remove the vial at the cathode end, and elute the desired peak by applying pressure [42-44].
It is also possible to use a continuous off-line atmospheric-pressure deposition interface [45, 46]. The CE capillary is held in direct contact with the target plate, which carries a cellulose membrane precoated with matrix. The stainless steel body of the target is connected to the ground of the CE high-voltage power supply. The background electrolyte (BGE) is eluted from the separation capillary by electroendosmotic or hydrodynamic flow as the moveable target advances and forms a narrow track. Karger *et al.* implemented such as device in a stand-alone rough vacuum chamber in which separation streaks were deposited on conventional stainless steel MALDI targets [26]. As previously discussed with the on-line continuous vacuum deposition interfaces, the width of the deposited sample trace was sufficiently small compared to the laser spot size to allow the beam to encompass the entire trace.
Ojima *et al.* [47] developed another method to hyphenate CE with MALDI-TOF/MS. In fact, the droplet electrocoupling (DEC) approach was implemented as an intermediate method between the off-line and on-line modes. A droplet of an electroconductive solution was put on the sample plate of a MALDI-TOF/MS where the CE capillary end dipped in order to make an electro-connection and to apply a high voltage between the metallic sample plate and the counter pole of the CE. The droplet was used for electrocoupling and as the solute-collecting interface. Furthermore, the separated component was collected and concentrated in a droplet.

2.2.1.2. On-line approaches

On-line coupling permits direct acquisition for high-throughput sample information. Two main strategies are used in the domain of on-line CE-MALDI: the rotating ball inlet (ROBIN) and the continuous vacuum deposition interface
The ROBIN is an interface for the continuous introduction of a flowing liquid into a MALDI-TOF/MS [48, 49]. Musyimi *et al.* [48] reported an interface based on a rotating stainless steel ball that transports samples from atmospheric pressure to the high vacuum of the mass spectrometer for deposition and ionization. The sample was directly deposited from the CE capillary on the ball surface and dried. Then it was delivered into the laser ionization region

using a polymer gasket interface. Mass detection limits in the femtomole range were reached without a memory effect or electrophoretic peak broadening.
The continuous vacuum deposition interface was developed by Karger *et al.* [50-52]. At the beginning [51], the mixed solution of analyte and matrix was deposited *via* a fused silica capillary on a quartz wheel. The capillary was positioned in a probe made of a stainless steel tube. The probe was inserted into the chamber source in a position such that the end of the capillary was slightly bent while touching the wheel. The outlet of the capillary was tapered, using fine sandpaper. It was important to accurately adjust the position of the wheel to gently touch the repeller at the slit position. Improved versions consisted of an interface using a disposable moving tape onto which the sample was deposited from the infusion capillary [50]. Rapid evaporation of the solvent from the tape produced a uniform sample track of 60 μm width. For high-throughput analyses, the single infusion capillary of the moving-tap interface was replaced with a ten-capillary array [52].

2.2.2. ESI interfaces

Many processes occur during electrospray: the production of charged droplets at the nebulizer tip, shrinkage of the charged droplet by solvent evaporation, disintegration of the drops resulting from the highly charged droplets, and the formation of gas-phase ions [53]. One characteristic of ESI is the formation of highly charged ions without fragmentation, lowering the *m/z* values to a range easily measured by different types of mass analyzers [54]. ESI is one of the most commonly used atmospheric pressure interfacing techniques for coupling CE to MS. Because mass analysis can only be accomplished at low pressure, it is necessary to transfer ions from atmospheric pressure to a low-pressure region. To accomplish this ion transfer from high pressure to low pressure, differential pumping is generally employed, with successive chambers at decreasing pressures. These chambers are separated by orifices through which ions must pass before reaching the mass analyzer.
The success of an ESI-MS analysis depends not only on the characteristics of the analyte and its concentration, but also on the instrumental parameters. Stable ESI operation can be achieved by optimizing numerous interface parameters, including the effluent liquid (*e.g.* flow rate, surface tension) and the source itself (*e.g.* geometry, applied voltage) [55]. In contrast to LC-ESI-MS, two additional key requirements have to be considered for efficient CE coupling with ESI-MS: the electric CE circuit needs to be closed at the sprayer tip and very low and BGE-dependent flows need to be handled [56]. Interfacing CE with MS *via* an ESI source can roughly be performed in two different ways, with or without an additional liquid. The first approach, known as the sheath-flow interface, is the most common one due to its robustness and ease of implementation, while the second one (the sheathless approach) should feature a higher sensitivity.

2.2.2.1. Sheath-flow interfaces

The voltage is applied to the CE buffer *via* a supportive contact liquid. There are two groups of liquid-supported systems: the sheath liquid interface and the liquid junction. In the sheath liquid interface, the separation capillary is surrounded by a second tube of larger diameter in

a coaxial arrangement. The supportive liquid is guided through this outer tube and mixed with the CE buffer directly at the exit end of the capillary. This arrangement may be surrounded by a third tube, through which a stream of gas can be pumped to support droplet formation [53]. The sheath-liquid systems are relatively easy to implement and use, although they are demanding in terms of optimization of operational parameters, such as capillary tip position, flow-rate, and sheath liquid composition. In liquid junction systems, the CE column is partially disconnected from the ESI emitter. In fact, the liquid-junction interface provides the electrical connection to close the CE circuit *via* a liquid reservoir.
Post-capillary liquid introduction shows flexibility because the make-up liquid can be selected with an appropriate pH, flow-rate, and composition for optimized ESI operation. This arrangement decouples the CE separation process from the ESI, allowing the individual optimization of each of the two systems [57]. It is worth mentioning that dilution of the CE effluent by the sheath liquid flow rate may reduce sensitivity, but does not significantly affect it since the sheath liquid is also evaporated during the spray process.

2.2.2.2. Sheathless interfaces

In this approach, the voltage is directly applied to the CE buffer. The main difficulty is to close the electrical circuit required for any CE separation. This can be achieved by applying a metal coating to the end of a tapered separation capillary or by connecting a metal-coated, full metal or conductive polymeric sprayer tip to the CE outlet [3]. A recent review written by Zamfir describes advances in the sheathless interfacing of CE and ESI-MS [58]. In this review, the author separates the sheathless interfaces into two groups: the conductive emitters and the electrodeless interfaces. For the first group, the emitter can be either manufactured directly at the CE capillary outlet or produced separately and connected afterwards to the original CE capillary. Sassi *et al.* [59] reported the use of a conductive ESI emitter to couple CE to TOF/MS for the automated separation and detection of intact polypeptides in human serum. On the other hand, Vrouwe *et al.* [60] developed an electrodeless nanospray interface. In this configuration, the emitter was positioned very close to the mass analyzer. The voltage applied to the buffer compartment was able to drive the electrophoresis, while initiating and maintaining the nanospray.

3. APPLICATIONS OF CE-TOF/MS ANALYSIS

CE-TOF/MS applications mainly involve the domains of biomolecules and natural products. The latter, as well as other applications are discussed in the next section.

3.1. Biomolecules

There is a growing interest in the analysis of biomolecules (*i.e.*, proteins, peptides, amino acids, oligonucleotides), mainly because of the great developments in genomics, proteomics, metabolomics, and lipidomics. Various techniques are used for their analysis, such as slab-gel electrophoresis (SGE), LC, and CE [61], but the latter is particularly appropriate and offers many benefits: analysis times are reduced, minute amounts of sample are needed,

and high efficiencies are obtained. In protein analysis for instance, CE allows the analysis of intact forms, in contrast to LC analysis, which usually involves an upstream digestion of the protein sample [2]. Furthermore, CE features the possibility of analyzing them without causing conformational changes due to organic modifiers and/or a stationary phase.

A very important point to consider for biomolecule analysis by CE is their adsorption on the capillary wall. A recent review written by Lucy *et al.* [62] describes all of the mechanisms involved in the protein adsorption process and explains different ways to emphasize the type of adsorption. Numerous approaches have been explored to minimize this adsorption, including the use of extreme pH, high ionic strength, and zwitterionic additives. The most common approach consists of coating the capillary wall, of which, two types are used: dynamic and static polymer coatings. The dynamic ones are generated by the simple addition of water soluble polymers to the BGE during the separation. The static ones physically adsorb to the capillary wall through forces similar to those affecting biomolecule adsorption (coulombic and hydration forces, electrostatic and hydrophobic interactions). Higher efficiencies are achieved using static adsorbed polymer coatings. Elhamili *et al.* recently used a mono-quaternarized piperazine compound for capillary coating compatible with MS detection [63]. The surface showed good stability with no bleeding into the MS, and good separations were obtained for large proteins (up to 669 kDa).

All applications involving biomolecule analysis by CE-TOF/MS are reviewed in Table 1. Generally, an ESI source is used, but several applications with MALDI source have emerged [35-39, 42, 44, 82]. A few articles also describe CE-nanoESI-TOF/MS applications for biomolecules. In 1994, Fang et *al.* developed a nanospray interface inserting a thin gold wire into the capillary outlet to establish the capillary potential [7]. In 1999, Hsieh *et al.* developed a novel nanospray interface [84] that allowed stable electrical currents and high detection sensitivity for peptides. In 2004, Bindila *et al.* used a nanoelectrospray chip for off-line coupling of CE with Q-TOF for the analysis of glycoconjugates [77]. A detailed description of the highly heterogeneous glycopeptide mixture entirely collected in the CE fractions was obtained.

Table 1: Summary of CE-TOF/MS applications for the analysis of biomolecules

Analyte[a]	**Sample matrix**	**Ionization source**	**BGE**[b]	**Sheath liquid**	**Reference**
amino acids	urine	ESI	2M formic acid in 20% methanol	0.5% formic acid in isopropanol/H_2O (50/50)	[64]
apothioneins	water	ESI	100mM acetic acid + 100mM formic acid (pH 2.3)	0.05% acetic acid in isopropanol/H_2O (50/50)	[65]
rHuEPO (and its glycans)	water	ESI	1M acetic acid in 20% methanol ; 2M acetic acid ; 100mM 6-aminocaproic acid + 0.9M NH_3 in 70% methanol	1% acetic acid in isopropanol/H_2O (50/50)	[66]
biomarkers	serum	nanoESI	60mM acetic acid in 20% methanol	-	[59]
transferrin	serum	ESI	25mM ammonium acetate (pH 8.5)	0.5% formic acid in methanol/H_2O (90/10)	[67]
proteins	water	ESI	1% formic acid	1% acetic acid in ACN/H_2O (50/50)	[68]
biomarkers	urine	ESI	0.5% formic acid in ACN/H_2O (20/80)	0.4% formic acid in isopropanol/H_2O (30/70)	[69]
peptides	water	chip-based nanoESI	20mM ammonium acetate (pH 8.5)	-	[70]
α- and β-EPO	water	ESI	1M acetic acid in 20% methanol	1% acetic acid in isopropanol/H_2O (50/50)	[71]
biomarkers	urine	ESI	250mM formic acid in 20% ACN	?	[72]
biomarkers	urine	ESI ; MALDI off-line	0.5% formic acid in 20% methanol	0.5% formic acid in isopropanol/H_2O (30/70)	[35]

peptides	mold fermentation	ESI	12.5mM ammonium formate in methanol	1% formic acid in isopropanol/H_2O (50/50)	[73]
glycopeptides	water	ESI ; MALDI off-line	50mM ammonium formate (pH 2.7) ; 50mM triethylammonium acetate (pH 5.0) ; 50mM ammonium acetate (pH 8.0)	0.4% formic acid in isopropanol/H_2O (50/50)	[36]
biomarkers	urine	ESI	0.5% formic acid in 30% methanol	0.5% formic acid in methanol/H_2O (30/70)	[74]
glycoproteins	water	ESI	50mM ammonium acetate (pH 4.5)	2% acetic acid in methanol/H_2O (50/50)	[75]
oligonucleotides	water	ESI	0.2mM CDTA in 25mM ammonium carbonate (pH 9.7)	isopropanol/H_2O/ ammonium carbonate 5mM (80/15/5)	[76]
amino acids and peptides	urine	nanoESI	0.1M formic acid in methanol/H_2O (40/60) (pH 2.8)	-	[77]
proteins	liver tissue	MALDI off-line	50mM triethylamine	-	[37]
biomarkers	urine and serum	ESI	0.5% formic acid in methanol/H_2O (30/70)	0.5% formic acid in methanol/H_2O (30/70)	[78]
biomarkers	human cells	ESI	100mM formic acid (pH 3.0)	?	[79]
myoglobin and apomyoglobin	water	ESI	50mM ammonium acetate (pH 8.3)	10mM ammonium in acetate/methanol (50/50)	[80]
peptides and amino acids	water	nanoESI off-line	0.5M ammonium formate in methanol/H_2O (60/40)	-	[81]
peptides	*Aplysia californica*	MALDI off-line	80mM phosphate buffer (pH 2.5)	-	[38]

insulin	water	MALDI off-line	50mM phosphate (pH 11.0) ; 50mM borate (pH 10.6)	-	[82]
peptides and proteins	water	MALDI off-line	500mM acetic acid + 10mM triethylamine	-	[39]
peptides	bacteria	ESI	100mM ammonium formate (pH 3.0)	?	[83]
peptides	water	ESI	0.3% acetic acid in methanol/H_2O (50/50)	-	[84]
biomarkers	urine	MALDI off-line	20mM sodium tetraborate	-	[42]
oligonucleotids	water	ESI	0.1M ammonium carbonate (pH 9.68)	isopropanol/H_2O/ ammonium carbonate 0.01M (80/15/5)	[85]
peptides and proteins	water	ESI off-line	1-10% acetic acid in H_2O	-	[86]
ovalbumin and DSPA	water	MALDI off-line	ovalbumin : 100mM borate + 3mM DAB (pH 8.5) DSPA : 100mM borate (pH 3)	-	[44]
biomarkers	urine	ESI	0.25M formic acid in 20% ACN	0.5% formic acid in isopropanol/H_2O (30/70)	[87]
biomarkers	urine	ESI	0.5% formic acid in 30% methanol (pH 2.4)	0.5% formic acid in 30% methanol	[88]
biomarkers	urine	ESI	0.5% formic acid in 30% methanol (pH 2.4)	0.5% formic acid in methanol/H_2O (30/70)	[89]
peptides	water	nanoESI	50mM acetic acid in methanol/H_2O (50/50)	-	[7]

peptides and proteins	water	ESI	0.1% acetic acid in H_2O	methanol	[8]
zein proteins	water	ESI	ACN/isopropanol/ formic acid/H_2O (40/20/2/38)	isopropanol/H_2O (50/50)	[90]
amino acids	urine	ESI	1M formic acid	0.1% formic acid in methanol/H_2O (50/50)	[91]
biomarkers	urine	ESI	0.5% formic acid in methanol/H_2O (30/70)	0.5% formic acid in methanol/H_2O (30/70)	[92]
biomarkers	urine	ESI	0.25M formic acid in 20% ACN	0.5% formic acid in isopropanol/H_2O (30/70)	[93]
metabolites	urine	ESI	50mM ammonia/ ammonium acetate (pH 9.0)	isopropanol/BGE (80/20)	[94]

[a] rHuEPO : recombinant human erythropoietin ; EPO : erythropoietin ; DSPA : desmodus salivary plasminogen activator

[b] CDTA : trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid ; DAB : 1,4-diaminobutane

A number of recent reviews have covered the application of CE-MS in biomarker discovery [95-98]. A biomarker is a biomolecule found in blood or tissues that is a sign of a normal or abnormal process, condition or disease. It may be used to see how the body responds to a treatment. Proteome analysis is emerging as a key technology for deciphering biological processes and discovering biomarkers for diseases from tissues and body fluids. There are numerous challenges to achieve these purposes. Indeed, there is a high complexity and wide dynamic range of peptides or proteins in body fluids. Sample preparation has to be performed taking into account the fact that the major concerns of proteomics are to avoid polypeptide loss and protein degradation, and to guarantee the reproducibility of the data. A fast and sensitive mass spectrometer is also required. IT systems are adequate analyzers, but in full-scan mode the resolution of an IT is generally too low to resolve single isotope peaks of more than three-fold charged molecules. TOF instruments are particularly adaptable because they achieve a more detailed and reproducible display of the compound pattern in body fluids. The analysis should be realized with a high resolution mass spectrometer considering that numerous and highly charged ions can be formed with ESI. It should be noted that the high information content of CE-MS spectra requires sophisticated software handling, especially in the clinical task flow. Numerous softwares have been developed to extract information from these highly complex spectra [99]. In the domain of data treatment, Williams *et al.* [100] recently described a method for displaying CE-MALDI-TOF/MS data of proteolytic digests. This data display mode yields distinct charge-based

trends for plots of *m/z vs.* CE migration time. The trends observed in these plots were used together with two series of charge-state marker peptides to aid in charge-state assignment. The 2D plots were simple and clear, providing compositional information on each peptide based on the peptide's location on the plot.

Analytical protein mass spectrometry generally uses a so-called "bottom-up" approach, in which protein identification depends on initial treatment with a protease to break proteins into relatively small peptides whose *m/z* can be determined by MS. Reliable identification can be achieved based on so-called "peptide mass fingerprinting", or using peptide fragmentation spectra in a tandem mass spectrometer. In contrast to bottom-up mass spectrometry, "top-down" approaches involve transferring an undigested protein to a gas phase before dissociation. The mass of an intact protein allows identifying all splicing and post-translational modifications. Furthermore, top-down approaches have the major advantage of being able to recognize native small peptides that may be excellent markers of a given pathophysiological entity. CE-TOF/MS is particularly adapted to the top-down approach, and Chalmers *et al.* described a methodology for biomarker discovery based on both top-down and bottom-up strategies [74]. The aim of the study was to identify biomarkers of renal disease, and both approaches were used because biomarkers could not be easily isolated. Consequently, the identification had to be performed from a complex mixture. CE-ESI-TOF/MS was used to analyze thousands of intact polypeptides contained in human urine, while ESI-Q/FTICR was used for their identification after digestion.

Up to 10,000 proteins are commonly present in serum or plasma, and most of them are at very low relative abundances. Highly abundant proteins, such as albumin and immunoglobulins, can interfere in the identification and quantitation of low-abundance proteins. In this context, the sample preparation becomes a crucial step of the analysis process. Sassi *et al.* [59] developed an ultrafiltration method to deplete the serum of proteins larger than 50,000 MW. The filtrate was then concentrated and desalted by adsorption on a C8 column. The column was washed multiple times with 5% ACN/0.1% acetic acid to remove salts before eluting the sample with 20 µl of 70% ACN/0.1% acetic acid. With a sheathless CE-ESI-TOF/MS system, concentrations down to 10 nM in serum were detected, and approximately 500 serum components were identified.

In contrast to blood matrices, urine presents many advantages: collection of large quantities is easy and not invasive. Moreover, urine should only display the health status of a limited amount of organs (mostly kidney and bladder), which reduces complexity. This last point clearly indicates that CE-MS in combination with efficient sample preparation is a suitable approach towards the examination of polypeptides in urine. Recently, Zimmerli *et al.* developed a CE-ESI-TOF/MS method for the analysis of proteomic biomarkers in coronary artery disease (Figure 3) [72]. Urine sample preparation consisted of centrifugation followed by application of the supernatant on a C2 column (to remove salts, urea and electrolytes) [74, 88, 89]. Peptides were eluted with 50% acetonitrile in HPLC-grade water containing 0.5% formic acid. The eluate was lyophilized in a Speed-Vac and resuspended in 50 µL of HPLC-grade water immediately before injection. The CE-ESI-TOF/MS method allowed the

highlighting of *ca.* 800 peptides. With a complementary CE-MALDI-TOF-TOF/MS off-line method, some of these biomarkers were identified.

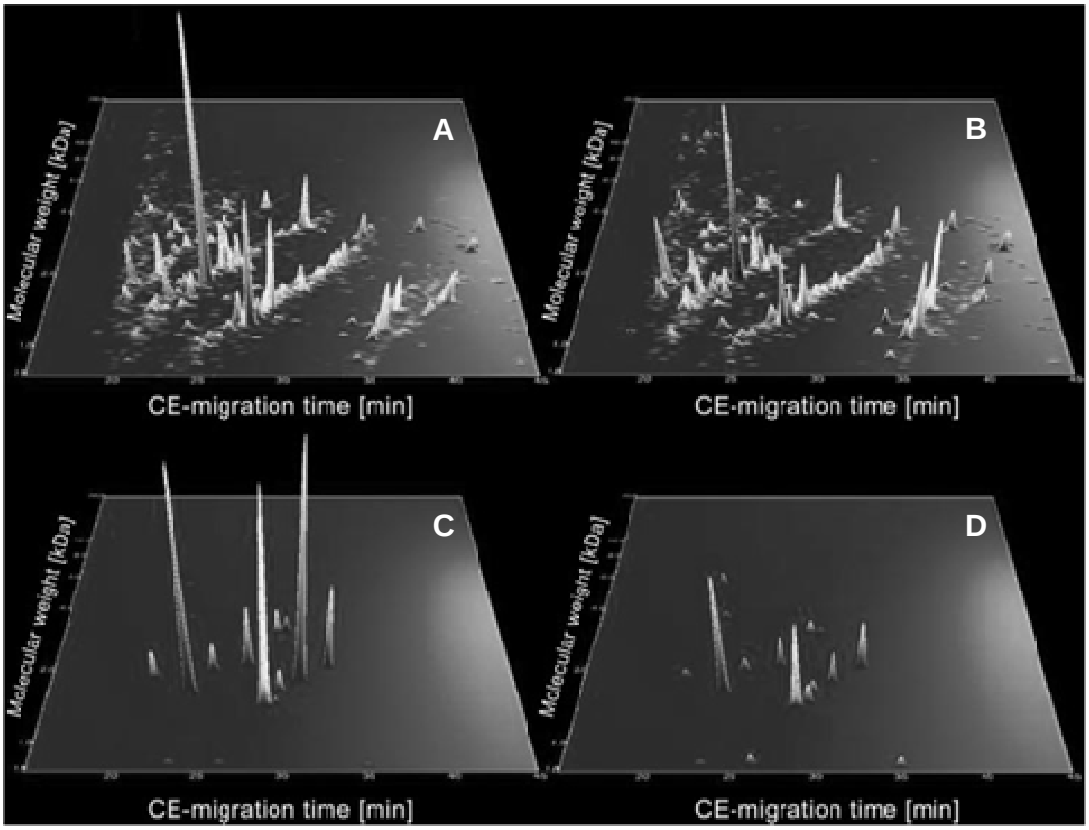

Figure 3: Polypeptide patterns distinguishing patients with coronary artery disease from controls: compiled data sets of 30 coronary artery disease samples (A), 20 control subjects (B), 15 indicative polypeptides defining the specific pattern for coronary artery disease (C), and controls (D). Reprinted from [72] with permission from Wiley-VCH Verlag GmbH.

3.2. Natural compounds

Table 2 reviews applications related to natural compound analysis, which is another field of great interest for CE-ESI-TOF/MS.

Table 2: Summary of CE-TOF/MS applications for the analysis of natural compounds

Analyte	Sample matrix	Ionization source	BGE	Sheath liquid	Reference
phenolic compounds	pollen	ESI	80mM ammonium acetate (pH 10.5)	0.1% triethanolamine in isopropanol/H_2O (60/40)	[77]
phenolic compounds	olive oil	ESI	25mM ammonium carbonate (pH 9.0)	isopropanol/H_2O (50/50)	[78]
glucosinolates	*Arabidopsis thaliana*	ESI	100mM ammonium acetate (pH 5.4) in 30% methanol	0.2% formic acid in isopropanol/H_2O (50/50)	[76]
anthocyanins	*Hibiscus sabdariffa*	ESI	200mM ammonium borate (pH 9.0)	0.1% formic acid in isopropanol/H_2O (60/40)	[79]
anthocyanins	red onion	ESI	15mM formic acid (pH 1.9)	methanol/H_2O/acetic acid (80/19.9/0.1)	[80]
alkaloids	*Rhizoma coptidis*	ESI	50mM ammonium acetate in methanol/ACN (80/20)	0.5% acetic acid in methanol/H_2O (50/50)	[81]
alkaloids	*Atropa belladonna*	ESI	60mM ammonium acetate in 5% isopropanol (pH 8.5)	0.5% formic acid in isopropanol/H_2O (50/50)	[82]
metabolites	soybean	ESI	50mM ammonium carbonate (pH 9.0)	0.1% ammonium hydroxide in isopropanol/H_2O (50/50)	[83]
biogenic amines	wine	ESI	1M formic acid (pH 2.0)	isopropanol/H_2O (50/50)	[84]

metabolites	maize	ESI	2.5% formic acid (pH 1.9) ; 25mM ammonium carbonate in 10% methanol (pH 7.82)	isopropanol/H_2O (50/50)	[85]
phenolic and other polar compounds	walnut	ESI	40mM ammonium acetate (pH 9.5)	isopropanol/H_2O (50/50)	[118]

Bringmann *et al.* used this technology to analyze the glucosinolate pattern of *Arabidopsis taliana* (Figure 4) [103]. Sheath liquid and background electrolyte were optimized for the best selectivity and sensitivity and were based on formate and acetate, respectively. The ESI-TOF/MS system allowed the determination of the elemental composition of the CE-separated glucosinolates.

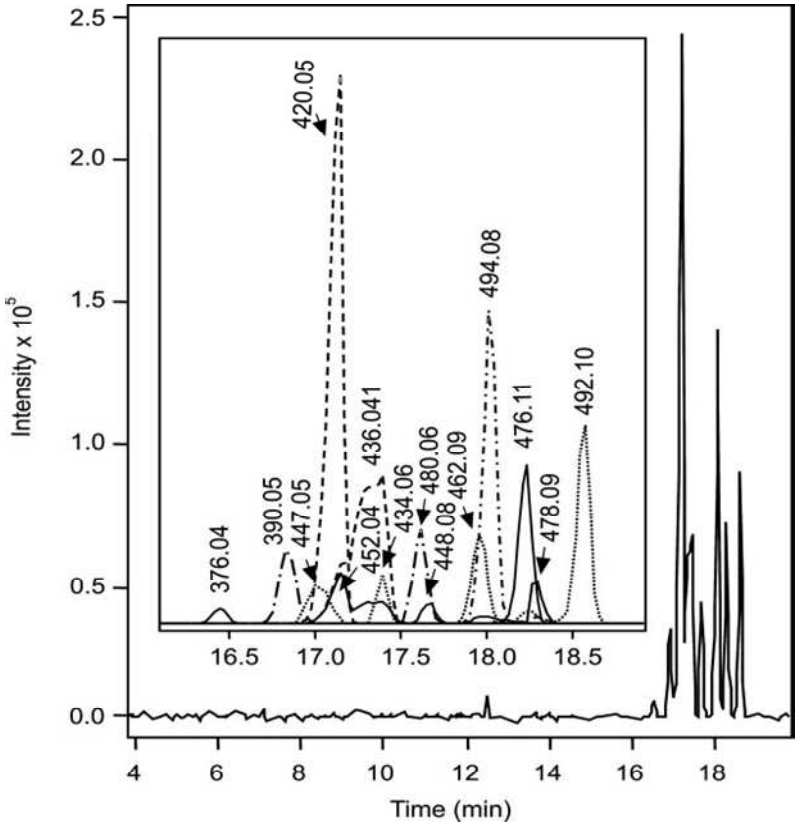

Figure 4: Electropherogram obtained by CE-ESI-TOF/MS of crude *Arabidopsis Thaliana* seed extract. The insert shows a detailed view of extracted ion electropherograms (*m/z* values) of the major compounds. Reprinted from [103] with permission from American Society for Biochemistry & Molecular Biology.

Arráez-Roman *et al.* and Carrasco-Pancorbo *et al.* worked on the analysis of phenolic compounds by CE-ESI-TOF/MS from pollen extracts and olive oil, respectively [101, 102]. The first group fully optimized experimental conditions (*e.g.*, buffer type and concentration, pH, use of organic modifiers, voltage, and injection time) according to resolution, sensitivity,

analysis time, and peak shape. The accurate mass values were better than 5 ppm. The second group used CE and LC, both coupled with ESI-TOF/MS. Each method allowed the determination of more than 45 analytes with acceptable repeatability. More recently, many groups have worked on the analysis of natural compounds by CE-ESI-TOF/MS [31, 104-108]. An interesting study was performed by Simó *et al.* [108], who compared IT and TOF, both hyphenated with CE-ESI for the analysis of biogenic amines in wine. Although both analyzers detect amines directly in wines without any previous treatment, CE-TOF/MS permitted the identification of a higher number of amines with better sensitivity. Finally, CE-TOF/MS has emerged as a very powerful technique in metabolic studies to separate and detect as many endogenous metabolites as possible in a single run. For instance, a large number of metabolites were tentatively identified by Levandi *et al.* in transgenic maize based on the high mass accuracy provided by the TOF/MS analyzer together with the isotopic pattern and expected electrophoretic mobility of these compounds [109]. They concluded that metabolomics procedures based on CE-TOF/MS could open new perspectives in the study of transgenic organisms in order to corroborate (or not) their substantial equivalence with their conventional counterparts.

3.3. Other applications

Table 3 summarizes various applications most of them in the toxicological or pharmaceutical fields.

Table 3: Summary of other CE-TOF/MS applications

Analyte[a]	Sample matrix	Ionization source	BGE	Sheath liquid	Reference
pharmaceutical and toxicological compounds	hair, blood and urine	ESI	25mM ammonium formate (pH 9.5)	0.5% formic acid in isopropanol/H_2O (50/50)	[89]
pharmaceutical and toxicological compounds	hair	ESI	25mM ammonium formate (pH 9.5)	0.5% formic acid in isopropanol/H_2O (50/50)	[88]
DMTLT	water	ESI	46.5mM ammonium carbonate (pH 10) in 15% methanol	0.5% formic acid in isopropanol/H_2O (50/50)	[90]
opium powder	water	ESI	25mM citrate (pH 3.0)	0.1% formic acid in methanol/H_2O (80/20)	[87]
octyphenol ethoxylates	water	MALDI off-line	60mM Tris-TAPS (pH 8.3)	-	[42]
oligosaccharides	water	MALDI off-line	10mM ammonium acetate (pH 4.75)	-	[39]
pharmaceutical and toxicological compounds	water	ESI	25mM citrate (pH 3.0)	0.1% formic acid in methanol/H_2O (80/20)	[86]
melamine resins	water	ESI	100mM trifluoroacetic acid in 50% methanol	0.6% formic acid in isopropanol/H_2O (80/20)	[115]
melamine-formaldehyde resins	water	ESI	500mM formic acid in 50% ACN	0.1% formic acid in isopropanol/H_2O (50/50)	[116]
toxicological compounds	blood	ESI	25mM ammonium formate (pH 9.5)	0.5% formic acid in isopropanol/H_2O (50/50)	[117]

[a] DMTLT : dimethacrylate-tyrosine-lysine-tyrosine

For instance, Lazar *et al.* used CE-ESI-TOF/MS for the analysis of drugs of abuse (morphine, cocaine, heroine, methamphetamine, amphetamine, or opium) [114, 115]. More recently, Gottardo *et al.* worked on toxicological analysis of hair based on CE-ESI-TOF/MS [112]. The high resolution of the TOF/MS analyzer permitted the unambiguous confirmation of the main drugs of abuse. In 2008, Polettini *et al.* presented a database of chemical formulae for the screening of pharmacologically and toxicologically relevant compounds in biological samples [111]. The database contained more than 50,000 records and allowed fast and automated searches for different measured masses. CE-ESI-TOF/MS is also used in the industrial field. Barry *et al.* used CE off-line hyphenated with MALDI-TOF/MS for the analysis of ethoxylated polymers [43]. Simó *et al.* described a method for the analysis of a cross-linker with an acrylic function that was synthesized in their lab [113]. They showed that CE-TOF/MS and CE-IT/MS were adequate tools to characterize charged compounds and polymers that could be used as biomaterials.

4. CONCLUDING REMARKS

Since the beginning of CE-TOF coupling in the middle of the 90's, many papers have appeared in the literature. CE-TOF coupling is particularly interesting, due to the important efficiency and small sample consumption afforded by CE, as well as the features of the TOF analyzer (*i.e.*, high mass accuracy, infinite mass range, and high mass resolution) combined with recent technical progress (such as orthogonal acceleration and the ion mirror). Regarding CE-TOF/MS applications, this analytical system was found to be useful in the analysis of biomolecules, particularly in the domain of biomarkers. Indeed, TOF high mass resolution and accuracy allowed for the detailed and reproducible display of compound patterns in body fluids. Lately, many applications have also been described in the domain of natural compounds with CE-TOF/MS. The latter provided low mass errors and the identification of empirical formulae with higher accuracy compared to normal quadrupole/MS techniques. Furthermore, it emerged as a complementary technique to analyze very polar fractions containing hydrophilic analytes, which are usually lost in the injection peak of conventional reversed phase liquid chromatography. Finally, a growing interest for CE-TOF/MS concerns the comprehensive analysis of low-molecular weight metabolites in biological systems (*i.e.*, metabolomics) [119]. CE-TOF/MS is a very powerful technique for this type of profiling and may become a dominant force in the very near future to understand physiological links from the cellular level to the whole organism as it perfectly couples the concept of limited sample amount with the requirements for high separation efficiency and high mass accuracy.

5. ABBREVIATIONS

MS^n	multistage MS analysis
oa	orthogonal acceleration

6. REFERENCES

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II.3 Approche méthodologique pour le contrôle de l'adsorption des protéines en CE-MS

Lors du développement d'une méthode d'analyse basée sur le couplage entre l'électrophorèse capillaire et la spectrométrie de masse, deux aspects fondamentaux sont à étudier: l'optimisation des conditions de source et la minimisation de l'adsorption des protéines aux parois du capillaire de silice fondue. L'**article III** concerne le second problème. Le phénomène d'adsorption des protéines aux parois du capillaire est un problème connu. Globalement, les protéines s'adsorbent au capillaire *via* des interactions électrostatiques et des interactions hydrophobes. Si peu de changements structuraux se produisent lors de l'interaction, la protéine peut retourner dans le tampon de séparation et on parle alors d'adsorption réversible. Au contraire, lorsque la protéine subit des modifications structurales qui l'amènent à un état stable « adsorbé », l'adsorption devient alors irréversible. Quoi qu'il en soit, les deux types d'adsorption sont délétères pour la qualité de la séparation CE et doivent être minimisés.

Pour cela, plusieurs méthodologies sont largement utilisées: l'ajout de petites molécules chargées ou de tensioactifs directement dans le tampon de séparation et/ou les revêtements de capillaire. Ces derniers sont les plus utilisés que ce soit en mode dynamique (ajout du revêtement dans le tampon à chaque analyse) ou en mode statique (revêtement semi-permanent). Cependant, ces trois stratégies présentent le désavantage de ne pas être forcément compatible avec une détection MS. En effet, il peut se produire de la suppression ionique et l'encrassement de la source. Les revêtements statiques semblent être moins concernés par ces problèmes mais un relargage n'est cependant pas à exclure. Dans l'optique d'une meilleure compatibilité MS, l'utilisation de solvant organique ajouté en petite proportion directement dans le tampon a été développée. Les solvants organiques peuvent avoir de nombreux effets potentiellement favorables à la diminution des phénomènes d'adsorption. Ainsi, ils modifient le pH apparent du tampon et par conséquent provoquent des changements au niveau des interactions électrostatiques entre la protéine et le capillaire. De plus, l'hydrophobicité du tampon est aussi modifiée ce qui peut induire des changements au niveau des interactions hydrophobes entre les mêmes partenaires. Finalement, il a aussi été remarqué que la structure même de la protéine était modifiée par l'ajout de solvant organique.

L'article présente différents tests qui ont été réalisés sur trois protéines modèles (insuline, hormone de croissance et hémoglobine) afin d'acquérir des données en CE-UV permettant de classer l'adsorption (réversible ou irréversible) et de quantifier de façon relative l'influence positive, neutre ou négative du solvant organique. Il a été mis en évidence une influence non négligeable des solvants organiques sur l'adsorption des protéines, particulièrement avec l'acétonitrile. Ces influences sont difficilement prédictibles et dépendent largement des caractéristiques physicochimiques des protéines (pI, masse moléculaire, flexibilité). Cependant dans plusieurs situations, l'adsorption a été fortement minimisée voire annulée. Les meilleures conditions en termes d'adsorption minimale ont été déterminées pour les trois

protéines et ces conditions ont été testées en CE-TOF/MS. Il s'avère que le transfert est facile et que les électrophérogrammes obtenus sont très satisfaisants.

L'évaluation de l'adsorption doit donc faire entièrement partie du développement de méthode en CE-TOF/MS des protéines intactes et l'ajout de solvants organiques s'avère être une option intéressante, seule ou en combinaison avec d'autres modes de minimisation de l'adsorption.

II.3.1 Article III

A. Staub, S. Comte, S. Rudaz, J.L. Veuthey, J. Schappler. **Use of organic solvent to prevent protein adsorption in CE-MS experiments**, *Electrophoresis* 2010, *31*, 3326-3333.

Use of organic solvent to prevent protein adsorption in CE-MS experiments

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ABSTRACT

Protein adsorption onto capillary wall often hampers CE separations, particularly in the CZE mode. Electrostatic interactions are not the only factor affecting adsorption, as hydrophobic interactions and/or protein conformational changes are also involved in the adsorption phenomenon. Numerous methods can be used to reduce or avoid adsorption, such as (i) addition of low molecular weight molecules in the BGE, (ii) use of surfactants, or (iii) capillary coatings. However, most of these methods are not MS-compatible. In this study, we evaluated the addition of organic solvent as an alternative MS-compatible method to decrease protein adsorption. The effect of solvent addition was emphasized using classical methods for estimating reversible and irreversible adsorption. In many cases, organic solvents were effective at decreasing adsorption. However, the influence of organic solvent on protein adsorption should be evaluated case-by-case in CE method development.

KEYWORDS

Adsorption, capillary electrophoresis, organic solvent, proteins

1. INTRODUCTION

Intact proteins analysis has become a very challenging field due to recent developments in protein chemistry, proteomics, and biotechnology [1]. Analyzing proteins by mass spectrometry (MS) in their intact form refers to the “top-down” approach, which involves transferring an undigested protein to the gas phase, in contrast to the “bottom-up” procedure [2]. The main advantage offered by the “top-down” strategy is its ability to directly obtain the molecular weight of the intact protein. Consequently, most splicing and post-translational modifications could be identified. In this context, capillary electrophoresis (CE) coupled to MS is particularly adapted due to the advantages of CE (i.e., reduced analysis times, minute amounts of samples needed and the high efficiencies obtained) and MS (i.e. sensitivity, selectivity and compounds identification). Electrospray ionization (ESI) is the most commonly ionization source used for coupling CE to MS with a sheath flow interface. This method allows intact protein analysis since it produces multicharged ions [3].

Various modes of CE can be used for protein separation, such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), micellar electrokinetic chromatography (MEKC), and capillary gel electrophoresis (CGE). However, volatile buffers are needed for ESI-MS detection, which often prevents direct coupling with CIEF, MEKC, and CGE modes, while CZE is more easily compatible. However, the analysis of intact proteins by CZE is often impaired by adsorption onto the negatively charged surface of fused silica capillaries [4,5], inducing a reduction in CE performance. Thus, protein adsorption must be evaluated and prevented at the early stages of the analytical method development.

The protein adsorption phenomenon comprises three steps (Figure 1). The first one involves protein transport to the surface, and the second step consists of the interaction and attachment of the protein to the surface. Adsorbed proteins can reversibly desorb from the surface (reversible adsorption), but in case of further conformational rearrangements (step three), protein could be definitively adsorbed on the surface (irreversible adsorption). The main driving forces for protein adsorption are electrostatic and hydrophobic interactions, as well as conformational changes in the protein [4,5]. Electrostatic interactions depend on surface and protein charges, which are mainly driven by the BGE composition (e.g., pH, ionic strength, and electrolyte nature). Hydrophobic interactions between proteins and the capillary wall could occur, while conformational changes in the protein are directly dependent on their physicochemical properties (e.g., size, charge, amino acid composition, and steric conformation) [4,5].

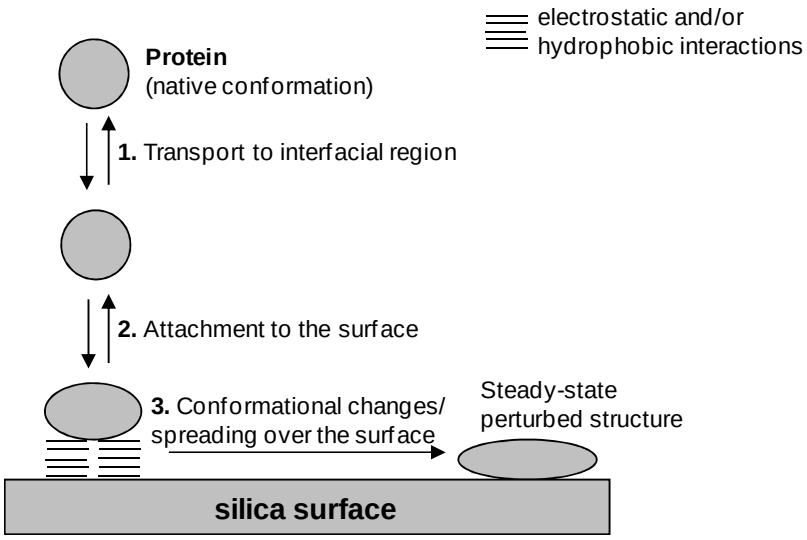

Figure 1: Schema of the protein adsorption phenomenon (adapted from References [4, 5]).

Reversible and irreversible adsorptions induce several consequences on CE separations. Reversible adsorption of proteins onto the capillary wall retards their migration time (MT) and decreases separation efficiency due to adsorption/desorption events. Irreversible adsorption causes loss of proteins within the capillary and an alteration of the electroosmotic flow (EOF) velocity.

Numerous approaches have been explored to decrease adsorption, such as the use of extreme pH, high ionic strength, and zwitterionic additives, but the most common approach is capillary wall coating. Two coatings are typically used, the static and the dynamic coating [6, 7]. However, for the latter, coating material entering the mass spectrometer can give background noise, suppression of analyte signals, and/or contamination of the ion source and MS optics. This requirement limits the usefulness for CE-MS dynamic coatings and involves minimizing the bleeding of the static coatings [8]. Thus, the addition of organic solvents to the BGE, as preventive agents for protein adsorption, should also be considered. Indeed, in the context of CE-MS analysis, the addition of organic solvent could assist the desolvatation process of the analyte in the ESI source (although due to substantial dilution, the sheath liquid composition plays the major role for the assistance in the ionization process), as well as influence protein adsorption through different possible mechanisms. First, the protein itself can be influenced by direct interactions of solvent molecules (modification of hydrophobic interactions), indirect effects *via* modification of the hydration shell (change of electrostatic interactions), or a combination of both these mechanisms [9,10]. These modifications could lead to conformational changes, which can influence protein adsorption. It is noteworthy that, besides disruption of the protein tertiary structure, organic solvents often leave secondary structure interactions largely undisturbed [11]. This influence depends on the surface topology of the protein and on the hydration shell, as well as on the concentration of the solvent. Second, the addition of organic solvent could lead to modifications on the capillary wall, such as zeta potential changes. Furthermore, the organic solvent present in the BGE could involve interesting selectivity and efficiency modifications, well known in non-aqueous capillary electrophoresis (NACE) [12].

The effect of capillary wall coatings on protein adsorption was already evaluated in the literature by monitoring reversible and irreversible adsorption based on different methodologies [4]: monitoring peak efficiencies [13], estimation of migration time (MT) repeatability [13, 14, 15] and electroosmotic flow conservation [16, 17, 18], and calculation of area recoveries [19, 20].
The aim of this study was to find a generic approach to determine optimal CE-MS conditions for intact protein analysis, involving pH of the BGE (basic vs. acidic), as well as the addition of organic solvent. In each case, adsorption was evaluated and characterized. Three model proteins of different size were selected: insulin (INS), growth hormone (GH), and hemoglobin (Hb). Protein sizing experiments [21, 22] were also performed in order to estimate the influence of organic solvent on this parameter. Finally, the best conditions were selected for each protein and the MS-transfer performed.

2. MATERIALS AND METHODS

2.1. Chemicals and samples

Acetonitrile (ACN) and methanol (MeOH) were of analytical reagent grade from Panreac (Barcelona, Spain). Ammonium hydroxide solution, formic acid, acetone, and ethanol (EtOH) were of analytical grade from Fluka (Buchs, Switzerland). Ultrapure water was supplied by a Milli-Q purification unit from Millipore (Bedford, USA).
Somatropin (GH) and insulin (INS) were purchased from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). Hemoglobin (Hb) was purchased from Sigma-Aldrich (St Louis, USA). The three proteins were dissolved in a 50 mM Tris-phosphate buffer (pH 7.4) at a concentration of 2.0, 1.7, and 0.8 mg/mL for Hb, GH, and INS, respectively (stock solutions). Standard solutions of GH, Hb, and INS at desired concentrations were prepared daily by appropriate dilution of the stock solutions with water.

2.2. CE-UV instrumentation

CE experiments were performed with an HP ^{3D}CE System (Agilent, Waldbronn, Germany) equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. Separation was performed in an uncoated FS capillary (BGB Analytik AG, Böckten, Switzerland) with a 50 µm I.D, a total length of 64.5 cm, and an effective length of 56 cm. Samples were hydrodynamically injected at 50 mbar for 8 s (equivalent to 1% of the effective capillary length). Prior to each sample injection, the capillary was rinsed at 2 bar for 1 min with fresh BGE. Experiments were carried out in positive polarity mode with the anode at the inlet and the cathode at the outlet. A constant voltage of 30 kV, with an initial ramping of 5000 $V \cdot s^{-1}$ (6 s), was applied during the analysis, and the capillary temperature was maintained at 25°C. UV detection was performed at 200 nm.
New bare FS capillaries, used under basic pH conditions, were rinsed at 1 bar with MeOH, 1 M HCl, water, 0.1 M NaOH, water, and BGE for 5 min each. New bare FS capillaries, used under acidic pH conditions, were rinsed at 1 bar with MeOH, 0.1 M NaOH, water, 1 M HCl,

water, and BGE for 5 min each. When not in use, the capillary was rinsed with water and then stored dry.

For protein sizing and area recovery experiments, an ActiPix™ D100 UV Area Imaging System (Paraytec, York, United Kingdom) was coupled with CE to perform analyses with two passes through the detector [22]. Experiments were also performed in uncoated FS capillaries (BGB Analytik AG, Böckten, Switzerland) with a 50 µm I.D. Capillaries with a total length of 115 cm and effective lengths of 32 and 65.5 cm were used for area recovery experiments. Capillaries with a total length of 98 cm and effective lengths of 32 and 49.5 cm were used for protein sizing experiments. Samples were hydrodynamically injected at 50 mbar for 16 s for area recovery experiments (equivalent to 2% of the effective capillary length, taking into account the first window). Samples were hydrodynamically injected at 50 mbar for 60 s (equivalent to 8.4% of the effective capillary length, taking into account the first window) and flushed at 1 bar for 5 min for sizing experiments. Prior to each sample injection, the capillary was rinsed at 2 bar for 4 min with fresh BGE. UV detection was performed at 200 nm.

Separations were carried out with 75 mM ammonium formate buffers (pH 2.5 and 9.0). The addition of different percentages of organic modifiers was tested (5-60%, *v/v,* of ACN, MeOH, or EtOH) under both basic and acidic conditions.

2.3. CE-MS instrumentation

CE was coupled to an Agilent Technologies 6210 LC/MS TOF mass spectrometer (Agilent, Palo Alto, USA) *via* a tri-axial sheath flow ESI interface from Agilent. The coaxial sheath liquid was optimized in terms of composition (water with MeOH, isopropanol or ACN at different percentages from 25 to 75%, formic or acetic acid at different proportions, from 0.05 to 10%) and delivery rates (from 2 to 8 $\mu L \cdot min^{-1}$) for each protein. The fragmentor voltage was optimized for each protein. Other MS parameters were identical for the three proteins: ESI capillary voltage was set at +4500 V, the nebulizing gas pressure at 4 psi, the drying gas flow rate at 4 $L \cdot min^{-1}$, and the drying gas temperature at 150°C. MS detection was carried out in the positive ion mode and 1 $spectrum \cdot s^{-1}$ was acquired (9742 transients/spectrum).

CE-MS experiments involved capillaries with a length of 80 cm and an internal diameter of 50 µm. Samples were hydrodynamically injected at 50 mbar for 30 s (equivalent to 2% of the capillary length).

2.4. Measurements and calculations

2.4.1 Reversible adsorption

Relative standard deviations of migration times (MT RSDs, n=5) were calculated for successive injections.

2.4.2 Irreversible adsorption

2.4.2.1 Protein recovery

A procedure for protein recovery was adapted from one reported by Towns and Regnier [19,20]. Successive injections of protein samples were performed in the same capillary (n=5) with two passes through the detector (ActiPix™ System). The decrease in area of the protein between the first and the second pass through the detector provided a measure of the irreversible adsorption on the capillary, and is given as the area recovery percentage.

2.4.2.2 EOF

Electrosmotic flow (EOF) mobilities (n=3) were calculated with and without protein injection. At basic pH, a strong cathodic EOF was observed and its mobility was calculated with acetone as a neutral marker. At acidic pH, acetone migrated around 110 minutes, due to the weak EOF observed at this pH. Therefore, the methodology of Williams and Vigh [17], with the assistance of pressure was used.

2.4.3 Protein sizing

The methodology for protein sizing experiments was based on the Taylor dispersion analysis (TDA) [23]. A looped capillary was used with the ActiPix™ System, providing two detection windows. A plug of the sample was hydrodynamically injected and driven by the application of external pressure along the capillary. UV absorption of the protein zone was recorded during the 1st and 2nd pass. Taylor dispersion induced greater width and lower amplitude of the signal recorded in the second window. Based on standard deviations associated with the solute peak broadening, the diffusion coefficient and the hydrodynamic radius of proteins could be determined with the equation 1 [21, 22, 23]:

$$R_h = \frac{4k_B T(\tau_2^2 - \tau_1^2)}{\pi\eta r^2(t_2 - t_1)} \quad [1]$$

where R_h = hydrodynamic radius, k_B = Boltzmann constant, T = absolute temperature, t_1 and t_2 = peak center times at the first and the second capillary windows, τ_1 and τ_2 = their corresponding standard deviations, η = viscosity, and r = capillary internal radius.

2.5. Software

Buffer solutions were prepared with the help of PHoEBuS software (version 1.3, Analis, Namur, Belgium). CE ChemStation (version A.10.02, Agilent, Waldbronn, Germany) was used for CE instrument control. ActiPix™ D100 (Paraytec, York, United Kingdom) was used for ActiPix™ control and hydrodynamic radii calculations.

3. RESULTS AND DISCUSSION

Two types of adsorption can occur, namely reversible and irreversible adsorption, each was distinguished from the other to clearly characterize the organic solvent influence. In this study, as reported below, three parameters were measured. Migration time (MT) was selected for reversible adsorption and a MT RSD higher than 3% was considered to reflect non-repeatable “chromatographic” retention due to adsorption. For irreversible adsorption, peak area recoveries and EOF conservation were monitored. Area recovery was considered as a measure of protein loss between the first and second pass through a detector. Non-conservation of EOF reflected adsorption of proteins onto the capillary wall due to permanent zeta potential modification.

Because adsorption phenomenon greatly depends on the physicochemical properties of a protein (*e.g.*, structure, size, pI, hydrophobicity), three different proteins were chosen (Table 1): insulin (INS), growth hormone (GH), and hemoglobin (Hb). It should be noted that Hb is a globular protein, well-known to be a flexible protein, that tends to adsorb easily onto capillary wall, in contrast to GH and INS, which are quite “rigid” proteins. Several organic solvents were tested at two different pH values (2.5 and 9.0, measured in aqueous solutions): methanol (MeOH), ethanol (EtOH), and acetonitrile (ACN). For sake of clarity, only the results for ACN are presented because this solvent gave a maximal effect at a minimal concentration. Indeed, MeOH and EtOH induced similar effects but at higher concentrations, corresponding to twice the ACN content. Initial experiments were carried out between 5 and 60% ACN, but for comparison purposes, 10% ACN was selected throughout the entire study, as it gave repeatable results for all proteins at each pH investigated.

Table 1: Characteristics of the three model proteins

	p*I*	nAA	MW (kDa)
Insulin	5.2	51	5.8
Growth hormone	5.2	191	22.1
Hemoglobin	7.0	574	66.8

The classical effects of organic solvents on CE should be noted, as reported in previous NACE studies [12]. For example, the addition of 10% ACN changed the dielectric constant ε and the viscosity η of the BGE [24, 25], as ε decreased from 80 to 70 (*ca.* -13%) and η increased from 890 to 970 [µPas] (*ca.* +9%). These modifications could influence selectivity and efficiency. Indeed, efficiency was related to the square root of the dielectric constant

over the BGE viscosity, ε^2/η [12]. Since ε^2/η with ACN was 1.4 time lower than without ACN, this could explain the eventual loss in efficiency when ACN was used. As the addition of organic solvent to the BGE could change peak efficiency, the monitoring of this parameter as a reversible adsorption indicator was excluded of this study. Selectivity was related to the dielectric constant over the viscosity ratio, ε/η.

Besides these classical effects, numerous mechanisms can account for the potential effects of organic solvents on protein adsorption onto capillary wall. Adding organic solvent to the BGE changes the apparent pH, which can alter the charge density of the protein, as well as the capillary surface, modifying electrostatic interactions between them. Moreover, organic solvents can influence the hydration shell of the protein, inducing structural modifications, particularly in its tertiary structure. The polarity of the BGE is also modified with organic solvents, which can affect hydrophobic interactions of proteins with the silica wall.

3.1. Study at basic pH

CE analyses of intact proteins are often carried out at alkaline pH. Besides the common advantage of great EOF, electrostatic repulsion at this pH could occur between globally negatively charged proteins and the capillary wall. Adsorption *via* electrostatic interactions could thus be reduced.

First experiments were performed at basic pH without ACN. For INS (200 µg/mL) and GH (400 µg/mL), the “rigid” proteins, no reversible adsorption was observed. As shown in Table 2, MT RSDs (n=5) were lower than 0.70%. Irreversible adsorption was very low, as peak area recovery was higher than 95% (Figure 2A) and EOF magnitude was equivalent in both the presence and absence of protein in the injected sample (Table 3A).

For Hb (450 µg/mL), no reversible adsorption was observed, as the corresponding MT RSD was low (Table 2). Furthermore, no irreversible adsorption occurred as peak area recovery was 100% (Figure 2A) and EOF magnitude was conserved.

Next, 10% ACN was added to the BGE particularly to try to circumvent the small irreversible adsorption observed for INS and GH. Adsorption was again evaluated in these new conditions. For INS and GH, MT RSDs were quite similar to those without ACN and thus, no reversible adsorption occurred. Peak area recoveries were, in contrast, improved with 100% recovery reached for both proteins (Figure 2A). EOF magnitude was equivalent in the presence and absence of proteins in the injected sample. Therefore, the addition of 10% ACN permitted the circumvention of the small irreversible adsorption.

The same experiments were conducted for Hb, but the addition of ACN was found to be adverse in terms of reversible and irreversible adsorptions. The MT RSD was higher than 10% (Table 2), while peak area recovery fell to 82% (Figure 2A) and EOF magnitude was significantly affected (-32%, Table 3A). ACN was observed to induce both reversible and irreversible adsorptions under these conditions. The large flexibility of the Hb could account for this phenomenon.

Table 2: Effect of ACN on MT repeatability

basic pH			
MT RSD (%), n=5	INS	GH	Hb
no ACN	0.68	0.30	0.30
10% ACN	0.69	0.50	13.00
acidic pH			
no ACN	0.62	n.p.d.	n.p.d.
10% ACN	0.60	1.28	18.00

n.p.d., no peak detected.

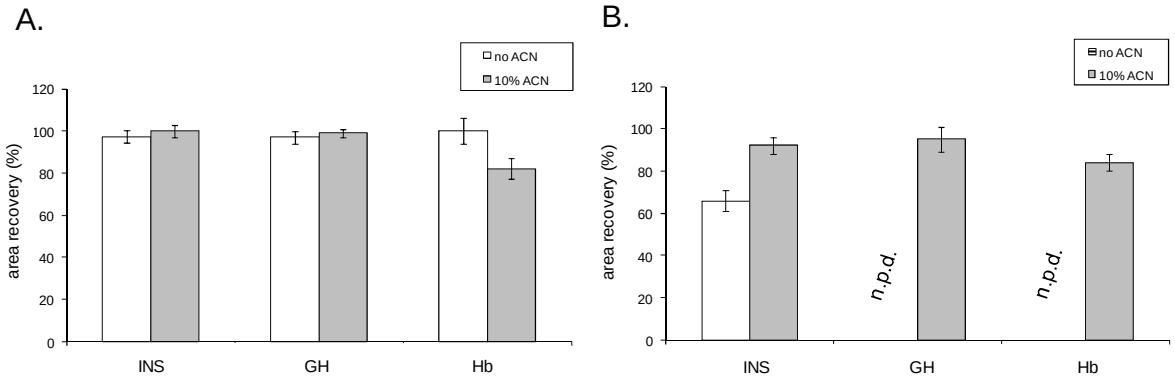

Figure 2: Effect of ACN on area recovery. (A) at basic pH; (B) at acidic pH; n.p.d.: no peak detected.

Table 3: Effect of ACN on EOF mobility before and during protein analysis at basic pH (A.) and acidic pH (B.).

A.

proteins	BGE	EOF measurements			
		Immediately after capillary conditonning		In the course of protein analysis	
		μEOF (10^{-4}•cm^2•V^{-1}•s^{-1})	RSD (%, n=3)	μEOF (10^{-4}•cm^2•V^{-1}•s^{-1})	RSD (%, n=3)
INS	no ACN	6.56	1.92	6.43	1.69
	10% ACN	5.66	1.25	5.52	1.01
Hb	no ACN	6.52	1.83	6.40	1.72
	10% ACN	5.57	1.34	3.79	19.72
GH	no ACN	6.63	1.97	6.63	1.42
	10% ACN	5.59	1.29	5.27	1.22

B.

proteins	BGE	EOF measurements			
		Immediately after capillary conditionning		In the course of protein analysis	
		μEOF (10^{-4}•cm^2•V^{-1}•s^{-1})	RSD (%, n=3)	μEOF (10^{-4}•cm^2•V^{-1}•s^{-1})	RSD (%, n=3)
INS	no ACN	1.00	1.95	0.99	1.93
	10% ACN	1.03	2.00	1.08	1.54
Hb	no ACN	1.12	2.02	n.p.d	n.p.d
	10% ACN	1.22	1.80	n.p.d	n.p.d
GH	no ACN	1.18	1.92	n.p.d	n.p.d
	10% ACN	1.08	1.45	1.11	5.02

n.p.d., no peak detected.

Two behaviors were thus highlighted at basic pH. For Hb analysis, data were satisfactory without organic solvent while its addition adversely affected the results. In contrast, while INS and GH analysis in aqueous conditions was acceptable, the addition of ACN decreased irreversible adsorption, further improving the results. It was thus interesting to investigate through which parameters organic solvent modified these behaviors. As mentioned above, the addition of ACN to the BGE changed the parameters of viscosity, global charge of the proteins (due to an increase in apparent basic pH and pKa values of acidic and basic moieties of the protein [26, 27, 28]), dielectric constant, zeta potential, and the molecular radius of the protein. The first four parameters were definitely modified, but due to the very small percentage of ACN present in the BGE, these changes should not be significant.
The last parameter to investigate was the molecular radius. An estimation of this value was obtained through the calculation of the hydrodynamic radius. Sizing experiments were thus conducted to emphasize the influence of ACN on this parameter. Figure 3A shows a large influence of organic solvent on the protein hydrodynamic radius. For the three proteins studied, decreases in their radii were observed (up to -35%), which could have been the result of tridimensional rearrangement in the protein structure. This change of hydrodynamic radius reflected the influence of organic solvents on protein structure. Exact influences of these structural modifications on protein adsorption are difficult to ascertain, due to the complexity of the adsorption phenomenon. Nevertheless, these changes could lead to a decrease of adsorption for rigid proteins and thus, organic solvent addition to the BGE could greatly improve CE experiments. Figure 4A presents electropherograms obtained for INS with and without ACN for comparison.

3.2. Study at acidic pH

In contrast to basic pH, the three proteins were globally positively charged at acidic pH and thus electrostatic interactions could occur to a greater extend with the capillary wall, which should still exhibit some deprotonated silanol moieties. For this reason, fewer applications were performed at this pH in comparison with alkaline conditions. However, as acidic pH could permit different selectivity, its use should be considered for intact protein analysis [29].

Experiments were first conducted without ACN. For INS, a peak was detected and the MT RSD was lower than 0.65% (Table 2). According to the MT repeatability, no reversible adsorption occurred for this protein. However, the INS peak was asymmetric (tailing, Figure 4B). Furthermore, Hb and GH were not observed, most likely due to irreversible adsorption.
The μ_{EOF} determination at acidic pH induced very long analysis times (> 100 min.). To overcome this limitation, the procedure of Williams and Vigh [17] was adopted. Three plugs of a neutral marker (acetone) were successively injected, followed by either flushes of BGE or voltage application. Thanks to the plug length estimation, as well as the flush and the voltage, μ_{EOF} could be calculated according to classical equations of CE connecting effective and total capillary lenghts to velocity and the times between the three plugs.
Electroosmotic flow mobility was conserved (Table 3B) and peak area recovery of INS was lower than 70% (Figure 2B) under these conditions. Therefore, ACN addition to the BGE was tested to overcome the irreversible adsorption occurring in aqueous media.

Addition of 10% organic solvent permitted peak detection for both GH and Hb. Low MT RSD (1.28%, n=5, Table 2) was obtained for GH, which emphasized negligible reversible adsorption. Hb presented less favorable results with a MT RSD of 18% (Table 2). In this case, ACN allowed for peak detection but reversible adsorption was still present. It should be noted that the further increase in ACN up to 60% did not reduce this Hb adsorption. For irreversible adsorption, addition of ACN permitted a very satisfying area recovery for GH (>90%, Figure 2B), as well as a good conservation of EOF magnitude (Table 3B). Hb area recovery with organic solvent was not as satisfying (<80%, Figure 2B), confirmed by EOF determination, which was not possible to estimate with injection of the protein in the presence of ACN. Irreversible adsorption was thus decreased but not enough. It should be noted that increasing the ACN percentage up to 60% did not bring any improvement to these results.

For INS, the MT RSD was similar to the RSD obtained without ACN (0.60 *vs*. 0.62%), showing again no reversible adsorption. INS presented an increase of area recovery with ACN (from <70% to >90%, Figure 2B), and μ_{EOF} was conserved. Therefore, irreversible adsorption of INS was drastically reduced with the addition of organic solvent. As presented in Figure 4B, INS peak shape was greatly improved.

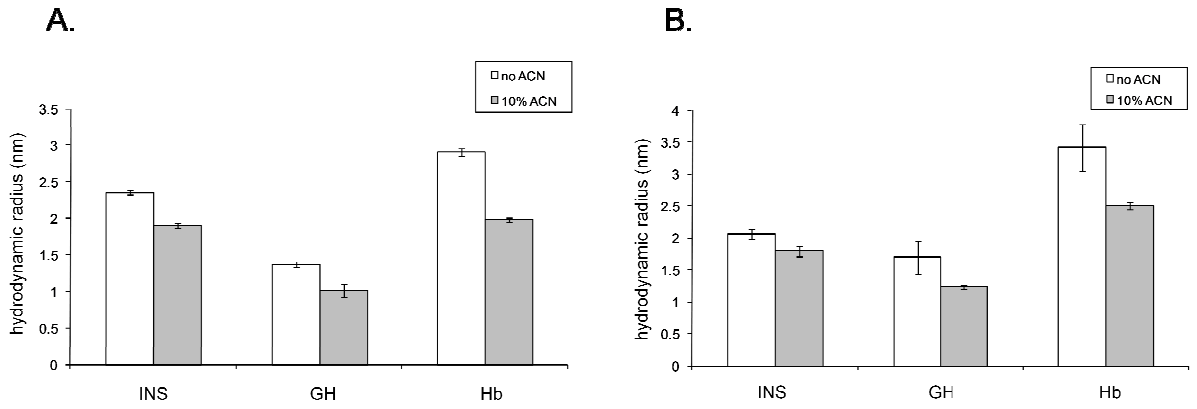

Figure 3: Effect of ACN on protein size. (A) at basic pH; (B) at acidic pH.

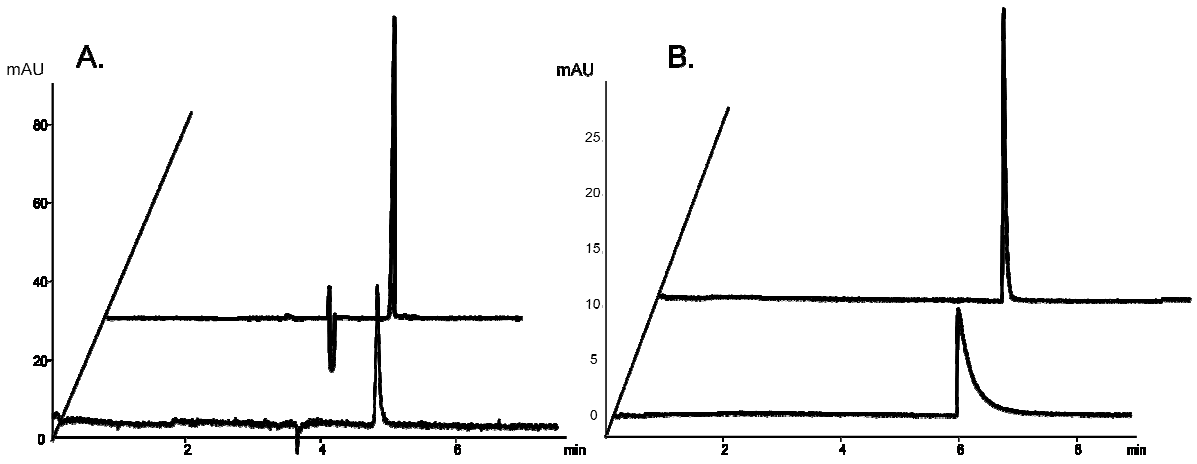

Figure 4: CE-UV electropherograms of INS at 200 µg/mL obtained with (A) alkaline BGE and (B) acidic BGE. Upper trace: with 10% ACN. Lower trace: without ACN. See text for experimental conditions.

At the acidic pH, a more complex situation was encountered. However, the addition of 10% ACN was found to be always favorable, despite the improvement was different according to initial situations. As observed, it was possible either to improve area recovery (INS case) or to obtain an electrophoretic peak (GH and Hb cases) when using ACN. Thanks to ACN addition, acidic BGE conditions could be considered more often for intact protein analysis by CE.

It was also interesting to evaluate the effect of organic solvent on protein hydrodynamic radius. As reported in section 3.1, ACN seemed to play a major role on this protein size parameter (up to -28% of the hydrodynamic radius, Figure 3B) and these modifications could partially explain the observed decrease in protein adsorption.

3.3. Global discussion

ACN had a net impact on protein adsorption, either improving or deteriorating the electrophoretic behavior. Thus, the use of organic modifiers has to be considered during CE method development for any intact protein analysis experiment. However, it is difficult to define a generic approach to this development, so a case-by-case approach is recommended. For example, in this study the addition of ACN was rather adverse for Hb (the most flexible protein), often and always beneficial for GH and INS, respectively. This alternative approach could be very useful in the case of CE-MS coupling because ACN is a MS-compatible solvent, in contrast to some classical adsorption prevention agents, such as some dynamic coating agents. Furthermore, this approach could permit the analysis of intact proteins at acidic pH, exploring other interesting selectivities. Regarding the method development strategy, initial experiments should be carried out in both acidic and basic conditions. The addition of an appropriate amount of organic solvent (*e.g.,* 10% ACN) should then be tested and, depending on results, greater amounts could be investigated. When different conditions give a good performance, the adsorption measurements could then be considered to determine the optimal analytical conditions.

3.4. Application to CE-MS experiments

The method development strategy proposed above was used to determine optimal CE-UV conditions directly compatible with MS for each protein. The best results in terms of sensitivity (with the signal-to-noise ratio) and efficiency were obtained at basic pH without ACN for Hb, at basic pH with 10% ACN for INS, and at acidic pH with 20% ACN for GH. MS parameters were adapted from results obtained from the experimental design carried out in a previous study [29]: the ESI capillary voltage was set at +4500 V, the nebulizing gas pressure at 4 psi, the drying gas flow rate at 4 $L \cdot min^{-1}$, and the drying gas temperature at 150℃. The sheath liquid was composed of isopropanol-water-formic acid in all cases, but in different proportions and delivered at different flow rates. For Hb, the sheath liquid consisted of isopropanol-water-formic acid in the proportion 48.5:48.5:3, (*v/v/v*) and was delivered at a flow rate of 4 $\mu L \cdot min^{-1}$ by a syringe pump system. For GH, the proportion was 50:50:0.05 (*v/v/v*) and the flow rate was 5 $\mu L \cdot min^{-1}$, while for INS, the ratio was 49.5:49.5:1 (*v/v/v*), delivered with a flow rate of 4 $\mu L \cdot min^{-1}$. The corresponding electropherograms and extracted

mass spectra are shown in Figure 5. Mass spectrum of Hb corresponded to the monomers of Hb, due to the dissociation of the tetramer in the ESI source [30].

The method development strategy proposed here allowed a rapid screening of CE conditions and an efficient method transfer from CE-UV to CE-MS.

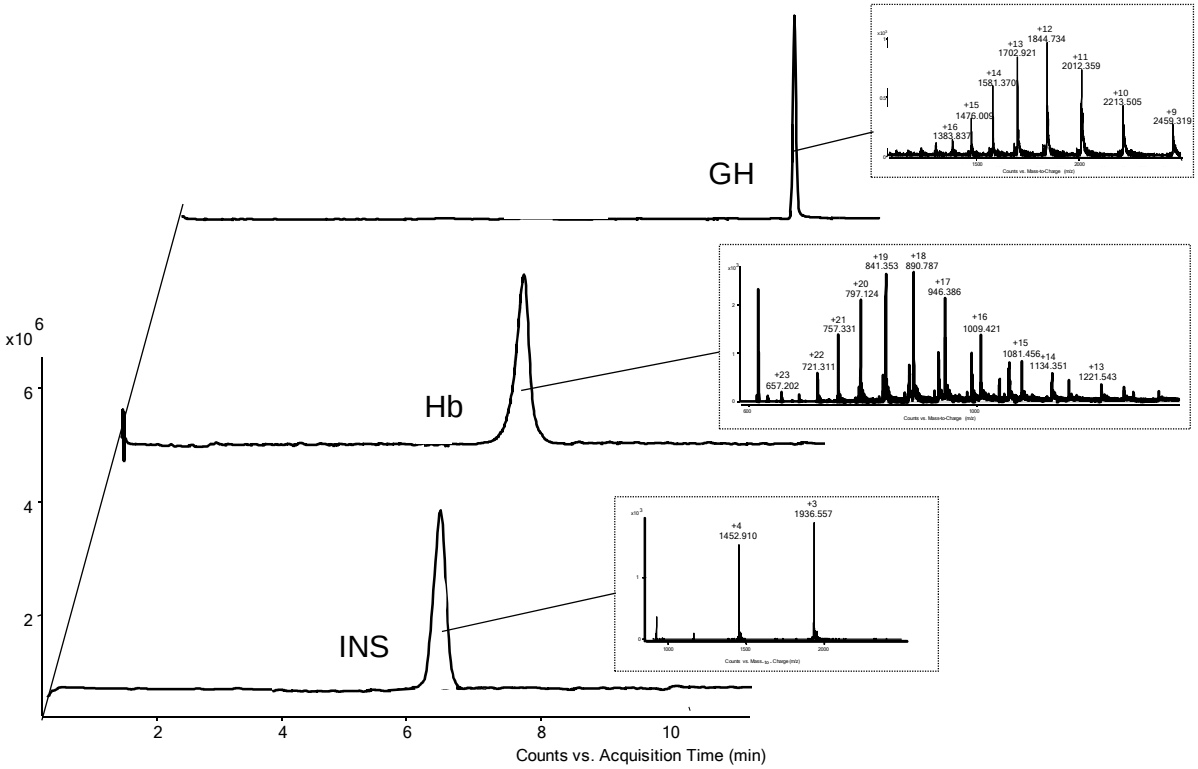

Figure 5: CE-TOF/MS electropherograms and extracted mass spectra obtained for (A) INS (200 µg/mL) in basic BGE with 10% ACN, (B) Hb (450 µg/mL) in basic BGE without ACN, and (C) GH (400 µg/mL) in acidic BGE with 20% ACN. See text for other experimental conditions.

4. CONCLUDING REMARKS

The aim of this study was to establish a generic CE-UV approach for intact protein analysis and its transfer to CE-MS. In this context, the addition of ACN as adsorption-preventing agent was evaluated. The effects of ACN addition were studied according to several descriptors of both reversible and irreversible adsorptions. Three model proteins were tested, insulin, growth hormone, and hemoglobin. Results showed an important effect of ACN on protein adsorption, but this effect was difficult to predict. A major effect of ACN was highlighted especially for rigid proteins from protein sizing experiments, as it strongly reduced the size of proteins.

Finally, due to the great number of proteins and their very different physicochemical characteristics, no prediction of organic solvent effect on adsorption could be done, but a generic strategy for method development could be proposed. The addition of an organic solvent should be considered on a case-by-case basis during method development as an alternative or additionnal method to reduce or prevent adsorption, particularly for CE-MS experiments.

5. ACKNOWLEDGMENTS

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6. ABBREVIATIONS

EtOH	ethanol
GH	growth hormone
Hb	hemoglobin
INS	insulin
MeOH	methanol
MT	migration time
n_{AA}	number of amino acids

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II.4 Application d'une méthode CE-TOF/MS à l'analyse de l'hormone de croissance dans un contexte forensique

L'hormone de croissance est une hormone polypeptidique qui se trouve naturellement dans le corps humain sous plusieurs isoformes qui diffèrent de par leur poids moléculaire (22'000, 20'000, 17'000 Da,…). Elle est également utilisée pour le traitement des retards de croissance. Depuis les problèmes liés à l'épidémie de la maladie de Creutzfeld-Jakob, cette hormone n'est plus extraite de cadavres humains mais est produite par génie génétique. La séquence peptidique de l'hormone recombinante est la même que celle de l'hormone naturelle mais elle ne présente qu'une isoforme à 22'000 Da. L'hormone de croissance est aussi employée illégalement comme produit dopant car elle permet d'augmenter la masse musculaire et la puissance en réduisant la masse graisseuse. Son trafic est donc largement répandu.

L'**article IV** présente une méthode d'analyse par CE-TOF/MS des hormones de croissance humaines naturelles et recombinantes et permet leur différenciation par deux niveaux de sélectivité. L'adsorption est minimisée par l'ajout de solvant organique, selon le principe expliqué dans l'article III. De plus, un tampon de séparation à pH acide est employé et ces conditions hydro-organiques acides permettent d'obtenir une sélectivité électrophorétique entre l'hormone recombinante et la naturelle. La deuxième sélectivité est obtenue grâce à l'exactitude de masse du TOF qui assure la distinction entre les deux isoformes majoritaires de l'hormone naturelle (22'000 et 20'000 Da) et par conséquent assure aussi la différenciation entre les formes naturelle et recombinante, cette dernière ne possédant pas la seconde isoforme.
La méthode a ensuite été appliquée à des échantillons saisis en douane et suspectés de contenir de l'hormone de croissance. Les résultats ont confirmé que les échantillons étaient constitués d'hormone de croissance recombinante, ceci par comparaison du comportement électrophorétique et du spectre de masse déconvolué avec ceux de standards naturels et recombinants. De plus, l'exactitude de masse du TOF a aussi mis en évidence une dégradation de l'un des échantillons. Ce dernier montrait une différence de masse moléculaire de +32 Da, traduisant une double oxydation complète de la protéine. Cette dégradation est certainement due à de mauvaises conditions de stockage de l'échantillon (température, lumière).

II.4.1 Article IV

A. Staub, S. Giraud, M. Saugy, S. Rudaz, J.L. Veuthey, J. Schappler. **CE-ESI-TOF/MS for human growth hormone analysis**, *Electrophoresis* 2010, *31*, 388-395.

CE-ESI-TOF/MS for human growth hormone analysis

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ABSTRACT

CE is a powerful analytical tool used to separate intact biomolecules such as proteins. The coupling of CE with TOF/MS produces a very promising method that can be used to detect and identify proteins in different matrices. This paper describes an efficient, rapid, and simple CE-ESI-TOF/MS procedure for the analysis of endogenous human growth hormone and recombinant human growth hormone without sample preparation. Operational factors were optimized using an experimental design, and the method was successfully applied to distinguish human growth hormone and recombinant human growth hormone in unknown samples.

KEYWORDS

CE, human growth hormone, MS, TOF

1. INTRODUCTION

Human growth hormone (hGH) consists of a single polypeptide chain containing 191 amino acid residues and two disulfide bridges (Figure 1). The pI for hGH is approximately 5.1. Endogenous hGH is secreted by the anterior pituitary gland under several isoforms, the 22 kDa variant being the most abundant one. Other forms, including the 20 kDa isoform, are commonly called non-22-kDa isoforms. hGH is prescribed to treat growth failure, hGH deficiency, and Prader-Willi syndrome [1,2]. Historically, hGH was extracted from human cadavers. However, since the end of the 1980s, several recombinant hGH (rhGH) variants have been obtained through genetic engineering to overcome the problems of contamination by Creutzfeldt-Jakob disease. Due to their anabolic action, rhGHs are often misused as doping agents to increase muscle mass and power and decrease fat mass [3]. Because rhGHs are quite expensive, their trafficking is relatively widespread [4]. Fast and simple analytical methods are thus needed to identify rhGH, in drug seizures for instance. rhGH possesses an identical sequence to the naturally occurring 22 kDa hormone, yet some modifications, such as deamidation, oxidation, or cleavage, may arise during the purification process and during protein storage [5]. Several methods have been developed to analyze these products. Among them, reversed-phase [5,6], ion exchange [6,7], and size-exclusion [6,8] liquid chromatography (LC) have been described. An immunological method has also been developed [9,10] to detect rhGH abuse using a change in hGH isoform ratios [11]. However, these methods suffer from some drawbacks, such as an incompatibility for intact protein analysis or MS detection and a lack of efficiency.

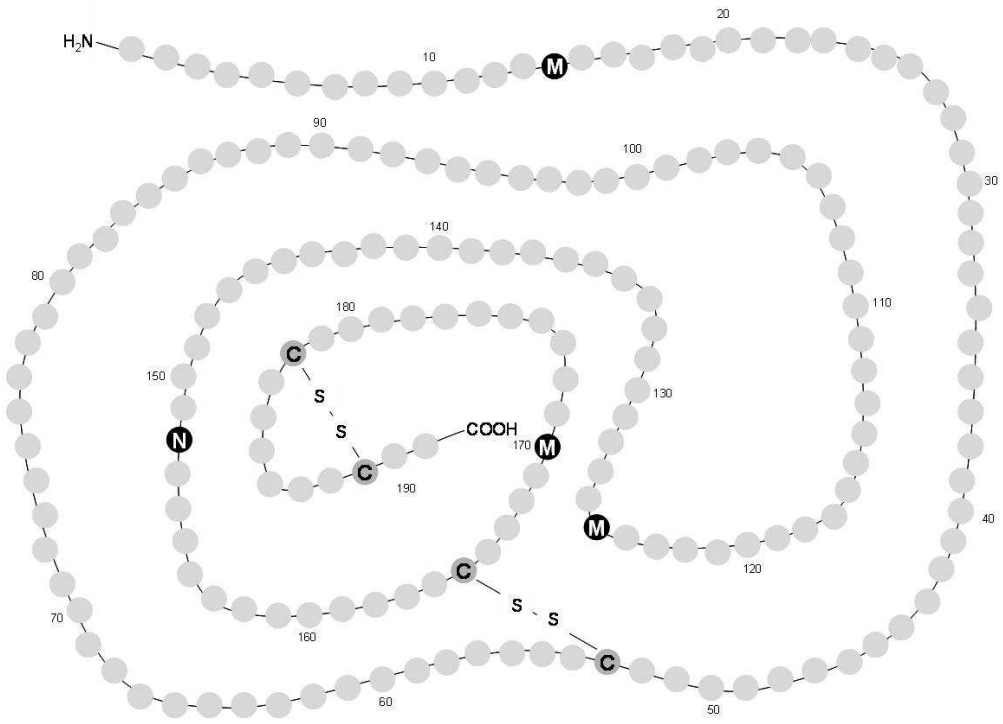

Figure 1: Chemical structure of hGH, with M: methionine; N: asparagines; and C: cysteine.

In this context, the on-line combination of capillary electrophoresis (CE) with mass spectrometry (MS) is an attractive option for analyzing intact proteins, such as hGH. First, CE has several appealing features, such as high speed, great efficiency, and low solvent and sample consumptions [12]. Moreover, in contrast to LC, CE separation is achieved in an empty capillary in aqueous conditions. This is a great advantage because conformational changes, resulting from organic modifiers in the mobile phase and/or stationary phase, should sometimes be avoided [13]. Second, MS provides sensitivity, selectivity, and the ability to identify. A time-of-flight (TOF) analyzer is particularly well suited for examining intact proteins because of numerous assets, including high mass range and accuracy [14]. As a drawback, a very important point to consider, with respect to the analysis of intact proteins by CE, is the adsorption of protein onto the capillary inner surface when fused silica (FS) is employed. A recent review written by Lucy *et al.* [15] described the numerous mechanisms involved in the protein adsorption process and reported different ways to overcome them (*e.g.*, extreme pH, high ionic strength, zwitterionic additives, polymeric coatings). However, only a few of these procedures are compatible with electrospray ionization-mass spectrometry (ESI-MS) detection.

CE methods have already been developed for the analysis of hGH and rhGH [16-20]. In 2005, the European Pharmacopeia incorporated a CE-based approach for detecting charge variants in rhGH [21], while in 2007, an improved method was presented [22]. It included a pre-conditioning step for dynamically coating the capillary wall with hexadimethrine bromide and poly(vinylsulfonate), which decreased migration time variability. All these CE methods were performed at basic pH, where hGH, as well as the capillary inner surface, was negatively charged, hindering the adsorption process by electrostatic repulsion.

The present study describes an improved CE method with ESI-TOF/MS detection for the characterization of hGH and rhGH. Particular attention was paid to selecting CE conditions that were fully compatible with MS detection, while avoiding adsorption issues.

2. MATERIALS AND METHODS

2.1. Chemicals and samples

Acetonitrile (ACN) and methanol (MeOH) were of analytical reagent grade from Panreac (Barcelona, Spain). Benzylamine, ammonium hydroxide solution, formic acid, phosphoric acid, and ethanol (EtOH) were of analytical grade from Fluka (Buchs, Switzerland). Isopropanol was of analytical grade from Acros Organics (Geel, Belgium). Tris(hydroxymethyl)aminomethane (Tris) was of analytical reagent grade from Riedel-de-Haën (Buchs, Switzerland). Ultrapure water was supplied by a Milli-Q purification unit from Millipore (Bedford, MA, USA).

Somatropin (hGH) was purchased from the NIBSC (National Institute for Biological Standards and Control, Potters Bar, UK) and was dissolved in a 50 mM Tris-phosphate buffer (pH 7.4) at a concentration of 1.7 mg/mL (stock solution). Standard solutions of hGH, at desired concentrations, were prepared daily by dilution of the appropriate stock solution with water. The lyophilized powder Humatrope (Eli Lilly, Vernier, Switzerland) was reconstituted before analysis with the provided solution. The reconstituted sample solution

contained rhGH (1.9 mg/mL), metacresol, glycerin, glycine, mannitol, and disodium hydrogen phosphate. Two lyophilized samples, whose composition was unknown, were obtained from the Swiss Laboratory for Doping Analysis (LAD, Epalinges, Switzerland). 10 mg of each powder were solubilized with an appropriate volume of water and aliquoted. One aliquot was directly injected into the CE-ESI-TOF/MS system, while the other was analyzed using the immunological test.

2.2. CE-ESI-TOF/MS instrumentation

2.2.1. CE system

CE experiments were performed with an HP ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. Separation was performed in an uncoated FS capillary (BGB Analytik AG, Böckten, Switzerland) with a 50 µm I.D. Experiments were carried out in positive polarity mode, with the anode at the inlet and the cathode at the outlet. A constant voltage of 30 kV, with an initial ramping of 5000 $V \cdot s^{-1}$ (6 s), was applied during analysis, and the capillary temperature was maintained at 25°C. New bare FS capillaries, used under basic pH conditions, were rinsed at 1 bar with MeOH, 1 M HCl, water, 0.1 M NaOH, water, and background electrolyte (BGE) for 5 minutes each. New bare FS capillaries, used under acidic pH conditions, were rinsed at 1 bar with MeOH, 0.1 M NaOH, water, 1 M HCl, water, and BGE for 5 minutes each. Prior to each sample injection, the capillary was rinsed at 2 bar for 1 minute with fresh BGE. When not in use, the capillary was rinsed with water and then dry stored.

CE-UV experiments involved capillaries with a total length of 64.5 cm and an effective length of 56 cm. Samples were injected hydrodynamically at 50 mbar for 16 s (equivalent to 2% of the effective capillary length). UV detection was performed at 200 nm.

Separations were performed with 25-100 mM ammonium formate buffers (pH 2.0-3.5 and 8.0-10.0). The addition of different percentages of organic modifiers was tested (5-80%, *v/v,* of ACN, MeOH, or EtOH) under both basic and acidic conditions.

2.2.2. CE-MS system

CE was coupled to an Agilent Technologies 6210 LC/MS TOF mass spectrometer (Agilent, Palo Alto, CA, USA) *via* a tri-axial sheath flow ESI interface from Agilent. The coaxial sheath liquid was optimized in terms of the water-isopropanol-formic acid ratio. The delivery rate, by a syringe pump system, was also optimized (2-8 $\mu L \cdot min^{-1}$). Nebulizing gas pressure and drying gas flow rate were tested between 1 to 10 psi and 1 to 8 $L \cdot min^{-1}$, respectively. Drying gas temperature was optimized between 150 and 350°C. ESI voltage and fragmentor voltage were set at +4500 V and +170 V, respectively. MS detection was carried out in the positive ion mode, and 2 $spectra \cdot s^{-1}$ were acquired (4871 $transients \cdot spectrum^{-1}$).

CE-MS experiments involved capillaries with a length of 80 cm. Samples were injected hydrodynamically at 50 mbar for 30 s (equivalent to 2% of the capillary length).

2.3. Immunological detection

Unknown samples were analyzed using a commercially available immunological test (CMZ-Assay GmbH, Berlin, Germany). The complete methodology, according to Bidlingmaier *et al.* [9,10], was followed. First, samples were diluted $2x10^6$ times with the human zero serum provided with the kit (*i.e.*, non-containing hGH serum, recommended for sample dilution). Then, a 50 µL diluted sample was incubated for 2 hours with the provided buffer. After intensive washing, detection antibody was added and the mixture was incubated for 2 hours. Finally, the sample was washed and measurements were made using a luminometer (AutoLumat Plus LB 953, Berthold Technologies GmbH, Wildbad, Germany).

2.4. Software

Buffer solutions were prepared with the help of PHoEBuS software (version 1.3, Analis, Namur, Belgium). CE ChemStation (version A.10.02, Agilent, Waldbronn, Germany) was used for CE instrument control. MassHunter (version B.02.00, Agilent, Waldbronn, Germany) was used for data acquisition, data handling, and spectral deconvolution. LBIS (version 3.3, Berthold Technologies GmbH, Wildbad, Germany) was used to calculate hGH concentration from immunological results. Modde software (version 7.0, Umetrics AB, Umea, Sweden) was used to generate experimental designs and to process the data.

3. RESULTS AND DISCUSSION

3.1. Initial CE conditions

According to the literature, almost every electrophoretic method for the analysis of hGH has used basic pH conditions [16,18-20]. Because the protein and the capillary wall were both negatively charged under these conditions, adsorption mediated by electrostatic interactions was reduced. However, Catai *et al.* [22] showed that bare FS capillaries did not provide stable migration times (which increased as a function of performed runs) because other adsorption mechanisms could occur. This was confirmed in our preliminary experiments with a 75 mM ammonium formate buffer at pH 9.0. The addition of organic modifiers (ACN, MeOH, and EtOH) to the BGE did not significantly reduce adsorption. Furthermore, peak degradation occurred under alkaline conditions. For both hGH and rhGH, a minor peak was observed migrating next to the major peak (Figure 2) as a result of the deamidation of a specific asparagine residue (amino acid 152) into aspartic acid, promoted by the alkaline pH [5,7,17,23]. Because the carboxylic acid on the side chain of the aspartic acid possesses a pK_a value around 3.66 [24], the deamidated-hGH had an absolute mobility that was higher than the non-degraded hGH because of a supplementary negative charge at pH 9. According to the mass spectra (Figure 2) obtained by the TOF analyzer, the same ions were detected for deamidated-hGH and hGH. Therefore, the same deconvoluted mass was calculated for both compounds, namely 22125 *amu*. However, a difference of 1 Da should be observed between deamidated-hGH and hGH, arising from the difference between the mass of the carboxylic acid group (45 Da) on the aspartic acid side chain compared to that of the amide

group (44 Da) on the asparagine side chain. MS resolution (<10000 FWHM) was not sufficient to distinguish between the small *m/z* differences of the highly charged ions, and thus, hGH and deamidated-hGH exhibited a similar mass. Finally, it can be noted that no electrophoretic selectivity was observed between hGH and rhGH (Figure 2).

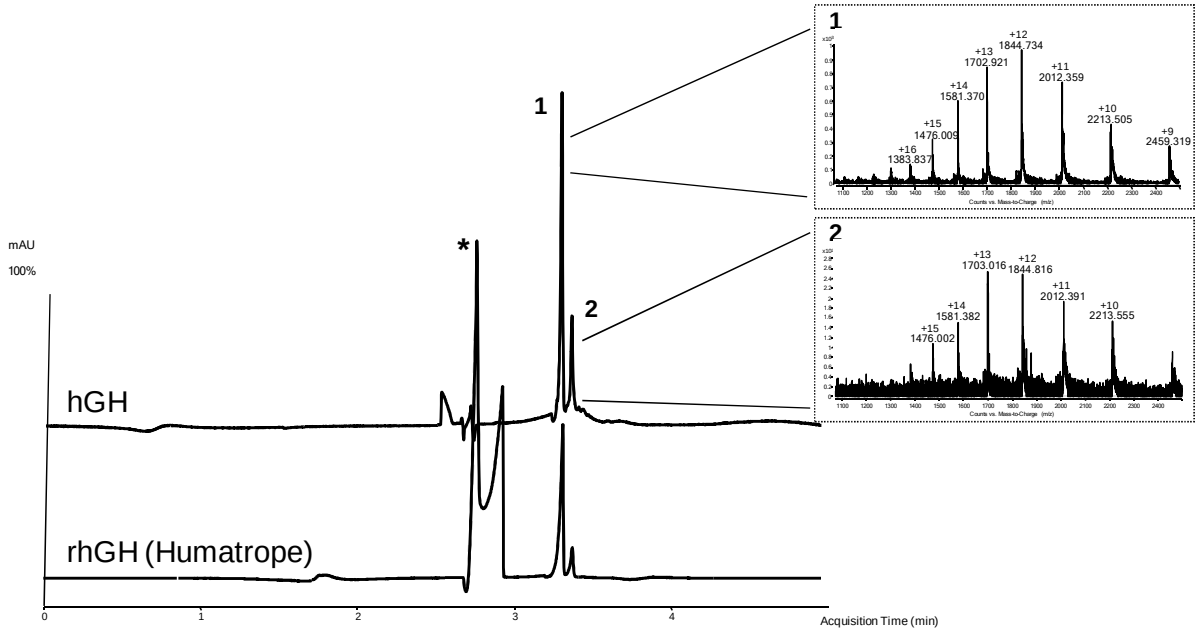

Figure 2: CE-UV electropherograms obtained with alkaline BGE and mass spectra obtained from CE-TOF/MS analyses. Upper trace: hGH at 200 µg/mL. Lower trace: rhGH (Humatrope) at 200 µg/mL. 1: intact hGH; 2: deamidated-hGH; *: excipients. See text for experimental conditions.

Therefore, alkaline conditions induced hGH degradation and adsorption on capillary wall. No electrophoretic discrimination was reached between hGH and rhGH. Other BGEs were subsequently considered to avoid protein degradation and adsorption, in order to decrease analysis variability and enhance selectivity.

3.2. Improved CE conditions

3.2.1. CE-UV

In order to improve the electrophoretic separation, acidic BGEs from pH 2.0 to 3.5 were evaluated. However, under these conditions, adsorption of proteins to the inner capillary surface occurred (Figure 3). The addition of organic modifiers to the BGE was then investigated. An organic solvent (ACN, MeOH, or EtOH) was added to the BGE between 5 and 80% (*v/v*). For the sake of clarity, only the best results obtained with 20% ACN in 75 mM ammonium formate buffer set at pH 2.5 are reported in Figure 3. Under these conditions, the adsorption was strongly reduced, which suggests that adsorption could be mainly due to hydrophobic interactions. Only one peak was detected and migration times were repeatable (RSD<2%, N=10). Degradation such as deamidation could not be excluded, with deamidated-hGH possessing a similar mobility to that of hGH because the carboxylic

acid would be mostly protonated at acidic pH and migrate as a neutral. It is noteworthy that in case of identification (in contrast to degradation studies), resolution between degraded and non-degraded forms is not mandatory, particularly if a powerful identification tool, such as TOF/MS detection, is used. The most interesting point was the ability to differentiate between hGH and rhGH because they possessed distinct apparent mobilities (Figure 3).

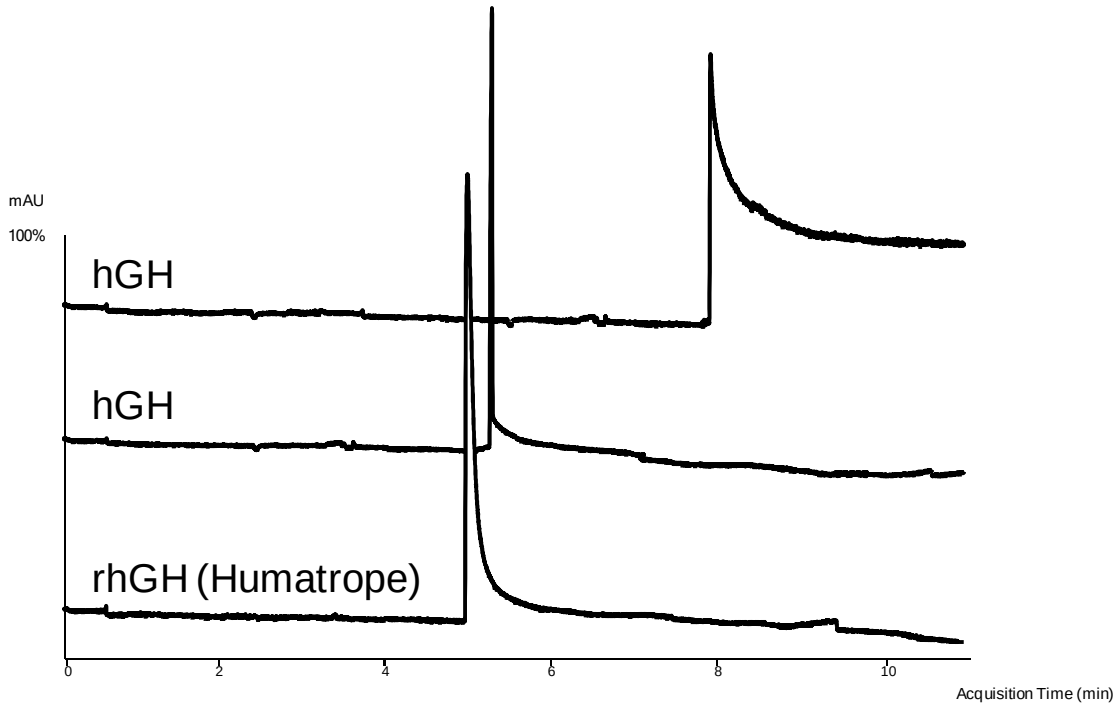

Figure 3: CE-UV electropherograms obtained with acidic BGE. Upper trace: hGH at 200 µg/mL without ACN. Middle trace: hGH 200 µg/mL with 20% ACN in BGE. Lower trace: rhGH (Humatrope) at 200 µg/mL with 20% ACN in BGE. See text for experimental conditions.

The simultaneous injection of a small molecule (benzylamine) as internal standard to correct for potential fluctuations in migration times confirmed that calculated mobilities were distinct in these conditions. It should be recalled that effective mobilities were identical for hGH and rhGH in basic conditions. To the best of our knowledge, it is the first description of a separative method that was able to distinguish endogenous hGH from recombinant hGH in their intact forms. Because recombinant hGH possesses an identical sequence to the naturally occurring 22 kDa hormone, both hGH and rhGH should have the same primary and secondary structure. However, the former is secreted by anterior pituitary gland, while the latter is produced by recombinant DNA technology. Differences in terms of conformation could thus not be excluded, particularly with the use of organic solvent and acidic condition. Consequently, hGH and rhGH could exhibit distinct size, leading to discrepancy in electrophoretic mobilities. In contrast to selectivity, the difference observed in efficiency might be explained by the respective compositions of the injected solutions. For instance, the dissolution solvent, provided by the manufacturer with rhGH, contained several additives in non-negligible concentrations that might not be appropriate for an optimal CE injection. On the contrary, hGH was extracted from anterior pituitary glands and did not contain excipients. Furthermore, it was dissolved in a buffer that perfectly matches in terms of stacking and

electrodispersion. The same discrepancy in efficiency was highlighted between other commercial rhGH and hGH (data not shown). Under these conditions, limits of detection (LOD, estimated with *H/N*=3) of approximately 5 $\mu g \cdot mL^{-1}$ (equivalent to 226 nM) were obtained with UV detection at 200 nm.

3.2.2. CE-ESI-TOF/MS

The improved CE-UV method was directly compatible with CE-MS. In order to implement a rugged and sensitive CE-ESI-TOF/MS method, ESI source parameters were optimized by a design of experiments (DOE) with a commercially available tri-axial sheath liquid interface already used for CE-ESI-MS experiments [25-27]. Sensitivity (*H/N*), peak height, noise, and peak efficiency were chosen as analytical responses, to reflect the fact that the aim of this study was to maximize sensitivity with a limited negative effect on efficiency.

In the first step, ESI voltage, capillary outlet position, and sheath liquid composition were determined by an unvaried procedure. The ESI voltage and the capillary outlet position were carefully adjusted to supply a stable ionization current in the ESI source [28]. The sheath liquid composition was optimized in terms of the nature and proportion of organic solvent and acid. The best ionization yield was obtained with an isopropanol-water-formic acid mixture (50:50:0.05, *v/v/v*). In the second step, a screening procedure was carried out, within a defined experimental range, to evaluate the influence of the following experimental variables on sensitivity and efficiency: sheath liquid flow rate (F_{Shea}, 2-8 $\mu L \cdot min^{-1}$), drying gas flow rate (F_{Dry}, 1-8 $L \cdot min^{-1}$), nebulizing gas pressure (P_{Neb}, 1-10 psi), and drying gas temperature (T_{Dry}, 150-350°C). Assessments of the experimental ranges for these variables were determined during the preliminary study, to ensure a measurable MS response (unless limited by instrumental values). Fourteen runs were randomly carried out using a half fractional factorial design (FFD) with 2^{5-2} runs and 6 central points. The screening study confirmed that all four parameters (F_{Shea}, P_{Neb}, F_{Dry}, and T_{Dry}) were influencing variables for either efficiency or sensitivity.

Hence, a second-degree design was applied to those relevant factors to obtain a response surface for sensitivity (*H/N*) and efficiency using a Box-Behnken (BB) design. The four-parameter design was developed according to the following pattern: two variables were set in a combination of their extreme values, while the other two variables were set to the center values. This mapping avoided edged situations that occurred with the regular FFD. This experimental design consisted of 24 trials and 6 trials at the center of the investigated ranges, which were narrowed compared to the previous FFD. A statistical study revealed the response fit, with R^2 values of 74% and 70% for *H/N* and efficiency, respectively. These values were considered sufficient to explain the observed responses, presented in Figure 4. As observed, surface responses exhibited curvature, indicating the importance of quadratic terms in the proposed model. Best responses were always obtained for a temperature fixed at its lowest value (*i.e.*, 150°C). Higher temperatures were found to be inappropriate for protein analysis because they might denature or even precipitate the protein. The optimal response in terms of sensitivity (Figure 4A) was achieved at 4 $L \cdot min^{-1}$ for F_{Dry} while maintaining an intermediate F_{Shea} (*ca.* 5 $\mu L \cdot min^{-1}$), with any P_{Neb}. However, the latter had a strong influence on efficiency (Figure 4B). It was thus set at 3 psi. The best result in terms of

efficiency was obtained with a high F_{Shea}. However, this was kept at 5 µL·min^{-1} to avoid a loss in sensitivity, maintaining an acceptable efficiency. As shown in Figure 4C, the mass spectrum obtained in optimized conditions showed a broader charge distribution than that obtained with the basic BGE (Figure 2). Consequently, a better sensitivity was obtained. The uncertainty of mass determination was calculated and was lower than 0.05 Da. With optimized conditions (150°C for T_{Dry} 4 L·min^{-1} for F_{Dry}, 5 µL·min^{-1} for F_{Shea}, and 3 psi for P_{Neb}), efficiency values up to 750000 plates were obtained, while LOD values of 50 µg·mL^{-1} (equivalent to 2.3 µM) were reached for both hGH and rhGH.

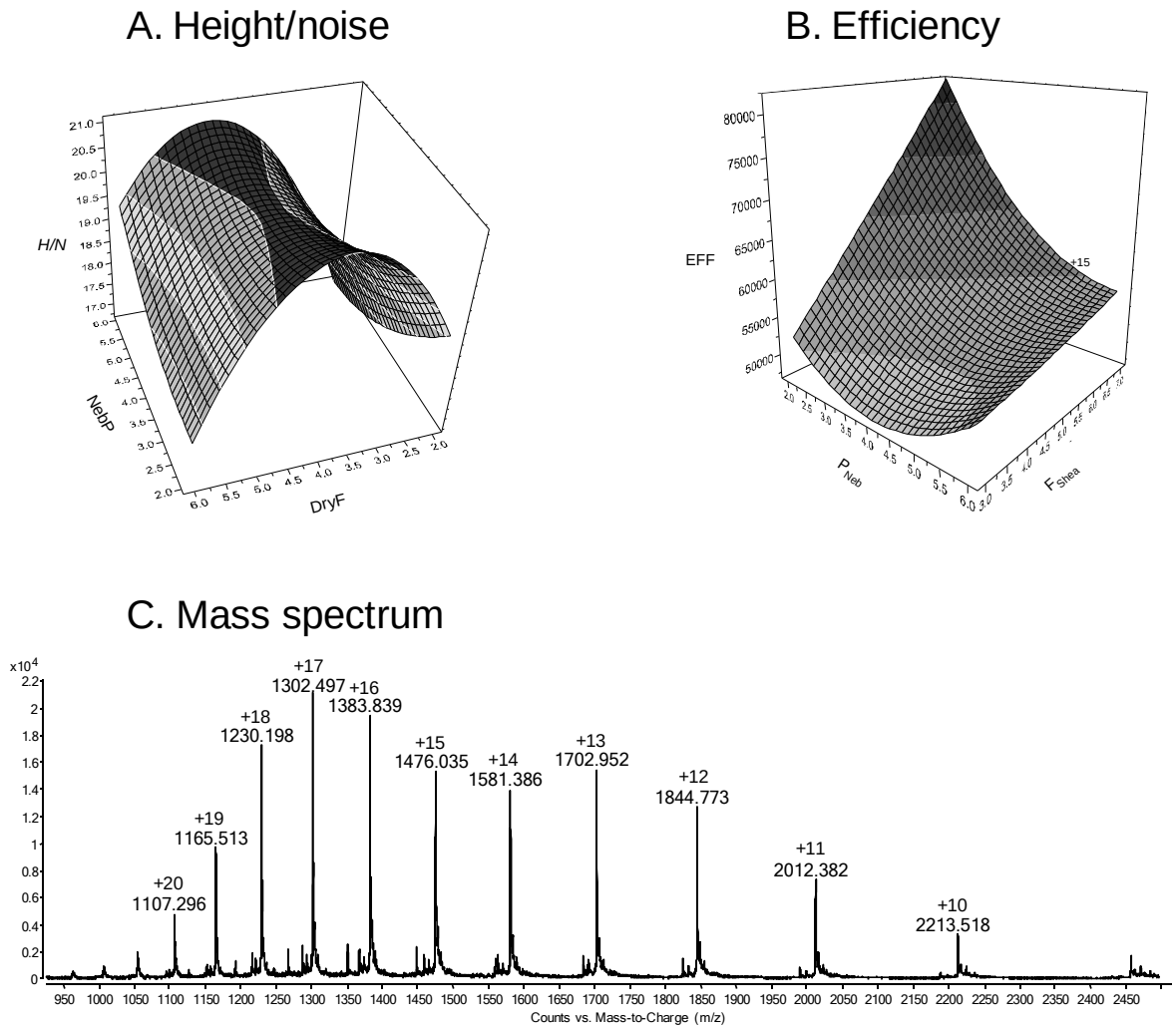

Figure 4: CE-ESI-TOF/MS optimization. A. Response surface modelling for sensitivity (*H/N*), depending on F_{Dry} and P_{Neb}, with 150°C T_{Dry} and 5 µL·min^{-1} F_{Shea}. B. Response surface modelling for efficiency, depending on F_{Shea} and P_{Neb}, with 150°C T_{Dry} and 4 L·min^{-1} F_{Dry}. C. Mass spectrum. See text for experimental conditions.

As observed in CE-UV, hGH mobility was lower than that of rhGH. Moreover, two deconvoluted peaks were emphasized in the hGH mass spectrum. A major peak possessed a mass of 22125 *amu*, while a minor peak presented a mass of 20270 *amu*, attributable to the 20 kDa isoform of hGH. This isoform accounted for 5% of the total protein quantity,

according to the manufacturer, and this was confirmed by the ratio of peak heights. The minor peak was totally absent from the MS deconvolution spectra for rhGH. This is in agreement with the fact that rhGH has an identical sequence to the only naturally occurring 22 kDa hormone.

To summarize, the CE method, under acidic conditions with ACN, allowed the discrimination of hGH from rhGH according to their respective mobilities in less than 10 min without adsorption or sample preparation. The TOF/MS detection confirmed this information, giving the exact mass of the hGH and allowing for a second distinction between hGH and rhGH by the presence (respectively absence) of the 20 kDa isoform.

3.3. Application to unknown samples

The immunological test, applied to unknown samples, allowed hGH detection but hindered to clearly indicate whether the detected signal came from recombinant or endogenous hGH variants.

These two unknown samples were analyzed with the aforementioned CE-ESI-TOF/MS methodology. No particular sample preparation was mandatory and the diluted samples were directly injected into the analytical system. Both standard hGH and rhGH (Humatrope) solutions were also analyzed under the same conditions. Figure 5A shows the electropherograms obtained for each sample. Both unknown samples presented the same electrophoretic mobility as the standard rhGH sample, and respective deconvoluted mass spectra did not reveal any 20 kDa isoform (Figure 5B). In addition to these observations, the first sample possessed a deconvoluted mass of 22125 *amu* and was thus confirmed as rhGH. The second sample possessed a deconvoluted mass of 22157 *amu*. The difference of +32 *amu* was attributed to the dioxidation of rhGH. In fact, methionine residues at positions 14 and 125 (Figure 1) could be prone to oxidation, while a third methionine residue at position 170 could not be oxidized in the native protein, as it is located in the interior of the protein [5,22,29]. In the context of drug seizure for instance, the hypothesis of inappropriate storage conditions could be raised with oxidative agents (oxygen), UV light exposure (photolysis), and/or heat (microbial growth) as possible causes.

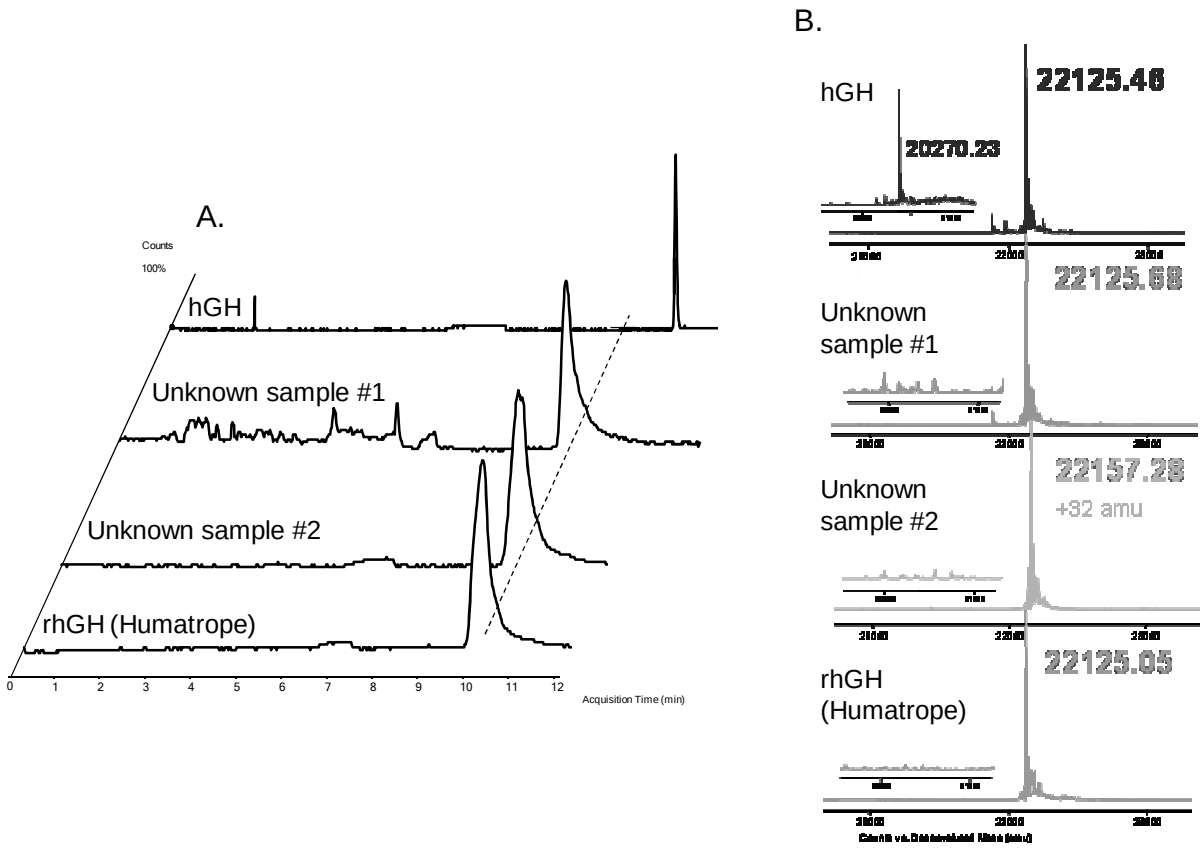

Figure 5: Analysis of unknown samples. A. CE-ESI-TOF/MS electropherograms. Upper trace: hGH at 200 µg·mL^{-1}. Upper middle trace: unknown sample #1. Lower middle trace: unknown sample #2. Lower trace: rhGH (Humatrope) at 200 µg·mL^{-1}. B. Respective deconvoluted mass spectra. See text for experimental conditions.

4. CONCLUDING REMARKS

A CE-ESI-TOF/MS method was developed for the rapid analysis of human growth hormone. Attention was focused on optimizing CE and ESI conditions for enhancing selectivity, efficiency, and sensitivity, while hindering protein adsorption on the capillary wall. The best CE conditions were obtained at acidic pH values with the addition of acetonitrile. In order to optimize CE-ESI-MS interfacing, a chemometric approach was implemented to emphasize the most relevant factors and to determine the optimal conditions. The optimized method presented several features: (i) rapid identification without sample preparation and (ii) ability to distinguish endogenous hGH from recombinant hGH. The latter was accomplished according to two levels of selectivity. First, hGH and rhGH possessed different electrophoretic mobilities. Second, deconvolution of the respective mass spectra emphasized that hGH contained both the 22 kDa and 20 kDa isoforms, whereas rhGH only exhibited the 22 kDa isoform. The CE-ESI-TOF/MS method was subsequently applied to successfully identify rhGH in unknown samples. This method was more efficient at unambiguously attributing the origin of hGH than the commercial immunological method.

5. ACKNOWLEDGMENTS

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The authors also wish to thank Dr Neil Robinson for helpful and valuable comments.

6. ABBREVIATIONS

EtOH	ethanol
F_{Dry}	drying gas flow rate
F_{Shea}	sheath liquid flow rate
FFD	fractional factorial design
hGH	human growth hormone
H/N	height-to-noise ratio
MeOH	methanol
P_{Neb}	nebulizing gas pressure
rhGH	recombinant human growth hormone
T_{Dry}	drying gas temperature

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II.5 Application de méthodes CE-UV et CE-TOF/MS à la détection de transporteurs d'oxygène à base d'hémoglobine

Le dopage sanguin est particulièrement répandu dans les sports d'endurance tels que le cyclisme, la natation ou la course à pieds. Il consiste en l'augmentation de la prise, du transport ou de la mise à disposition de l'oxygène. La prise d'érythropoïétine ou l'utilisation de transfusions sanguines sont les modes les plus utilisés. Un troisième mode consiste à prendre des produits à base d'hémoglobine polymérisée, à savoir les transporteurs d'oxygène à base d'hémoglobine (HBOC). L'hémoglobine est une protéine globulaire composée de quatre sous-unités, chacune contenant une chaîne et un hème. Il existe plusieurs variants présents naturellement, ceux-ci différant de par leur composition en chaînes. L'uilisation directe de l'hémoglobine a été dans un premier temps envisagée, mais celle-ci, une fois injectée directement dans le sang, provoque une toxicité rénale. C'est donc tout d'abord pour cette raison que la polymérisation de l'hémoglobine a été testée. Le fait que le transport d'oxygène soit de plus augmenté jusqu'à trois fois a ajouté une plus-value à cette étape de polymérisation. Développés initialement pour le traitement des cas lourds d'anémie, les HBOCs sont maintenant détournés dans un cadre de dopage afin d'améliorer les performances. Plusieurs d'entre eux sont actuellement en essais cliniques mais une seule spécialité est disponible sur le marché, l'Oxyglobin®. Cette dernière est composée d'hémoglobine bovine polymérisée.

L'**article V** présente une nouvelle approche pour la détection de ce type de dopage, basée sur la CE, couplée soit à un détecteur UV/Vis soit à un spectromètre de masse à temps de vol (TOF). La détection UV/Vis à 415 nm présente une sélectivité des plus intéressantes dans la mesure où elle détecte sélectivement les protéines contenant un hème, ce qui est le cas de l'hémoglobine et ses dérivés, au contraire de la plupart des autres protéines sanguines. Ce mode de détection peut donc être employé ici à condition d'être capable de séparer l'hémoglobine qui peut être naturellement présente dans l'échantillon plasmatique du sportif (hémolyse mécanique) de l'éventuelle présence du dérivé polymérisé d'hémoglobine, signe de dopage. La CE permet cela grâce à une méthode développée avec un tampon à pH basique, sans autres additifs, l'adsorption étant minime sur standards.
Le TOF permet bien évidemment une sélectivité supérieure. Un fait intéressant a pu être noté. L'hémoglobine et ses dérivés migrent dans le capillaire CE sous leur forme intacte, mais une fois dans la source électrospray, au contact des composants acides et organiques du liquide additionnel, il se produit une dissociation des globines de l'hémoglobine dans le cône de Taylor. Ainsi, après déconvolution et grâce à l'exactitude de masse du TOF, les masses moléculaires des chaînes composant l'hémoglobine sont détectées individuellement. Il est possible de différencier les variants naturels de l'hémoglobine ainsi que les hémoglobines de différentes espèces animales car les masses de leurs chaînes sont suffisamment différentes. Ce point est particulièrement important de par la nature de l'Oxyglobin® car *via* le TOF une sélectivité d'espèce est obtenue entre le produit dopant d'origine bovine et l'hémoglobine naturelle humaine. Cela ajoute une dimension supplémentaire à la sélectivité déjà possible grâce à la CE.

Le dernier point à aborder était le problème de la préparation des échantillons sanguins. En effet, si l'adsorption était minime avec les standards d'hémoglobine et d'Oxyglobin®, les protéines abondantes du plasma rendaient les temps de migration très variables et la qualité de séparation moindre. Une approche d'immunodéplétion sur cartouche a été utilisée. *Via* des interactions avec des anticorps spécifiques, vingt des protéines les plus abondantes ont ainsi été déplétées avec un rendement de l'ordre de 50%, ce qui s'avère suffisant pour permettre des analyses CE répétables.

Les limites de détection obtenues sont compatibles avec les valeurs-seuils spécifiées par les autorités de lutte antidopage et la méthode pourrait donc être appliquée à des échantillons réels.

II.5.1 Article V

A. Staub, S. Rudaz, M. Saugy, J.L. Veuthey, J. Schappler. **Analysis of hemoglobin-based oxygen carriers (HBOCs) by CE-UV/Vis and CE-ESI-TOF/MS**, *Electrophoresis* 2010, *31*, 1241-1247.

Analysis of hemoglobin-based oxygen carriers (HBOCs) by CE-UV/Vis and CE-ESI-TOF/MS

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Electrophoresis 2010, *31*, 1241-1247.

ABSTRACT

Blood doping involves the use of products that enhance the uptake, transport, or delivery of oxygen to the blood. One approach uses artificial oxygen carriers, known as hemoglobin-based oxygen carriers (HBOCs). This paper describes an analytical strategy based on CE for detecting intact HBOCs in plasma samples collected for doping control. On-capillary detection was performed by UV/Vis at 415 nm, which offered detection selectivity for hemoproteins (such as hemoglobin and HBOCs). On-line ESI-MS detection with a TOF analyzer was further used to provide accurate masses on CE peaks and to confirm the presence of HBOCs. An immunodepletion sample preparation step was mandatory prior to analysis, in order to remove most abundant proteins that interfered with CE separation and altered the ESI process. This analytical method was successfully applied to plasma samples enriched with Oxyglobin®, a commercially available HBOC used for veterinary purposes. Detection limits of 0.20 $g \cdot dL^{-1}$ and 0.45 $g \cdot dL^{-1}$ were achieved in plasma for CE-UV/Vis at 415 nm and CE-ESI-TOF/MS, respectively.

KEYWORDS

Blood doping, CE, Hemoglobin-based oxygen carriers, TOF/MS

1. INTRODUCTION

Blood doping is defined by the WADA (World Anti-Doping Agency) as the use of products that enhance the uptake, transport, or delivery of oxygen to the blood. Most commonly, this involves either erythropoietin (EPO) uptake or blood transfusion. Another approach uses artificial oxygen carriers, known as hemoglobin-based oxygen carriers (HBOCs) [1]. HBOCs were initially developed as artificial blood replacement products to substitute the oxygen-carrying functions of erythrocytes [2]. However, they are now misused as performance enhancers. These products are made of bovine or human hemoglobin (Hb), which is intra- or intermolecularly cross-linked, polymerized, or conjugated. These procedures are necessary to prevent the Hb tetrameric structure from dissociating into dimers, leading to nephrotoxicity. Furthermore, these stabilized Hb molecules improve oxygen off-loading due to their decreased oxygen affinity [3,4]. Although several HBOCs are under development or clinical trials (*e.g.,* Polyheme®, Northfield) [5], few such compounds of bovine origin have been approved by authorities. Among them, Oxyglobin® (Biopure) is the only one approved by the FDA for veterinary purposes, while Hemopure® (Biopure) was approved for human use in South Africa and is currently in clinical trials in Europe and elsewhere. Oxyglobin® and Hemopure® being both made of glutaraldehyde polymerized bovine hemoglobin, the only difference between both products is the final filtration of low molecular weight components, which is applied to Hemopure®.

Three qualitative methods have been developed as screening or confirmatory procedures for doping control analysis in human serum: a slab-gel electrophoretic technique coupled to the selective detection of heme-containing molecules [6], a method based on size exclusion liquid chromatography (SEC-LC) [7], and an LC-ESI-MS/MS method [3]. More recently, Simitsek *et al.* have presented methodological modifications to increase the selectivity of the latter [8]. Capillary electrophoresis (CE) appears to be an interesting alternative technique for HBOC analysis in the context of doping control, since different methods have already been developed for the analysis of Hb variants [9,10,11,12]. In addition, the on-line combination of CE with MS is an attractive option for intact protein analysis (*i.e.*, no digestion, no derivatization step required) [13]. On the one hand, CE offers features such as high speed, great efficiency, and low solvent and sample consumptions. Moreover, CE allows working under aqueous conditions and without stationary phase. On the other hand, MS provides selectivity and ability to identification. Time-of-flight (TOF) instruments are particularly well suited to protein analysis, due to their high mass range and mass accuracy [14].

For analyzing Oxyglobin® in plasma samples, the removal of a high amount of undesirable proteins is necessary, because these can interfere with the separation and the detection of the proteins of interest, particularly when the latter are present at low concentrations. It is well known that a few proteins (*ca.* 22) account for approximately 99% of the total amount of plasma proteins (*e.g.*, albumin accounts for 50%) [15]. Therefore several approaches have been developed to remove these interfering proteins: among them, affinity-based methods (*e.g.*, protein A/G, peptide-based ligands, etc.), size-based methods (ultrafiltration), antibody-based methods (with monoclonal or polyclonal antibodies), or isoelectric focusing techniques (*e.g.*, Off-Gel™) [16]. In this study, a sample preparation based on immunodepletion was

performed prior to CE-UV/Vis and CE-ESI-TOF/MS analysis of intact Oxyglobin® in plasma samples collected for doping control.

2. MATERIALS AND METHODS

2.1 Chemicals and samples

Ammonium hydroxide solution (25%, *m/m*) and formic acid (98%, *m/m*) were of analytical grade from Fluka (Buchs, Switzerland). Isopropanol and sodium hydroxide were of analytical grade from Acros Organics (Geel, Belgium). Ultrapure water was supplied by a Milli-Q gradient A10 purification unit from Millipore (Bedford, MA, USA).
Oxyglobin® stock solution at 13 $g \cdot dL^{-1}$ was purchased from Biopure (Cambridge, MA, USA). Standard solutions of Oxyglobin® at the desired concentration were prepared daily by appropriate dilution of stock solution in water. Haptoglobin (Hp), albumin from human serum (HSA), hemoglobin A_0 from bovine serum, hemoglobin A_0 (Hb A_0) and A_2 (Hb A_2) from human serum were obtained from Sigma-Aldrich (St Louis, MO, USA). Blank plasma was obtained from the Blood Center of the Geneva Hospital (Geneva, Switzerland). CEofix® HbA_2 coating was purchased from Analis (Suarlee, Belgium).

2.2 Sample preparation

2.2.1 Procedures

Two sample preparation procedures were investigated, starting with 40 µL of plasma spiked with the appropriate amount of Oxyglobin®. The first procedure consisted of a 2:1 dilution of plasma with water (*v/v*). The second procedure was an immunodepletion step, using a ProteoPrep® 20 Plasma Immunodepletion Kit (Sigma-Aldrich, St Louis, MO, USA). This kit could be regenerated up to 100-fold. The depletion procedure was carried out on a spin column according to the manufacturer's instructions, except that the sample concentration step was carried out using a Minicon CS15 system (Millipore, Bedford, MA, USA). This last step saved time and avoided some problematic manipulations (*e.g.,* unrepeatable multiple ultracentrifugation steps). Thirty microliters of the final immunodepleted sample were obtained, transferred to an appropriate vial, and directly injected into the CE system.

2.2.2 Performance

Protein depletion efficiency (DE) was evaluated by monitoring blank plasma UV absorbance at 280 nm before and after immunodepletion by UV.
Oxyglobin® recovery without matrix (R1), Oxyglobin® recovery with matrix (R2) and overall process efficiency (PE) were evaluated according to the method developed by Marchi *et al.* [17] (Figure 1). Four types of samples were prepared. Sample A was used to evaluate the 415 nm UV/Vis absorbance of neat standard of Oxyglobin® in water. Sample B consisted of blank plasma spiked with Oxyglobin® after immunodepletion. Sample C was prepared with blank plasma spiked with Oxyglobin® before immunodepletion. Sample D was prepared in

water spiked before immunodepletion. The respective concentration of Oxyglobin® in each sample was calculated to reach an equivalent concentration after immunodepletion (0.30 $g \cdot dL^{-1}$), taking into account dilution factors and a theoretical recovery of 100%. Samples B, C and D were first immunodepleted with one loading on the ProteoPrep® 20 spin column and then with two loadings in order to evaluate and compare both processes. By comparing UV/Vis absorbance at 415 nm of sample C over A, PE could be estimated. The ratio of D over A allowed the evaluation of R1. Finally, the ratio of C over B allowed the estimation of R2.

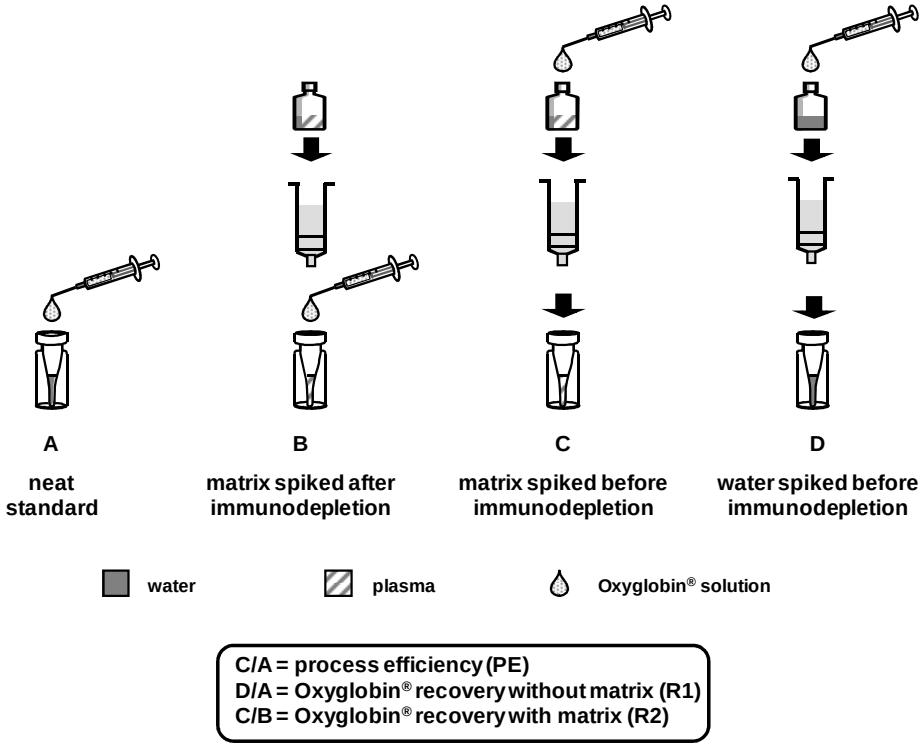

Figure 1: Schematic illustration of experiments for the evaluation of immunodepletion performance (adapted from [17]).

2.3 Instrumentation

2.3.1 CE system

CE experiments were performed with an HP ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. Separations were performed using a BGE consisting of 75 mM ammonium formate (pH 9.5) in an uncoated fused silica (FS) capillary (BGB Analytik AG, Böckten, Switzerland) with a 75 µm I.D. Experiments were carried out in positive polarity mode, with the anode at the inlet and the cathode at the outlet. A constant voltage of 30 kV, with an initial ramping of 5000 $V \cdot s^{-1}$, was applied during analysis, and the capillary temperature was maintained at 25°C. New FS capillar ies were rinsed with MeOH, 1 M HCl, water, 0.1 M NaOH, water, and BGE at 1 bar for 5 minutes each. Prior to each sample

injection, the capillary was rinsed at 2 bar for 1 minute with fresh BGE. When not in use, the capillary was rinsed with water and stored dry.
CE-UV experiments involved capillaries with a total length of 64.5 cm and an effective length of 56 cm. Samples were injected hydrodynamically for 12 s at 25 mbar (equivalent to 1.6% of effective capillary length). UV/Vis detection was performed at 415 nm.

2.3.2 CE-MS system

The CE instrument was coupled to a 6210 LC/MS TOF mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) *via* a coaxial sheath flow ESI interface from Agilent. The sheath liquid consisted of isopropanol-water-formic acid (48.5:48.5:3, *v/v/v*) and was delivered at a flow rate of 4 $\mu L \cdot min^{-1}$ by a syringe pump system. ESI capillary voltage was set at +4500 V, the nebulizing gas pressure at 4 psi, the drying gas flow rate at 4 $L \cdot min^{-1}$, the drying gas temperature at 150°C, and the fragmentor voltage at 170 V. MS detection was carried out in the positive ion mode and 1 $spectrum \cdot s^{-1}$ was acquired (9742 transients/spectrum).
CE-MS experiments involved capillaries with a length of 80 cm. Samples were injected hydrodynamically for 10 s at 50 mbar (equivalent to 1.5% of capillary length).

2.3.3 UV/Vis spectrophotometer

UV/Vis experiments were performed with a Lambda 20 UV/VIS Spectrometer (PerkinElmer, Waltham, MA, USA). The wavelength was set at 280 nm for protein DE experiments and at 415 nm for Oxyglobin® recovery (R1 and R2) and PE evaluation experiments.

2.4 Software

BGE solutions were prepared with the help of PHoEBuS software (version 1.3, Analis, Namur, Belgium). CE ChemStation, (version A.02.10 Agilent, Waldbronn, Germany) was used for CE instrument control. MassHunter (version B.02.00, Agilent, Waldbronn, Germany) was used for data acquisition, data handling, and spectral deconvolution.

3. RESULTS AND DISCUSSION

3.1 Method development

3.1.1 Electrophoretic selectivity

Oxyglobin® and Hb can be found simultaneously in plasma since the former is a circulating polymerized protein and the latter can be released from erythrocytes due to mechanical hemolysis [18] and/or sampling conditions [19]. Therefore, the first aim of this work was to obtain sufficient electrophoretic selectivity between Oxyglobin® and the main Hb variants. Several CE conditions were tested (*e.g.,* pH, electrolyte nature and concentration) and the best results were obtained using a BGE consisting of ammonium formate (75 mM at pH 9.5,

Figure 2). Analyses lasted less than 4 minutes with repeatable migration times (RSD<2%, N=10). Because Oxyglobin® and Hb both have pI values between 6.9 and 7.4, they migrated as negatively-charged species at pH 9.5.

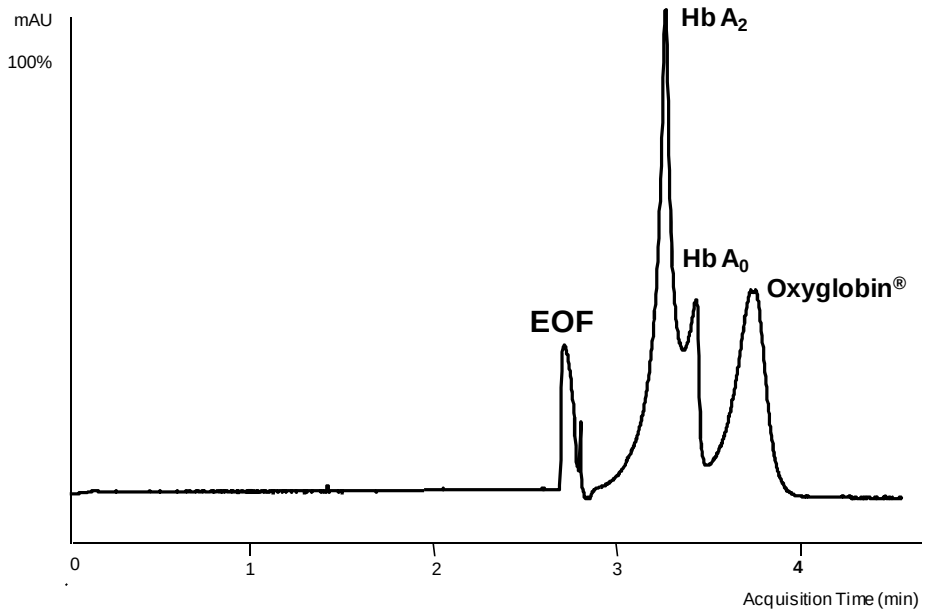

Figure 2: CE-UV electropherogram obtained by injecting a mixture of Hb A_2 (0.1 g·dL^{-1}), Hb A_0 (0.1 g·dL^{-1}), and Oxyglobin® (0.2 g·dL^{-1}). UV/Vis at 415 nm online. See text for experimental conditions.

3.1.2 Detection selectivity

Some selectivity was obtained using UV/Vis absorbance at 415 nm. At this wavelength, hemoproteins such as Oxyglobin® and Hb were easily differentiated from the other proteins by the absorbance of their heme moieties.

Hb and Oxyglobin® were also detected by ESI-TOF/MS. The source conditions were optimized to reach maximal sensitivity, obtained in positive electrospray polarity mode (ESI+). Negative mode (ESI-) was tested, but without success. The use of a sheath liquid interface allowed for tuning ionization parameters independently from CE conditions [13]. Thus, electrophoretic selectivity could be optimized by varying the composition of the BGE, while ionization conditions were independently optimized by adjusting the sheath liquid composition. Consequently, two interesting features were emphasized.

First, because Hb and Oxyglobin® were separated according to the electrophoretic mobility of their anionic forms (pH 9.5), but detected in ESI+ as cationic species, a high percentage of formic acid was added to the sheath liquid (*i.e.*, 3%, *v/v*), in order to protonate proteins in the source.

Second, while Hb and Oxyglobin® migrated in their intact forms, they were detected as their respective dissociated chains. Specifically, the quaternary structure of Hb comprises two pairs of heme-containing α- and β- or δ-subunits in a tetrahedral arrangement. The non-

covalent bonds stabilizing the complex (*via* non-polar and van der Waals interactions, hydrogen bonds, and salt bridges) can be easily overcome, leading to the reversible dissociation of Hb and its synthetic analogues into dimers and monomers [12,20-22]. Under acidic and alcoholic conditions, such as those in the sheath liquid, monomers of Hb were detected due to in-Taylor cone dissociation of the tetramers. Figure 3 shows deconvoluted spectra of human Hb A_0 and A_2. Both molecules possess α chains (15126 *amu*) but differ regarding other chains: Hb A_0 contains β chains (15867 *amu*), while Hb A_2 contains δ chains (15924 *amu*). Figure 4 shows deconvoluted spectra of human Hb A_0, bovine Hb A_0, and Oxyglobin®. Similar ions were detected by the TOF analyzer for bovine Hb A_0 and Oxyglobin® chains. The same deconvoluted masses were thus calculated, *i.e.*, 15053 and 15954 *amu* for α and β chains, respectively. Taken into account the theoretical values for Oxyglobin® chains, the mass determination uncertainty was equal to 15.3 ppm and 3.8 ppm for α and β chains, respectively.

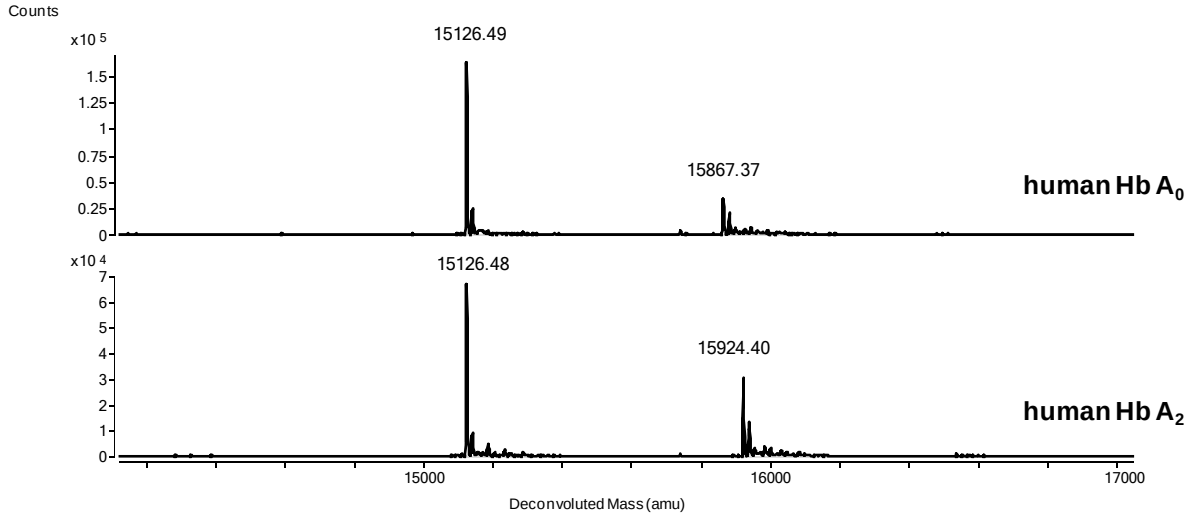

Figure 3: CE-ESI-TOF/MS deconvoluted mass spectra of human Hb. Upper trace: Hb A_0 (0.1 $g \cdot dL^{-1}$). Lower trace: Hb A_2 (0.1 $g \cdot dL^{-1}$). See text for experimental conditions.

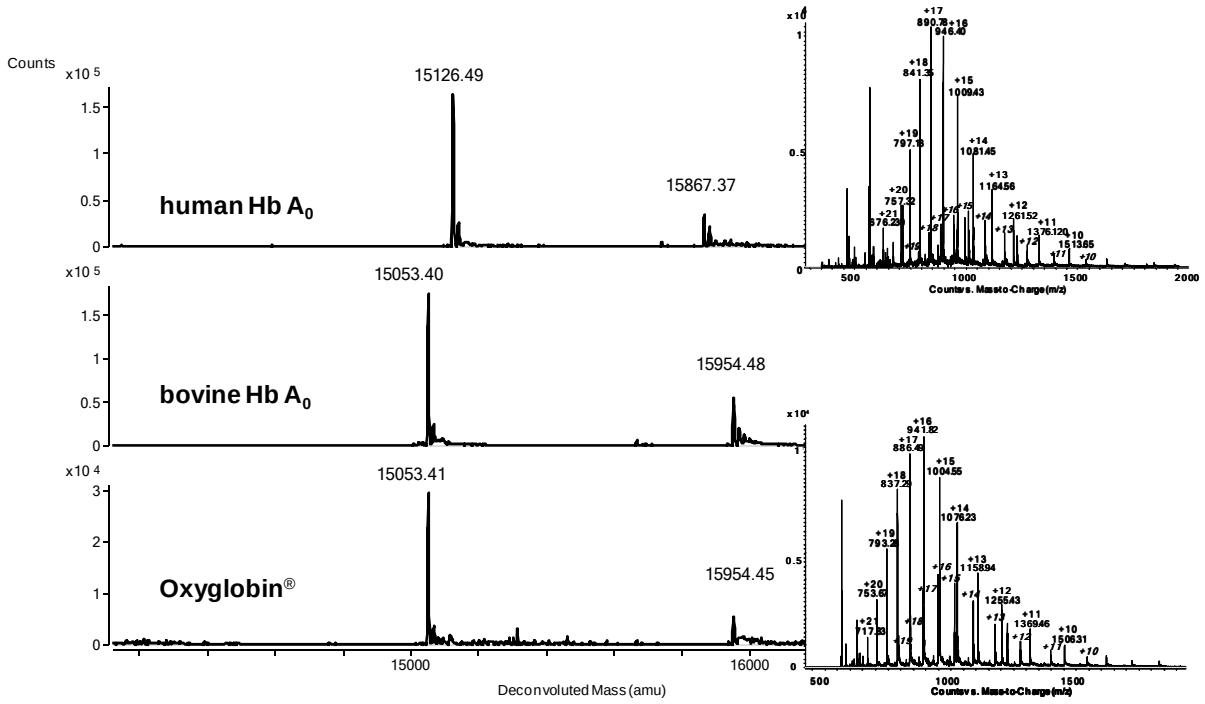

Figure 4: CE-ESI-TOF/MS deconvoluted mass spectra and respective MS spectra. Upper trace: Hb A_0 (0.1 g·dL^{-1}). Middle trace: bovine Hb A_0 (0.1 g·dL^{-1}). Lower trace: Oxyglobin® (0.2 g·dL^{-1}). See text for experimental conditions.

While CE enabled selectivity between intact Hb variants and Oxyglobin® in less than 6 minutes, UV/Vis at 415 nm and ESI-TOF/MS detections allowed for increased selectivity between hemoproteins and other proteins (by UV/Vis at 415 nm), and between bovine and human Hb, as well as between different Hb variants (by ESI-TOF/MS).

3.2 Application to plasma samples

3.2.1 Dilute and shoot

First, a dilute and shoot procedure was tested. Plasma spiked with Oxyglobin® at 0.3 g·dL^{-1} was diluted with water in a 2:1 ratio (*v/v*). The sample was directly injected into the CE system. As shown in Figure 5A, even if endogenous proteins, such as albumin and immunoglobulins, were not observed at 415 nm, their presence at high concentrations in plasma interfered with the separation. Their putative adsorption on the capillary wall has induced: (i) a degradation of Oxyglobin® peak, (ii) a reduction in the degree of Hb-Oxyglobin® resolution, and (iii) a decrease of repeatability (RSD>5%, N=3). To overcome problems due to adsorption, a dynamic coating (CEofix® HbA_2) was tested. The repeatability was improved, but only when a post-washing step (NaOH 0.1 M followed by BGE) was implemented between each analysis to regenerate the capillary surface. It is worth mentioning that these procedures were not compatible with MS detection. Furthermore, these additional conditions increased the analysis time and did not resolve other issues. In addition, a ghost peak appeared next to the Oxyglobin® peak. This degradation did not appear with blank plasma

(Figure 5B) or with standard Oxyglobin® solutions (Figure 5C) treated by the same procedure.

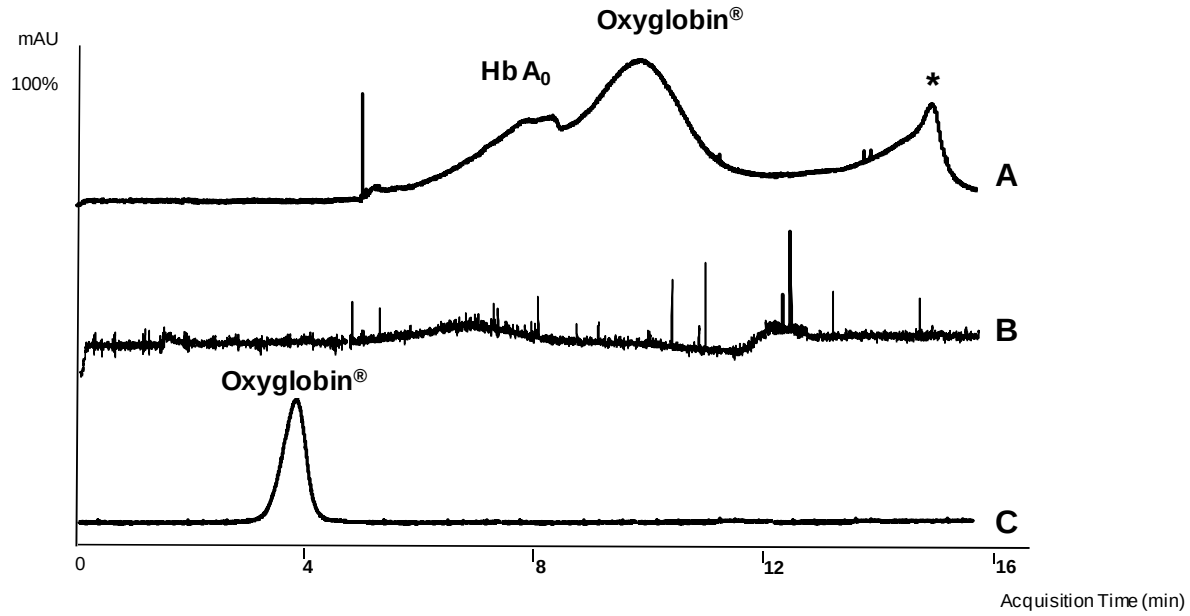

Figure 5: CE-UV electropherograms after dilute and shoot procedure. Trace A: Hb A_0 (0.1 $g \cdot dL^{-1}$) and Oxyglobin® (0.3 $g \cdot dL^{-1}$) in plasma, *: ghost peak. Trace B: blank plasma. Trace C: Oxyglobin® (0.2 $g \cdot dL^{-1}$) in water. UV/Vis 415 nm online. See text for experimental conditions.

3.2.2 Proteins removal by immunodepletion

Immunodepletion with a ProteoPrep® 20 Plasma Immunodepletion Kit was selected to remove interfering proteins, based on various criteria, including: (i) the ability to remove interfering proteins, (ii) Oxyglobin® recovery, (iii) CE and CE-MS compatibility, (iv) cost, and (v) process length. This kit is reported able to remove 20 of the most abundant proteins from human plasma or serum, *via* a high density conjugation process using small recombinant immunoaffinity ligands and conventional antibodies. The system was tested with blank plasma between Oxyglobin® analyses and no carry-over was observed. The kit also permitted up to 2 loadings of the same plasma sample on the spin column to increase the degree of protein depletion. In this study, the samples treated with one *vs.* two loadings were compared on the basis of protein depletion efficiency (DE), Oxyglobin® recovery (R1 and R2), and sample processing time.

Immunodepletion performance was evaluated by monitoring the 280 nm absorbance of blank plasma before and after immunodepletion. After one loading on the ProteoPrep column, 50% of total proteins were removed. The second loading removed an additional 21% of proteins, resulting in a total DE of 71%.

Similarly, the ProteoPrep® performance was evaluated in terms of Oxyglobin® recovery, without (R1) or with (R2) the matrix, and process efficiency (PE) by UV/Vis absorbance at 415 nm (Figure 1). After one loading on the spin column, a PE of 72% was attained, along with R1 and R2 values of 82% and 75%, respectively. Overall, these results were satisfactory for the present application. After two loadings, R2 was found to be 60%, R1 was 66%, and

PE of 59% was observed. The preparatory procedure itself was thus the main source of Oxyglobin® loss during the sample preparation (R1), because of adsorption of Oxyglobin® on the cartridge medium and the Minicon® system. On the other hand, the matrix had only a limited effect (6-7%, R2) on Oxyglobin® recovery, regardless of the number of loadings on the spin column. Since a very low matrix effect was observed for UV/Vis measurements, PEs were found to be similar to R2s.

While a single loading on the immunodepletion column resulted in a 50% depletion of interfering proteins, with 75% recovery of Oxyglobin®, the second loading increased the amount of proteins depletion (71%), but also lowered the Oxyglobin® recovery to 60%. On the basis of these data, a single loading on the column was considered optimal, as it provided appropriate levels of depletion, without requiring any coating or post-washing steps. Moreover, a single loading provided adequate Oxyglobin® recovery, and reduced the overall processing time by 35%.

Figure 6 shows the electropherograms obtained following immunodepletion. In contrast to the “dilute and shoot” procedure, immunodepletion avoided adsorption problems. Furthermore, the migration time repeatability was satisfactory (RSD <3%, N=5), and no capillary coating or post-washing procedures were required, resulting in reduced analysis time and greater MS-compatibility. The ghost peak next to the Oxyglobin® peak remained present (Figure 6A) and was not found to be due to interfering proteins, as immunodepleted blank plasma did not exhibit such a peak (Figure 6B). Similarly, no peak appeared when an aqueous Oxyglobin® standard was treated by immunodepletion (Figure 6C).

An important point concerned the removal of haptoglobins (Hp), because these proteins have a great affinity for circulating Hb. As previously mentioned, the presence of Hb in plasma samples could not be avoided due to potential hemolysis as a result of mechanical stress and/or sampling conditions. The Hb-Hp complex exhibited the same mobility as Oxyglobin® under experimental conditions (Figure 6D), and demonstrated significant UV/Vis absorbance at 415 nm, due to the Hb component of the complex. The Hb-Hp complex also dissociated during the ionization process and individual Hb globin chains were detected. In case of Oxyglobin® uptake, the discrimination was achieved because of the mass differences. False positive results could thus be excluded thanks to MS measurements. In presence of HBOCs made of human polymerized hemoglobin, no discrimination could be achieved by MS. Therefore, Hb-Hp complex had to be removed to eliminate false positive results. Since the ProteoPrep® 20 Plasma Immunodepletion Kit contains specific antibodies for human Hp, its potential for Hb-Hp complex removal was evaluated based on maximal circulating Hp concentrations (2360 $mg \cdot mL^{-1}$). Because the matrix had a limited effect on immunodepletion performance, a water sample containing 2500 $mg \cdot mL^{-1}$ of Hp and 1920 $mg \cdot mL^{-1}$ of Hb was treated by the immunodepletion procedure (an Hp/Hb ratio of 1.3 was chosen on the basis of the manufacturer’s information). Because no peak was detected at 415 nm, it was concluded that the immunodepletion procedure was sufficiently effective at removing Hb-Hp complexes for the present application.

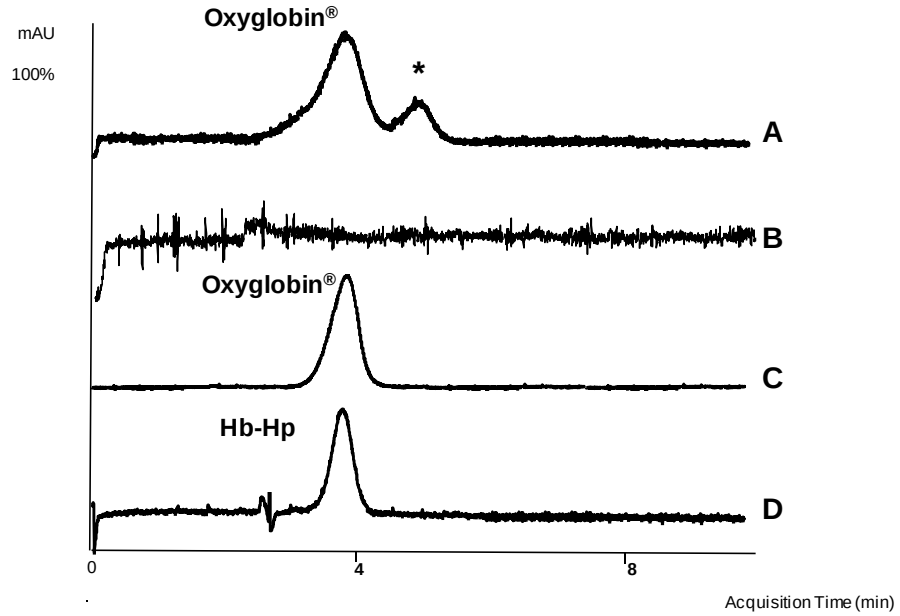

Figure 6: CE-UV electropherograms after immunodepletion procedure. Trace A: Oxyglobin® (0.3 g·dL^{-1}) in plasma, *: ghost peak. Trace B: blank plasma. Trace C: Oxyglobin® (0.2 g·dL^{-1}) in water. Trace D: Hb-Hp complex (0.192 and 0.250 g·dL^{-1}, respectively) in water. UV/Vis 415 nm online. See text for experimental conditions.

Thus, the immunodepletion sample procedure removed 50% of plasma proteins, while its gentle conditions allowed direct injection of processed samples into the CE system, and permitted Oxyglobin® to be analyzed in its intact form. Furthermore, the degree of protein depletion permitted the repeated use of a bare-fused silica capillary, which was readily compatible with ESI-MS detection. A concentration range of 0.10 g·dL^{-1} to 1.20 g·dL^{-1} was tested and detection limits (estimated with a signal-to-noise ratio of 3) of 0.20 g·dL^{-1} and 0.45 g·dL^{-1} were achieved in plasma for CE-UV/Vis at 415 nm and CE-ESI-TOF/MS, respectively. This methodology thus appears suitable for implementation as a doping control screening method for Oxyglobin® analysis.

4. CONCLUDING REMARKS

In the context of doping analysis, the ability to detect HBOC doping agents such as Oxyglobin® is critical. In this study, CE-UV/Vis and CE-ESI-TOF/MS methods were developed for the analysis of Oxyglobin® in plasma. Optimal CE conditions were obtained at basic pH, resulting in full resolution of Hb and Oxyglobin® peaks without the need for time-consuming capillary coating and washing procedures. Additional selectivity was gained from ESI-TOF/MS detection due to protein dissociation in the ESI source, which resulted in the ability to distinguish bovine and human hemoglobin by the differences in their monomeric chain molecular weights. Thus, the differentiation of Oxyglobin®, a polymerized form of bovine hemoglobin, from human hemoglobin was possible. UV/Vis absorbance at 415 nm offered another selectivity level, since only hemoproteins absorb at this wavelength. Sample preparation was identified as a key aspect of the method: removal of plasma proteins by immunodepletion was necessary to reduce adsorption onto the capillary walls and eliminate Oxyglobin® peak degradation and signal suppression. This procedure also achieved excellent haptoglobin removal, thus eliminated Hb-Hp complexes with the potential to interfere with Oxyglobin® detection.

5. ACKNOWLEDGMENTS

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6. ABBREVIATIONS

DE	depletion efficiency
HBOC	hemoglobin-based oxygen carrier
Hb	hemoglobin
Hp	haptoglobin
PE	process efficiency
R1	recovery without matrix
R2	recovery with matrix

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II.6 Identification et quantification de formulations d'insuline à l'aide de la technique d'injections multiples et par CE-TOF/MS

Dans un contexte de contrôle qualité de formulations pharmaceutiques, il peut être intéressant d'avoir une méthode analytique permettant une quantification et une identification simultanées. Pour les deux aspects, le TOF apporte une solution. L'identification est rendue possible grâce à l'exactitude de masse de cet analyseur et par comparaison des ions majoritaires multichargés du produit à identifier avec ceux d'un standard de référence. L'aspect quantification paraît aussi possible en utilisant ces mêmes ions majoritaires, mais le problème du choix d'un standard interne reste posé.

Les standards internes les plus employés dans le domaine de la CE-MS sont ceux marqués isotopiquement ainsi que les analogues de structure. Dans le cas des protéines, leur emploi est compliqué car ces derniers sont difficiles à obtenir et/ou très onéreux. L'**article VI** propose une technique alternative de quantification basée sur l'injection multiple. Pour cela, un lot de la formulation de protéine à quantifier est choisi comme matériel de référence et injecté en premier dans le système. Une seconde injection est ensuite effectuée lors du même run avec la formulation à identifier et quantifier. Les tests de faisabilité ont été effectués avec l'insuline comme protéine modèle. L'identification est faite sur les spectres de masse extraits du pic du standard et de la protéine « inconnue ». Par comparaison des masses exactes des ions majoritaires, l'identité est confirmée ou infirmée. La quantification est effectuée sur l'électrophérogramme ionique extrait en faisant le rapport de l'aire du pic de la protéine sur l'aire du pic du standard. Il s'est avéré que la correction d'ionisation n'était pas suffisante (CV>8%). Ainsi, un standard d'injection a été ajouté aux deux échantillons protéiques (standard et « inconnu »). L'aire de ces standards a été acquise *via* la détection UV en ligne et les aires des protéines obtenues en TOF corrigées par les aires respectives de leur standard d'injection. La combinaison des deux corrections a permis de diminuer le CV à moins de 2% et la méthode de quantification a ensuite pu être validée. Les performances quantitatives sont satisfaisantes et le profil d'exactitude est compris dans les limites de spécification de ± 5% autour de la valeur cible de 100% exigée pour les formulations pharmaceutiques. Finalement, quatre lots de formulations d'insuline ont été analysés avec la méthode. Trois provenaient du marché régulier tandis qu'un autre a été obtenu sur internet sans ordonnance. Les quatre lots ont donné des résultats conformes tant en termes d'identité que de concentration.

Par la suite, cette approche méthodologique devra être confrontée à des protéines plus complexes et testées pour le contrôle des contrefaçons.

II.6.1 Article VI

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Multiple injection technique for the determination and quantitation of insulin formulations by capillary electrophoresis and time-of-flight mass spectrometry

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ABSTRACT

This paper describes an efficient CE-UV-ESI-TOF/MS method for the determination and quantitation of intact insulin (INS) in a pharmaceutical formulation. The CE conditions were optimized to avoid the adsorption of proteins onto the capillary wall. Particular attention was paid regarding the choice of the internal standard (IS). A strategy based on multiple injections was selected and the methodology was validated according to international guidelines. The optimized method was applied with success to the analysis of INS formulations obtained from regular and parallel markets.

KEYWORDS

Capillary electrophoresis, intact protein, multiple injection, quantitation, time-of-flight mass spectrometry

1. INTRODUCTION

In the pharmaceutical area, recombinant proteins produced by biotechnology have grown considerably with a market evaluated at over $70 billion *per* year by a 2010 estimate [1]. These proteins comprise antibodies, hormones, biological response modifiers to stimulate cell growth, enzymes, and vaccines [2,3]. During the biopharmaceutical development process, several parameters are needed for regulatory purposes, regarding the identity, quantity (concentration), quality, and purity of the products [4,5]. Determining the identity and concentration of the therapeutic proteins is also important after their release on the market from a quality control perspective. Since unofficial channels exist to obtain these products without prescription or without extensive evidence for quality control, therapeutic protein analysis is also relevant for the parallel market. Moreover, biopharmaceuticals available in the parallel market can be counterfeit drugs. These include products with or without the correct ingredients, without the active ingredients, with insufficient or too much active ingredients, or with fake packaging [6]. Consequently, from public health perspective, it is essential to develop analytical methods to quickly monitor the identity, quality, and quantity of these biopharmaceuticals.

For the identification and quantitation of protein formulations, the analysis of proteins in their intact form is a promising approach because no tedious sample preparation, such as a digestion step, is required. Various methods for determining the quantity of intact protein exist, and the choice of the assay mainly depends on parameters such as the quantity of protein available or the required throughput [7]. Commonly, these assays are based on UV-VIS spectroscopy (*e.g.*, UV absorbance at 280 nm, Bradford protein assay, Lowry assay) or fluorescent detection after derivatization with a fluorescent probe (*e.g.*, fluorescamine, 3-(4-carboxybenzoyl)quinoline- 2-carboxaldehyde) [7,8]. The lack of specificity is the main bottleneck of these assays. In the contrary, mass spectrometry (MS) allows for a higher level of selectivity; often, confirmation of the product's identity is obtained through the accurate determination of its molecular mass, when high resolution mass analyzers are used [4]. To perform the simultaneous identification and quantitation of the active protein in its intact form, whether in a pharmaceutical formulation or in another matrix, it is necessary to couple a separation technique to an appropriate detector. Therefore, the hyphenation of capillary electrophoresis (CE) and MS *via* an electrospray ionization (ESI) source is an attractive option [9,10]. CE offers high speed, great efficiency, and low solvent and sample consumptions, while MS provides selectivity, sensitivity, and specificity.Due to its high mass range and mass accuracy, the time-of-flight (TOF) analyzer is particularly well suited for the detection of intact proteins that are multi-charged as a result of ESI [11].

Capillary zone electrophoresis (CZE) is widely used given its versatility and compatibility with ESI-MS. However, the analysis of proteins by CZE is often impaired by the tendency of the proteins to adsorb onto the negatively charged surface of fused silica (FS) capillaries [12,13], thus degrading CE performance. The evaluation of protein adsorption and its prevention must be considered during the analytical method development, particularly when accurate quantitation is attempted. The choice of the internal standard (IS) is an important point to consider in quantitative analysis. Even if matrix effects seem negligible in the case of pharmaceutical formulations analysis involving good separation of active ingredient(s) and

excipient(s), stable isotopically labeled (SIL) compounds and structural analogues remain the gold standards. However, for intact proteins, SIL compounds are not commonly available and/or could be very expensive [14,15]. Structural analogues differ from the intact protein by an exchange or removal/addition of amino acids, or a small modification in one or more side chains. These analogues are not easy to obtain for all proteins, can be expensive, and may present a different ionization behavior than that of the protein of interest. An alternative methodology to the IS concept was adapted from the multiple injection technique [16]. Initially developed to reduce the analysis time, this technique could be used to overcome the lack of satisfactory IS for intact proteins. In this approach, two injections are performed in the same analytical run, the first one with a standard of the protein of interest at a known concentration and the second one with the protein to be quantified. Therefore, the IS would be a standard of the protein, considered as the reference material.

In this study, a CE-UV-ESI-TOF/MS method was developed for the analysis of a recombinant human insulin (INS) as a model protein. INS was selected because of the numerous pharmaceutical formulations available on the market. Furthermore, since 1999, INS has been prohibited in sports for athletes who do not suffer from diabetes mellitus [17]. In addition, patients with this chronic disease often buy INS online without prescription because of the potentially lower cost. Due to these misuses, the risk of finding counterfeit drugs on the parallel market has increased dramatically. In the context of public health, analytical methods for quality control of these pharmaceutical formulations are needed. INS was already analyzed by CE [18,19,20], also coupled with MS detection [21], but never with identification and quantitation by MS. Quantitation was here attempted, using a multiple injection technique based on the successive injection of a reference standard of INS and the sample in one single run. The complete methodology was fully validated according to the guidelines of the International Conference of Harmonization (ICH) and applied to pharmaceutical formulations obtained in pharmacies and on the web without a formal prescription.

2. MATERIAL AND METHODS

2.1 Chemicals and samples

Ammonium hydroxide solution (25%, *m/m*) and formic acid (98%, *m/m*) were of analytical grade and were purchased from Fluka (Buchs, Switzerland). Isopropanol (iprOH) and sodium hydroxide were also of analytical grade and were obtained from Acros Organics (Geel, Belgium). Acetonitrile (ACN) and methanol (MeOH) were of analytical reagent grade from Panreac (Barcelona, Spain). Ultrapure water was supplied by a Milli-Q gradient A10 purification unit from Millipore (Bedford, MA, USA). Procaïne (PROC) was obtained from Sigma-Aldrich (St Louis, MO, USA).

The human insulin (INS) used for adsorption measurements was purchased from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, United Kingdom). The protein was dissolved in a 50 mM Tris-phosphate buffer (pH 7.4) at a concentration of 0.8 mg/mL (stock solution). Standard solutions of INS of the desired concentrations were prepared daily by appropriate dilution of the stock solution with water.

Actrapid® formulations of INS (batch XS61290, Novo Nordisk A/S, Bagsvaerd, Denmark) at 100 $IU \cdot mL^{-1}$ (stock solution), used for the validation process, were obtained from the Geneva Hospital Pharmacy (Geneva, Switzerland). Standard solutions of Actrapid®, at desired concentrations, were prepared daily by appropriate dilution of the stock solution with water. Three other batches of Actrapid® (TS62996 or batch 1, VS63735 or batch 2, and VS64228 or batch 3), used as routine samples, were obtained from the Geneva Hospital Pharmacy. One Actrapid® drug sample was purchased online (XS63170 or batch 4) without prescription.

2.1.1 Solutions used for calibration

Although it is mandatory to insure INS stability, the composition of the Actrapid® formulation (the exact nature and concentration of the excipients) was not known; for instance, the zinc and glycerine quantities were not mentioned in the manufacturer's datasheet, hindering the reconstitution of the formulation. To overcome this issue, a reference batch of Actrapid® (XS61290) was used as a stock solution and an independent sample from this batch was used on each day for the validation process (two ampoules *per* day).
The calibration function selected was the linear regression without intercept (Y=aX), with the target level of the calibration standard (CS) at 100%. The stock solution was diluted 20-fold to fall in the MS range and was independently prepared in three different series (j=3). The CS was injected as the first injection in the multiple injections procedure (see section 2.4). Procaine (PROC) at 50 $\mu g \cdot ml^{-1}$ was added to each CS sample as an injection standard.

2.1.2 Solutions used for validation

According to the guidelines of the ICH, as well as recommendations from the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP), three series (j=3) of three independent replicates (n=3) were prepared at each concentration level.
Three levels (k=3) were selected for the validation standards (VS), corresponding to 75%, 100%, and 125% of the concentration. The stock solution of INS was thus diluted 25-fold, 20-fold, and 15-fold, respectively. The VS were injected as the second injection in the multiple injections procedure (see section 2.4). Procaine (PROC) at 50 $\mu g \cdot ml^{-1}$ was added to each VS sample as an injection standard.

2.1.3 Real samples

Two independent samples were prepared for each real sample by a 20-fold dilution to fall in the MS range. The CS was injected as the first injection in the multiple injections procedure (see section 2.4). Procaine (PROC) at 50 $\mu g \cdot ml^{-1}$ was added to each prepared real sample as an injection standard.

2.2 Instrumentation

2.2.1 CE system

All CE experiments were performed with an HP ^{3D}CE system (Agilent Technologies, Waldbronn, Germany) equipped with an on-capillary diode array detector, an autosampler, and a power supply able to deliver up to 30 kV. Separations were performed using a backgroung electrolyte (BGE) consisting of 75 mM ammonium formate (pH 9.0) with 10% of ACN. An uncoated fused silica (FS) capillary (BGB Analytik AG, Böckten, Switzerland) was used, with an I.D. of 50 µm, a total length of 80 cm, and an effective length of 22 cm for UV detection (performed at 195 nm). The experiments were carried out in the positive polarity mode, with the anode at the inlet and the cathode at the outlet. A constant voltage of 30 kV, with an initial ramping of 5000 $V \cdot s^{-1}$, was applied during analysis; the capillary temperature was maintained at 25°C. New FS capillaries were con ditioned with MeOH, 1 M HCl, water, 0.1 M NaOH, water, and BGE at 1 bar for 5 minutes each. Prior to each sample injection, the capillary was conditioned at 2 bar for 1 minute with fresh BGE. When the capillary was not in use, it was rinsed with water and dry-stored.
For area recovery experiments, an ActiPix™ D100 UV Area Imaging System (Paraytec, York, United Kingdom) was coupled with CE to perform analyses with two passes through the detector [22]. Experiments were also performed in uncoated FS capillaries with a 50 µm I.D. Capillaries with a total length of 115 cm and effective lengths of 32 and 65.5 cm were used. Samples were hydrodynamically injected at 50 mbar for 16 s (equivalent to 2% of the effective capillary length, taking into account the first window). Prior to each sample injection, the capillary was conditionned at 2 bar for 4 min with fresh BGE. UV detection was performed at 195 nm.

2.2.2 ESI-MS system

The CE instrument was coupled to a 6210 LC/MS TOF mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) *via* a coaxial sheath flow ESI interface from Agilent. The sheath liquid consisted of iprOH-water-formic acid (49.5:49.5:1, *v/v/v*) and was delivered at a flow rate of 4 $\mu L \cdot min^{-1}$ by a syringe pump system. The ESI voltage was set at +4500 V, the nebulizing gas pressure at 4 psi, the drying gas flow rate at 4 $L \cdot min^{-1}$, the drying gas temperature at 150°C, and the fragmentor voltage at 400 V. MS detection was carried out in the positive ion mode and one spectrum was acquired *per* second (9742 transients/spectrum) in the 900 to 2500 *m/z* range.

2.3 Adsorption measurements and calculations

Adsorption measurements were carried out according to a previous study performed in our laboratory (see reference [23] for experimental details).
Reversible adsorption was measured *via* the relative standard deviations of migration times (MT RSDs, n=5), calculated for successive injections [24,25,26].

Irreversible adsorption was measured by the peak area recovery and the EOF mobility. For the area recovery, a procedure adapted from Towns and Regnier [27,28] was used. Briefly, successive injections of the protein samples were performed in the same capillary (n=5) with two passes through the detector (ActiPix™ System). The decrease in area of the protein between the first and the second pass through the detector provided a measure of the irreversible adsorption on the capillary, and is given as the percentage of area recovery. Electroosmotic flow (EOF) mobilities (n=3) were calculated with and without protein injection [29,30,31]. At a basic pH, a strong cathodic EOF occurred, and its mobility was calculated with acetone as a neutral marker. At an acidic pH, acetone migrated at around 110 minutes due to the weak EOF observed at this pH. Therefore, the methodology of Williams and Vigh [30] with the assistance of pressure was used.

2.4 Multiple injections procedure

The multiple injection procedure involved a series of two successive injections into the CE-UV-ESI-TOF/MS system with injection of BGE plugs in between. The CS was injected at 50 mbar for 10 s (equivalent to 0.68% of the total capillary length) as the first injection. Then, a plug of BGE was injected at 50 mbar for 130 s (8.90% of the total capillary length). The sample to be quantified was injected at 50 mbar for 10 s (0.68% of the total capillary length) as the second injection. Finally, a plug of BGE was injected at 50 mbar for 4 s (0.27% of the total capillary length).

2.5 Software

BGE solutions were prepared with the help of the PHoEBuS software (version 1.3, Analis, Namur, Belgium). CE ChemStation (version A.02.10, Agilent, Waldbronn, Germany) was used for CE instrument control. MassHunter (version B.02.00, Agilent, Waldbronn, Germany) was used for data acquisition, data handling, and spectral deconvolution.

3. RESULTS AND DISCUSSION

3.1 Method development

3.1.1 CE-ESI-MS conditions

According to previous results [23], initial experiments were carried out under acidic and basic conditions. Both acidic and basic BGE were made of 75 mM ammonium-formate, at pH 2.5 and 9.0, respectively. Volatile BGEs were chosen to be directly ESI-MS compatible. Two capillaries were conditioned, and experiments were performed at each pH with and without acetonitrile (ACN) in the BGE. Different proportions of ACN were tested to improve CE performance and to reduce the adsorption of the protein onto the capillary wall. Adsorption experiments were thus performed to determine the final analytical conditions.

At an acidic pH, reversible adsorption was negligible regardless of the addition of ACN, but ACN addition was beneficial for decreasing irreversible adsorption. Globally, an acidic BGE with 10% ACN was acceptable for qualitative purposes, but in the case of quantitation,

irreversible adsorption was still too important to insure acceptable repeatability. In basic conditions, there was no reversible adsorption, even in aqueous conditions; the addition of ACN eliminated irreversible adsorption. A BGE at a basic pH with 10% ACN was thus selected for the quantitative study.

ESI-MS conditions were selected according to a previous work [32], which emphasized the most important parameters for protein analysis through an experimental design. The starting conditions were finely tuned following a univariate optimization to obtain the best sensitivity for INS (estimated by the signal-to-noise ratio, S/N). To summarize, the sheath liquid made of iprOH-water-formic acid (49.5:49.5:1, *v/v/v*) was delivered at a flow rate of 4 $\mu L \cdot min^{-1}$, the ESI and fragmentor voltages were set at +4500 V and 400 V, respectively, the nebulizing gas pressure was set at 4 psi, the drying gas flow rate at 4 $L \cdot min^{-1}$, and the drying gas temperature at 150°C. Under these conditions, the limit of detection (LOD, determined for a S/N = 3) was 5 $\mu g \cdot mL^{-1}$. The response function was also evaluated over a concentration range of 5 to 250 $\mu g \cdot mL^{-1}$, where a linear relationship was observed.

3.1.2 Quantitative aspects

The most important issue in quantitative analysis of proteins by CE-ESI-MS is the choice of the IS, as it should feature the same ionization behavior as that of the protein to be quantified. As neither SIL nor structural analogues are easily available for all proteins and in order to obtain a methodology adaptable to several proteins, an alternative procedure was implemented. In the same run, a standard of INS at a known concentration was first injected ("IS"), followed by an injection of the sample to be quantified ("unknown"). This procedure is based on a multiple injection technique that allows the decrease of the run-to-run variability identified as a major contributor of dispersion in the results.

Initial experiments were performed with dilute Actrapid® reference solutions of 100 $\mu g \cdot mL^{-1}$ for the IS and of approximately 175 $\mu g \cdot mL^{-1}$ for the unknown sample. Figure 1 shows the total ion current (TIC), the extracted mass spectrum, and the extracted ion current (EIC) for a typical CE-ESI-TOF/MS analysis obtained with this double injection approach. The $[M+3H]^{3+}$ and $[M+4H]^{4+}$ multicharged ions were detected as the major extracted ions (1937 and 1453 *m/z*, respectively). The EIC was reconstructed using both ions, and integration was achieved on the EIC. The peak area of the unknown sample was corrected by the peak area of the IS, but the repeatability was not acceptable for quantitative purposes (RSD ≥8%, N=5). Two main reasons could account for this result. Firstly, the short-term variability of ionization process could occur since both peaks did not co-migrate, as reported when structural analogues are used as IS. Secondly, the hydrodynamic injection was not repeatable enough.

Therefore, an injection standard was added to both samples (*i.e.*, "IS" and "unknown" samples). To avoid an additional source of variability, an analyte easily detected by UV was selected. However, because UV is a non-selective detection technique, the co-migration of the injection standard with interfering peaks had to be excluded. Procaine (PROC), a compound that migrated as a cation before the EOF in the analytical conditions, was selected. PROC was added in both IS and unknown samples at a concentration of 50 $\mu g \cdot mL^{-1}$; online UV detection was performed at 195 nm for both PROC peaks at an effective length of 22 cm (Figure 2A). A BGE plug was injected between both injections to insure sufficient

resolution at 22 cm between all peaks, namely PROC (*i.e.*, the cationic compound migrating before the EOF, peaks c. and d.), excipients (*i.e.*, metacresol and glycerol, the neutral compounds detected in the EOF, peaks *), and INS (*i.e.*, the anionic compound migrating after the EOF, peaks e. and f.). With a BGE plug corresponding to approximately 9% of the capillary length between both injections, the PROC peaks were sufficiently resolved to be easily integrated. Each TOF peak area of INS (IS and unknown samples, corresponding to peaks b and a in Figure 2B) was corrected by its respective UV PROC peak (peaks d and c in Figure 2A) for quantitative purposes. Finally, the ratio of the corrected area of the unknown sample (b/d) over the corrected area of IS (a/c) was calculated. The corresponding equation was (b/d)/(a/c). With this optimized procedure, the overall variability of the corrected areas was greatly improved (RSD ≤2%, N=5).

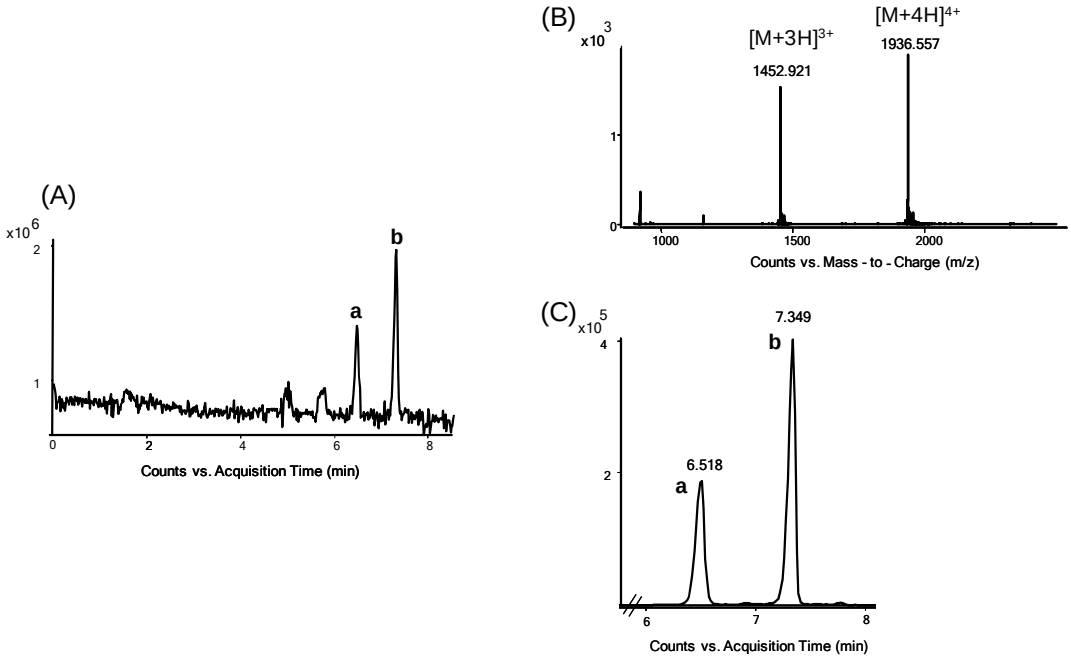

Figure 1: CE-ESI-TOF/MS electropherograms and mass spectrum of INS obtained with the multiple injection approach: (A) total ion current (TIC), (B) extracted mass spectrum, and (C) extracted ion current (EIC). (a) IS (INS at 100 µg·mL^{-1} from the first injection) and (b) unknown sample (INS at around 175 µg·mL^{-1} from the second injection).

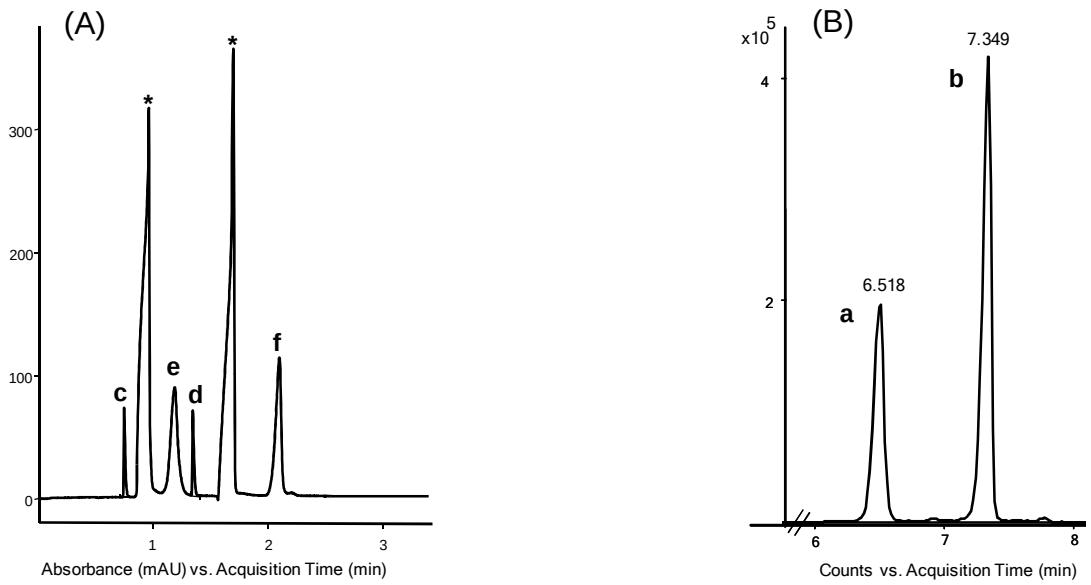

Figure 2: Electropherograms of Actrapid® obtained with the multiple injection approach: (A) UV detection and (B) ESI-TOF/MS detection. (a) IS (INS at 100 µg·mL^{-1} from the first injection), (b) unknown sample (INS at around 175 µg·mL^{-1} from the second injection), (c) PROC at 50 µg·mL^{-1} from the first injection, (d) PROC at 50 µg·mL^{-1} from the second injection, (e) IS (INS at 100 µg·mL^{-1} from the first injection), and (f) unknown sample (INS at 175 µg·mL^{-1} from the second injection). * Neutral excipients migrating with EOF.

3.2 Method validation

The quantitative performance of the CE-ESI-TOF/MS method was estimated on three separate series (j=3). According to the SFSTP 2003 recommendations [33], as well as the identification of linear response function without significant intercept (student's t test, α=0.05) during the pre-validation process, validation protocol V1 was selected. Protocol V1 recommended a calibration out of matrix using two CS at the same concentration level (for example: at the target concentration or at a slightly higher concentration) and involved three concentration levels (k=3) with two repetitions (n=2) for VS, which was injected as the second injection sample ("unknown"). CS was set at a concentration corresponding at 100% of the formulation's concentration and was injected as the first injection sample ("IS"). The double role of the first injection (CS and IS) improved the throughput of the validation process, resulting in fewer injections. The trueness and precision were estimated for each concentration level. The former was expressed as relative bias while for the latter, the variances of repeatability and intermediate precision were computed from the estimated concentrations; the precision was expressed by RSD, as described in Rozet *et al.* [34]. The accuracy profile was then obtained according to the SFSTP 2003 recommendations (β=80%). The upper and lower tolerance limits represented the total error of the method, based on the tolerance interval.

3.2.1 Selectivity

The method selectivity was performed by comparing typical electropherograms obtained by injecting water (CAL 00), water spiked with PROC at 50 µg·mL^{-1} (CAL 0), and a VS at 125%. As illustrated in Figure 3A and 3B, no interference was observed at the migration time (MT) corresponding to the PROC and INS peaks in UV or ESI-TOF/MS measurements.

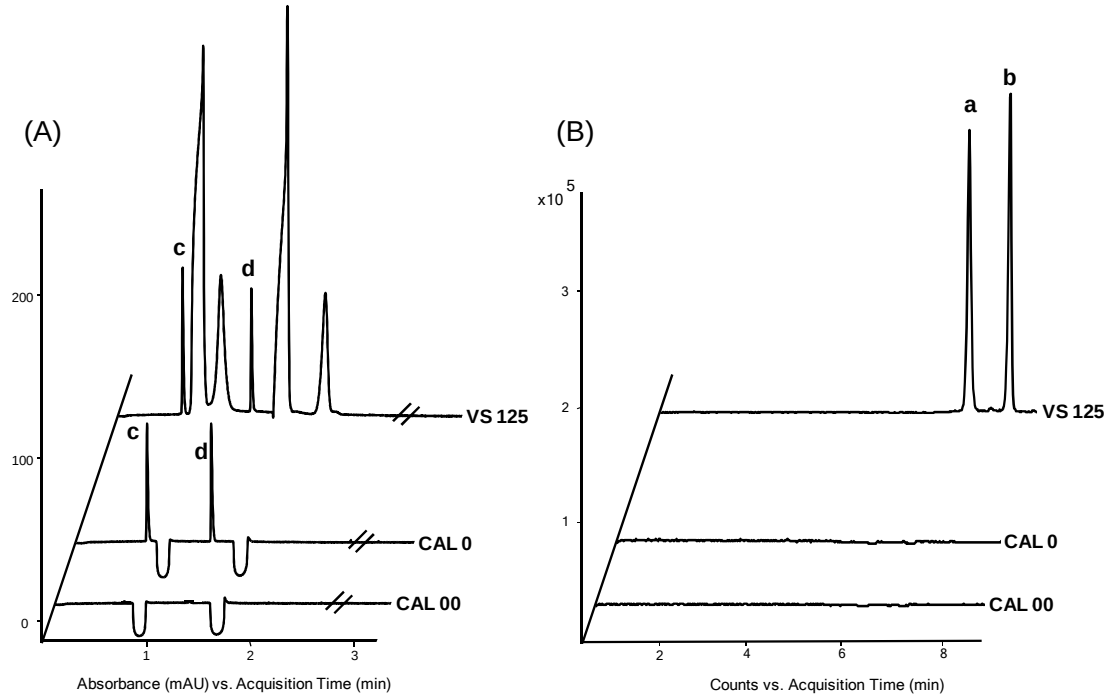

Figure 3: Electropherograms obtained with the multiple injection approach: (A) UV detection and (B) ESI-TOF/MS detection. Injection of: water (CAL 00), water spiked with PROC at 50 µg·mL^{-1} (CAL 0), and VS at 125%. (a) IS (INS at 100 µg·mL^{-1} from the first injection), (b) unknown sample (INS at 175 µg·mL^{-1} from the second injection), (c) PROC at 50 µg·mL^{-1} from the first injection, and (d) PROC at 50 µg·mL^{-1} from the second injection.

3.2.2 Trueness and precision

The results for trueness were expressed as relative bias (%) and assessed from the VS. As described in Table 1, the trueness was acceptable for the field of pharmaceutical formulation analysis since all values were lower than ±1.6%.

The precision was estimated by calculating the repeatability and intermediate precision at each concentration level of the VS. The RSD values presented in Table 1 were also acceptable for the field of pharmaceutical formulation analysis; they were in the range of 0.4 to 1.2% for repeatability, and between 0.8 and 1.6% for intermediate precision.

3.2.3 Accuracy

Accuracy takes into account the total error of the method and includes the combination of systematic (trueness) and random (precision) errors. The accuracy profile was selected as the decision tool to evaluate the method's capacity to quantify samples over the expected concentration range (75-125%). The accuracy profile for INS is illustrated in Figure 4. As shown in Table 1, the upper and lower tolerance limits of the relative bias (%) were included into the acceptance limits (±5%). Consequently, the CE-UV-ESI-TOF/MS method could be considered accurate for INS over the investigated concentration range [131-219 $\mu g \cdot mL^{-1}$].

Table 1: Validation results for INS in Actrapid® formulation obtained with the multiple injection approach (j=3, k=3, n=2).

Validation criterion	INS
Trueness	
Relative bias (%)	
131 $\mu g \cdot ml^{-1}$ (75%)	-0.3
175 $\mu g \cdot ml^{-1}$ (100%)	-0.7
219 $\mu g \cdot ml^{-1}$ (125%)	+1.6
Precision	
Repeatability/Intermediate precision (RSD, %)	
131 $\mu g \cdot ml^{-1}$ (75%)	0.7/1.0
175 $\mu g \cdot ml^{-1}$ (100%)	0.4/0.8
219 $\mu g \cdot ml^{-1}$ (125%)	1.2/1.6
Accuracy	
Lower and upper tolerance limits of the total error (%)	
131 $\mu g \cdot ml^{-1}$ (75%)	[-2.0;1.5]
175 $\mu g \cdot ml^{-1}$ (100%)	[-2.2;0.7]
219 $\mu g \cdot ml^{-1}$ (125%)	[-1.2;4.4]

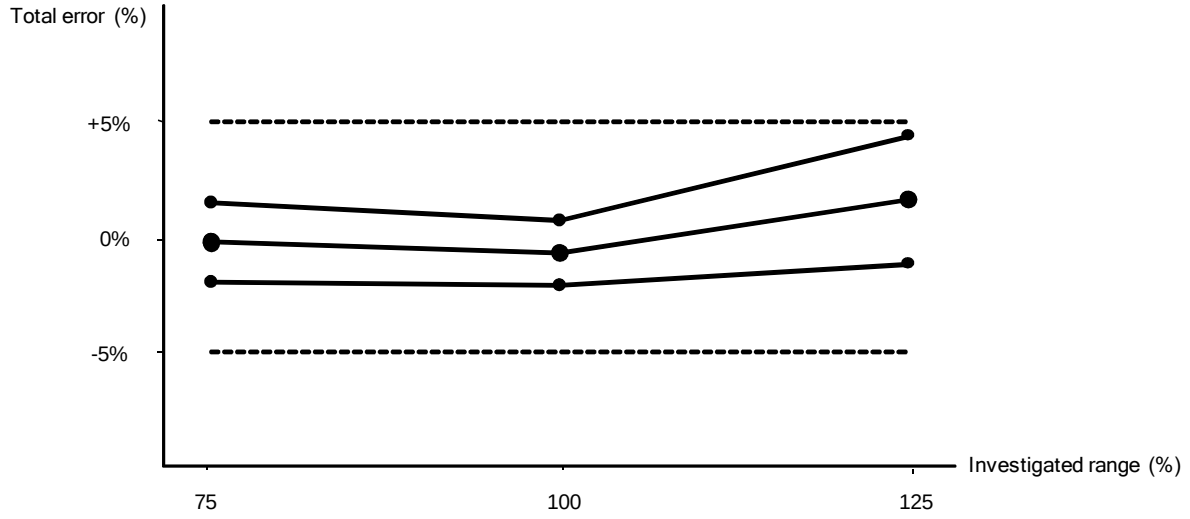

Figure 4: Accuracy profile of INS in Actrapid® formulation obtained with the multiple injection approach (j=3, k=3, n=2).

3.3 Application to real cases

To demonstrate the applicability of the CE-UV-ESI-TOF/MS method to real samples, the identification and quantitation of INS was achieved on pharmaceutical formulations of INS. Three samples were received from the Geneva Hospital Pharmacy (batches 1, 2, and 3, shipped from Switzerland), and one was purchased from the web (batch 4, shipped from Greece).

The unknown concentrations of INS samples, injected as second injection, were calculated with reference to CS at 100%, which was injected as the first injection. Two independent samples of each batch were prepared (N=2), using the previously described methodology (20-fold dilution and addition of PROC as injection standard).

Figure 5 shows the EIC and extracted mass spectra of the four INS samples. The identity was confirmed by the *m/z* of both major extracted ions, owing to the mass accuracy determination afforded by the TOF analyzer. Concentrations of 99.9 ± 2.2%, 98.2 ± 2.2%, and 101.2 ± 2.2% (relative concentration to the reference batch ± confidence interval [%]) were calculated for the three batches obtained from the Pharmacy of the Geneva Hospital. A concentration of 100.2 ± 2.2% was calculated for the sample purchased on the web. All batches were complied with the expected specifications as their identities were confirmed and their concentrations fell within the expected limits (± 5% around the target value).

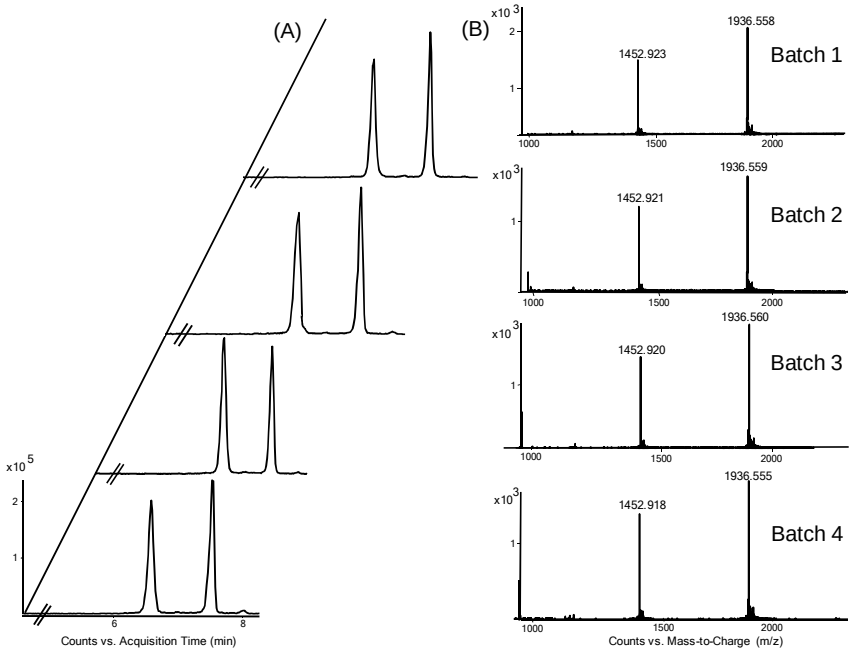

Figure 5: CE-ESI-TOF/MS electropherograms and mass spectra of different samples of Actrapid® obtained with the multiple injection approach: (A) extracted ion currents (EIC) and (B) extracted mass spectra.

4. CONCLUSIONS

A CE-UV-ESI-TOF/MS method was developed for the rapid identification and quantitation of pharmaceutical formulations containing intact proteins, such as insulin (INS). Our attention was first focused on estimating and preventing adsorption of the protein onto the capillary wall. The optimal CE conditions were obtained at basic pH values with the addition of acetonitrile to enhance CE performance and decrease adsorption. To improve CE-ESI-TOF/MS quantitation, a multiple injection approach and UV detection were chosen for correcting both ionization and injection variabilities. A fully validated strategy based on the accuracy profile was selected to demonstrate the ability of the CE-UV-ESI-TOF/MS method to quantify INS in Actrapid® formulations within a ±5 % acceptance range. Four batches of INS formulation were successfully identified and quantified by the CE-UV-ESI-TOF/MS procedure. This strategy could be implemented in the field of quality control, as well as in the detection of counterfeits.

5. ACKNOWLEDGMENTS

The authors wish to thank Dr. Sandrine Fleury from the Geneva Hospital Pharmacy for the kind gift of the Actrapid® batches and for fruitful discussions.

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Chapitre III | CONCLUSIONS ET PERSPECTIVES

III.1 CONCLUSIONS ET PERSPECTIVES

Ce travail de thèse a permis de mettre en évidence le potentiel du couplage électrophorèse capillaire de zone (CZE) et spectrométrie de masse à temps de vol (TOF) pour l'analyse des protéines sous leur forme intacte. Les atouts de la CZE, tels que l'efficacité et la sélectivité de séparation, alliés à ceux du TOF que sont l'exactitude de masse et la haute résolution permettent d'obtenir des résultats intéressants dans des domaines variés. Au travers de l'article de revue I, il a été mis en évidence la nécessité de pouvoir compter sur des techniques analytiques aux principes et aux formats très différents. Au sein de ce large éventail analytique, cette thèse permet de présenter le potentiel de la CZE couplée au TOF, ceci dans le but d'amener des informations supplémentaires et d'obtenir une image plus complète de la protéine étudiée.

Une première étude fondamentale a été conduite pour tenter de comprendre certains phénomènes d'adsorption entre les protéines et le capillaire de CZE et pouvoir ensuite les minimiser voire les annuler. L'optique d'un couplage de la CZE avec la spectrométrie de masse (MS) a toujours été conservée et dans ce contexte-là, une méthode alternative de minimisation de l'adsorption a été testée. Les méthodes classiques (tensioactifs, revêtements de capillaire) peuvent poser des problèmes de compatibilité avec le système de détection (suppression d'ionisation). Pour cela, l'utilisation d'un solvant organique dans le tampon de séparation a été employée. Les effets ont été estimés par des tests simples permettant à la fois de qualifier le type d'adsorption rencontré et de quantifier l'influence du solvant organique. Les résultats ont globalement montré une influence positive du solvant organique sur les phénomènes d'adsorption. Cependant, ces effets ne sont pas prévisibles et dépendent fortement du type de protéine analysée (caractéristiques physicochimiques, flexibilité). Quoi qu'il en soit, l'ajout de solvant organique dans le tampon doit être intégré dans le processus global de développement d'une méthode d'analyse CE-TOF des protéines intactes au vu des influences positives possibles et de la complète compatibilité MS.

Une première application a permis d'identifier des échantillons saisis en douane et contenant effectivement de l'hormone de croissance recombinante humaine. Une méthode CE-TOF a pour cela été développée et a mis en évidence deux niveaux de sélectivité entre l'hormone de croissance naturelle et la recombinante. La première est obtenue par la migration électrophorétique différente des deux hormones à pH acide et le TOF permet la seconde en mettant en évidence l'absence d'isoformes dans les échantillons d'hormone recombinante. Grâce à ces deux niveaux de sélectivité, le contenu des échantillons de douane a été identifié comme étant de l'hormone de croissance recombinante.

La seconde application concernait le domaine du dopage sanguin, plus particulièrement le dopage à l'aide de composés dérivés de l'hémoglobine. Le TOF a permis ici de détecter sélectivement les hémoglobines issues d'espèces différentes, ceci après un phénomène intéressant de dissociation des sous-unités globulaires des hémoglobines dans la source electrospray. Cette sélectivité s'est avérée d'autant plus importante que le produit dopant à

base d'hémoglobine est constitué d'hémoglobine bovine. Le TOF a donc permis de distinguer la présence naturelle d'hémoglobine dans l'échantillon de la présence illégale de produit dopant à base d'hémoglobine.

L'aspect quantification a été abordé dans la troisième application. Il a été démontré qu'à l'aide d'une approche d'injections multiples, il est possible de quantifier et d'identifier en une seule analyse une protéine présente dans une formulation pharmaceutique. L'insuline a été utilisée comme protéine modèle. L'exactitude de masse du TOF est particulièrement utile pour cela. En effet, les masses exactes des ions majoritaires de la protéine sont employés pour l'identification (comparaison avec les ions du standard de la protéine) et la quantification (reconstruction d'un électrophérogramme à partir de ces ions et quantification par le rapport des aires entre le standard et la protéine à quantifier). La méthode de quantification a été validée et appliquée à des formulations pharmaceutiques d'insuline obtenues sur le marché régulier ou sur internet.

Finalement, ces résultats permettent d'envisager d'intéressantes perspectives. S'il a été démontré au travers des applications et de l'étude plus fondamentale que chaque protéine est unique et qu'il est par conséquent difficile de mettre en oeuvre des approches analytiques génériques, la singularité des protéines permet par contre d'entrevoir de nombreuses applications.

Ainsi, en ce qui concerne l'application quantitative, il sera important de voir si elle peut être employée pour des protéines plus complexes que l'insuline testée ici, que ce soit par une masse moléculaire plus grande ou la présence de modifications post-traductionnelles et donc de nombreuses isoformes. Les problèmes seront surtout rencontrés au niveau du traitement du signal dans la mesure où, contrairement au cas de l'insuline, le nombre d'ions multichargés sera considérable. Il faudra alors déterminer quels ions sont à prendre en compte pour l'identification et lesquels pour la quantification. En ce qui concerne l'aspect identification, il est à noter qu'en complexifiant l'analyte étudié, il est évident qu'une seule méthode ne suffira plus pour garantir l'identité de la protéine de manière non équivoque. De plus, il serait certainement nécessaire d'effectuer ces tests avec un TOF possédant un plus grand pouvoir résolutif ou avec une sélectivité supplémentaire (quadripôle-TOF), les isoformes des protéines ne différant souvent que de très peu en termes de masse moléculaire.

Le domaine des analyses en milieu biologique n'a été que peu abordé au cours de cette thèse (exception faite de l'application liée au dopage sanguin). Ce domaine implique en effet une étude approfondie de l'étape de préparation d'échantillon. Plus particulièrement, deux approches différentes sont alors à considérer: (i) déplétion des protéines interférentes et/ou (ii) extraction sélective de la ou des protéine(s) d'intérêt. L'aspect de préparation d'échantillon est un point à étudier et qui nécessite un travail approfondi. Les résultats obtenus seront complémentaires aux résultats de cette thèse et pourront ensuite être intégrés en amont des principes mis en évidence lors de ce travail de thèse.

Comme évoqué ci-dessus, un autre point à considérer est l'instrumentation elle-même. En effet, des limitations en termes de sensibilité et de résolution du TOF ont été rencontrées durant les différents développements de méthode. La sensibilité pourrait par exemple être quelque peu améliorée par des techniques appropriées de pré-concentration en CE, mais de grandes augmentations de sensibilité et de résolution ne semblent pouvoir être atteintes que par l'utilisation de spectromètres de masse plus performants. Ainsi, des TOF plus récents permettent d'atteindre des résolutions de l'ordre de 40'000, ce qui pourrait s'avérer nécessaire pour des applications impliquant l'identification d'isoformes ne différant que d'un dalton par exemple. La sensibilité sera aussi certainement améliorée dans le futur avec les progrès techniques au niveau de la transmission ionique. Par conséquent, le couplage CE-TOF pourrait par la suite être plus largement employé dans des domaines nécessitant une grande sensibilité comme le domaine de la lutte anti-dopage ou de la recherche de biomarqueurs.

Une fois ces obstacles technologiques et méthodologiques franchis, un grand défi consistera certainement en l'utilisation du couplage CZE-TOF pour l'analyse des anticorps monoclonaux qui se positionnent assurément comme les candidats d'intérêt thérapeutique les plus importants dans le futur.

Finalement, en considérant la problématique globale de l'analyse des protéines sous leur forme intacte et au vu des récents progrès des supports chromatographiques dédiés, une étude des performances atteignables avec la chromatographie en phase inverse voire à interaction hydrophile pour l'analyse de ces molécules constitue également une perspective prometteuse. L'électrophorèse capillaire s'est souvent positionnée en technique orthogonale à la chromatographie liquide en phase inverse et il serait dès lors intéressant de tester cette complémentarité avec les protéines intactes.

Chapitre IV | ANNEXES

ANNEXE 1

Article VII

J. Schappler, A. Staub, J.L. Veuthey, S. Rudaz. **Highly sensitive detection of pharmaceutical compounds in biological fluids using capillary electrophoresis coupled with laser-induced native fluorescence**, *J. Chromatogr. A* 2008, *1204*, 183-190.

Highly sensitive detection of pharmaceutical compounds in biological fluids using capillary electrophoresis coupled with laser-induced native fluorescence

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Journal of Chromatography A 2008, *1204*, 183-190.

ABSTRACT

Due to its selectivity and sensitivity, capillary electrophoresis coupled to laser-induced fluorescence has evolved as a useful analytical tool for determining drugs in biological samples. This paper describes a simple, sensitive, efficient, and rapid method for analyzing propranolol without derivatization in plasma by CE-LIF using a diode solid-state laser at 266 nm. An experimental design methodology was assessed for the investigation of electrophoretic parameters and a Box-Behnken design was selected to optimize both sensitivity and efficiency. The CE-LIF method was linear over the concentration range of 50-850 $ng \cdot mL^{-1}$ and successfully applied to a real forensic sample.

KEYWORDS

Capillary electrophoresis, design of experiments, laser-induced native fluorescence, optimization, plasma, validation

1. INTRODUCTION

Capillary electrophoresis (CE) is a powerful separation technique that has found numerous applications for a wide range of compounds in various analytical fields. This technique has several advantages, such as a high efficiency, rapid method development, simple instrumentation and low solvent and sample consumption; these features are the main reasons for its success. UV-VIS spectrophotometry is likely the most widely used detection technique in CE because of its on-line configuration simplicity and quasi-universality, particularly in the pharmaceutical field. However, its sensitivity, which is directly related to the optical pathlength afforded by the capillary I.D. (in the µm range), remains the major bottleneck of this technique. UV detection requires relatively high analyte concentrations and is often unsuitable for bioanalytical applications, since many drugs present a high volume of distribution, resulting in low concentration levels [1]. To overcome this sensitivity issue, appropriate sample preparation procedures for analyte preconcentration and/or highly sensitive detectors [2], such as fluorescence techniques, should be considered. Indeed, the latter are generally more sensitive than absorption-based methods, since fluorescence signals are directly proportional to excitation power. Lamp-based fluorescence provides detection limits below 10^{-6} M, although focusing inside the capillary could be difficult. Because lasers are easily focused and allow high efficient excitation, laser-induced fluorescence (LIF) is particularly adaptable for CE [3]; furthermore, the high irradiance (I) provided by a laser leads to better signal-to-noise ratio (S/N), since signal intensity and noise are proportional to I and $I^{1/2}$, respectively. LIF detection provides very low limits of detection (LOD), with an improvement up to 10^5 compared to conventional UV detection [4]. While LOD down to 10^{-12} M have been reported [5], this sensitivity level is rather difficult to achieve for numerous reasons. Chemical derivatization is generally required, particularly for non-fluorescent analytes and fluorophores that cannot be excited at the available laser wavelength [6-8], and the kinetics of derivatization reactions often contribute to detection limitations (sometimes already at the 10^{-7} M level). Furthermore, co-migrating interferences may also be labeled and provide fluorescence properties comparable to those of the analyte [9]. LIF detection is only advantageous if the background noise is not increased by the same factor as the analyte signal. Some lasers exhibit power instability and increase the background noise, leading to a low increase in the overall sensitivity. However, interesting improvements have been recently implemented to overcome this issue. Diode lasers that feature very low output power fluctuations have been introduced, which limit intensity fluctuations, background noise amplification and spatial instability. Furthermore, their reduced size combines a moderate price with a long life span. Most diode lasers were first developed for the infrared and visible wavelengths but UV diode lasers are now commercially available.

As already mentioned, because chemical structures do not always possess a strong chromophore or fluorophore, derivatization procedures with a suitable fluorescent label, which matches the laser excitation wavelength, are often mandatory for sensitive detection [10]. A major feature of UV diode lasers is their ability to directly implement CE-LIF methods for natively fluorescent analytes, which is particularly advantageous because a variety of drugs contain a fluorophore that can be excited in the UV region. Numerous lasers have

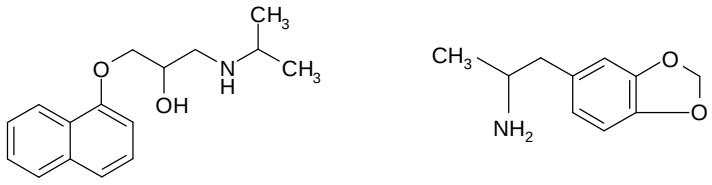
been developed for generating excitation wavelengths in the UV region. Initially, continuous wave argon-ion lasers, which presumably were widespread over various laboratories, were modified to provide UV excitation wavelength. For instance, frequency-doubled Ar lasers were tuned to obtain excitation wavelengths at 244 nm [11], 257 nm [12,13] or 275 nm [14]. Other materials were also shown to provide UV excitation wavelengths with comparable performance, *e.g.* pulsed KrF [15-17] or pulsed NeCu [18] lasers both emit at 248 nm, and XeCl laser coupled to a frequency-doubled dye laser allows generation of a 280 nm excitation wavelength [19]. Nevertheless, the aforementioned gas lasers present several previously listed restrictions, such as power instability, which ultimately lead to sensitivity limitations. Diode solid-state lasers, such as the pulsed Nd:YAG laser emitting at 266 nm [20], have emerged to address this constraint. Numerous publications were dedicated to experiments achieved on a conventional CE-LIF system with a 266 nm laser (CE-LIF 266). Most of them considered the determination of neurotransmitters in urine [21,22] or brain microdialysis samples [23], while few papers were dedicated to pharmaceutical drug analysis [24]. Furthermore, only univariate optimization was carried out for developing CE-LIF 266 procedures, without considering potential interactions that could occur between experimental factors.

In this paper, a multivariate optimization approach was applied to CE-LIF 266 analysis of propranolol as a model pharmaceutical compound present in biological matrices. The chemometric methodology involves the simultaneous investigation of the selected experimental factors, attributable to an adapted design of experiments (DOE), allowing high quality information from a relatively limited number of trials [25-28]. To our knowledge, this is the first application of this methodology to CE-LIF 266 optimization. The quantitative performance of the CE-LIF 266 method was assessed with an appropriate procedure and performed in agreement with regular guidelines and validation protocols [29-32]. The performance criteria evaluated were selectivity, response function, trueness, precision (repeatability and intermediate precision), accuracy, linearity and limit of quantitation (LOQ). The methodology was finally applied to the quantitation of propranolol in a real plasma sample issued from a forensic case.

2. MATERIALS AND METHODS

2.1 Chemicals

Propranolol (PROP) hydrochloride was obtained from Aldrich (Schnelldorf, Germany) and 3,4-methylenedioxyamphetamine (MDA) was purchased from Lipomed AG (Arlesheim, Switzerland). Chemical structures are reported in Fig. 1.



PROP MDA

Figure 1: Chemical structures of investigated compounds.

Acetonitrile (ACN) and methanol (MeOH) were of analytical reagent grade from Panreac (Barcelona, Spain). Ethanol (EtOH) was of analytical reagent grade from Fisher Scientific (Leicestershire, UK). Phosphoric acid, formic acid, acetic acid, 2-(N-morpholino)ethanesulfonic acid (MES), 3-morpholinopropanesulfonic acid (MOPS), sodium hydroxide (NaOH), formamide (FA) and N-methylformamide (NMF) were of analytical reagent grade from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane (TRIS) was of analytical reagent grade from Riedel-de-Haën (Buchs, Switzerland). α-, β- and γ-cyclodextrins (CD) were purchased from Sigma (Buchs, Switzerland), while hydroxypropyl β-CD (HP β-CD) and carboxymethyl β-CD (CM β-CD) were obtained from Roquette (Lestrem, France) and Cyclolab (Kötlichen, Switzerland), respectively. Ultrapure water was supplied by a Milli-Q RG purification unit from Millipore (Bedford, MA, USA). Blank plasma was obtained from the Blood Center of the Geneva Hospital (Geneva, Switzerland). Actual blood samples were obtained from the Institut Universitaire de Médecine Légale (IUML, Geneva, Switzerland).

2.2 Background electrolyte (BGE) and sample preparation

The BGE consisted of a 25 mM (ionic strength) TRIS-phosphate buffer at pH 2.8. The pH value was measured with a SevenMulti pH meter (Mettler-Toledo, Schwerzenbach, Switzerland), calibrated daily with four aqueous solutions at pH 2.00, 4.00, 7.00 and 10.00 from Riedel-de-Haën (Buchs, Switzerland). Stock standard solutions of analytes were prepared by dissolving each compound in MeOH to obtain a concentration of 1 $mg \cdot mL^{-1}$, which were stored at 4°C until use. Standard solutions of analytes at the desired concentrations were prepared daily by appropriately diluting stock solutions in water. Sample solutions were stable for more than two days at 4°C, and no degradation was observed for the tested analytes during analysis.

2.2.1 Solutions used for calibration

The calibration standards (CS) were samples with the matrix containing known concentrations of analytes, which were prepared in an independent way. Three levels (k=3) were selected, corresponding to low (estimated limit of quantitation, LOQ), medium and high concentrations of the investigated range. CS were replicated twice (n=2) and independently prepared in three different series (j=3). Human blank plasma was spiked with the drug mixture at varied concentrations: 50, 500 and 1000 $ng \cdot mL^{-1}$ of PROP and 500 $ng \cdot mL^{-1}$ of MDA as I.S.

2.2.2 Solutions used for validation

Validation standards or quality control samples (QC) consisted of reconstituted samples in the matrix containing known concentrations of analytes and independently prepared from the CS. According to the international conference on harmonization (ICH) and FDA guidelines as well as recommendations from the “société française des sciences et techniques pharmaceutiques” (SFSTP), three series (j=3) of three independent replicates (n=3) were prepared at each concentration level (k=4). Human blank plasma was spiked with the drug mixture at following concentrations levels: 50, 100, 500 and 850 $ng \cdot mL^{-1}$ of PROP and 500 $ng \cdot mL^{-1}$ of MDA as I.S.

2.2.3 Protein precipitation (PP)

For PP procedure, 400 µL of ACN was added to 200 µL of spiked plasma. After vortex-mixing for 30 s, the sample was centrifuged for 5 minutes at 6000 g. Next, 100 µL of the supernatant was transferred into an appropriate vial and injected in hydrodynamic mode (See § 2.3.1 for injection conditions).

2.2.4 Application to biological samples

A frozen post-mortem plasma sample (sample #TA88/05) was received from the Institut Universitaire de Médecine Légale (IUML, Geneva, Switzerland). The sample was defrosted at 4°C and vortex-mixed for 1 minute. To 200 µL of this solution, 400 µL of ACN, containing 500 $ng \cdot mL^{-1}$ of MDA (I.S.), was added. The applied PP procedure was identical to the previously described methodology (See § 2.2.3) and repeated twice (N=2).

2.3 Instrumentation

2.3.1 Capillary electrophoresis

CE experiments were performed with an HP ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with on-capillary diode array detector, autosampler and power supply able to deliver up to 30 kV. Separation was performed in an uncoated fused silica (FS) capillary (BGB Analytik AG, Böckten, Switzerland) with a 50 µm I.D., total length of 47 cm and effective length of 33.5 cm for LIF detection. Experiments were carried out in positive polarity mode, with the anode at the inlet and the cathode at the outlet. A constant voltage of 30 kV, with an initial ramping of 1000 $V \cdot s^{-1}$ (30 s), was applied during analysis, and the capillary temperature was maintained at 30°C. Samples were kept at ambient temperature in the autosampler and injected in hydrodynamic mode at 480 mbar·s (equivalent to 2.4 % of capillary length). Before its first use, the capillary was sequentially rinsed with MeOH, NaOH (0.1 M), water and fresh BGE for 5 minutes each. Prior to each sample injection, the capillary was rinsed under pressure (2 bar) for 1 minute with fresh BGE. After each run, the capillary was rinsed under pressure (1 bar) with NaOH (0.1 M) and fresh BGE for 1 minute each, and a voltage of 30 kV was applied for 1 minute to refresh the capillary wall and obtain repeatable

electroosmotic flow [33]. When not in use, the capillary was rinsed with water and then dry stored. As the electrophoretic process alters the running buffer pH by electrolysis and subsequently changes migration times, the separation buffer was refreshed every four runs.

2.3.2 Fluorescence

Fluorescence measurements were performed with an LS 50B fluorescence spectrometer (Perkin Elmer, Waltham, MA, USA) equipped with a pulsed xenon discharge lamp producing 7.3 W of average power at 50 Hz.

2.3.3 Laser induced fluorescence

LIF detection was performed with a ZetaLIF Evolution system (Picometrics, Ramonville, France) hyphenated to the CE system. A NanoLaser diode-pumped solid-state (DPSS) UV laser (JDS Uniphase, San Jose, CA, USA) provided fluorescence excitation. The laser cavity consisted of a 2 mm^3 Nd:YAG producing a 1064 nm output radiation, which was converted to 266 nm using harmonic generation stages integrated in the laser head. The laser produced 1 mW of average power and operated in quasi-continuous wave mode, with a repetition rate that ranged between 8 and 13 kHz and ~800 ps pulse width. A photomultiplier detector was used to measure fluorescence intensity.

2.4 Softwares

Buffer and BGE solutions were prepared with the help of PHoEBuS software version 1.3 (Analis, Namur, Belgium). CE ChemStation (Agilent, Waldbronn, Germany) was used for CE instrument control and Azur version 4.0 (Datalys, Saint Martin d'Hères, France) was used for data acquisition, data handling and analytical parameter calculation (efficiency, *S/N*). Modde software version 7.0 (Umetrics AB, Umea, Sweden) was used to generate experimental designs and data processing.

3. RESULTS AND DISCUSSION

PROP was selected as the model compound for this study. PROP is a non-cardioselective beta-blocker, mostly used in the treatment of hypertension, angina, and for the prevention of re-infarction in patients who have suffered from myocardial infarction [34]. PROP is also used in the treatment of extrapyramidal disorders and in the prophylaxis of migraine headaches, as well as in acute stress reactions, somatic anxiety and panic reactions [35,36]. Because PROP is extensively metabolized by the liver, plasma concentrations, and therefore pharmacological effects, differ depending on patient metabolism and/or drug interactions. Since toxicity can be correlated with PROP concentration in plasma, rapid quantitation with a robust methodology is required. In aqueous solution, PROP exhibited a strong UV absorbance in the range of 260-310 nm, including the excitation wavelength of 266 nm. After excitation at this wavelength, maximum fluorescence was determined at 350 nm.

3.1 Optimization of separation conditions

3.1.1 BGE composition

Since PROP is considered a weak base (pK_a value of 9.1), capillary zone electrophoresis (CZE) analysis could be performed in either acidic or neutral conditions. Because BGE constituents could have an influence on LIF sensitivity, *i.e.*, on both the absorption coefficient and the fluorescence quantum yield, preliminary experiments were conducted to determine the impact of the buffer and selectivity modifiers on PROP detection.
Six buffers, reported in Table 1, covering a pH range from 2.0 to 7.0 and five types of electrolyte (phosphate, formate, acetate, MES and MOPS), were selected. The absorbance spectrum was found to be pH independent from pH 2 to pH 7. As described in the literature, the buffer nature did not induce any change in the emission spectra, and no influence of pH was observed on the relative fluorescence intensity. In fact, a red shift in the emission wavelength, as well as an increase in the fluorescence intensity, were only observed at pH higher than 7 for compounds possessing functions with pK_a values higher than 9 [37].

Table 1: Buffers' preparation at 50 mM ionic strength and pH in the range of 2-7

Buffer composition		Theoretical pH (25°C)	Measured pH (25°C)	Buffer capacity (mM/pH unity)
Buffering species (mM)	NaOH (mM)			
H_3PO_4 (103.9)	37.7	2.0	2.0	89.3
HCOOH (278.9)	48.8	3.0	3.0	105.1
HCOOH (72.9)	49.9	4.0	4.0	37.3
CH_3COOH (73.1)	50.0	5.0	5.0	37.3
MES (104.7)	50.0	6.0	6.0	63.0
MOPS (114.0)	50.0	7.0	7.0	39.3

The influence of selectivity modifier addition was investigated, since it could affect both fluorescence intensity and peak efficiency (in addition to selectivity changes). Five different organic modifiers were selected: MeOH, EtOH, ACN, FA and NMF added from 10 to 50 % (v/v) to a phosphate buffer solution (pH 2.5). Efficiency and sensitivity, expressed as *S/N* ratio, were compared with and without solvent addition. No significant changes were observed with the alcohols and ACN. A moderate increase in the relative fluorescence intensity, usually observed for most amine compounds due to modification of the molecule dipole moment after adding organic solvent, was not observed in our experiments [24]. FA and NMF were investigated because they present interesting physico-chemical properties, especially in terms of the dielectric constant. For instance, the high ε^2/η ratio of NMF (about 20'000 at 25°C) resulted in higher efficiency than aqueous buffer, which implied a lower LOD [38,39]. However, their use in CE-LIF with an excitation wavelength at 266 nm led to enhanced background noise, most likely related to their high UV cutoff (*ca.* 250 nm) [40,41]. Finally, addition of CD into the BGE was investigated because it was reported that it could

significantly enhance the fluorescence quantum yield, mainly by decreasing rotation motion of the entrapped molecule [42]. Although β-CD was the most referenced CD for enhancing fluorescence, other CD molecules were tested because they were identified to form 1:1 inclusion complexes with PROP [43]. Native α-, β- and γ-CD as well as HP β-CD and CM β-CD were used in the range of 5-30 $mg \cdot mL^{-1}$, according to solubility limits and optimal complexation concentrations [44]. When comparing *S/N* with and without CD, no fluorescence enhancement was observed. It has to be noted that the CD influence on relative fluorescence of investigated analytes was not predictable, and fluorophore/CD inclusion complexes do not systematically lead to a fluorescence increase. Indeed, steric constraints of the guest molecule, as well as nature and intensity of interactions, rule the fit between host and guest and are responsible for the subsequent emission change of the guest. For instance, a fluorescence increase of model compounds was reported upon addition of CD, whereas no improvement in fluorescence of their structural isomers was obtained under the same conditions [45].

It could be concluded that the fluorescence detection of PROP was independent of the retained BGE *via* changes in the absorption coefficient or the fluorescence quantum yield. Particularly, addition of an organic solvent or CD did not significantly enhance CE-LIF sensitivity and efficiency. Therefore, a conventional acidic BGE was maintained for further investigation. The phosphate buffer was selected as it leads to higher efficiencies compared to other tested electrolytes, attributable to an optimal mobility matching between analytes and BGE [46]. TRIS counter ion was selected as it generated little electrophoretic current without any observed difference in fluorescence detection towards NaOH.

3.1.2 Response surface modeling (RSM)

In order to optimize electrophoretic parameters for PROP analysis, an experimental design methodology was selected. A second-degree design was applied to four relevant factors, namely pH (X_1), ionic strength (X_2), temperature (X_3) and voltage (X_4). To determine the relationship between the experimental response mainly expressed as PROP sensitivity (*S/N*), and efficiency, an investigation was performed with a Box-Behnken (BB) design. The latter is not based on a full factorial design, such as the face-centered composite design [47], as it uses center points instead of corner points. The experimental plan for a four-parameter design was developed according to the following pattern: two variables were set in a combination of their extreme values, while the other two variables were set to the center values (Table 2). Another feature of the BB structure is its sequential implementation toward the experimental parameters : three factors (X_1, X_2 and X_3) could be assessed in the first set of experiments and the last factor (X_4) could be investigated in a further sequence, which included results obtained during the first step. Because optimization of the first three factors could generate unwanted Joule heating, particularly at high ionic strength and temperature values, voltage (X_4) was maintained in the middle of the experimental domain in the initial series of experiments. Voltage was studied in a second series of experiments to determine its influence on analysis time without compromising efficiency. The sequential 3+1 BB design was built on a hypercube with 4 dimensions. All design points (24 trials: 1-12/16-27) were located at the center of the edges and six trials were performed in both series at the center of

the investigated ranges (13-15/29-30) to evaluate the standard method error. The investigated range for each factor is summarized in Table 3. Assessment of low (-1) and high (+1) levels was determined by considering (i) buffer capacity of the selected BGE, (ii) instrumental limits (iii) generated Joule effect, and (iv) total analysis time to run the whole DOE in one day, thereby avoiding inter-day variability. Sensitivity (*S/N*), signal height (*S*), background noise (*N*) and efficiency of PROP were chosen as analytical responses. Experiments were fully randomized to calculate quadratic regression coefficients able to fit a model, which would reveal the main trends (slope, curvature) in the sensitivity- and efficiency-factor relationships.

Table 2: Coded values of experimental factors for Box-Behnken design, and responses in terms of signal, noise, signal-to-noise ratio and efficiency

Exp no.	X_1 pH	X_2 ionic strength (mM)	X_3 temperature (°C)	X4 voltage (kV)	Signal PROP (S)	N oise PROP (N)	(S/N) PROP	Efficiency PROP x 10^4
1	-1	-1	0	0	8	0.065	125	9.65
2	-1	+1	0	0	8	0.078	101	10.3
3	+1	-1	0	0	9	0.065	135	10.8
4	+1	+1	0	0	12	0.075	160	13.8
5	-1	0	-1	0	7	0.067	110	9.11
6	+1	0	-1	0	9	0.068	130	10.5
7	-1	0	+1	0	10	0.074	129	12.8
8	+1	0	+1	0	10	0.069	150	12.0
9	0	-1	-1	0	8	0.065	117	7.55
10	0	+1	-1	0	9	0.068	134	10.6
11	0	-1	+1	0	10	0.062	162	11.1
12	0	+1	+1	0	10	0.066	156	12.8
13	0	0	0	0	9	0.066	134	12.3
14	0	0	0	0	10	0.066	152	12.9
15	0	0	0	0	9	0.063	140	11.8
16	0	0	-1	-1	10	0.063	166	15.7
17	0	0	+1	-1	11	0.069	164	15.8
18	0	0	-1	+1	5	0.075	71	7.90
19	0	0	+1	+1	8	0.068	112	10.1
20	0	-1	0	-1	10	0.062	165	14.7
21	0	+1	0	-1	10	0.068	144	16.7
22	0	-1	0	+1	6	0.075	85	8.85
23	0	+1	0	+1	7	0.079	89	11.2
24	-1	0	0	-1	11	0.077	142	15.3
25	+1	0	0	-1	10	0.078	133	16.0
26	-1	0	0	+1	6	0.065	86	8.75
27	+1	0	0	+1	9	0.077	113	10.3
28	0	0	0	0	9	0.066	141	12.1
29	0	0	0	0	9	0.064	147	12.5
30	0	0	0	0	9	0.066	143	11.5

Responses obtained for a voltage fixed at its middle value (*i.e.*, 22.5 kV) are presented in Fig. 2, corresponding to the first set of experiments (Table 2, assays 1-15). For clarity, only *S/N* and efficiency response surfaces were reported. A statistical study of the coefficient values revealed that the response fit adequately, with determination coefficients (R^2) higher than 91 % for *S/N* and efficiency. Therefore, the selected models were used to find optimal conditions for sensitivity and efficiency. As observed, surface responses exhibited curvature, indicating the importance of quadratic terms in the proposed model. The optimal response in terms of sensitivity (Fig. 2A) was achieved at the highest temperature (40 °C), while maximum efficiency (Fig. 2B) was obtained between 22 and

40 °C. Because the capillary was not thermoregulate d over its whole length (detection cell outside CE instrument), the temperature was set to 30 °C. This value ensured high efficiency while maintaining acceptable sensitivity. Moreover, the baseline was unstable when the temperature was increased, most likely due to a temperature gradient in the detection cell. The pH was set at 2.8 for a maximum sensitivity (Fig. 2A), as well as an acceptable buffer capacity (9.5 mM/pH unit, considering 25 mM ionic strength). Although pH could be increased up to 3 without loss of sensitivity, lower buffer capacity could induce unstable electrophoretic conditions.

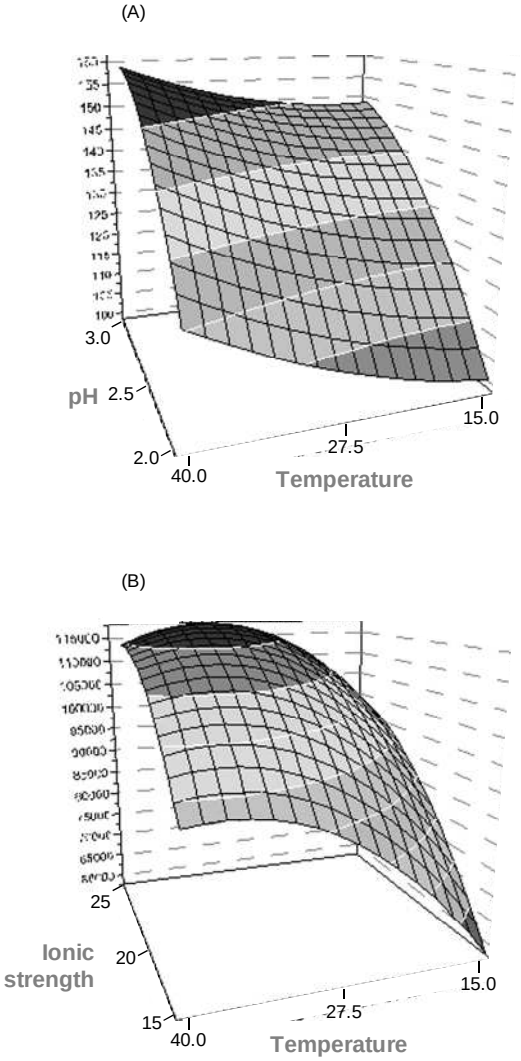


Figure 2: CE-LIF 266 Box-Behnken response surface modeling obtained for: (A) propranolol sensitivity (S/N) depending on pH and temperature, with 22.5 kV voltage and 25 mM ionic strength, and (B) proparanolol efficiency depending on ionic strength and temperature, with 22.5 kV voltage and pH 2.8.

Because the observed current was acceptable during the first set of experiments, a second series of analysis allowing the evaluation of the last experimental parameter, voltage (X_4), was achieved (Table 2, assays 16-30). The complete results were merged to obtain the

response surfaces presented in Fig. 3, where *S/N* and efficiency results are modeled at the maximum voltage value (30 kV). A statistical study of the coefficient values revealed the response fit, with R^2 of 91 % and 96 % for *S/N* and efficiency, respectively. Therefore, the selected models were valid and explained the observed responses. With the highest voltage value (30 kV), fast analyses were achieved, without compromising efficiency (Fig. 3B), while maintaining sufficient sensitivity (Fig. 3A). The best sensitivity (Fig. 3A) and efficiency (Fig. 3B) were obtained at the highest values of ionic strength, specifically 25 mM. This was attributed to the stacking effect when a high conductivity difference occurred between the sample zone and BGE. As a result, the peak height was directly proportional to ionic strength. Furthermore, high ionic strength ensured a high buffer capacity.

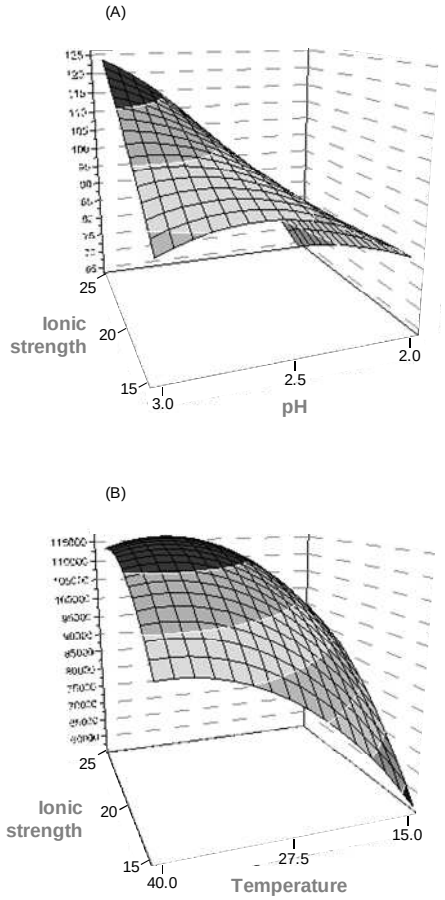


Figure 3: CE-LIF 266 Box-Behnken response surface modeling obtained for: (A) propranolol sensitivity (S/N) depending on ionic strength and pH, with 30 kV voltage and 30℃ temperature, and (B) propranolol efficiency depending on ionic strength and temperature, with 30 kV voltage and pH 2.8.

Finally, the response surfaces allowed visualization of optimal zones where robustness (*i.e.*, low surface curvature) of the response could be established for further validation processes [48]. In fact, estimated optimum sensitivity and efficiency corresponded to the following experimental conditions: pH 2.8, ionic strength 25 mM, temperature 30℃ and voltage 30 kV. In these conditions, the sensitivity obtained in CE-LIF 266 was in agreement with estimated

S/N value from the model. The complete set of optimized parameters obtained for CE-LIF 266 is presented in Table 3.

Table 3: Experimental values of factors for Box-Behnken design, and optimized values for maximal sensitivity and efficiency

Level	X_1 pH	X_2 ionic strenght (mM)	X_3 temperature (°C)	X_4 voltage (kV)
-1	2.0	15.0	15.0	15.0
0	2.5	20.0	27.5	22.5
+1	3.0	25.0	40.0	30.0
Optimized conditions	2.8	25.0	30.0	30.0

3.2 Validation

Quantitative performance of the CE-LIF 266 method was estimated, according to SFSTP validation guidelines, in three separate series (j=3). According to SFSTP 2003 recommendations [31], validation protocol V5 was selected. This protocol involved three (k=3) and four concentration levels (k=4) with two (n=2) and three (n=3) repetitions for CS and QC, respectively, each prepared in the biological matrix (plasma). MDA was chosen as I.S. because its physico-chemical properties, as well as its electrophoretic behavior, were close to that of PROP. Furthermore, after excitation at 266 nm, MDA exhibited a similar fluorescence spectrum with a maximum value at 330 nm. The ratio of the normalized area (area/migration time) of PROP to the normalized area of MDA (I.S.) was used for calculation. After selecting the most appropriate regression model for the response function, the trueness and precision of the whole procedure were estimated for each concentration level. The former was expressed as a relative bias, while for the latter, variances of repeatability (s^2_r) and intermediate precision (s^2_R) were computed from the estimated concentrations, and the precision was expressed by relative standard deviation (*RSD*). Confidence intervals were then calculated according to SFSTP 1997 recommendations [29], with fixed degrees of freedom (df=k*j-n) at an α=5 % level of risk. Upper and lower confidence limits represented the total error of the method, based on the confidence interval of the obtained results, and enabled the construction of accuracy and linearity profiles.

3.2.1 Selectivity

As the selected sample preparation procedure (protein precipitation) was known to be not selective, interfering substances could not be sufficiently removed from the biological matrix and may alter the compound analysis. The method selectivity was therefore assessed by comparing typical electropherograms obtained by injecting human blank plasma (CAL 00), plasma spiked with MDA at 500 ng·mL^{-1} (CAL 0) and a CS at medium concentration (500 ng·mL^{-1}). Six independent sources of plasma were tested. As illustrated in Fig. 4, no interference was observed in migration times corresponding to PROP and I.S.

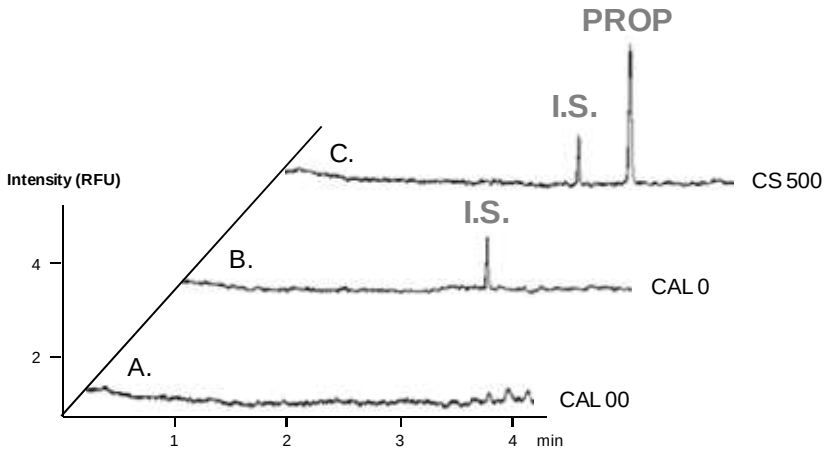


Figure 4: Typical electropherograms obtained by injecting: (A) treated blank plasma (CAL 00), (B) treated plasma spiked with MDA (I.S.) at 500 ng·mL^{-1} (CAL 0), and (C) calibration standard at 500 ng·mL^{-1} (CS 500). See text for experimental conditions.

3.2.2 Selection of the most appropriate regression model

Since reliability of the validation results strictly depends on the selected calibration curve, different regression models were evaluated. These regression models included ordinary least squares linear regression (OLS), OLS forced through zero, external standard with the highest level of CS concentration, OLS after logarithm transformation of both concentration (x) and response (y), OLS after square root transformation of both concentration (x) and response (y), and weighted least squares linear regression (WLS) with two weighting factors (1/x, 1/x^2). From the data, different accuracy profiles were plotted, and the regression model was selected, corresponding to the best accuracies obtained for PROP covering the entire concentration range. The acceptance limits were set at ±30 %, according to the last proposals and the conference report on quantitative bioanalytical methods [49]. The best calibration model was the OLS after square root transformation, with R^2 above 99.8 % and a confidence interval fully included inside the acceptance limits of the whole investigated concentration range.

3.2.3 Trueness and precision

Results for trueness were expressed in terms of relative bias (%) and assessed from the QC. As described in Table 4, the trueness was acceptable, as the relative biases did not exceed the individual threshold of ±15 %, despite the concentration level, and most of the values were lower than ±8 %.

The *RSD* values presented in Table 4 were acceptable for the field of bioanalytical studies and were in the range of 7.5 to 10.8 % for repeatability, and 9.0 to 12.5 % for intermediate precision, demonstrating good precision of the method.

Table 4: Validation results of PROP in plasma (j=3; k=4; n=3)

Validation criterion	PROP
Trueness	
Relative bias (%)	
50 ng mL^{-1}	-5.9
100 ng mL^{-1}	-7.9
500 ng mL^{-1}	-2.2
850 ng mL^{-1}	-7.2
Precision	
Repeatability/intermediate precision (RSD, %)	
50 ng mL^{-1}	9.0/12.5
100 ng mL^{-1}	9.4/10.4
500 ng mL^{-1}	7.5/9.0
850 ng mL^{-1}	10.8/10.8
Accuracy	
Lower and upper confidence limits of the total error (%)	
50 ng mL^{-1}	[-28.2;17.0]
100 ng mL^{-1}	[-27.0;11.1]
500 ng mL^{-1}	[-18.8;14.4]
850 ng mL^{-1}	[-26.9;12.6]
Linearity	
Range (ng mL^{-1})	[50;850]
Slop	0.936
Intercept	0.0036
R^2	0.9988
LLOQ (ng mL^{-1})	50

3.2.4 Accuracy and linearity

The accuracy profile was selected as the decision tool to evaluate the method's capability to quantify samples with an accepted risk (α=5 %) [50]. The accuracy profile for PROP is illustrated in Fig. 5A. As shown in Table 4, the upper and lower confidence limits of the mean bias (%) did not exceed the acceptance limits (±30 %) for each concentration level. Consequently, the CE-LIF 266 method could be considered accurate for PROP over the investigated concentration range [50-850 ng·mL^{-1}]. Since the accuracy profile was included within the acceptance limits (Fig. 5A), the lowest evaluated concentration level (50 ng·mL^{-1}) was confirmed as the lower limit of quantitation (LLOQ) for PROP. Lower estimated LOQ (S/N : 10) were achieved in LC with MS [51] or tandem MS detection [52] where

concentrations at the ppb level were detected (5.5 ng·mL^{-1} and 0.8 ng·mL^{-1}, respectively). To assess the method linearity, an OLS was fitted on the back-calculated concentrations of the QC *vs.* the theoretical concentrations (Fig. 5B). The slope and intercept, as well as determination coefficient, are presented in Table 4. Since the slope value was equal to 0.94 and R^2 was higher than 99.9 %, the method could be considered linear.

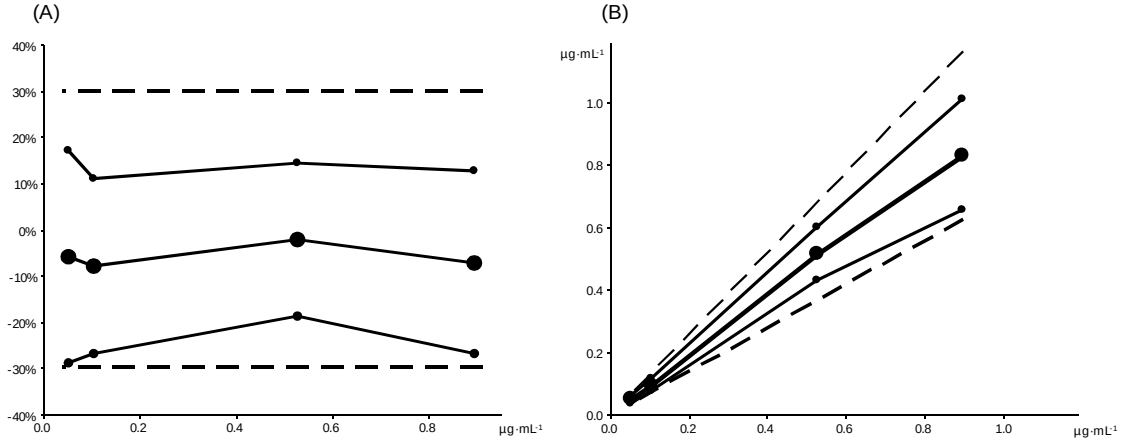


Figure 5: Validation of propranolol in plasma obtained by considering the OLS after square root transformation with α=5%. (A) Accuracy profile and (B) linearity graph.

3.3 Application to biological sample

In order to demonstrate the applicability of the CE-LIF 266 method to real samples, quantitation of PROP was achieved on a plasma sample from a forensic case (post-mortem sample #TA88/05). A screening CE-ESI-MS method [53] emphasized PROP present in the plasma sample as well as mirtazapine, zolpidem and fluoxetine (data not shown). The latter were fluorescent at 266 nm as illustrated in Fig. 6C, and the presence of PROP was confirmed by spiking (Fig. 6D).

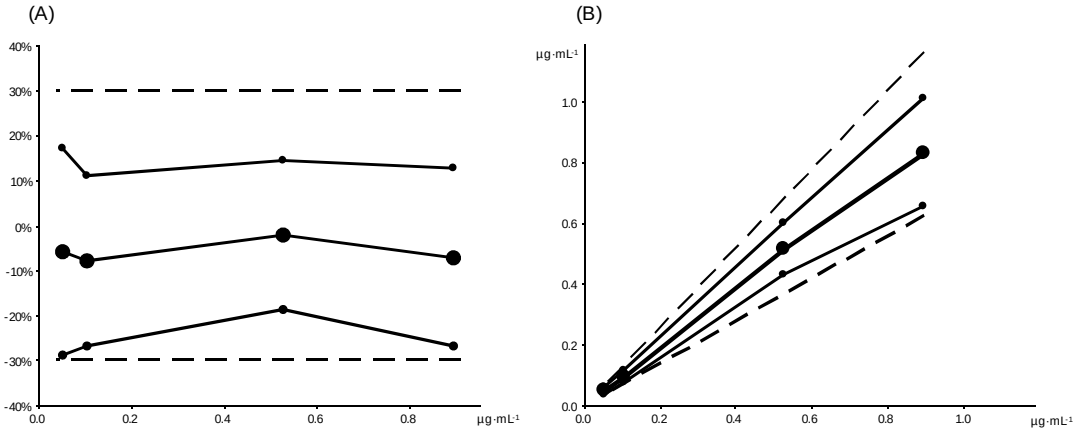


Figure 6: Electropherograms obtained by injecting: (A) treated human blank plasma (CAL 00), (B) treated plasma spiked with MDA (I.S.) at 500 ng·mL^{-1} (CAL 0), (C) treated actual sample issued from sample #TA88/05, and (D) treated actual sample spiked with propranolol and MDA (I.S.) at 10 µg·mL^{-1} each. See text for experimental conditions.

As the selectivity afforded by CE was sufficient, PROP quantitation was performed. The unknown concentration of PROP was calculated with reference to a calibration curve constructed the same day. CS at three concentration levels (k=3) were replicated twice (n=2) and OLS was applied after square root transformation of both concentration (x) and response (y). Since two independent analyses (N=2) were performed on the post-mortem #TA88/05 sample, the confidence interval associated to the result mean could be expressed as :

$$\bar{x} = t_{df,\alpha}\sqrt{\frac{s_r^2}{N} + s_g^2}$$

where N is the number of analyses and $\bar{x}$ the mean result. $t_{df,\alpha}$ (Student constant depending on df and α), s_r^2 and s_g^2 variances were determined during validation with the regular ANOVA-based variance decomposition. The analysis repetition was particularly useful to obtain a smaller confidence interval, since most of the variability came from repeatability (s_r^2). In sample #TA88/05, a PROP concentration of 156±28 ng·mL^{-1} was determined. Hence, PROP uptake could not be held for patient death, since blood concentration was included within therapeutic limits (*ca.* from 20 up to 300 ng·mL^{-1}) while toxic doses are higher than 1 µL·mL^{-1} [54,55]. Qualitative and quantitative results were confirmed by the Institut Universitaire de Médecine Légale (IUML).

4. CONCLUDING REMARKS

CE was coupled with laser-induced native fluorescence detection for analyzing PROP in plasma at low concentration levels. A DPSS laser, emitting at 266 nm, allowed direct detection of unlabelled PROP, which avoided limitations related to derivatization. Furthermore, the high selectivity afforded by the 266 nm detection approach allowed the use of numerous BGE constituents without any interference. As a result, a simple, generic and reliable sample preparation procedure (protein precipitation) could be applied before injecting plasma samples. The CE-LIF 266 procedure for PROP analysis was optimized by a sequential DOE methodology, using a BB second-degree design. This strategy allowed the response surfaces to be obtained, which described electrophoretic and detection properties. A full validation strategy based on accuracy profiles was then selected to demonstrate the ability of the CE-LIF 266 method to quantify PROP in plasma. With an appropriate data transformation and linear calibration model, LLOQ, trueness and precision were determined. The methodology demonstrated good accuracy performance included within the ±30 % acceptance range. Finally, a forensic plasma sample containing PROP was successfully analyzed with the proposed CE-LIF 266 procedure.

5. ACKNOWLEDGMENTS

Authors wish to acknowledge Jocelyne Tahar from Picometrics (Toulouse, France) for the kind loan of DPSS UV laser and the Institut Universitaire de Médecine Légale (Geneva) for the forensic case. Josiane Prat is also acknowledged for her technical assistance.

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ANNEXE 2

Article VIII

A. Staub, S. Rudaz, M. Saugy, J.L. Veuthey, J. Schappler. **Blood doping detection – A new analytical approach with capillary electrophoresis**, *Chimia* 2010, *64*, 886.

Blood doping detection – A new analytical approach with capillary electrophoresis

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Chimia 2010, *64*, 886.

KEYWORDS
Capillary electrophoresis, HBOC, blood doping, time-of-flight mass spectrometry

Blood doping is defined by WADA (World Anti-Doping Agency) as the use of products and methods that enhance the uptake, transport, or delivery of oxygen to the blood. One approach uses artificial oxygen carriers, known as hemoglobin-based oxygen carriers (HBOC). These products are made of bovine or human hemoglobin (Hb) and were developed to treat some types of severe anaemia. They are claimed to provide a third time more efficient oxygen transport and consequently to potentially improve sports performance, particularly in endurance disciplines such as long distance running, cycling or swimming.

Capillary electrophoresis (CE) appears to be a promising technique for HBOC analysis in the context of doping control, since different CE protocols have already been developed for the analysis of Hb variants. In addition, the on-line combination of CE with mass spectrometry (MS) is an attractive option for intact protein analysis (*i.e.*, no digestion, no derivatization step required). On the one hand, CE offers features such as high speed, great efficiency, and low solvent and sample consumptions. On the other hand, MS provides selectivity and specificity.

In this context, a complete analytical strategy based on CE was developed to detect intact HBOC in plasma samples. This methodology implied four distinct steps (Figure 1):

1) Plasma samples preparation based on immunodepletion to remove most abundant proteins (e.g. albumin and immunoglobulin) that can interfere with CE separation and alter electrospray ionization.
2) CE separation to obtain sufficient electrophoretic resolution between HBOC and Hb that could be released from mechanical hemolysis.
3) Online UV-visible detection at 415 nm to selectively detect hemoproteins such as HBOC and Hb.
4) TOF/MS detection to provide accurate mass on analytes and unambiguous determination of HBOC uptake (Figure 2).

The limits of detection were in agreement with doping control requirements. This methodology appears thus suitable for implementation as a doping control screening method for HBOC analysis.

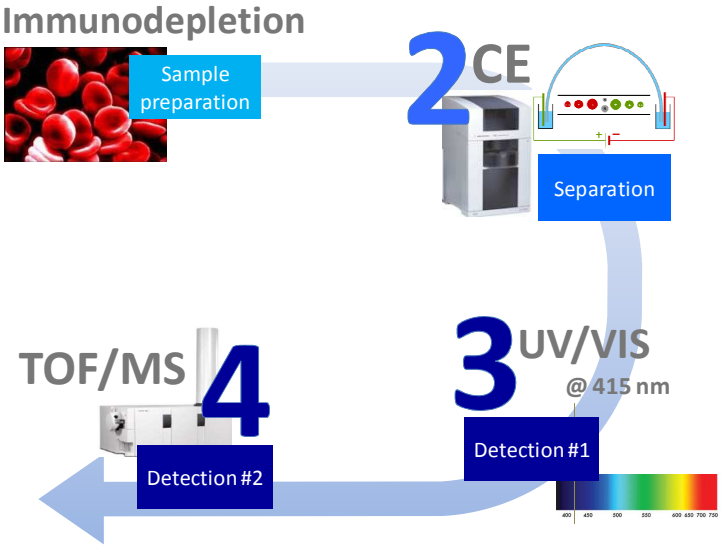

Figure 1: The four selectivity levels obtained with the methodology.

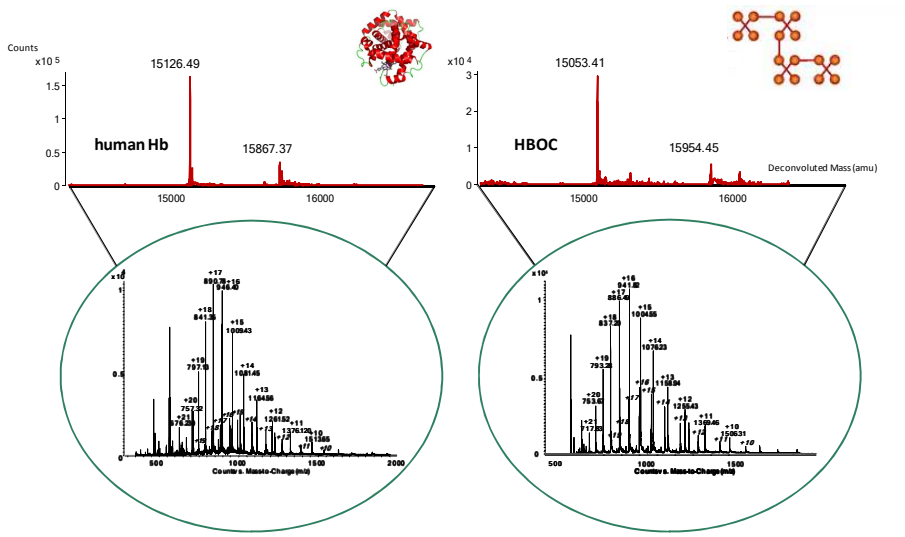

Figure 2: Mass spectra and deconvoluted mass spectra of Hb and HBOC by CE-TOF/MS.

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ANNEXE 3

Communication orale SCS Fall Meeting 2009

New insights in protein analysis with CE-TOF/MS; A. Staub*, J. Schappler, M. Saugy, S. Rudaz, J.L. Veuthey; Septembre 2009, Lausanne (Suisse).

The on-line combination of capillary electrophoresis (CE) with mass spectrometry (MS) is an attractive option for intact protein analysis (*i.e.*, no digestion, no derivation). On the one hand, CE presents features such as high speed, great efficiency, and low solvent and sample consumptions. Moreover, CE allows working under aqueous conditions and without stationary phase. On the other hand, MS provides selectivity and ability to identification. TOF (time-of-flight) analyzer is particularly well suited to protein analysis, due to high mass range and mass accuracy. This lecture presents two CE-TOF/MS developments for intact proteins analysis.

In a first application, an efficient, rapid, and simple CE-TOF/MS method was developed to analyze natural human growth hormone (hGH) and recombinant growth hormones (rhGH) without sample preparation. The method presented original analysis conditions that allowed distinguishing hGH from rhGH. It was successfully applied to seized samples in a forensic case. In a second application, a complete analytical strategy based on CE-TOF/MS was developed to detect intact hemoglobin-based oxygen carriers (HBOC) in plasma samples collected for doping control. HBOC (such as Oxyglobin®) are purified proteins obtained from polymerized bovine hemoglobin that are misused as performance enhancers. A sample preparation based on immunodepletion was mandatory to remove most abundant proteins that interfered with CE separation and altered electrospray ionization (ESI).

ANNEXE 4

Communication orale SEP09

Analyse de protéines intactes par électrophorèse capillaire couplée à un spectromètre de masse à temps de vol (CE-TOF/MS), A. Staub*, J. Schappler, S. Rudaz, J.-L. Veuthey; Décembre 2009, Marseille (France).

Les récents développements dans les domaines de la protéomique et des biotechnologies ont considérablement augmenté la demande en termes de méthodes d'analyse des protéines. Trois techniques analytiques sont couramment utilisées : l'électrophorèse sur gel, la chromatographie liquide, et l'électrophorèse capillaire [1].
Le couplage en ligne de l'électrophorèse capillaire (CE) et de la spectrométrie de masse (MS) est une stratégie très attractive pour l'analyse de protéines sous forme intacte. De plus, les atouts de la CE tels que la vitesse, la haute efficacité ainsi que la faible consommation de solvants et d'échantillons, sont ajoutés à ceux de la MS, c'est-à-dire la sélectivité et la grande capacité d'identification, particulièrement lorsqu'un spectromètre de masse à temps de vol (TOF) est employé [2].
Cependant, le couplage CE-MS n'est aisément obtenu qu'en utilisant le mode électrophorèse capillaire de zone. Si le couplage avec la MS est possible grâce à l'utilisation de tampons de séparation volatils, le phénomène d'adsorption des protéines aux parois du capillaire doit être évalué et minimisé. Des stratégies classiques de revêtements du capillaire (statique ou dynamique) sont envisageables pour s'en soustraire, mais des méthodes alternatives telles que l'usage de modificateurs organiques dans le tampon sont aussi à considérer.
Tous ces aspects importants ont été pris en compte lors du développement de méthodes CE-TOF/MS pour l'analyse de protéines intactes dans différents domaines d'applications. Une première application concerne le domaine de la lutte anti-dopage et plus particulièrement le dopage sanguin. Une méthode d'analyse complète des transporteurs d'oxygène à base d'hémoglobine dans le plasma a été mise au point et remplit les critères anti-dopage en termes de sélectivité et de sensibilité. Une seconde application s'inscrit dans le domaine forensique et a permis sans ambiguïté et sans préparation d'échantillon l'identification d'hormones de croissance dans des échantillons saisis par les douanes.

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ANNEXE 5

Communication orale HPLC 2010

Intact protein analysis by CE-ESI-TOF/MS, from method optimization to quantitation; A. Staub*, J. Schappler, S. Rudaz, J.-L. Veuthey; Juin 2010, Boston (Etats-Unis).

The on-line combination of capillary electrophoresis (CE) with mass spectrometry (MS) is an attractive option for intact protein analysis (*i.e.*, no digestion, no derivatization). On the one hand, CE presents features such as high speed, great efficiency, and low solvent and sample consumption. On the other hand, MS provides selectivity, ability to identification, and sensitivity. Due to its high mass range and mass accuracy, time-of-flight (TOF) analyzer is particularly well suited to the detection of intact proteins that are multi-charged with electrospray (ESI) ionization.
In this lecture, a CE-ESI-TOF/MS strategy is presented for intact protein analysis and quantitation, with human insulin as model protein. The method is intended for biopharmaceuticals quality control, as well as detection of counterfeits and illegally available biomedicines.
The optimization was performed taking into account major issues such as protein adsorption onto the capillary wall and ESI parameters (*e.g.,* sheath liquid composition and flow rate, temperature). The quantitation was then attempted but the choice of the internal standard was not trivial. A new approach was developed using a multiple injection technique, based on successive injections of a reference standard of insulin and the sample in one single run. The complete methodology was fully validated according to ICH guidelines and applied to pharmaceutical formulations obtained in drugstores and on the web without prescription.

ANNEXE 6

Communication orale 14èmes Journées Scientifiques du ccCTA et 3ème Journée Scientifique du Club Jeunes de l'AfSep (2010)

Nouveaux développements dans l'analyse de protéines intactes par CE-TOF/MS; A. Staub*, S. Rudaz, J.L. Veuthey, J. Schappler; Septembre et Octobre 2010, Les Diablerets (Suisse) et Paris (France)

Prix "Jeune Chercheur" de la meilleure présentation orale (3ème Journée Scientifique du Club Jeune de l'AfSep).

Les récents développements dans les domaines de la protéomique et des biotechnologies ont considérablement augmenté la demande en termes de méthodes d'analyse des protéines. Si les méthodes spectroscopiques sont encore largement employées, des techniques séparatives sont souvent nécessaires. Deux approches sont couramment utilisées : la chromatographie liquide et les techniques électrophorétiques.
Dans ce contexte, le couplage en ligne de l'électrophorèse capillaire (CE) et de la spectrométrie de masse (MS) est une stratégie très attractive pour l'analyse des protéines sous leur forme intacte. De plus, les atouts de la CE tels que la vitesse, la haute efficacité ainsi que la faible consommation de solvants et d'échantillons, sont ajoutés à ceux de la MS, c'est-à-dire la sélectivité et la grande capacité d'identification. Lorsqu'en outre un spectromètre de masse à temps de vol (TOF) est employé, une grande exactitude de masse et une résolution intéressante sont ajoutées.
Cependant, le couplage CE-MS n'est aisément obtenu qu'en utilisant le mode électrophorèse capillaire de zone (CZE). Si le couplage avec la MS est possible grâce à l'utilisation de tampons de séparation volatils, le phénomène d'adsorption des protéines aux parois du capillaire doit être évalué et minimisé. Des stratégies classiques de revêtements du capillaire (statique ou dynamique) sont envisageables pour s'en soustraire, mais des méthodes alternatives telles que l'usage de modificateurs organiques dans le tampon sont aussi à considérer.
Tous ces aspects importants ont été pris en compte lors du développement de méthodes CE-TOF/MS pour l'analyse de protéines intactes dans différents domaines d'applications. L'accent est mis sur l'intérêt d'un spectromètre de masse à masse exacte comme détecteur dans le contexte de l'analyse des protéines intactes. Une première application concerne le domaine de la lutte anti-dopage et plus particulièrement le dopage sanguin. Une méthode d'analyse complète des transporteurs d'oxygène à base d'hémoglobine dans le plasma a été mise au point et remplit les critères anti-dopage en termes de sélectivité et de sensibilité. Une seconde application s'inscrit dans le domaine du contrôle qualité et a permis *via* une approche d'injections multiples d'identifier et de quantifier simultanément l'insuline contenue dans une formulation pharmaceutique.

ANNEXE 7

Poster TIAFT 2009 et SCS Fall Meeting 2009

Blood doping with hemoglobin-based oxygen carriers (HBOC): analysis by CE-UV/Vis and CE-ESI-TOF/MS; A. Staub*, J. Schappler, S. Rudaz, J.-L- Veuthey; Août et Septembre 2009, Genève et Lausanne (Suisse).

1er prix pour “excellence in poster presentation” remis par the Youth Scientific Committee of TIAFT.

Blood doping with hemoglobin-based oxygen carriers (HBOC): analysis by CE-UV/Vis and CE-ESI-TOF/MS

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Swiss Centre of Applied Human Toxicology (SCAHT), University of Geneva, CMU, Rue Michel-Servet 1, 1211 Geneva 4, SWITZERLAND

Hemoglobin-based oxygen carriers (HBOC)

HBOC were initially developed as artificial blood replacement products to substitute the oxygen-carrying functions of erythrocytes. However, they are now misused in sport as performance enhancers. These products consist of **bovine or human hemoglobin (Hb)**, which is **polymerized**. Polymerizing of Hb molecules is necessary, otherwise Hb tetrameric structure dissociates into dimers, leading to nephrotoxicity.

OXYGLOBIN	approved for veterinary use in US and Europe	polymerized **bovine** hemoglobin
Hemopure	phase III trials	
PolyHeme	phase III trials	polymerized **human** hemoglobin
HEMOSPAN	phase III trials	

Analytical challenge

Oxyglobin as well as Hb can be found simultaneously in plasma since the former is a circulating polymer and the latter can be released from erythrocytes due to mechanical haemolysis (may occur from physical effort, such as running) and/or sampling conditions.

→ **False positive** results have to be eliminated.

Adapted from www.oxyglobin.com (accessed 10 August 2009)

Sample preparation

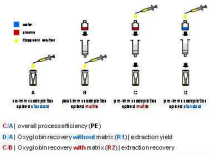
Endogenous proteins, such as albumin and immunoglobulins, are present at high concentrations in plasma and could interfere with the separation and the detection.

→ **Immunodepletion** can remove interfering proteins with the following features:
- specific interactions with **antibodies**
- the **20** most abundant proteins removed from plasma | haptoglobin (Hp) and Hp-Hb complexes totally removed
- spin column format | only **40 µL** of plasma
- **CE compatible** | direct injection, no desalting

1 Protein removal

50% protein removal
→ no more CE adsorption and ESI suppression

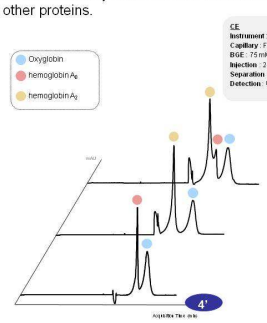
2 Oxyglobin recovery



→ Process efficiency (PE) = **72%**
Extraction yield (R1) = **82%**
Extraction recovery (R2) = **75%**

UV/Vis detection

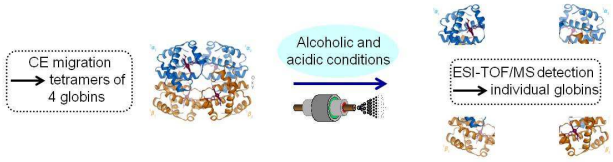
Capillary electrophoresis (CE) appears to be an interesting technique for HBOC analysis since different methods have already been developed for the analysis of Hb variants.
Sufficient electrophoretic selectivity between Oxyglobin and main Hb variants was reached with a BGE at pH 9.5.
An interesting selectivity was obtained with the UV/Vis detection at **415 nm**. At this wavelength, hemoproteins such as Oxyglobin and Hb were easily observed due to their heme moiety in contrast to other proteins.



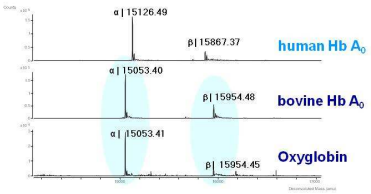
LOD (UV/Vis)
→ **0.20 g/dL in plasma** | 13 µM
1 pmol (16 ng) injected

ESI-TOF/MS detection

Although Hb and Oxyglobin migrated under their intact form, they were detected according to their respective dissociated chains. Monomers were detected because **in-Taylor cone dissociation** of the tetramers occurred afforded by the sheath liquid.



Because human and bovine Hb possess different polypeptide sequences, discrimination between both species was possible. Oxyglobin beeing constituted of bovine Hb, it presented a different deconvoluted mass spectra than human Hb.



LOD (TOF/MS)
→ **0.45 g/dL in plasma** | 29 µM
2 pmol (36 ng) injected

Human Hb presented α and β chains of 15126 and 15867 amu respectively, while bovine Hb and Oxyglobin presented α and β chains of 15053 and 15954 amu respectively. **Unambiguous identification of Oxyglobin was thus possible.**

Conclusions

- Immunodepletion prior to the CE-UV/Vis-ESI-TOF/MS analysis of Oxyglobin in plasma was achieved, providing four complementary analytical steps.
- The methodology could be implemented in doping control.

The authors wish to thank the Swiss Federal Council of Sports (Magglingen, Switzerland) and Dr Matthias Kamber, Director of Antidoping Switzerland (Bern, Switzerland) for financial support.
The World Anti-Doping Agency (Montreal, Canada) is also acknowledged for supporting the "forensic approach of fight against doping" project.

ANNEXE 8

Poster SCS Fall Meeting 2009

CE-ESI-TOF/MS for human growth hormone analysis; A. Staub*, J. Schappler, S. Rudaz, J.-L- Veuthey; Septembre 2009, Lausanne (Suisse).

CE-ESI-TOF/MS for human growth hormone analysis.

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Human growth hormone (hGH)

- Polypeptide of 191 amino acids (pI = 5.2).
- Endogenous hGH presents several isoforms : 22 kDa (most abundant one), 20 kDa, 17 kDa, etc.
- Recombinant hGH (rhGH) possesses an identical sequence to the naturally occurring 22 kDa hormone.

hGH is misused as doping agent due to its anabolic action → its trafficking is relatively widespread

- A rapid CE-ESI-TOF/MS method was developed to analyze hGH in unknown samples without sample preparation.

Interface optimization

In the first step, ionization voltage, capillary outlet position, and sheath liquid composition were determined by an unvaried procedure.

A **response surface** modeling was finally performed with the most significant parameters to find optimal values. **Box Behnken** design was used and 24 experiments were necessary.

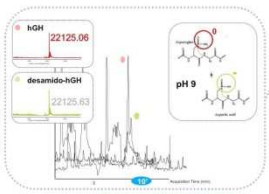
A **screening** methodology was then performed to reveal important interface factors for sensitivity and efficiency. A **fractional factorial** experimental design procedure was used with 22 experiments.

Optimized ESI parameters

- Sheath liquid : iPrOH-H_2O (50:50) + 0.05 % formic acid, **4 µL·min^{-1}**
- Nebulizing gas pressure : **4 psi**
- Drying gas : **4 L·min^{-1}, 150°C**
- Ionization voltage : + 4500 V

Basic pH conditions

In 2005, the European Pharmacopeia incorporated a CE-based approach for detecting charge variants in rhGH under alkaline pH conditions. However, adsorption on the capillary inner surface was observed, unless a coating procedure was implemented.



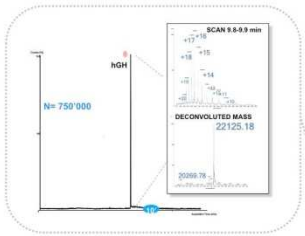
At basic pH, hGH was degraded and presented two peaks :
- peak 1 : hGH
- peak 2 : Asn^{152}-desamido-hGH

Adsorption
Degradation

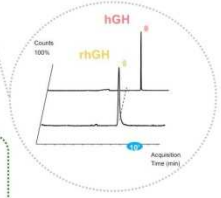
CE
Instrument : HP ^{3D}CE, Agilent (Waldbronn, Germany)
Capillary : FS capillary, 80 cm * 50 µm ID
BGE : 75 mM ammonium buffer pH 9.0
Injection : 50 mbar * 15 s (1 % cap.)
Separation : 25 °C, 30 kV (6 s ramping)
MS
Instrument : 6210 LC/MS TOF mass spectrometer, Agilent (Waldbronn, Germany)
Detection : scan range 900-2500 m/z, scan rate 1 spectrum/s

Acidic pH conditions

Because hGH can potentially adsorb on capillary inner surface, acidic pH conditions have never been reported in literature. The addition of ACN to acidic BGE appeared as an interesting alternative to avoid adsorption and obtain high efficiency. Furthermore, this BGE enabled selectivity between hGH and rhGH.



CE
Instrument : HP ^{3D}CE, Agilent (Waldbronn, Germany)
Capillary : FS capillary, 80 cm * 50 µm ID
BGE : 75 mM formate buffer pH 2.5 + 40% ACN
Injection : 50 mbar * 15s (1 % cap.)
Separation : 25 °C, 30 kV (6 s ramping)
MS
Instrument : 6210 LC/MS TOF mass spectrometer, Agilent (Waldbronn, Germany)
Detection : scan range 900-2500 m/z, scan rate 1 spectrum/s

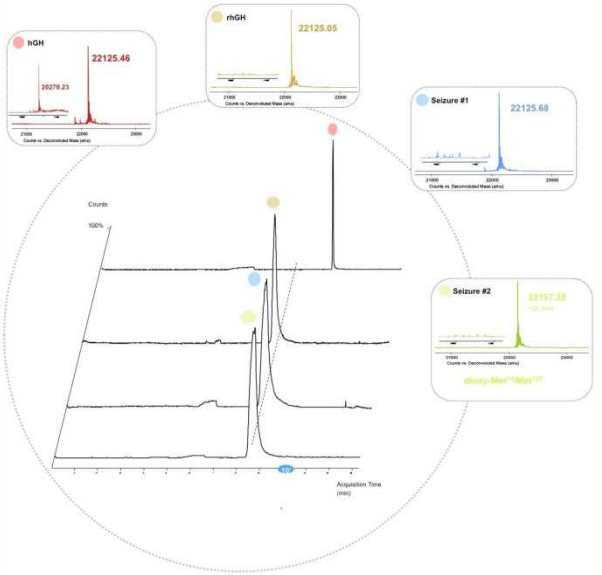


✓ No adsorption
High efficiency

✓✓ Selectivity between hGH and rhGH

Seized samples analysis

Unknown samples were seized and suspected to contain hGH. The CE-ESI-TOF/MS method at acidic pH was used to unambiguously identify the presence of hGH, in less than 10 minutes without sample preparation.



Unknown seized samples were identified as **rhGH**.

- Seizure #1:
  - ✓ same electrophoretic mobility as rhGH
  - ✓ same deconvoluted mass as rhGH

  → **rhGH**
- Seizure #2:
  - ✓ same electrophoretic mobility as rhGH
  - ✓ deconvoluted mass +32 Da compared to hGH

  → **rhGH**, which suffered a double oxidation, probably due to inappropriate storage conditions

Conclusions

- The developed CE-ESI-TOF/MS method at acidic pH allowed discrimination between hGH and rhGH with high efficiency and no adsorption.
- This original BGE composition could be an interesting alternative to commonly used basic pH BGE for the analysis of proteins.
- The method was successfully applied to unknown seized samples, which were unambiguously identified as recombinant human growth hormone.

The authors wish to thank the Swiss Federal Council of Sports (Magglingen, Switzerland) and Dr Matthias Kamber, Director of Antidoping Switzerland (Bern, Switzerland) for financial support.
The World Anti-Doping Agency (Montreal, Canada) is also acknowledged for supporting the "forensic approach of fight against doping" project.

ANNEXE 9

Poster MSB 2010

Intact protein analysis by CE-ESI-TOF/MS, from method optimization to quantitation; A. Staub*, J. Schappler, S. Rudaz, J.L. Veuthey; Mars 2010, Prague (République Tchèque).

Intact protein analysis by CE-ESI-TOF/MS, from method optimization to quantitation

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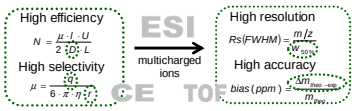
Introduction

Since the beginning of the 21th century, **proteins** have become important on the drugs market. In this study, an analytical procedure was developed to identify and quantify insulin (INS) in Actrapid® formulation. The **top-down approach** was chosen and thus proteins were analyzed under their intact form:

- simple sample preparation (no derivatization, no digestion)
- **CE-ESI-TOF/MS** system particularly well suited



Optimization and adsorption evaluation

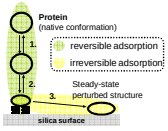
The principal issue, which occurs with CE-ESI-TOF/MS analysis of intact protein, is the adsorption of proteins onto capillary wall. This aspect should be considered and evaluated during method optimization.

Initial experiments were achieved in CE-UV in alkaline condition. The addition of acetonitrile (ACN) to the BGE was investigated, as it may play an important role for preventing adsorption.

→ adsorption evaluation to choose best analytical conditions

Adsorption, which can be reversible and/or irreversible, was evaluated with and without ACN addition.

- Reversible adsorption of proteins onto the capillary wall retards their **migration time** (MT) and decreases **efficiency** of separation due to adsorption/desorption events.
- Irreversible adsorption causes **loss of proteins** within the capillary and alteration of the **electroosmotic flow** (EOF) mobility.

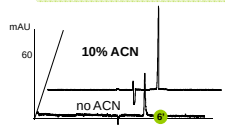


1. Transport to interfacial region
2. Attachment to the surface
3. Conformational changes/spreading over the surface

	Basic pH without ACN	Basic pH with 10% ACN
MT RSD [%] (n=5)	0.68	0.69
FWHM [min] (n=3)	0.09	0.08
peak area recovery [%] (n=5)	95	**100**
µEOF conservation (n=5)	OK	OK

→ no reversible adsorption regardless of ACN addition (low MT RSD and FWHM)
→ irreversible adsorption reduced with 10% ACN

→ Basic pH with 10% ACN

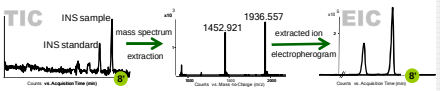


CE
Instrument : HP ^{3D}CE, Agilent (Waldbronn, Germany)
Capillary : FS capillary, L_{tot} 64.5 cm, L_{det} 56cm, 50µm ID
BGE : 75mM formate buffer pH 9.0
Injection : 50mbar * 8s (1 % cap.)
Separation : 25 C, +30kV (6s ramping)
Detection : UV 200nm (response time 0.2s)

Quantitation and validation

1) Ionization and injection corrections

The choice of the internal standard (IS) for quantitative purpose of intact proteins is not trivial. Indeed, isotopically labelled internal standards (IS) and structural analogues are not commonly available. The **multiple injections technique** was used as an alternative methodology to overcome the lack of IS. Two injections were performed sequentially in the same run, the first one with a standard of the protein of interest at a known concentration (reference material) and the second one with the sample to be quantified. Hence, the IS would be the protein itself, exhibiting the same ionization behavior.



CE
Instrument : HP ^{3D}CE, Agilent (Waldbronn, Germany)
Capillary : FS capillary, 80cm * 50µm ID
BGE : 75mM ammonium buffer pH 9.0 + 10% ACN
Multiple injections : 50mbar*15s standard (1% cap.), 50mbar*130s BGE (1.4% cap.), 50mbar*15s sample (1% cap.), 50mbar*4s BGE (0.2% cap.)
Separation : 25 C, +30KV (6s ramping)
MS
Instrument : 6210 LC/MS TOF mass spectrometer, Agilent (Waldbronn, Germany)
Detection : scan range 900-2500m/z, scan rate 1 spectrum/s, reconstruction EIC on ions 1452 and 1936m/z.

The ratio of area of INS sample on area of IS was performed on EIC trace.

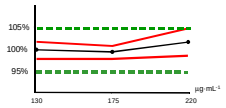
An injection standard (procaïne, PROC) was added to both injected samples in the same run to decrease the observed variability. Online UV detection at 195 nm was performed at the effective length of 22 cm. TOF peak areas of INS were corrected by the respective UV peak areas of PROC. Finally, the ratio of corrected area of INS sample on corrected area of IS was performed.

2) Validation

Quantitative performance of the CE-ESI-TOF/MS method was estimated, according to SFSTP 2003 validation proposal on three separate series (j=3). Validation protocol V1 was selected, involving three concentration levels (k=3) with two repetitions for validation standard (n=2). Calibration standard (CS) was at 100% of the concentration and injected at each run as the first injection plug.

Quantitative performance and accuracy profile

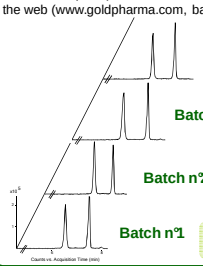
	Trueness *Relative bias*	Repeatability *RSD*	Intermediate precision *RSD*
80 % (131 ppm)	+0.3 %	0.7 %	1.0 %
100 % (175 ppm)	+0.7 %	0.4 %	0.8 %
120 % (219 ppm)	+1.6 %	1.2 %	1.6 %



→ CE-ESI-TOF/MS method can be applied to real samples

Application to real samples

Identification and quantitation of INS was achieved on four pharmaceutical formulations of Actrapid®. Three were obtained from the Pharmacy of the Geneva Hospital (batches n°TS62996, VS63735, and VS64228) and one on the web (www.goldpharma.com, batch n°XS63170), sent from G reece.



Quantitation procedure
- system suitability test (n=1)
- real samples (n=2)

batch n°1 (TS62996) 99.9 ± 1.5%
batch n°2 (VS63735) 98.2 ± 1.5%
batch n°3 (VS64228) 101.0 ± 1.5%
batch n°4 (XS63170) 100.2 ± 1.5%

→ All batches were found to comply with the expected specifications

Conclusions

- The addition of ACN to the BGE circumvented adsorption issue in CE.
- The CE-UV-ESI-TOF/MS methodology allowed the intact insulin quantitation in Actrapid® formulations.
- A multiple injections approach was used to overcome ionization variability. Insulin was used as standard.
- The CE injection variability was corrected by the use of an injection standard (procaïne) detected by UV.
- The complete methodology was fully validated and successfully applied to batches obtained from the Hospital and on the web without prescription.
- This approach could be applied to the identification and quantitation of other protein formulations as well as products obtained on the illegal market.

Acknowledgements

The authors wish to thank Dr Sandrine Fleury from the Pharmacy of the Geneva Hospital for the kind gift of Actrapid® batches.

A PDF copy of this poster can be downloaded on our website: http://www.unige.ch/sciences/pharm/fanal/lcap/index.htm