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NOVEL METHODS FOR QUANTITATIVE DRUG METABOLITE PROFILING BASED ON HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO INDUCTIVELY COUPLED PLASMA – (TANDEM) MASS SPECTROMETRY (HPLC-ICP-MS(/MS))

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LIST OF ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism, Excretion
AMS	Accelerator Mass Spectrometry
ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredient
A_s	USP Tailing Factor
CE	Capillary Electrophoresis
CMPI	2-Chloro-1-methylpyridinium iodide
CS-MAS	Continuum Source Molecular Absorption Spectrometry
DIHEN	Direct Injection High Efficiency Nebulizer
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
DRC	Dynamic Reaction Cell
DTPA	Diethylenetriaminepentaacetate
ECRS	Bis(ethylenediamine)-4´-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester
ELSD	Evaporative Light Scattering Detection
ESI	Electrospray Ionization
ESTD	External Standard Calibration
FDA	U.S. Food and Drug Administration
FEM	*N*-(2-ferroceneethyl)maleimide
FMEA	Ferrocenecarboxylic acid (2-maleimidoyl)ethylamide
GC	Gas Chromatography
GMP	Good Manufacturing Practice
HR	High Resolution
hTISIS	High-Temperature Torch-Integrated Sample Introduction System
HTLC	High Temperature Liquid Chromatography
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
ICP-MS(/MS)	Inductively Coupled Plasma (Tandem) Mass Spectrometry

ID	Internal Diameter
ISTD	Internal Standard Calibration
KED	Kinetic Energy Discrimination
LOD	Limit of Detection
LOQ	Limit of Quantification
MeCAT	Metal-Coded Affinity Tag Technique
NMR	Nucelar Magnetic Resonance Spectroscopy
NPSP	*N*-(phenylseleno) phthalimide
Online ID	Online Isotope Dilution
P	Peak Capacity
PHMB	*p*-Hydroxymercuribenzoate
RP-(U)HPLC	Reversed Phase (Ultra) High-Performance Liquid Chromatography
RSD	Relative Standard Deviation
S/N	Signal-to-Noise Ratio
SBR	Signal-to-Background Ratio
SC	Spray Chamber
SFP	Succinimidylferrocenyl propionate
SPE	Solid Phase Extraction
TBPA	Tetrabromophthalic anhydride
TCEP	Tris(2-carboxyethyl)phosphine
TEA	Triethylamine
TMPP	Tris(2,4,6-trimethoxyphenyl) phosphonium propylamine
TOF	Time-of-Flight
TXRF	Total Reflection X-ray Fluorescence
UCT	Uncontrolled Temperature
USP	United States Pharmacopoeia
UV	Ultraviolet detection
w_b	Baseline Peak Width
WHO	World Health Organization

OBJECTIVES AND OUTLINE

During drug discovery, the absorption, distribution, metabolism and excretion (ADME) behaviour of a candidate drug (active pharmaceutical ingredient - API) must be investigated at an early stage of development. In the pharmaceutical industry, the common way to explore the metabolite profile of APIs and to identify the different metabolites is presently mostly based on the combination of liquid chromatography with different detection methods (*e.g.,* mass spectrometry) and nuclear magnetic resonance spectroscopy (NMR). However, when it comes to quantification, the major disadvantages of these traditional analytical techniques are either the structure-dependent nature of the detector response (MS-based techniques), requiring individual analytical standards for each metabolite, or the possible lack of satisfactory sensitivity/selectivity (NMR). As individual standards are typically not available for (all of) the metabolites, especially in an early phase of drug development, an alternative strategy is indispensable for accurate quantification. An existing alternative for these methods is radio-labelling followed by radio-HPLC. In this approach, the drug molecule is labelled with a radionuclide, usually ^{3}H or ^{14}C. In this way, following the chromatographic separation, the metabolites containing the radionuclide are easily and selectively detected and quantitatively determined based on the radioactive decay, since this response is structure-independent. Major disadvantages of this technique are the need for the costly synthesis of a radiolabelled version of the parent drug and the need to handle and use radioactive materials. The latter also comes with ethical issues, such as the application of radioactive substances in human studies (especially if the drug has a long residence time in the body) and/or the handling of radioactive waste. Thus, the research and development of alternative strategies enabling quantitative metabolite profiling of pharmaceuticals is justified and highly desirable.

This research project therefore aimed to develop a more versatile and easily applicable alternative for metabolite profiling, based on the combination of liquid chromatography (LC) and inductively coupled plasma-mass spectrometry (ICP-MS). The main advantages of this technique are the element-selective detection, the high sensitivity and the fact that the response is independent of the molecular structure in which the target element is introduced. In spite of these significant advantages, the technique has not been widely employed in the pharmaceutical

R&D yet due to several difficulties. This PhD research project addressed the most important issues affecting and compromising the application of reversed phase (RP) HPLC-ICP-MS(/MS) for quantitative metabolite profiling purposes. Although the combination of RP-HPLC and ICP-MS(/MS) is not routinely applied in pharmaceutical industry yet, the two techniques, separately, are among the most important tools in pharmaceutical analysis. Due to the physicochemical properties (solubility, hydrophobicity etc.) of the majority of medical drugs, RP-HPLC coupled with UV-detection has become the most widespread technique for the quality control of APIs and drug products (*i.e.* API in its final formulation together with the necessary excipients) while ICP-MS plays a very important role in the determination of elemental (mainly metallic) impurities mostly in APIs. Therefore, it can be concluded that both principle techniques of the present research project, on their own, are able to provide an analytical performance of the highest quality (*i.e.* GMP-compliance). Thus, although being aware, that metabolite profiling activities do not always require a GMP environment in pharmaceutical R&D, the corresponding analytical GMP standards have still been considered as points of reference during the development and validation works performed in the context of the present research.

In **Chapter 1**, a critical review of the literature serves as an introduction to the present status of (U)HPLC-ICP-MS(/MS) in the field of quantitative metabolite profiling of pharmaceutical drugs not containing a metal, with special emphasis on quantification approaches. Next to the possibilities for the interference-free monitoring of the (non-metal) hetero-elements most typically present in pharmaceutical drugs using ICP-MS(/MS), also derivatization strategies for drugs originally not containing any ICP-MS-detectable hetero-element are discussed.

As a first step of addressing the main issues compromising the applicability of the technique, a novel approach for the determination of the total content of drug-related Cl and Br in human plasma based on RP-HPLC-ICP-MS/MS is presented in **Chapter 2.** Besides the development and successful validation of a novel strategy (including the interference-free determination of Cl and Br with a state-of-art triple-quadrupole ICP-(QQQ)MS/MS system), also a proof-of-concept study is detailed. In this proof-of-concept study, real-life clinical samples containing a newly developed API with both Cl and Br in its chemical structure were analysed aiming at a description of the variation of the content of the drug (and possible metabolites) in human plasma as a function of time.

Chapter 3 addresses one of the most critical challenges arising during the application of gradient RP-HPLC-ICP-MS(/MS), *i.e.* the development of a quantification strategy able to compensate for the detrimental effect of gradient elution on the ICP-MS response for the target element. Due to the continuously changing eluent composition, the application of gradient

elution is typically compromised by a continuously varying ICP-MS response throughout the chromatographic run. Several approaches for the compensation of this signal drift have already been developed and published in the literature, but each of them also shows significant disadvantages preventing the routine use of HPLC-ICP-MS(/MS) in pharmaceutical R&D. Within the context of this chapter, the effect of the organic solvent concentration present in a typical RP-HPLC eluent system on the ICP-MS/MS response obtained for Cl was explored. A novel, straightforward, simple, time- and cost-effective quantification strategy, based on mathematical correction of the aforementioned signal drift, was developed using diclofenac and its related compounds, including 4`-hydroxy-diclofenac (*i.e.* the major metabolite of diclofenac) as model compounds.

In addition to the interference-free ICP-MS(/MS) determination of the hetero-elements typically present in pharmaceuticals and a straightforward strategy for accurate quantification, also the chromatographic aspects must be taken into consideration when it is aimed to use RP-HPLC-ICP-MS(/MS) in the field of pharmaceutical analysis, for which strict GMP regulations for analytical validation may also apply. Therefore, **Chapter 4** is dedicated to the systematic investigation and comparison of the effects that different ICP sample introduction systems exert on the most critical chromatographic performance indicators with respect to the possible GMP regulations using diclofenac and 4`-hydroxy-diclofenac as model compounds once again.

In an effort to cover the most important hetero-elements present in APIs, **Chapter 5** deals with the possibility of F-determination with ICP-MS/MS and evaluates the suitability of an approach based on in-plasma BaF^+ formation for quantitative metabolite profiling purposes.

DOELSTELLING EN OVERZICHT

Bij de ontwikkeling van geneesmiddelen moet het ADME-gedrag (absorptie, distributie, metabolisme en excretie) van een kandidaat-geneesmiddel (actief farmaceutisch ingrediënt - API) reeds in een vroeg stadium van de ontwikkeling worden onderzocht. In de farmaceutische industrie is de meest gebruikelijke manier om het metabolietprofiel van API's te onderzoeken en om de verschillende metabolieten te identificeren momenteel grotendeels gebaseerd op de combinatie van vloeistofchromatografie met verschillende detectiemethoden (bijvoorbeeld massaspectrometrie) en NMR. Met het oog op kwantificering vertonen deze analytische technieken echter enkele belangrijke nadelen. Voor MS-gebaseerde technieken situeren deze zich in de detectorrespons die afhankelijk kan zijn van de structuur van de geanalyseerde verbindingen, zodat afzonderlijke analytische standaarden vereist zijn voor de verschillende metabolieten. NMR wordt dan eerder gekenmerkt door een mogelijk gebrek aan gevoeligheid / selectiviteit. Aangezien individuele standaarden meestal niet beschikbaar zijn voor (alle) metabolieten - vooral niet in een vroege fase van de ontwikkeling van geneesmiddelen - is een alternatieve strategie nodig voor accurate kwantificering.

Een bestaand alternatief voor deze methodes is radiolabeling, gevolgd door radio-HPLC. In deze benadering wordt de geneesmiddelmolecule gemerkt met een radionuclide, gewoonlijk ^{3}H of ^{14}C. Op deze manier worden de metabolieten die de radionuclide bevatten na de chromatografische scheiding gemakkelijk en selectief gedetecteerd en kwantitatief bepaald op basis van het radioactieve verval. Deze methode levert een structuuronafhankelijke respons op, maar kent als grote nadelen de noodzaak tot de (dure) synthese van een radioactief gemerkte versie van het oorspronkelijke medicijn en de noodzaak om met radioactieve materialen te werken. Dit brengt vaak ethische problemen met zich mee, o.a. wat betreft het gebruik van radioactieve stoffen in studies bij mensen (vooral als het medicijn een lange verblijftijd in het lichaam heeft) en / of de behandeling van radioactief afval. Daarom is het onderzoek naar en de ontwikkeling van alternatieve strategieën die kwantitatieve metabolietprofilering van geneesmiddelen mogelijk maken noodzakelijk.

Het doel van dit onderzoeksproject bestond erin een veelzijdig en gemakkelijk toepasbaar alternatief voor kwantitatieve metabolietprofilering te ontwikkelen, gebaseerd op de combinatie van vloeistofchromatografie (LC) en inductief gekoppeld plasma-massaspectrometrie (ICP-MS). De belangrijkste voordelen van deze techniek zijn de element-selectieve detectie, de hoge gevoeligheid en het feit dat de respons onafhankelijk is van de moleculaire structuur waarin het analietelement aanwezig is. Ondanks deze duidelijke voordelen wordt de techniek tot op heden nog niet op ruime schaal toegepast in de farmaceutische R&D, als gevolg van verschillende problemen. Het algemene doel van dit doctoraatsonderzoek was dus om de toepassing van HPLC-ICP-MS(/MS) voor farmaceutische metabolietprofilering uit te breiden en te vergemakkelijken door verschillende problemen aan te pakken die het gebruik van omkeerfase (RP) HPLC-ICP-MS(/MS) bemoeilijken. Hoewel de combinatie van omkeerfase (RP) met HPLC-ICP-MS(/MS) nog niet routinematig wordt ingezet in de farmaceutische industrie, behoren de twee technieken afzonderlijk wel tot de belangrijkste technieken in de farmaceutische analyse. Als gevolg van de fysicochemische eigenschappen (oplosbaarheid, hydrofobiciteit, enz) van de meeste geneesmiddelen is RP-HPLC met UV-detectie uitgegroeid tot de meest gebruikte techniek voor de kwaliteitscontrole van APIs en geneesmiddelen, terwijl ICP-MS een zeer belangrijke rol speelt in de bepaling van de (meestal metallische) elementaire onzuiverheden in APIs. De twee belangrijkste technieken aangewend in dit onderzoek bieden op zichzelf een analytische performantie van het hoogste niveau (m.a.w. GMP-compliance). Daarom werden – hoewel rekening houdend met het inzicht dat in farmaceutische R&D een GMP-omgeving niet altijd vereist is – de GMP standaarden toch steeds als referentiepunten beschouwd bij de ontwikkeling van methodes en hun validatie in de context van dit werk.

In hoofdstuk 1 dient een kritische review van de bestaande literatuur als inleiding tot de huidige status van het gebruik van (U)HPLC-ICP-MS(/MS) voor kwantitatieve metabolietprofilering van geneesmiddelen die geen metaal bevatten - met speciale nadruk op kwantificeringsmethodes. Naast een bespreking van de mogelijkheden voor interferentievrije ICP-MS(/MS) bepaling van de (niet-metallische) hetero-elementen die het meest typisch aanwezig zijn in farmaceutische producten, worden ook strategieën behandeld voor de derivatisatie van geneesmiddelen die oorspronkelijk geen ICP-MS-detecteerbare hetero-elementen bevatten.

Als een eerste stap in de richting van het uitbreiden van de inzetbaarheid van HPLC-ICP-MS(/MS), beschrijft hoofdstuk 2 de ontwikkeling van een nieuwe methode voor de bepaling van het totale gehalte aan Cl en Br afkomstig van een toegediend geneesmiddel in serum. Naast de ontwikkeling en succesvolle validatie van een nieuwe strategie (inclusief de

interferentievrije bepaling van Cl en Br met een triple-quadrupool ICP-MS/MS-systeem), werd daarbij ook een proof-of-conceptonderzoek uitgevoerd. In deze proof-of-conceptstudie werden klinische monsters geanalyseerd die een nieuw ontwikkelde API bevatten met zowel Cl als Br in de chemische structuur, met het oog op een beschrijving van de variatie in de concentraties van het geneesmiddel (en mogelijke metabolieten) in humaan plasma in functie van de tijd.

Hoofdstuk 3 behandelt één van de grootste uitdagingen die zich aandienen bij het gebruik van HPLC-ICP-MS(/MS) met gradiëntelutie, namelijk de ontwikkeling van een kwantificatiestrategie die compenseert voor het nadelige effect van gradiëntelutie op de ICP-MS respons voor het analietelement. Vanwege de continu veranderende samenstelling van het eluens wordt de toepassing van gradiëntelutie immers typisch gecompromitteerd door een continu variërende ICP-MS-respons gedurende de chromatografische scheiding. In het verleden werden reeds verschillende benaderingen ontwikkeld en beschreven in de literatuur voor de compensatie van dit fenomeen, maar elk daarvan vertoont aanzienlijke nadelen die het routinematige gebruik van HPLC-ICP-MS(/MS) in farmaceutische R & D in de weg staan. In de context van dit hoofdstuk werd een nieuwe, eenvoudige, tijds- en kostenefficiënte kwantificatiestrategie ontwikkeld, gebaseerd op wiskundige correctie voor bovengenoemde signaaldrift voor Cl als analietelement. Diclofenac en verwante verbindingen zoals 4'-hydroxy-diclofenac (de belangrijkste metaboliet van diclofenac) werden daarbij gebruikt als modelcomponenten.

Naast een interferentievrije ICP-MS(/MS) bepaling van de hetero-elementen die typisch aanwezig zijn in geneesmiddelen en een eenvoudige strategie voor accurate kwantificering, moet ook aandacht besteed aan de chromatografische aspecten die een invloed kunnen hebben wanneer het de bedoeling is HPLC-ICP-MS(/MS) in te zetten in de context van farmaceutische toepassingen waarvoor strikte GMP-voorschriften gelden voor analytische methodevalidatie. Hoofdstuk 4 is daarom gewijd aan het systematisch onderzoek van verschillende ICP-monsterintroductiesystemen en de vergelijking van de effecten die hun gebruik teweeg brengt op de meest kritische chromatografische prestatie-indicatoren met betrekking tot de mogelijke GMP-voorschriften. Diclofenac en 4'-hydroxy-diclofenac werden daarbij opnieuw als modelcomponenten gebruikt.

In een poging HPLC-ICP-MS(/MS) inzetbaar te maken voor elk van de belangrijkste hetero-elementen aanwezig in APIs, wordt in hoofdstuk 5 dieper ingegaan op de mogelijkheden voor F-bepaling met ICP-MS/MS. F is immers een frequent voorkomend hetero-element in API's, dat echter traditioneel zeer moeilijk te bepalen is door middel van ICP-MS. In dit werk werd de

mogelijkheid onderzocht om gebruik te maken van de vorming van BaF^+-ionen in het plasma om de bepaling van F mogelijk te maken in het kader van kwantitatieve metabolietprofilering.

IMPACT AND SCIENTIFIC OUTCOMES

The impact of this PhD research is reflected in international peer-reviewed publications:

1. **B. Klencsár**, C. Sánchez, L. Balcaen, J. Todolí, F. Lynen and F. Vanhaecke, Comparative evaluation of ICP sample introduction systems to be used in the metabolite profiling of chlorine-containing pharmaceuticals via HPLC-ICP-MS, *Journal of Pharmaceutical and Biomedical Analysis*, **153** (2018) 135-144.

2. **B. Klencsár**, S. Li, L. Balcaen, F. Vanhaecke, High-performance liquid chromatography coupled to inductively coupled plasma - mass spectrometry (HPLC-ICP-MS) for quantitative metabolite profiling of non-metal drugs, *TrAC Trends in Analytical Chemistry*, **104** (2018) 118-134.

3. **B. Klencsár**, L. Balcaen, F. Cuyckens, F. Lynen, F. Vanhaecke, Development and validation of a novel quantification approach for gradient elution reversed phase high-performance liquid chromatography coupled to tandem ICP-mass spectrometry (RP-HPLC-ICP-MS/MS) and its application to diclofenac and its related compounds, *Analytica Chimica Acta* **974** (2017) 43-53.

4. **B. Klencsár**, E. Bolea-Fernandez, M. R. Flórez, L. Balcaen, F. Cuyckens, F. Lynen, F. Vanhaecke, Determination of the total drug-related chlorine and bromine contents in human blood plasma using high performance liquid chromatography – tandem ICP-mass spectrometry (HPLC-ICP-MS/MS), *Journal of Pharmaceutical and Biomedical Analysis*, **124** (2016) 112-119.

Additional publications in peer-reviewed journals:

1. S. Li, **B. Klencsár**, L. Balcaen, F. Cuyckens, F. Lynen, F. Vanhaecke, A pre-column derivatization method allowing quantitative metabolite profiling of carboxyl and phenolic hydroxyl group containing pharmaceuticals in human plasma via liquid chromatography-inductively coupled plasma-tandem mass spectrometry (LC-ICP-MS/MS), *Journal of Analytical Atomic Spectrometry*, **33** (2018) 274-282.

2. S. Li, **B. Klencsár**, L. Balcaen, F. Cuyckens, F. Lynen, F. Vanhaecke, Quantitative metabolite profiling of an amino group containing pharmaceutical in human plasma via pre-column derivatization and high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS), *Analytical Chemistry* **89 (3)** (2017) 1907–1915.

The scientific outcomes of the PhD were presented on international conferences with the following oral presentations:

1. **B. Klencsár**, E. Bolea-Fernandez, M. R. Flórez, L. Balcaen, F. Cuyckens, F. Lynen, F. Vanhaecke, Determination of the total content of drug-related chlorine and chlorine speciation in human blood plasma using high performance liquid chromatography – tandem ICP-mass spectrometry (HPLC-ICP-MS/MS), EWCPS 2017 (European Winter Conference on Plasma Spectrochemistry), 2017, Sankt-Anton am Arlberg, Austria

2. **B. Klencsár**, E. Bolea-Fernandez, M. R. Flórez, L. Balcaen, F. Cuyckens, F. Lynen, F. Vanhaecke, Determination of the total drug-related chlorine and bromine contents in human blood plasma using high performance liquid chromatography – tandem ICP-mass spectrometry (HPLC-ICP-MS/MS), HTC-14 (14th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology), 2016, Gent, Belgium

3. **F. Vanhaecke**, Y. Anoshkina, M. Costas-Rodriguez, M. R. Flórez, A. A. M. B. Hastuti, B. Klencsár, S. Lauwens, S. Li, T. Van Acker and S. J. M. Van Malderen, Concentrations, speciation, images and isotope ratios in biomedical / pharmaceutical research … ICP-MS delivers it all!, Asia-Pacific Winter Conference on Plasma Spectrochemistry, 2017, Matsue, Japan

CHAPTER 1

Introduction

Adapted from:

B. Klencsár, S. Li, L. Balcaen, F. Vanhaecke, High-performance liquid chromatography coupled to inductively coupled plasma - mass spectrometry (HPLC-ICP-MS) for quantitative metabolite profiling of non-metal drugs, *TrAC Trends in Analytical Chemistry*, **104** (2018) 118-134.

1.1 Introduction

The absorption, distribution, metabolism and excretion (ADME) behaviour of a candidate drug (active pharmaceutical ingredient – API) must be investigated at a sufficiently early stage of drug development. For this often crucial and challenging step of drug development, several techniques are traditionally applied for the identification, characterization and quantification of drug metabolites (thus for monitoring the biotransformation of a novel API). These techniques can include (ultra)high-performance liquid chromatography combined with UV-detection and/or (tandem) mass spectrometry ((U)HPLC-UV/MS^n) [1-6], HPLC with radiodetection [7], gas chromatography - MS(/MS) (GC-MS(/MS)) [8], capillary electrophoresis – MS(/MS) (CE-MS(/MS)) [9-11], nuclear magnetic resonance spectroscopy (NMR) [12-15], accelerator mass spectrometry (AMS) [16] and with a continuously increasing significance, also inductively coupled plasma - (tandem) mass spectrometry (ICP-MS(/MS)) [17, 18]. While the identification of metabolites can usually be achieved by using traditional techniques (typically MS and/or NMR), the quantification is more challenging due to the typical lack of individual standards for (all of) the metabolites and the structure-dependent response (in case of MS-based techniques or UV-detection) or the possible lack of adequate sensitivity/selectivity (in the case of NMR) of the traditional techniques. Therefore, the present standard methodology for quantitative drug metabolite profiling is based on radiodetection, providing a selective and structure-independent analytical signal for the parent drug and its metabolites. However, HPLC followed by radiodetection suffers from several disadvantages. First of all, it requires the synthesis of a radiolabelled version of the parent drug (typically with ^3H or ^{14}C), which is very costly and time-consuming, in addition to the need of handling radioactive materials. Ethical concerns related to the administration of radioactive materials to human volunteers during clinical studies must also be taken into consideration, especially if the candidate pharmaceutical has a long residence time in the body. Therefore, alternative strategies to radiolabelling are being investigated. ICP-MS can serve as a possible alternative for this purpose owing to its high sensitivity and the element-specific analytical response, independent of the molecular structure. ICP-MS is not a new technique for the pharmaceutical industry, but so far it has been mainly used for the determination of inorganic (mainly metallic) impurities in APIs, *i.e.* for demonstrating that these products meet the continuously tightening requirements established by the pharmaceutical authorities [19-26]. When hyphenated with an adequate separation technique (*e.g.*, (U)HPLC), enabling the separation of the drug-related compounds of interest in biological matrices from endogenous compounds and from one another, ICP-MS can also be deployed in the context of quantitative drug metabolite profiling. This introduction provides an

overview of the current status of (U)HPLC-ICP-MS(/MS) in the field of pharmaceutical metabolite profiling, and discusses the benefits, but also the main limitations and challenges of the technique in this context. While being aware of the existence and great importance of metal-based drugs (*e.g.*, both the chemotherapeutic drugs cisplatin and carboplatin are included in the WHO Model List of Essential Medicines (EML) [27]), it must be noted that only a very small fraction of all pharmaceutical drugs contains a metal [28], while the utility of ICP-MS in this context is rather obvious and has already been reviewed in the recent past [29-37]. Therefore, the present chapter focuses on drugs not containing a metal, for which the application of ICP-MS as a detector is less self-evident and more challenging [38]. Hence, this introduction deals with drugs containing typical non-metal hetero-elements (with special emphasis on S, Cl, P, Br and I, all of which are within the top-10 constituting elements of APIs [28]) and on derivatization strategies for drugs without any ICP-MS detectable hetero-element, based on derivatization via reaction with the most abundant functional groups present in drug substances (-OH, -COOH, -NH_2 and -SH) [39].

1.2 Brief history of RP-HPLC and ICP-MS

As is well known, owing to the physicochemical properties (hydrophobicity, solubility, etc.) of the majority of the medical drugs, especially reversed phase (RP) HPLC is commonly used in the field of pharmaceutical analysis [40]. The fundamentals of modern liquid chromatography were established in the 1960s by Horváth at Yale University [41, 42]. This has also initiated intensive research concerning the improvement of different stationary phases by Kirkland [43-45] and Snyder [46-48]. Although the great potential of reversed phase chromatography was already demonstrated in 1950 by Howard and Martin [49], a wider spread of the technique was strongly compromised by the lack of appropriate stationary phases and of understanding of the separation mechanism. Owing to the pioneering work of Horváth *et al* to understand the physicochemical principles of RP-HPLC [50] and to the continuous development of bonded stationary phases by Kirkland *et al* and Snyder *et al*, RP-HPLC has soon become the most commonly applied chromatographic technique in various fields, including pharmaceutical analysis [40, 51-54]. Even though the principles were already laid down decades ago, RP-HPLC still remains intensively researched nowadays. As a result, the literature on the topic is continuously expanding and there is still continuous improvement of both the stationary phases (*e.g.,* BEH technology introduced by Waters Corp. or Core-shell technology by Phenomenex) and instrumentation (*e.g.,* introduction of UPLC™ by Waters Corp.) [55-58]. This progress and

the current state-of-art have been summarized in several excellent recent books and review papers [53, 54, 59, 60].

While RP-HPLC has become one of the most important techniques in the field of separation sciences, the same is true for ICP-MS in the field of atomic spectroscopy. ICP-MS is a relatively young analytical technique as the first coupling of an ICP ion source to an MS was described by Houk *et al* in 1980 only [61]. The first successful attempts were closely followed by the commercial introduction of the first ICP-MS instruments in 1983, owing to the enormous potential of the new technique. ICP-MS quickly became a widespread technique around the globe owing to its high ionization efficiency, thus very high sensitivity, high sample throughput, multi-element capability and the large flexibility in terms of sample introduction, enabling the trace element analysis of a huge variety of matrices. Simultaneously with the fast propagation of the technique, intensive research has explored and described the physicochemical principles of ICP-MS and the use of the technique was extended to various fields of application, which is perfectly demonstrated in several review papers and books in the field [62-66]. Similarly to RP-HPLC, also ICP-MS is characterized by continuous improvement of the instrumentation which is, for example, illustrated by the recent introduction of ICP-(tandem)MS/MS by Agilent Technologies [67-69], which was applied as the principle type of ICP-MS instrument in the present research project.

1.3 Coupling between HPLC and ICP-MS(/MS) and quantification approaches in HPLC-ICP-MS(/MS)

As mentioned above, ICP-MS(/MS) may be a sterling alternative to radiodetection in quantitative drug metabolite profiling. Therefore, a straightforward and efficient coupling between the two techniques (RP-HPLC and ICP-MS) is an obvious pre-requisite for the routine application of HPLC-ICP-MS in this field. At first sight, there seems to be a full compatibility between HPLC and the traditional sample introduction system of ICP-MS, as HPLC provides a typical flow rate in a range of 0.2 – 1.0 mL min^{-1}, which perfectly matches the flow rate range of the traditional nebulizers used (in combination with a spray chamber) for sample introduction in ICP-MS. However, it should be noted that ICP-MS is traditionally designed for analysis of aqueous solutions, while RP-HPLC typically deals with mixtures of organic solvents (typically acetonitrile and/or methanol) and water. The introduction of organic solvents into the ICP-MS without any modification of the traditional set-up may instantly douse the plasma or result in carbon deposition on the torch, interface cones, clogging of the torch injector and the sampling cone aperture, thus causing unstable conditions with the possibility of extinguishing the plasma.

The introduction of O_2 into the Ar-plasma [70, 71] can help stable conditions to be maintained. Most of the newer ICP-MS instruments are equipped with an additional gas line and built-in mass-flow controller that enable the introduction of O_2 or an Ar-O_2 mixture as an optional gas into the carrier gas flow [71, 72], rendering the instrumental set-up much more simple and user-friendly. While the use of O_2 seems to solve some of the major problems related to coupling RP-HPLC with ICP-MS, new difficulties may arise due to the modified and more "aggressive" plasma conditions. First of all, as a result of the introduction of O_2, the more traditional Ni cones have to be replaced by the more resistant (but also more expensive) Pt cones. To reduce the organic load of the plasma to the highest extent possible, a torch with a smaller ID (1.0-1.5 mm) injector is typically deployed instead of a standard injector with an ID of 2.0-2.5 mm and the spray chamber is cooled to around 0 °C or even a sub-zero temperature [73]. The significant reduction in sensitivity for the analytes of interest compared to the "O_2-free mode" and the possibility of an enhanced formation of newly formed interfering polyatomic entities (*e.g.*, oxide ions) must also be taken into consideration when O_2 is admixed to the plasma.

For the separation of metabolites and parent drug(s) from one another in the presence of a typically complex biological matrix, isocratic elution suffices in rare cases only; most often gradient elution is required [40]. As a consequence of gradient elution, a sample flow with continuously changing composition enters the plasma, resulting in continuously changing plasma conditions, thus giving rise to a continuously varying analytical response (sensitivity) throughout the chromatographic run. This varying response compromises the employability of a simple external or internal standard calibration approach for quantification. Therefore, this effect must be avoided or at least properly corrected for to realize accurate quantification. Several approaches have already been tested for the elimination/correction of this detrimental effect of gradient elution.

One of the most popular and reliable tools is the application of species-unspecific *online isotope dilution (ID)* as described by Rottmann and Heumann in 1994 [74-78]. This strategy is based on the post-column addition of an isotopically enriched spike solution containing the element of interest. The chromatogram displaying the variation in the isotope ratio monitored for the target element as a function of time can be easily converted into the corresponding mass flow chromatogram, enabling direct quantification of the parent drug molecule and its metabolites by peak integration. This approach has the ultimate benefit of providing outstanding accuracy (as both isotopes are equally affected by gradient elution and the high-temperature ICP provides isotopic equilibration). However, online ID also comes with some disadvantages and limitations, hindering its routine applicability. First of all, it can only be used for elements with

two or more stable isotopes, jeopardizing its application for the mono-isotopic elements, including P, which is the fourth most abundant hetero-element in FDA-approved pharmaceuticals [28]. Obviously, this also means, that online ID is applicable only for those multi-isotopic analyte elements for which interference-free ICP-MS monitoring is feasible for at least two of its isotopes, which may be especially challenging for light elements (*e.g.*, Cl, S), which are also among the most abundant hetero-elements in pharmaceuticals. Finally, due to its species-unspecific nature, any difference in the analyte transport efficiency of the entire sample introduction system between species would jeopardize accurate quantification using online ID. In addition to these limitations, online ID also requires a more complex instrument set-up, impeding its adoption into the daily routine of pharmaceutical development. Although the application of online isotope dilution is far from routine in pharmaceutical industry due the limitations mentioned above, several drug metabolite profiling studies using this quantification strategy have been reported in literature. Cuyckens *et al* [79] and Meermann *et al* [80] successfully demonstrated the applicability of online isotope dilution for the quantitative metabolite profiling of a novel Br-containing anti-tuberculosis drug *i.e.* bedaquiline (since then approved by the U.S. FDA and marketed under the brand name *Sirturo*) utilizing the $^{81}Br/^{79}Br$ isotope ratio. This work can be regarded as a milestone in the history of HPLC-ICP-MS used in the field of quantitative drug metabolite profiling, as it is the first ever application approved by a major pharmaceutical authority (U.S. FDA) during the registration procedure of a novel drug. A quantitative comparison between radio-HPLC (based on a ^{14}C-label) and HPLC-ICP-MS was provided, while HPLC-ESI-QTOF-MS/MS was used for the identification of the metabolites. Recognizing an additional significant drawback of online ID – *i.e.* the lack of selectivity for distinguishing between drug-related (target compounds) and possible endogenous species containing the target element (Br in this case) – Meermann *et al* [81] also presented a reversed online ID approach for the quantitative metabolite profiling of the same drug based on the application of a ^{81}Br-labelled version of the drug and a spike solution (introduced post-column) with natural Br isotopic composition. Although this approach also requires the synthesis of a labelled version of the drug, the application of radio-active isotopes is still not necessary, eliminating the corresponding ethical concerns.

Another popular and successful strategy for correction of the biasing effect of gradient elution is the application of a *post-column counter-gradient,* ensuring a sample flow with constant composition entering the ICP [82]. Similarly to online ID, this methodology also requires a more complex instrument set-up with two liquid flows perfectly harmonized in time. The main advantage of this strategy is its applicability to mono-isotopic analytes or analytes for which

interference-free determination is possible for one isotope only. Pereira *et al* [18] successfully applied a compensation gradient for the quantification of different hetero-element-containing pharmaceuticals using both ICP-MS and ELSD (evaporative light scattering detection) detection following RP-HPLC separation in a combined instrumental system. Pröfrock *et al* [83] implemented the same strategy for the quantification of phosphorylated peptides and tryptic protein digests using gradient capillary RP-HPLC coupled to ICP-MS. They have thoroughly investigated the effect of different gradient elution programs on the ICP-MS sensitivity obtained for P and the compensation for this effect was found necessary for accurate quantification. As a result, the quantification could be carried out by using a simple inorganic P standard, injected into the sample flow post-column during the final, isocratic washing period of the chromatogram.

Next to these methods, also *mathematical correction* for the effect of gradient elution can be considered as a straightforward approach. Siethoff *et al* [84] employed this strategy for the quantification of nucleotides based on P detection following RP-HPLC separation. To investigate the effect of the applied gradient on the ICP-MS sensitivity for P, 1 mg L^{-1} inorganic P (*ortho*-phosphoric acid) was added to both eluents and a correction function was determined based on the change of the P signal intensity throughout the chromatographic run. A very similar approach was followed by Wind *et al* [85] for the determination of the degree of phosphorylation of proteins on the basis of simultaneous detection of P and S using capillary HPLC-ICP-MS. Similarly to Siethoff *et al* [84], they also spiked the eluents with P and S (10 µM phosphate and 10 µM cysteine) to establish an appropriate function for both elements to be able to correct for the effect of gradient elution. As the study was utilizing the P/S ratio for the determination of phosphorylation degree, a correction function for the P/S ratio vs retention time was applied. Although the application of mathematical correction is technically very simple compared to the application of online ID or compensation gradient, there are several limitations characterizing this strategy. First of all, the reproducibility of this approach highly depends on the stability of the ICP-MS instrument between the subsequent chromatographic runs. 10-20% variation was reported by Siethoff *et al* [84], which may be sufficient for several applications, but hardly satisfactory for drug metabolite profiling. Another major drawback of the methodology applied in the studies detailed above, is the addition of the target analyte into the eluent system. Depending on the nature of the compound used for spiking, this practice can cause long-term contamination and serious memory effects in the HPLC system, possibly compromising future trace level determination of these analytes. A novel quantification approach based on mathematical correction was developed by Klencsár *et al* [86] (detailed in

Chapter 3) for the determination of diclofenac and its related compounds in blood plasma via RP-HPLC-ICP-MS/MS. The ICP-MS/MS sensitivity for Cl as a function of organic solvent content of the eluent was systematically investigated for both methanol and acetonitrile by means of flow injection experiments. By comparison of the signals obtained for inorganic Cl and diclofenac-Cl, it was confirmed that ICP-MS/MS indeed provides a structure-independent analytical response. Additionally, via a simple flow injection experiment in the relevant organic content range prior to the injection of the samples, a reliable and reproducible mathematical function describing how the Cl-sensitivity is affected by the eluent composition could be established. This function could be subsequently used for the compensation of the signal drift observed during gradient elution. Thus, by utilizing the excellent stability of the ICP-MS/MS instrument applied, the addition of the target analyte into the eluents could be avoided and an accurate and precise quantification of diclofenac and its related compounds, including its major metabolite, 4`-hydroxy-diclofenac, could be achieved in human plasma matrix. Naturally, it is worthwhile to note that in addition to an excellent instrument stability, also quantitative recovery of the species from the chromatographic column and an analyte introduction efficiency that is independent of the species in which the target element appears are, once again, required. Besides the most commonly used quantification strategies detailed above, also alternative approaches for the same purpose have been described. An interesting approach was described by Meermann and Kießhauer [87] in 2011. A novel instrumental set-up for providing an O_2-gradient, matched to the gradient programme of the HPLC, was developed and investigated for its ability to compensate for the signal drift during gradient elution. The introduction of O_2 into the ICP is inevitable to maintain stable plasma conditions when RP-HPLC is coupled to ICP-MS. Obviously - when gradient elution is applied - the addition of a constant O_2 flow results in O_2-excess during the largest fraction of the chromatographic run. By monitoring the CeO^+/Ce^+ ratio and maintaining it below 2%, Meermann and Kießhauer optimized the O_2 flow as a function of the organic content of the HPLC-eluent, in this way, matching the O_2 gradient to the chromatographic programme. For comparison of the figures of merit at constant O_2 flow and with a matched O_2 gradient, the signal stability for the $^{140}Ce^+$ signal was monitored throughout the whole chromatographic run by injecting a Ce-standard solution every 1.5 minutes. The results clearly showed that the signal drift could be significantly reduced by applying an O_2 gradient instead of a constant O_2 flow. However, as was also pointed out by Meermann and Kießhauer, the signal drift could not be completely eliminated as the nebulizer gas flow rate was not accordingly adjusted for the changing O_2 flow, thus resulting in a continuously varying sample gas flow rate reaching the plasma.

An alternative approach to deal with the issues related to the high organic content of the eluent and gradient elution is a significant reduction of the organic load of the plasma by miniaturization of the chromatographic system, alongside with modification of the sample introduction system for ICP-MS, ensuring the compatibility with the down-scaled (4-100 µL min^{-1}) flow rate range [73, 88, 89]. In a study by Stefánka *et al* [90], a systematic down-scaling of column IDs from 2.1 to 0.32 mm was evaluated for the speciation of the chemotherapeutic drugs carboplatin and oxaliplatin. A variety of ICP-MS sample introduction systems, including different nebulizers (also including the direct-injection high efficiency nebulizer; DIHEN) and spray chambers, were tested and the results were compared in terms of peak width, peak asymmetry and resolution for/between the peaks of carboplatin and oxaliplatin. The results showed that no improvement in resolution could be achieved by miniaturization, while significant improvement for peak asymmetry was observed with the DIHEN, which is characterized by the lowest dead volume. The effect of the methanol content on the ICP-MS signal was also investigated using different flow rates and sample introduction systems. It was clear, that the signal drift becomes less pronounced by reducing the organic load of the plasma (miniaturization), but is still far from negligible when aiming at accurate quantification. Therefore, it can be concluded, that miniaturization can contribute to a reduction of the detrimental effect of gradient elution, but an effective correction approach is still needed for accurate quantification, as was also stated by both Pröfrock *et al* [83] and Wind *et al* [85] for capillary HPLC.

1.4 Metabolite profiling of drugs containing (a) non-metal hetero-element(s)

As mentioned above, at first sight, ICP-MS is only applicable for drugs containing a suitable hetero-element. A metal would of course be ideal for ICP-MS detection, but only a very small fraction of the pharmaceuticals contain a metal, as mentioned previously. In fact, S, Cl, F, P, Br and I – in this order of incidence – are the most abundant hetero-atoms in the drugs approved by the U.S. Food and Drug Administration [28]. Therefore, the capability of ICP-MS for the quantification of these elements fundamentally determines the analytical merit of HPLC-ICP-MS for pharmaceutical metabolite profiling. The determination of these elements by means of ICP-MS is possible, but is typically seriously hampered by their possible endogenous presence at high concentration levels in biological matrices (*e.g.,* Cl, P, S) and/or by the occurrence of spectral overlap of the analyte signals with those of interfering ions having the same nominal mass, as summarized in Table 1.1 [38, 91-94]. However, over the years, several approaches to overcome spectral interference have been developed by the instrument manufacturers.

The fast evolution of quadrupole ICP-MS systems – especially the introduction of collision/reaction cells – has significantly improved the employability of ICP-MS for these non-metal elements [95, 96]. Spectral interference from polyatomic ions can be counteracted by utilizing kinetic energy discrimination (KED) in collision mode (typically using He as an inert collision gas) or by relying on selective ion-molecule chemistry in reaction mode (using a variety of reaction gases). In the first approach, the kinetic energy of the interfering polyatomic ions is reduced to a higher extent than that of the analyte ions as a consequence of the larger size of the former ones. Thus, the polyatomic ions with reduced energy are prevented from entering the mass spectrometer via an appropriate bias (decelerating potential) setting, while the target ions can pass towards the mass analyser. Chemical resolution on the other hand relies on selective and complete reaction between the interfering ion and the reaction gas (on-mass approach) or reaction between the analyte ion and the reaction gas (*e.g.*, H_2, NH_3, O_2, CH_3F etc.), converting the analyte ion into a reaction product ion that can be measured interference-free at another mass-to-charge ratio (mass-shift approach). In the latter case, the reaction needs to be selective but not necessarily complete, which is an important advantage. The more recent introduction of tandem ICP - mass spectrometry (ICP-MS/MS) with an octopole collision/reaction cell mounted in-between two quadrupole mass analysers provides an even more powerful tool to effectively overcome spectral overlap, as has been recently reviewed by Balcaen *et al* [68] and Bolea-Fernandez *et al* [69]. Considering elements of relevance in the context of this research project, Br can be effectively determined on-mass by removing Ar-based interferences via applying H_2 as a reaction gas [71, 72], utilizing the reactions described by Feldmann *et al* [97, 98] and Boulyga *et al* [99]. Similar strategies can be found for the on-mass determination of Se by using different reaction gasses, such as H_2, N_2O, CH_4 and NH_3 [100-105]. The mass-shift strategy is typically employed for the determination of S, Cl and P. S and P can be effectively converted to SO^+ and PO^+, respectively, shifting the monitored analyte m/z with 16 units [67, 92, 94, 106, 107]. However, it must be noted that while the overlap at m/z = 32 (S) and 31 (P) is easily eliminated by the reaction with O_2, the targeted reaction product ion masses (m/z = 48 and 47, respectively) can also be strongly compromised by polyatomic interferences, as can be seen from Table 1.1. The same is true for Cl, determined as ClH_2^+ using H_2 as a reaction gas [71, 107]. However, this problem is easily avoided with double mass selection in ICP-MS/MS, whereby the first quadrupole is set to the original m/z of the analyte ion, thus removing other ions at the m/z of the reaction product ion subsequently generated in the octopole collision/reaction cell. In this way, the interferences hampering the monitoring of the reaction product ions listed in Table 1.1 can be effectively eliminated. It can

thus be stated that the introduction of tandem ICP-MS/MS has particularly revolutionized the quantification of these light elements, of high importance in the field of pharmaceutical analysis. The major advantages of the application of quadrupole instruments equipped with collision/reactions cells are the high sensitivity and pronounced robustness. As drawbacks, the reduction in sensitivity as a result of the use of a collision/reaction gas and the need for method development/optimization for each novel analytical task can be mentioned.

Table 1.1: Summary of interfering ions (non-restrictive list) affecting the determination of non-metal elements typically present in pharmaceuticals and that of Se

Analyte ion	Relative abundance of the nuclide of interest (%)	First ionization energy (eV)	Typical interfering ions
$^{32}S^+$	94.99 [108]	10.36 [94, 109]	$^{16}O_2^+$, $^{14}N^{18}O^+$, $^{15}N^{17}O^+$, $^{15}N^{16}O^1H^+$, $^{14}N^{17}O^1H^+$, $^{14}N^{16}O^1H_2^+$ [38, 94]
$^{33}S^+$	0.75 [108]		$^{32}S^1H^+$, $^{16}O^{16}O^1H^+$, $^{14}N^{18}O^1H^+$, $^{15}N^{17}O^1H^+$, $^{16}O^{17}O^+$, $^{16}O_2{}^1H^+$, $^{15}N^{18}O^+$ [38, 94]
$^{34}S^+$	4.25 [108]		$^{15}N^{18}O^1H^+$, $^{16}O^{18}O^+$, $^{17}O_2^+$, $^{16}O^{17}O^1H^+$, $^{16}O^{16}O^1H_2^+$, $^{33}S^1H^+$, $^{32}S^1H_2^+$ [38, 94]
$^{35}Cl^+$	75.76 [108]	12.97 [91, 109]	$^{16}O^{18}O^1H^+$, $^{16}O^{17}O^1H_2^+$, $^{34}S^1H^+$ [38, 71]
$^{37}Cl^+$	24.24 [108]		$^{36}Ar^1H^+$, $^{36}S^1H^+$ [38]
$^{31}P^+$	100 [108]	10.49 [109]	$^{14}N^{16}O^1H^+$, $^{15}N^{15}N^1H^+$, $^{15}N^{16}O^+$, $^{14}N^{17}O^+$, $^{13}C^{18}O^+$, $^{12}C^{18}O^1H^+$ [38, 83, 85]
$^{79}Br^+$	50.69 [108]	11.84 [72, 109]	$^{38}Ar^{40}Ar^1H^+$, $^{40}Ar^{39}K^+$, $^{31}P^{16}O_3^+$ [38, 72]
$^{81}Br^+$	49.31 [108]		$^{40}Ar^{40}Ar^1H^+$, $^{32}S^{16}O_3{}^1H^+$, $^{33}S^{16}O_3^+$ [38, 72]
$^{74}Se^+$	0.89 [108]	9.75 [109]	$^{37}Cl_2^+$, $^{36}Ar^{38}Ar^+$, $^{38}Ar^{36}S^+$, $^{40}Ar^{34}S^+$ [38, 110]
$^{76}Se^+$	9.37 [108]		$^{40}Ar^{36}Ar^+$, $^{38}Ar_2^+$, $^{40}Ar^{36}S^+$, $^{31}P_2{}^{14}N^+$ [38, 110]
$^{77}Se^+$	7.63 [108]		$^{40}Ar^{37}Cl^+$, $^{36}Ar^{40}Ar^1H$+, $^{38}Ar_2{}^1H^+$, $^{12}C^{19}F^{14}N^{16}O_2^+$ [38, 110]
$^{78}Se^+$	23.77 [108]		$^{38}Ar^{40}Ar^+$, $^{38}Ar^{40}Ca^+$, $^{31}P_2{}^{16}O^+$ [38, 110]
$^{80}Se^+$	49.61 [108]		$^{40}Ar_2^+$, $^{32}S^{16}O_3^+$ [38, 110]
$^{82}Se^+$	8.73 [108]		$^{40}Ar_2{}^1H_2^+$, $^{12}C^{35}Cl_2^+$, $^{34}S^{16}O_3^+$, $^1H^{81}Br^+$, $^{82}Kr^+$ [38, 110]
Common examples of collision/reaction cell reaction and mass-shift analysis for dealing with spectral interference			
Reaction			**Interferences potentially affecting the reaction product ion (non-restrictive list)**
$^{32}S^+ + O_2 \rightarrow {}^{32}S^{16}O^+$ (m/z 48)			$^{48}Ti^+$, $^{48}Ca^+$, $^{36}Ar^{12}C^+$, $^{31}P^{16}O^1H^+$, $^{31}P^{17}O^+$, $^{34}S^{14}N^+$, $^{33}S^{15}N^+$, $^{14}N^{16}O^{18}O^+$, $^{14}N^{17}O_2^+$, $^{12}C_4^+$ [38, 67, 92-94, 106]
$^{33}S^+ + O_2 \rightarrow {}^{33}S^{16}O^+$ (m/z 49)			$^{49}Ti^+$, $^{32}S^{17}O^+$, $^{32}S^{16}O^1H^+$, $^{35}Cl^{14}N^+$, $^{34}S^{15}N^+$, $^{14}N^{17}O_2{}^1H^+$, $^{36}Ar^{13}C^+$, $^{36}Ar^{12}C^1H^+$, $^{12}C^{37}Cl^+$, $^{31}P^{18}O^+$ [38, 67, 94, 106]
$^{34}S^+ + O_2 \rightarrow {}^{34}S^{16}O^+$ (m/z 50)			$^{50}Ti^+$, $^{50}Cr^+$, $^{50}V^+$, $^{33}S^{17}O^+$, $^{32}S^{18}O^+$, $^{32}S^{17}O^1H^+$, $^{38}Ar^{12}C^+$, $^{36}Ar^{14}N^+$, $^{35}Cl^{15}N^+$, $^{36}S^{14}N^+$, $^1H^{14}N^{35}Cl^+$ [38, 67, 92, 94, 106]
$^{35}Cl^+ + H_2 \rightarrow {}^{35}Cl^1H_2^+$ (m/z 37)			$^{37}Cl^+$, $^{36}Ar^1H^+$, $^{36}S^1H^+$ [38, 71, 107]
$^{31}P^+ + O_2 \rightarrow {}^{31}P^{16}O^+$ (m/z 47)			$^{47}Ti^+$, $^{32}S^{14}N^1H^+$, $^{32}S^{15}N^+$, $^{15}N^{16}O_2^+$, $^{12}C^{35}Cl^+$, $^{30}Si^{16}O^1H^+$, $^{33}S^{14}N^+$, $^{14}N^{16}O_2{}^1H^+$ [38, 67]
$^{78}Se^+ + O_2/N_2O \rightarrow {}^{78}Se^{16}O^+$ (m/z 94)			$^{40}Ar^{54}Fe^+$, $^{94}Mo^+$, $^{39}K_2{}^{16}O^+$ [38, 105]
$^{80}Se^+ + O_2/N_2O \rightarrow {}^{80}Se^{16}O^+$ (m/z 96)			$^{40}Ar^{56}Fe^+$, $^{96}Mo^+$, $^{96}Ru^+$, $^{96}Zr^+$, $^{40}K_2{}^{16}O^+$, $^{39}K^{41}K^{16}O^+$, $^{79}Br^{17}O^+$, $^{79}Br^{16}O^1H^+$ [38, 105]

An alternative approach to solve the problem of spectral interference is the application of high resolution (HR) sector-field ICP-MS [111, 112], also offering high sensitivity and satisfactory robustness for HPLC-ICP-MS analysis. This technique has the ultimate advantage of significantly less need for method development for different analytical tasks, but the ion transmission is reduced when higher mass resolution is applied, resulting in a considerable reduction of sensitivity. It must also be noted that – even at the highest resolution setting of $m/\Delta m \approx 10000$ – some spectral interferences cannot be avoided in this way [113].

Although the determination of non-metallic light elements with ICP-MS can be seriously compromised, numerous successful applications can be found in the literature for the HPLC-ICP-MS(/MS) speciation of these elements, also including studies aiming at quantitative drug metabolite profiling.

1.4.1 Iodine

Gradient RP-HPLC-ICP-MS was applied by Duckett *et al* [114] to investigate the metabolism of 2-fluoro-4-iodoaniline in earthworms based on I-selective detection. A quadrupole ICP-MS system equipped with a hexapole collision/reaction cell was relied on for the determination of I at *m/z* 127 while applying a mixture of H_2 and He in the reaction cell. No special approach was applied for the compensation of the detrimental effect of gradient elution, but instead only semi-quantitative results were aimed at by post-column injection of a 2-fluoro-4-iodoaniline standard after each run as a basis for quantification. 25 ng I / peak was reported as an absolute LOQ with the methodology applied. The quantitative metabolite profiling and characterization of an iodine-containing anti-arrhythmic agent, *i.e.* amiodarone, was achieved by Lohmann *et al* [115] in a comparative study with isocratic HPLC-ICP-MS and HPLC-ESI-MS. Interference-free detection of I could be carried out at *m/z* 127 using a quadrupole ICP-MS system. Both the metabolite profiles as generated upon electrochemical oxidation and rat liver microsomal incubation were successfully described and quantified.

1.4.2 Bromine

As Br is the fifth most abundant hetero-element in FDA-approved pharmaceuticals [28], several applications aiming at the metabolite profiling of Br-containing organic compounds, also including medical drugs, can be found in the literature. In one of the first studies, the metabolite pattern of 4-bromoaniline in rat urine samples was characterized by applying RP-HPLC-ICP-MS by Nicholson *et al* [116]. Both Br isotopes could be determined on mass by using a quadrupole ICP-MS instrument with a mixture of H_2 and He in the collision/reaction cell to

overcome the possible Ar-based spectral interferences at *m/z* 79 and 81. Also among the first studies, Marshall *et al* [117] applied HPLC-ICP-MS for the investigation of the metabolic fate of bradykinin by using a Br-labelled version of bradykinin and determine the corresponding compounds in rat and human plasma. Similarly to the previous study, Br could be determined with a quadrupole ICP-MS instrument using a mixture of H_2 and He in the collision/reaction cell. While gradient RP-HPLC was relied on for the chromatographic separation of the metabolites, simple external standard calibration could be applied for quantification owing to the use of a desolvating sample introduction system to get rid of the organic solvent content of the eluent. Jensen *et al* [118] explored the metabolic fate of 2-, 3-, and 4-bromobenzoic acids by analyzing rat bile and urine using isocratic RP-HPLC-ICP-MS. Both Br isotopes could be determined with a quadrupole ICP-MS instrument using He as an inert collision gas in the hexapole reaction/collision cell and kinetic energy discrimination. Cuyckens *et al* [79] and Meermann *et al* [80] successfully applied gradient RP-HPLC-ICP-MS for the quantitative metabolite profiling of a novel anti-tuberculosis drug (bedaquiline) in dog, rat and human faeces extracts utilizing its Br-content. Both Br isotopes were determined on-mass using a quadrupole ICP-MS system equipped with a quadrupole-based collision/reaction cell (termed dynamic reaction cell or DRC by the manufacturer) in no gas (vented) mode. Gradient elution was applied, thus making correction for the signal drift inevitable. This correction was accomplished by using post-column online isotope dilution for quantification, as detailed in section 1.3. A comparative study between HPLC-ICP-MS and radio-HPLC was performed by analysing samples coming from *in vivo* experiments with a ^{14}C-labelled version of the drug. Good agreement was found between the results (rel. dif. < 16%) obtained using the two principally different approaches. The limit of detection (LOD) values were also found to be of the same order of magnitude (5 and 1 ng drug with HPLC-ICP-MS and radio-HPLC, respectively) for the two different strategies, proving HPLC-ICP-MS as a sterling alternative to radio-HPLC in this context. Recognising the lack of the capability of online ID to distinguish between endogenous Br and drug-related Br, a reverse online isotope dilution approach was also developed and validated by Meermann *et al* [81], as detailed in section 1.3. A quantitative comparison was carried out between the results obtained via online ID, reverse online ID and radio-HPLC strategies. Good agreement was found again among the results, with HPLC-ICP-MS LODs better than or in the same order of magnitude as those obtained with radio-HPLC (0.05, 0.04 and 0.09 mg L^{-1} Br in faeces extract for reverse online ID, online ID and radiodetection, respectively), further confirming the great potential of HPLC-ICP-MS in this context. It is worthwhile to note however, that although the overall analytical capability of

radio-HPLC and HPLC-ICP-MS was found similar in the aforementioned two studies, the detection sensitivity of radio-HPLC is still significantly higher, but it is highly compromised in real-life *in-vivo* studies by the strong dilution of the radio-labelled drug with the "cold" version. Vlieger *et al* [93] applied high-temperature liquid chromatography (HTLC) coupled to ICP-MS for the metabolite profiling of two kinase inhibitors, SB-203580-Iodo and p38 MAP kinase inhibitor VIII, based on their I and S, and Br and Cl contents, respectively. HTLC was proved to be a successful tool to eliminate the issues related to gradient elution, as the separation could be achieved by using a temperature gradient and an isocratic elution with 5% acetonitrile or 15% methanol. All four hetero-elements (Cl, Br, I, S) were determined on-mass in no gas mode at *m*/*z* 35, 79, 127 and 32, respectively, using a quadrupole ICP-MS instrument equipped with a collision/reaction cell. S was also determined as $^{32}S^{16}O^{+}$ at *m*/*z* 48 utilizing the enhanced oxide formation in the plasma due to the presence of O_2 admixed to prevent carbon deposition on the cones. LODs of 130, 0.5, 0.08 and 230 µM were found for Cl, Br, I and S, respectively, corresponding to values of 4.5, 0.04, 0.01 and 7.3 mg L^{-1} for the different elements. The LOD for S could be significantly improved to 18 µM (0.6 mg L^{-1}) by monitoring $^{32}S^{16}O^{+}$ at *m*/*z* 48 instead of $^{32}S^{+}$. However, in the absence of an effective strategy to eliminate the spectral interferences especially affecting Cl and S (both at *m/z* 32 and 48) determination, only the LODs obtained for Br and I were sufficiently low for quantitative metabolite profiling for the two kinase inhibitors mentioned above. Nevertheless, in contrast to ESI-MS, ICP-MS was able to provide a structure-independent analytical response, thus enabling the quantitative metabolite profiling based on Br and I.

1.4.3 Chlorine

Among the halogens, Cl is of highest importance in the context of medical drugs, as it is the second most abundant hetero-element in the FDA-approved pharmaceuticals after S [28]. Nevertheless, appreciably less papers describing Cl-speciation using HPLC-ICP-MS have been published so far than for *e.g.,* Br. This can be explained by the fact that the determination of Cl with ICP-MS is significantly more challenging than that of I or Br due to the even higher ionization potential of Cl and the more pronounced problem of spectral interference, as indicated in Table 1.1. Nevertheless, a couple of studies in the literature address the determination of Cl using HPLC-ICP-MS in a pharmaceutical context. As mentioned previously, the absence of an effective strategy for the elimination of the spectral interferences can strongly compromise the Cl-determination, resulting in an insufficient LOD for real-life drug metabolite profiling, as was also observed by Vlieger *et al* [93]. Among the first studies,

Corcoran *et al* [119] investigated the metabolic profile of the Cl-containing drug diclofenac by analysing rat urine samples by gradient RP-HPLC-UV separation, followed by simultaneous detection with ICP-MS and ESI-TOF-MS in a single instrumental set-up. A quadrupole ICP-MS system equipped with a hexapole collision/reaction cell was applied for the simultaneous detection of $^{35}Cl^+$, $^{37}Cl^+$ and $^{32}S^+$ while using a mixture of H_2 and He in the cell. Although diclofenac does not contain sulphur, $^{32}S^+$ was also monitored as metabolic sulfation of hydroxylated metabolites could occur. No extra effort was done for compensating for the signal drift due to gradient elution as no accurate quantification was aimed at. Duckett *et al* [120] also demonstrated the great potential of HPLC-ICP-MS for the speciation of the Cl-containing pharmaceuticals diclofenac and chlorpromazine by investigating the quantification possibilities in both isocratic and gradient elution conditions when applying methanol (between 10-50 % (*v*/*v*)) in the eluent system. Cl was determined on mass with a quadrupole ICP-MS system resulting in a similarly high degree of spectral interference as documented by Vlieger *et al* [93]. No significant change was observed in the Cl-response in the aforementioned methanol concentration range, as has been recently confirmed by Klencsár *et al* [86] (detailed in Chapter 3), enabling straightforward quantification despite the use of gradient elution.

The ICP-MS determination of Cl was revolutionized by the introduction of tandem ICP-mass spectrometry (ICP-MS/MS): as this type of instrumentation provides an effective means for overcoming the spectral interference, adequate Cl quantification is enabled at lower concentration levels. The first study using reversed phase HPLC-ICP-MS/MS for Cl-speciation in a pharmaceutical context was published by Klencsár *et al* [71] (detailed in Chapter 2). The total drug-related Cl and Br contents were determined in human plasma via RP-HPLC-ICP-MS/MS with the aim of assessing the total drug exposure for a novel medical drug containing both elements. Cl was determined as $^{35}ClH_2^+$ at *m*/*z* 37 using H_2 in the reaction/collision cell, while Br could be determined on mass at *m*/*z* 79 under the same conditions. Reversed phase HPLC was employed for the separation of inorganic (endogenous) Cl and Br from the drug-related compounds, which were eluted in one "peak group" applying 85% (*v*/*v*) methanol in isocratic conditions. Owing to the constant eluent composition at the elution position of the compounds of interest, both simple external and internal standard (using the two elements as internal standards for each other) calibration approaches could be applied for quantification. This work also demonstrated the applicability of ICP-MS detection in the absence of individual standards, as the quantification was carried out by using model compounds (diclofenac for Cl and 4-bromobenzoic acid for Br) as external standards, due to the lack of analytical standards for the parent drug and its metabolites present in the human plasma samples analysed.

Significantly improved LOQ values were reported (50 and 10 µg L^{-1} for Cl and Br, respectively) in HPLC-ICP-MS/MS conditions compared to the works where no special emphasis was put on overcoming spectral interference (> 5 mg L^{-1} for Cl and > 40 µg L^{-1} for Br [79-81, 93]).

1.4.4 Sulphur and phosphorus

The determination of the most abundant hetero-element in FDA-approved drugs, *i.e.* S, is also strongly compromised by spectral interference (see Table 1.1), as thoroughly reviewed by Martínez-Sierra *et al* [94]. However, as S (similarly to P) is present in crucial biomolecule types, several applications attempting at S- and P-speciation by using HPLC-ICP-MS can be found in the literature. Wind *et al* [85] investigated the degree of protein phosphorylation based on the P/S ratio as obtained via gradient elution reversed phase capillary HPLC-ICP-MS with simultaneous detection of $^{32}S^+$ and $^{31}P^+$. A sector-field high resolution (HR) ICP-MS system was applied at medium mass resolution ($m/\Delta m$ = 4000) to avoid the spectral interferences affecting both nuclides. Quantification was performed by applying a mathematical function correcting for both the S and P signal drifts caused by gradient elution (as detailed in section 1.3). A similar approach was reported by Siethoff *et al* [84] for the quantification of DNA-adducts based on $^{31}P^+$ detection also using the combination of gradient RP-HPLC and sector field ICP-MS operated at higher mass resolution. A quadrupole ICP-MS system equipped with a collision/reaction cell was successfully applied by Pröfrock *et al* [83] for the quantification of phosphorylated peptides based on $^{31}P^+$ detection while using He as a collision gas to get rid of the polyatomic interferences. An LOD of 6 µg L^{-1} P was achieved in gradient capillary RP-HPLC-ICP-MS conditions, while assuring accurate quantification via the use of a counter-gradient. Jensen *et al* [92] successfully carried out the metabolite profiling of the S-containing compound omeprazole in rat urine samples from rats dosed with a 1:1 mixture of ^{34}S-labelled and unlabelled drug. S-monitoring was accomplished using a quadrupole ICP-MS system equipped with a hexapole collision/reaction cell. Both on-mass monitoring (^{32}S and ^{34}S) and monitoring of the corresponding SO^+ ions, generated by adding O_2 into the hexapole cell for conversion of S^+ to SO^+, was evaluated. A 100-fold improvement in the LOD was reported for SO^+ monitoring, thus allowing the metabolite profiling of omeprazole with gradient RP-HPLC-ICP-MS (which was impossible with on-mass determination). Quadrupole DRC-ICP-MS and sector-field HR-ICP-MS coupled to RP-HPLC were compared by De Wolf *et al* [121] for the investigation of the reactive metabolites of the Cl-containing clozapine obtained by *in vitro* electrochemical metabolite generation in the presence of the S-containing glutathione. Glutathione readily forms complexes with reactive metabolites, protecting them from

degradation and further reaction. Therefore, both the non-reactive and reactive metabolite profile of clozapine could be explored by the simultaneous detection of Cl and S with ICP-MS. Similarly to the studies previously mentioned, O_2 was applied as a reaction gas in the cell of the quadrupole ICP-MS system to convert S^+ into SO^+, enabling S detection at *m*/*z* 48 ($^{32}S^{16}O^+$) and 50 ($^{34}S^{16}O^+$), while the interference-free determination of both S isotopes could be achieved at medium mass resolution by using a sector-field ICP-MS instrument. LODs of 10 and 300 µg L^{-1} were obtained based on $^{32}S^{16}O^+$ and $^{34}S^{16}O^+$, respectively, when using the quadrupole system, while values of 1 and 2 µg L^{-1} were found in the case of sector-field ICP-MS, jeopardizing the metabolite profiling in the former case and enabling it in the latter one. Similarly to Cl, the ICP-MS determination of S and P was also revolutionized by the introduction of tandem ICP-MS/MS. The first application on the topic by Fernández *et al* [67] described the simultaneous speciation and quantification of S- and P-containing peptides using gradient capillary RP-HPLC coupled to ICP-MS/MS by means of the detection of S and P as SO^+ and PO^+ (at *m*/*z* 48 and 47), respectively, by applying O_2 in the collision/reaction cell. While with a single quadrupole ICP-MS system, not all spectral interferences at *m*/*z* 48 and 47 can be eliminated, the ICP-MS/MS system ensures an interference-free determination at these masses as the first quadrupole filter (in front of the collision/reaction cell) prevents the entrance of ions with mass 48 and 47 into the reaction cell. Fernández *et al* used two model compounds (methionine and bis(4-nitro-phenyl)phosphate for S and P, respectively) as standards for quantification and added a post-column make-up flow with constant composition to reduce the signal drift deriving from the gradient elution. Detection limits of 0.18 and 0.10 µg L^{-1} were obtained for S and P, respectively (corresponding to 11 and 6.6 fmol S and P, respectively), which meant two and one orders of magnitude improvement for S and P, respectively, in comparison to previous works relying on the use of single-quadrupole ICP-MS. A comparative study between a single-quadrupole ICP-MS system equipped with a DRC and a tandem ICP-MS/MS unit has been recently performed by Møller *et al* [122] for the quantification of S-containing pharmaceutical peptides in human plasma applying O_2 in the reaction cell in both cases. In the first case (single-quadrupole ICP-MS with a DRC), a membrane desolvation system was applied to remove the organic content of the sample flow, while O_2 was admixed to the plasma gas when the ICP-MS/MS system was employed. In both cases, online ID was applied for quantification, adding a ^{34}S-enriched sulphate standard solution post-column to the sample flow. 0.6 and 0.5 pmol S were realized as LOD values for the single-quadrupole and tandem ICP-MS/MS setup, respectively, which is representing a much lower difference between the two systems than reported before. Møller *et al* explained this by the application of

the desolvation system in the case of single-quadrupole ICP-MS, even though the use of a desolvation system brought about a slightly worse chromatographic performance due to an increased dead volume of the system. The higher LOD obtained with HPLC-ICP-MS/MS in this work compared to the results reported by Fernández *et al* [67] detailed above was explained by the application of traditional HPLC conditions with a flow rate of 0.2 mL min^{-1} instead of capillary HPLC.

Table 1.2 provides an overview of selected applications targeting the speciation of the non-metal hetero-elements typically present in active pharmaceutical ingredients.

1.4.5 Selenium

When reviewing the literature [103, 110, 123, 124], it becomes clear that a great share of the speciation works targeting non-metal hetero-elements is devoted to Se, mainly because of its essential nature to animals and humans and its possible protective effect against cancer [125]. But, Se has a rather moderate importance in the pharmaceutical context, taking the number of Se-containing drugs into account [28, 31]. Therefore, no special emphasis is put on Se speciation in this introduction chapter, but only a couple of examples related to pharmaceutical research are mentioned. Gammelgaard *et al* [104] quantified two Se-metabolites, *i.e. Se*-methylseleno-*N*-acetylgalactosamine and *Se*-methylselenoacetylgalactosamine, in human urine samples before and after the consumption of Se-supplements by using HPLC coupled to a quadrupole ICP-MS system equipped with a DRC. Methane was applied as reaction gas in the DRC to eliminate the Ar-based spectral interferences while monitoring the ion signals of the ^{78}Se, ^{80}Se and ^{82}Se isotopes on-mass. Three different ICP-MS sample introduction systems, *i.e.* a microconcentric nebulizer mounted onto a cyclonic spray chamber, a direct injection nebulizer (DIN) and an ultrasonic nebulizer, were compared in the context of HPLC-ICP-MS analysis of Se-species. The microconcentric nebulizer combined with a cyclonic spray chamber was found to be the optimal sample introduction system taking chromatographic peak shape, sensitivity and LODs into account. The LODs varied between 0.1-0.5 µg L^{-1} Se for the two species of interest and for the different isotopes. Lunøe *et al* [126] studied the ability of different Se compounds to induce cell death in different cancer cell lines by using the same ICP-MS approach as Gammelgaard *et al* [104]. As several Se species, also including inorganic forms (selenite, selenate) were tested, different orthogonal separation strategies, including reversed phase, anion exchange and size exclusion HPLC, were used in combination with ICP-MS for metabolite profiling. Flouda *et al* [127] have recently applied HPLC-ICP-MS for the quantification of low molecular weight Se compounds in human plasma from cancer patients

subjected to selenite treatment in a clinical trial. Two different DRC-ICP-MS systems were applied with methane in the reaction cell for the interference-free determination of ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se relying on post-column online isotope dilution using a ^{77}Se-enriched standard solution for quantification purposes. Similarly to the study previously mentioned, different chromatographic approaches were employed for the quantification of both organic and inorganic Se-metabolites. The LOD varied between 0.4-2.1 $\mu g\ L^{-1}$ in human plasma depending on the chemical form of Se and the chromatographic strategy applied.

Table 1.2: Summary of selected studies aiming at the speciation of the most abundant hetero-elements (S, Cl, P, Br and I) present in pharmaceuticals

Target element	Target analyte	Sample type	ICP-MS detection mode	Separation method	LOD/LOQ	Reference
I	2-fluoro-4-iodoaniline	Earthworm tissue extract	Quadrupole ICP-MS with H_2/He mixture in the reaction/collision cell, as $^{127}I^+$	Gradient RP-HPLC	25 ng I / peak LOQ	[114]
	Amiodarone	Electrochemical oxidation Rat liver microsomal incubation	Quadrupole DRC-ICP-MS, no-gas mode, as $^{127}I^+$	Phase-optimized LC (POPLC)	Not reported	[115]
	Kinase inhibitors: SB-203580-Iodo and p38 MAP kinase inhibitor VIII	Electrochemical oxidation, metabolic incubations with bacterial cytochrome P450 BM3 and human liver microsomes	Quadrupole ICP-MS with a reaction/collision cell, no gas mode, as $^{127}I^+$	HTLC	0.01 mg L^{-1} I LOD	[93]
Br	4-bromoaniline	Rat urine	Quadrupole ICP-MS with H_2/He mixture in the reaction/collision cell as $^{79}Br^+$ and $^{81}Br^+$	Gradient RP-HPLC	Not reported	[116]
	Br-labelled bradykinin	Rat and human plasma	Quadrupole ICP-MS with H_2/He mixture in the reaction/collision cell as $^{79}Br^+$ and $^{81}Br^+$	Gradient RP-HPLC	Not reported	[117]
	2-, 3-, and 4-bromobenzoic acid	Rat bile and urine	Quadrupole ICP-MS with He in the reaction/collision cell as $^{79}Br^+$ and $^{81}Br^+$	Isocratic RP-HPLC	Not reported	[118]
	Bedaquiline	Dog, rat and human faeces	Quadrupole DRC-ICP-MS, no gas mode, as $^{79}Br^+$ and $^{81}Br^+$	Gradient RP-HPLC	0.04 mg L^{-1} Br LOD	[79-81]
	Kinase inhibitors: SB-203580-Iodo and p38 MAP kinase inhibitor VIII	Electrochemical oxidation, metabolic incubations with bacterial cytochrome P450 BM3 and human liver microsomes	Quadrupole ICP-MS with a reaction/collision cell, no gas mode, as $^{79}Br^+$	HTLC	0.04 mg L^{-1} Br LOD	[93]
	A novel Br- and Cl-containing drug with unrevealed chemical structure	Human plasma	ICP-(QQQ)MS/MS with H_2 in reaction/collision cell, as $^{79}Br^+$	Isocratic RP-HPLC	0.01 mg L^{-1} Br LOQ	[71]
Cl	Kinase inhibitors: SB-203580-Iodo and p38 MAP kinase inhibitor VIII	Electrochemical oxidation, metabolic incubations with bacterial cytochrome P450 BM3 and human liver microsomes	Quadrupole ICP-MS with a reaction/collision cell, no gas mode, as $^{35}Cl^+$	HTLC	4.5 mg L^{-1} Cl LOD	[93]

	Diclofenac	Rat urine	Quadrupole ICP-MS with H_2/He mixture in the reaction/collision cell, as $^{35}Cl^+$ and $^{37}Cl^+$	Gradient RP-HPLC	Not reported	[119]
	Diclofenac and chlorpromazine	Standard solutions	Quadrupole ICP-MS, no gas mode, as $^{35}Cl^+$	Gradient RP-HPLC	Not reported	[120]
	A novel Br- and Cl-containing drug with unrevealed chemical structure	Human plasma	ICP-(QQQ)MS/MS with H_2 in reaction/collision cell, as $^{35}Cl^1H_2^+$	Isocratic RP-HPLC	0.05 mg L^{-1} Cl LOQ	[71]
	Diclofenac and its related compounds	Human plasma	ICP-(QQQ)MS/MS with H_2 in reaction/collision cell, as $^{35}Cl^1H_2^+$	Gradient RP-HPLC	0.03-0.05 mg L^{-1} Cl LOQ	[86]
	Kinase inhibitors: SB-203580-Iodo and p38 MAP kinase inhibitor VIII	Electrochemical oxidation, metabolic incubations with bacterial cytochrome P450 BM3 and human liver microsomes	Quadrupole ICP-MS with a reaction/collision cell, no gas mode, as $^{32}S^+$ and $^{32}S^{16}O^+$	HTLC	7.3 mg L^{-1} S as $^{32}S^+$ 0.6 mg L^{-1} S as $^{32}S^{16}O^+$ LOD	[93]
	α- and β-casein and phosphopeptides	Standard solutions	SF-ICP-MS at medium mass resolution, as $^{32}S^+$	Gradient RP-HPLC	Not reported	[85]
	Omeprazole	Rat urine	Quadrupole ICP-MS with a reaction/collision cell, both no gas mode as S^+ and O_2 reaction mode as SO^+	Gradient RP-HPLC	Not reported	[92]
S	Clozapine in the presence of the S-containing glutathione	*In-vitro* electrochemical generation	Comparison of quadrupole DRC-ICP-MS with O_2 in the DRC and SF-ICP-MS at medium mass resolution	Gradient RP-HPLC	10 and 300 µg L^{-1} S as $^{32}S^{16}O^+$ and $^{34}S^{16}O^+$ with DRC-ICP-MS and 1 and 2 µg L^{-1} S as $^{32}S^+$ and $^{34}S^+$ with SF-ICP-MS LOD	[121]
	P- and S-containing peptides	Standard solution	ICP-(QQQ)MS/MS with O_2 in reaction/collision cell, as SO^+	Gradient capillary RP-HPLC	0.18 µg L^{-1} (11 fmol) S LOD	[67]

	S-containing pharmaceutical peptides	Human plasma	Comparison of quadrupole DRC-ICP-MS and ICP-(QQQ)MS/MS with O_2 in reaction/collision cells, as SO^+	Gradient RP-HPLC	0.6 pmol for DRC-ICP-MS and 0.5 pmol for ICP-MS/MS LOD	[122]
P	α- and β-casein and phosphopeptides	Standard solutions	SF-ICP-MS at medium mass resolution, as $^{31}P^+$	Gradient RP-HPLC	Not reported	[85]
	Modified nucleotides and DNA	*In-vitro* modified DNA samples	SF-ICP-MS at medium mass resolution, as $^{31}P^+$	Gradient RP-HPLC	Not reported	[84]
	Phosphorylated peptides	Tryptic digested phosphoprotein samples	Quadrupole ICP-MS with He in the reaction/collision cell, as $^{31}P^+$	Gradient capillary RP-HPLC	6 µg L^{-1} P LOD	[83]
	P- and S-containing peptides	Standard solution	ICP-(QQQ)MS/MS with O_2 in reaction/collision cell, as PO^+	Gradient capillary RP-HPLC	0.10 µg L^{-1} (6.6 fmol) P LOD	[67]

1.5 Derivatization strategies for drugs without any ICP-MS detectable hetero-element

As described above, the application of HPLC-ICP-MS requires the drug and its metabolites to contain a hetero-element detectable with ICP-MS. However, the largest fraction of pharmaceuticals does not contain any other element apart from carbon, hydrogen, nitrogen, and oxygen [28, 128, 129]. To extend the applicability of the technique, chemical derivatization via the introduction of an ICP-MS detectable hetero-element by utilizing the chemical reactivity of the compounds of interest can be a powerful strategy. Although the derivatization reaction is preferred to be simple and straightforward, challenges still arise, for example, the formation of intermediates and by-products, the harsh reaction conditions required, the potential instability of reaction products, non-quantitative reaction etc., all of which negatively affect the accuracy and precision of this analytical approach. Nevertheless, in the pharmaceutical sciences and in proteomics, several derivatization strategies have been successfully developed, mostly focusing on the application of elemental labeling combined with HPLC-ICP-MS detection for biopharmaceuticals and proteins [130, 131]. The common basis of these reactions is targeting the reactive functional groups *i.e.* -OH, -COOH, $-NH_2$, -SH, typically present in pharmaceuticals.

1.5.1 Derivatization of the –OH functional group

Although the -OH group is the most common functional group in the currently approved drug substances, especially among anti-cancer drugs of which 41.4% contains this functional group [39], only a few studies aiming at the derivatization of -OH containing compounds for HPLC-ICP-MS analysis have been discussed in the literature. Harigaya *et al* [132] reported a method for the sensitive determination of 4-chloro-1-butanol, a genotoxic alkylating agent possibly present in active pharmaceutical ingredients, by using 3-iodobenzoyl chloride as a derivatization reagent. Acyl halides readily react with the -OH group and the reaction requires no catalyst, as presented in Table 1.3, line 1. The excellent ICP-MS sensitivity for I enabled determination of 4-chloro-1-butanol with an LOD of 0.2 mg L^{-1}. Obviously, the sensitivity attainable and thus the LOD can be further improved if a more suitable hetero-element, preferably a metal, is introduced into the target molecule by utilizing the reactivity of the -OH group. The metal-coded affinity tag technique (MeCAT) is the most common strategy to introduce a metallic hetero-element into the target component allowing for the sensitive quantification of biomolecules [128]. The essence of this method is the application of chelating compounds with different incorporated metals to improve the detection characteristics of biomolecules. Various metal elements (mainly 3-fold positively charged lanthanides) can be

applied. 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-based agents are commonly employed metal chelators to label different conjugate functional groups in peptides and proteins [133-143]. Yan *et al* [138] employed HPLC-ICP-MS for absolute protein quantification by means of a selective labeling strategy targeting the -OH groups of the serine residue in serine protease enzymes by an amide derivative of 4-(2-aminoethyl) benzenesulfonyl fluoride and 6-heptynoic acid (referred to as an SF-alkyne), followed by a coupling reaction with azido-DOTA-Eu, as shown in Table 1.3, line 2. The method was successfully applied to the determination of the active serine proteases in real biological samples with an absolute LOD of 0.2 fmol relying on species-unspecific isotope dilution for quantification. As can be seen, some successful applications for the derivatization of -OH group followed by HPLC-ICP-MS determination have already been published. However, taking into account the abundance and significance of -OH group in pharmaceuticals, which is further stressed by the fact that oxidation is the most important bio-transformation pathway of pharmaceuticals (potentially enabling the derivatization of the metabolites of those drugs that originally do not contain an -OH group), further research is urgently needed to further exploit the possibilities of quantitative metabolite profiling of medical drugs based on the derivatization of their -OH group.

1.5.2 Derivatization of the –COOH functional group

Carboxyl groups are also found in a large fraction of the pharmaceutical drugs, as well as in endogenous metabolites [39, 144]. Profiling analysis of these compounds has great significance since they are believed to play an important role in immune regulation and signal transduction [145]. Derivatization of carboxylic acids can be carried out by using a variety of reagents. However, only a few are suited to be used for subsequent ICP-MS analysis of the derivatized components. The phosphorus-containing reagent tris(2,4,6-trimethoxyphenyl) phosphonium propylamine (TMPP) was used by Cartwright *et al* [129] for the determination of carboxylic acids with RP-HPLC-SF-ICP-MS. The introduction of P allowed $^{31}P^{+}$ selective detection at medium mass resolution for those acids that are normally not accessible to ICP-MS detection. A membrane desolvation system was applied to eliminate the adverse effects of the high organic content of the eluent and of the gradient elution. 2-Chloro-1-methylpyridinium iodide (CMPI) and triethylamine (TEA) were used as coupling reagents for the activation of the carboxyl group prior to the reaction with TMPP, as shown in Table 1.3, line 3. An LOD of 0.05 nmol TMPP (corresponding to 1.4 ng P) was observed, while a 5-fold worse LOD, *i.e.* 0.25 nmol maleic acid, was obtained when the derivatization reaction was applied. This was explained by the

relatively low (approx. 20%) reaction efficiency achieved for this specific reaction. Marshall *et al* [146] developed a capillary HPLC-ICP-MS method for the determination of carboxylic acids with pre-column derivatization by using N-(2-aminoethyl) ferrocene carboxamide, as shown in Table 1.3, line 4. The derivatization reaction was carried out under mild conditions using CMPI/TEA for the activation of the -COOH group, similarly to the study previously mentioned. The introduction of Fe can significantly improve the sensitivity for ICP-MS, while the conjugation can also be beneficial for the chromatographic separation due to the reduced polarity of the derivatives. However, it is also worthwhile to mention, that the introduction of Fe as a target element for HPLC-ICP-MS can be considered rather unfortunate as the ICP-MS determination of $^{56}Fe^{+}$ is compromised by spectral interference from $^{40}Ar^{16}O^{+}$ [38, 147], while the traditional HPLC systems are commonly made of stainless steel, resulting in an elevated Fe-background due to leaching, thus compromising the LOD and LOQ achievable. Although the former issue can be eliminated by using HR-ICP-MS or chemical resolution in a collision/reaction cell and the latter by applying bio-inert LC systems, these modifications significantly increase the cost and complexity of the analysis. Therefore, similarly to -OH group, also the derivatization of -COOH group requires additional research aiming at the introduction of further hetero-elements.

1.5.3 Derivatization of the –SH functional group

Although the -SH group is rare among the approved pharmaceuticals [39], the determination of –SH containing compounds is still of great interest since they are critical physiological components and are also related to several human diseases [148]. In fact, thiol compounds are visible to ICP-MS due to the presence of sulfur atoms [122]. However, S detection by ICP-MS is hampered by its low ionization efficiency and the occurrence of spectral interference, as detailed in section 3, such that the expectations in terms of sensitivity and LOD cannot always be met. The introduction of an element with higher ICP-MS sensitivity via chemical derivatization has proved to be a judicious decision. Several derivatization reagents have been reported for improving the ICP-MS sensitivity for thiol compounds. Based on the high affinity between mercury and sulfur, mercury-containing reagents have been widely used to study the biochemical function of -SH groups. As a first attempt, Takatera *et al* [149] developed a novel HPLC-ICP-MS approach for the determination of chicken ovalbumin by the derivatization of -SH groups with organic mercury compounds. The reactivity of five Hg-containing reagents (p-(chloromercuri) benzenesulfonic acid, mersalyl acid, ethylmercuric chloride, p-chloromercuribenzoate and fluorescein mercuric acetate) was investigated, as detailed in Table

1.3, line 5. The results indicated that the reaction rate is strongly influenced by steric hindrance around the mercury atom, rendering ethylmercuric chloride to be the most reactive agent. Two orders of magnitude improvement in the LOD was achieved by Hg-based derivatization in comparison with S-based detection. *p*-Hydroxymercuribenzoate (PHMB) was also widely used to derivatize SH-containing compounds in various samples [150-152]. Bakirderea *et al* [152] developed a method for the determination of thiol groups in biological samples by HPLC-ICP-MS and HPLC-ESI-MS after derivatization with PHMB. PHMB reacts with -SH groups at room temperature in less than 90 s with high affinity and specificity, forming a PHMB-SH complex which is stable for at least one day at room temperature.

The application of selenamide reagents in the derivatization of biological thiols is becoming increasingly popular. Similarly to Hg-based agents, these reagents usually also show high selectivity, reaction rate and conversion yield. The reaction mechanism is based on the cleavage of the Se–N bond and the formation of a selenenyl sulfide (Se–S) bond between the selenamide reagent and the thiol-containing molecule [153], as shown in Table 1.3, line 6. 2-Phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen) and *N*-(phenylseleno) phthalimide (NPSP) are currently commonly used selenamide reagents [153-155]. Espina *et al* [154] developed a method to determine reduced homocysteine in human serum by RP-HPLC coupled to ICP-MS and ESI-MS detection. The use of ebselen as a derivatization reagent enables a selective and sensitive detection based on Se monitoring to quantify derivatized reduced homocysteine in real samples. Post-column online ID was relied on for accurate quantification with an LOD of 9.6 nM obtained for the derivatization product with monitoring based on Se, which is better than the LODs obtained using fluorescence detection or ESI-MS detection.

Ferrocene-based reagents also play an important role as organometallic derivatizing agents and have been developed for the modification of several functional groups. These compounds display numerous advantages as derivatization reagents, such as stability, cost-effectiveness and availability. Moreover, they are also able to convert highly polar compounds into less polar derivatives, thus facilitating the use of RP-HPLC. A few ferrocene-based reagents are reported for the analysis of thiol functionalities, such as *N*-(2-ferroceneethyl)maleimide (FEM) [156, 157] and ferrocenecarboxylic acid (2-maleimidoyl)ethylamide (FMEA) [156, 158, 159]. FMEA was used to derivatize thiol groups in phytochelatins to avoid oxidation of the free thiol functions during analysis [159]. *N*-substituted maleimide reacts rapidly and selectively with the thiol group, forming a stable thioether bond, and the Fe atom enables ICP-MS quantification, as shown in Table 1.3, line 7.

As mentioned above, DOTA-based agents are also commonly used for the derivatization of various functional groups, including -SH [133, 134, 137, 142, 143]. Two thiol-specific DOTA-based MeCAT reagents have been synthesized and used for the absolute quantification of peptides and proteins by Ahrends *et al* [133]. The maleimido part of the DOTA reagent reacts with the thiol group, followed by chelation between the macrocycle of DOTA and a lanthanide metal ion (M^{3+}), resulting in a stable metal chelate complex, as shown in Table 1.3, line 8. The results showed that a quantitative labeling of peptides and proteins could be obtained under optimized reaction conditions, and no metal loss or exchange was observed during the analyses, demonstrating the suitability of the MeCAT approach for quantitative proteomics. Due to the voluminous DOTA complexes and spatial hindrance in peptides and proteins, some of the active sites are inaccessible for these reagents. To deal with this problem, a novel two-step labeling strategy was applied for the efficient tagging of thiol-containing proteins by He *et al* [142]. Firstly, the thiol group of cysteine was modified with a terminal alkyne group containing reagent avoiding the direct introduction of DOTA-based compound resulting in steric hindrance. In the second step, DOTA-azide (as a metal complex) was introduced to form the final derivatization product, as presented in Table 1.3, line 9. Due to the lower steric hindrance, the labeling efficiency of cysteine was around 96%, offering significant advantages over other MeCAT labeling strategies using reagents such as MeCAT-maleimide [133, 134] and MeCAT-iodoacetamide [137, 143].

1.5.4 Derivatization of the –NH_2 functional group

Amino functional groups are also very common in both approved pharmaceuticals and endogenous metabolites. Amino-containing pharmaceuticals play an important role in the fight against infections and tumors [39], while endogenous amino-containing metabolites like amino acids and neurotransmitters are critical components of biological organisms [160, 161]. Tetrabromophthalic anhydride (TBPA) was used to derivatize free NH_2-groups for the quantitative metabolite profiling of an amino group containing pharmaceutical (levothyroxine) in human plasma by Li *et al* [72], introducing Br as an ICP-MS detectable element. The derivatization is based on the simple and straightforward reaction between an acid anhydride and a primary amino-group and can be performed at room temperature without the use of a catalyst, as shown in Table 1.3, line 11. The formation of the derivatives was found to be quantitative, providing a 4:1 stoichiometric ratio of Br/NH_2. A cross-validation was also possible based on the I-content of the model drug (levothyroxine) and its metabolites and demonstrated the applicability of the derivatization approach. An LOQ of 0.1 mg L^{-1} Br was

achieved for HPLC-ICP-MS in the chemical form of the different derivatives obtained for the parent drug and its metabolites. This study also emphasized that the sensitivity, and thus the attainable LOQ, could be significantly improved by using tetraiodophthalic anhydride (also commercially available) instead of tetrabromophthalic anhydride due to the better detection characteristics for I with ICP-MS compared to Br. Harigaya *et al* [162] developed a 2D-HPLC-ICP-MS method for the quantification of residual phenylhydrazine in antipyrine. 3-Iodophenyl isocyanate and 2,3,5-triiodobenzoyl chloride were investigated as derivatization reagents, as shown in Table 1.3, lines 12 and 13. Since isocyanates and acyl halides are both highly active towards amino groups, both reactions demonstrated a quantitative recovery. The LOD for the target analyte when using 2,3,5-triiodobenzoyl chloride was one third (0.29 µg L^{-1}) of that when 3-iodophenyl isocyanate (0.95 µg L^{-1}) due to the higher number of iodine atoms introduced (3 and 1, respectively).

As indicated before, ferrocene-based reagents are also widely used for the derivatization of various functional groups for detection via different techniques, including ICP-MS. Among these reagents, a considerable number are dedicated to the derivatization of amino groups, such as ferrocenoyl chloride, ferrocenecarboxaldehyde, ferrocenyl isothiocyanate, 3-ferrocenylpropionic anhydride and succinimidylferrocenyl propionate (SFP), etc [158, 163, 164]. SFP was used as labeling agent for the quantification of lysozyme, ß-lactoglobulin A and insulin by Bomke *et al* [158] via gradient RP-HPLC-ICP-MS. As presented in Table 1.3, line 14, this derivatization approach is utilizing the simple and straightforward reaction between an *N*-succinimidyl reagent and an amino group, as also widely used for the derivatization of amines and amino acids with the intention to improve their UV-absorbance and/or to induce a fluorescent effect [165-168]. Within the context of this study, a dual-labelling strategy of thiol and amino groups was also tested by the subsequent derivatization with FMEA reagent, targeting the -SH groups (detailed above), followed by the derivatization of the -NH_2 groups with SFP. The use of multiple metallocene labels for different functional groups of one molecule was reported for the first time here and it may be a promising tool for bioanalysis in the future. The determination of amino acids is extremely important due to their role in various physiological contexts. Similarly to most of the organic compounds, they cannot be detected with ICP-MS without any chemical modification. Iwahata *et al* [169-171] performed several studies to improve the applicability of HPLC-ICP-MS for amino acids analysis. A six-coordinated complex of ruthenium, *i.e.* bis(2,2´-bipyridine)-4´-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester-bis(hexafluorophosphate) (Rub_2m–O–Su) was used to derivatize free amino acids (with the reaction presented in Table 1.3, line 15) extracted from a

single adult fly, the pupa, the larva and the ovary [169, 170], followed by RP-HPLC-ICP-MS analysis. As mentioned above, *N*-succinimidyl esters are highly reactive towards the amino group, and ruthenium is an ideal element for ICP-MS detection with almost no spectral interference. By comparing the HPLC-ICP-MS results with the more traditional ninhydrin-based amino acid analyzer, the excellent accuracy of the novel methodology could be demonstrated. A novel metal tag with a modified chelating ligand *i.e.* bis(ethylenediamine)-4´-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester (ECRS) was also synthesized and applied in amino acids analysis using RP-HPLC-ICP-MS by the same research group [171], as shown in Table 1.3, line 16. The same chemical principle is used for derivatization, while the application of ethylenediamine as a metal chelator instead of bipyridine results in an even more suitable (reduced) hydrophobicity compared to Rub_2m–O–Su for RP-HPLC separation. The detection limit was found to be 1.5 nM for branched amino acids in the standard solution.

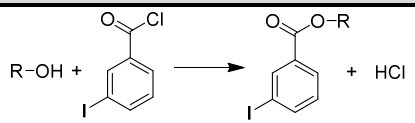
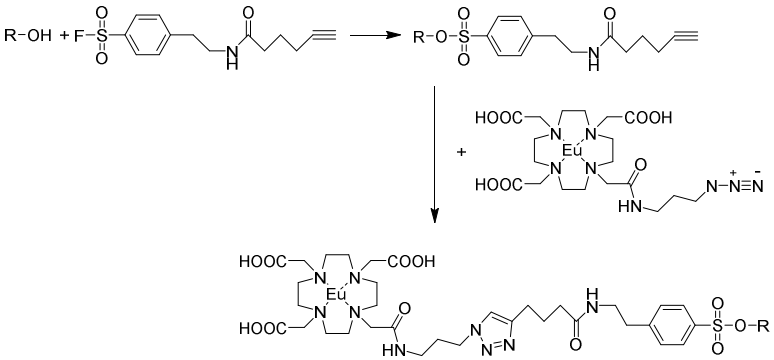
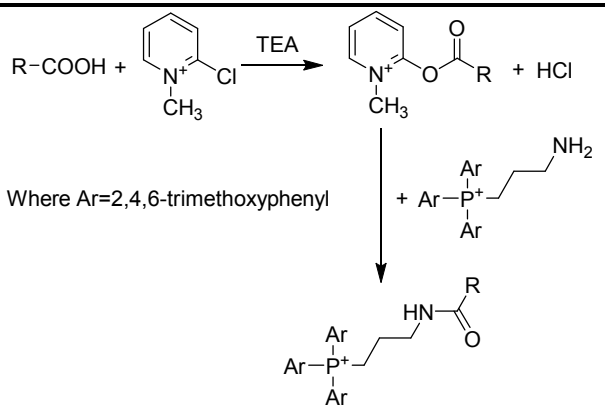
1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(*N*-hydroxysuccinimide ester) (DOTA-NHS ester) is also widely used to derivatize amino groups [136, 137, 139, 140] as presented in Table 1.3, line 17. El-Khatib *et al* [137] investigated a dual labeling strategy targeting the $-NH_2$ and -SH groups of peptides. The amino groups were derivatized with DOTA-NHS ester, followed by metallisation with ions of a lanthanide, while keeping the peptides retained on a thiol-specific resin. Subsequently, the peptides were eluted from the resin by using tris(2-carboxyethyl)phosphine (TCEP) solution, followed by the labelling of thiol groups by applying lanthanide-containing metal-coded affinity tags (MeCAT). By applying different lanthanides in the two subsequent procedures, an accurate assessment of amino/thiol ratio in peptides becomes possible, while alternatively, the sensitivity can be improved if the same lanthanide is employed. Liu *et al* [140] reported an HPLC-ICP-MS method for the absolute quantification of peptides (using bradykinin as a model peptide) by Eu tagging with DOTA-NHS ester using species-unspecific isotope dilution for quantification. The labeling efficiency was proved to be almost 100%, resulting in a 1:1 stoichiometric ratio of Eu to bradykinin. The LOD was found to be 7.2 fmol for bradykinin in HPLC-ICP-MS conditions.

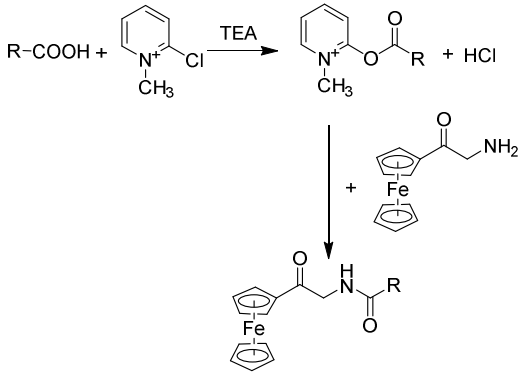
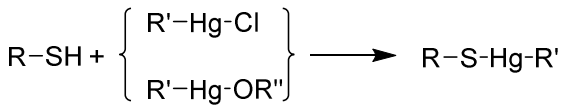
Diethylenetriaminepentaacetate (DTPA) and its derivatives are commonly used chelating agents for introducing metals into amino group containing compounds. Among them, cyclic diethylenetriaminepentaacetic anhydride (cDTPA) is an inexpensive and commercially available reagent which has been commonly used for the covalent modification of the $-NH_2$ group in both proteins and small molecules [146, 172-174], as presented in Table 1.3, line 18. A novel method for the determination of *n*-octylamine and benzylamine using gradient capillary RP-HPLC-ICP-MS following the derivatization with cDTPA, aiming at the introduction of Cu

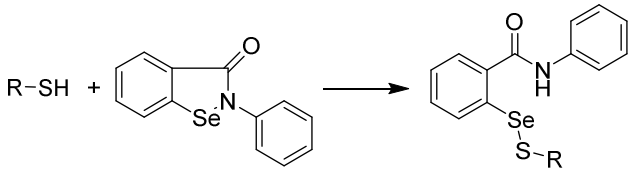
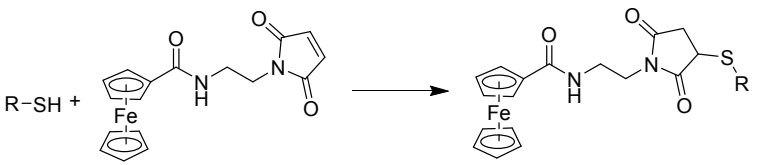
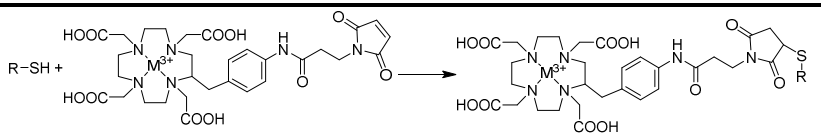
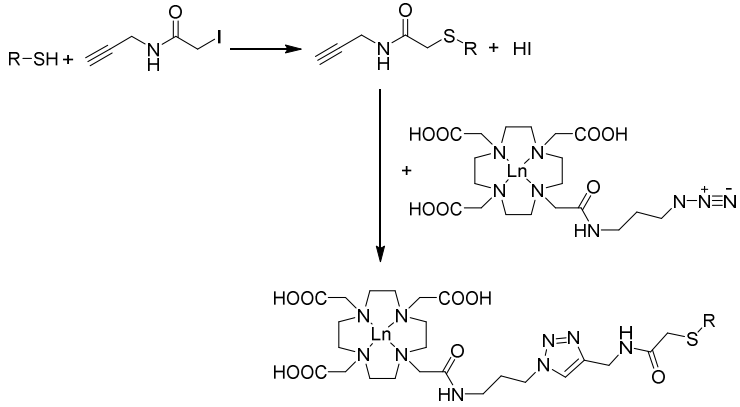
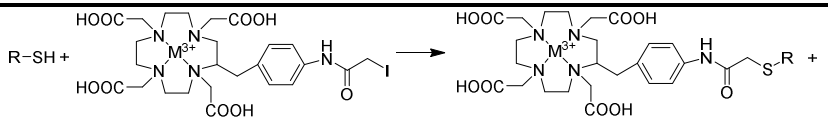
was proposed by Marshall *et al* [146]. Patel *et al* [173] developed an RP-HPLC-ICP-MS method for the quantification of bradykinin and substance P. cDTPA was used to derivatize the $-NH_2$ groups in the target compounds followed by chelation with Eu^{3+}, allowing the use of ICP-MS as an element-selective detector for the quantification of the peptides. An LOD of 10 μM bradykinin was obtained, while it was also noted that an improvement could be achieved by the further optimization of the reaction efficiency.

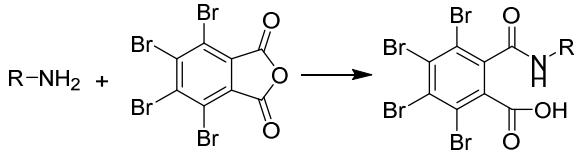
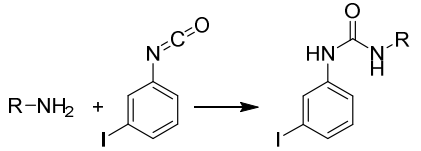
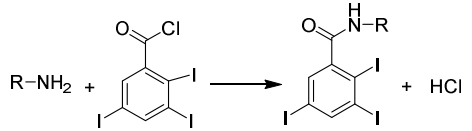
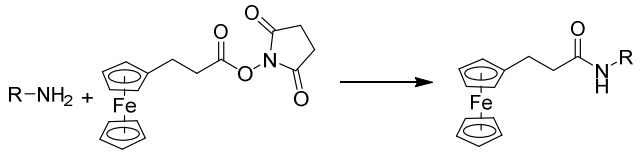
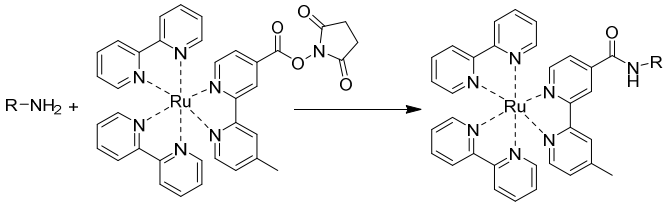
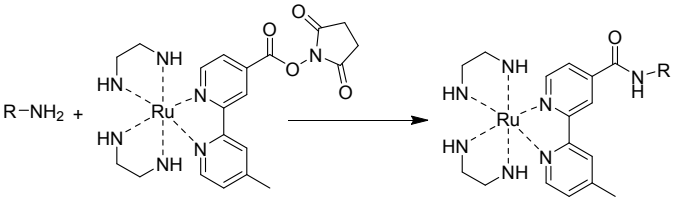
As can be seen, several successful applications aiming at the introduction of ICP-MS detectable hetero-elements via derivatization of different functional groups of pharmaceuticals, biomolecules and their related compounds have already been described. Nevertheless, further research, especially for the quantitative drug metabolite profiling of both small molecule drugs and biopharmaceuticals subsequent to their derivatization is considered strongly necessary due to the highly complex biological matrices typically dealt with and selectivity issues in chemical derivatization. Owing to the fact that the most common functional groups in pharmaceuticals also occur in endogenous compounds in biological matrices, advanced chromatographic strategies are typically needed for the separation of the trace level derivatized drug-related compounds from the derivatized matrix components. Depending on the hydrophobicity of the target compounds, this chromatographic challenge may require the application of UHPLC systems instead of HPLC or even 2D-LC, which significantly increases the complexity of the method. Furthermore, quantitative conversion of the target analyte compounds should be ensured, even in the presence of a large excess of a biological matrix, and under the mildest conditions possible to avoid the degradation of the potentially non-stable metabolites. As a result, the development of derivatization strategies preferably proceeding at room temperature and without the addition of strong acids or bases is of utmost importance in the context of quantitative metabolite profiling.

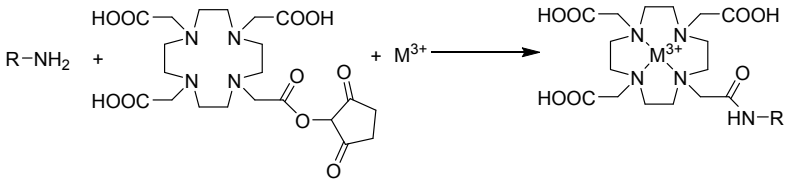
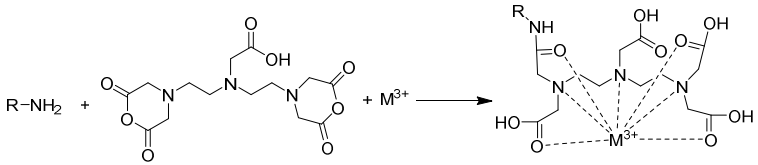
Table 1.3: The application of elemental labeling combined with HPLC-ICP-MS for various functional groups

Line	Reagent	Target analyte	Target functional group	Analytical method	Derivatization reaction	Reference
1	3-iodobenzoyl chloride	4-chloro-1-butanol	-OH	2D-LC-ICP-MS and 2D-LC-MS		[162]
2	SF–alkyne and azido-DOTA-Eu	serine residue	-OH	HPLC-ICP-MS and ESI-MS		[138]
3	TMPP	maleic, fumaric, sorbic and salicylic acid	-COOH	HPLC-SF-ICP-MS and LC-ESI-MS/MS		[129, 175]

4	N-(2-aminoethyl) ferrocene carboxamide	Phenylacetic and octanoic acid	-COOH	capillary LC-ICP-MS		[146]
5	p-(chloromercuri) benzenesulfonic acid p-chloromercuriben zoate ethylmercuric chloride mersalyl acid fluorescein mercuric acetate	chicken ovalbumin	-SH	HPLC-ICP-MS and ICP-AES		[149]
		ovalbumin	-SH	capillary LC-ICP-MS, MALDI-MS and ESI-MS		[151]
	PHMB	cysteine, homocysteine, selenocysteine, glutathione, selenomethionin e and cysteinyl-glycine	-SH	HPLC-ICP-MS and HPLC-Orbitrap MS		[152]

6	ebselen	homocysteine and cysteine	-SH	HPLC-ICP-MS, HPLC-UV/VIS and HPLC-ESI-MS/MS		[154]
7	FMEA	tripeptide glutathione and insulin	-SH	LC-ICP-MS and LC-ESI-MS		[158]
		phytochelatins	-SH	LC-ICP-MS, LC-ESI-MS and ICP-OES		[159]
8	MeCAT-maleimide	peptides	-SH	HPLC-ICP-MS and nano-LC-ESI-MS		[133, 134]
9	2-iodo-N-(prop-2-yn-yl)acetamide alkyne and Ln–DOTA–azide	cysteine residue	-SH	SDS-PAGE, nanoLC(RP)–ESI-FT-ICR-MS and LC-ICP-MS		[142]
10	MeCAT-iodoacetamide	cysteine residue	-SH	LC-ICP-MS and LC-ESI-FTICR-MS		[137]

11	TBPA	levothyroxine and its metabolites	-NH_2	HPLC-ICP-MS and UPLC-MS		[72]
12	3-iodophenyl isocyanate	phenylhydrazine	-NH_2	2D-LC-ICP-MS and 2D-LC-MS		[162]
13	2,3,5-triiodobenzoyl chloride					
14	SFP	lysozyme, ß-lactoglobulin A and insulin	-NH_2	LC-ICP-MS and LC-ESI-MS		[158]
15	Rub2m–O–Su	amino acids	-NH_2	HPLC-ICP-MS		[169] [170]
		branched amino acids	-NH_2	HPLC-ICP-MS and LC-MS/MS		
16	ECRS	proteinogenic amino acids	-NH_2	HPLC-ICP-MS and LC-Q-TOFMS		[171]
17	DOTA-NHS ester and Ho, Tb or Tm	peptides	-NH_2	MALDI-MS, LC-ESI-MS/MS and LC-ICP-MS		[136]

	DOTA-NHS ester and Ln	cysteine-containing peptides, bovine serum albumin and human serum albumin	-NH_2	LC-ICP-MS and LC-ESI-FTICR-MS		[137]
18	cDTPA and Cu	*n*-octylamine and benzylamine	-NH_2	capillary LC-ICP-MS		[146]
	cDTPA and Eu	bradykinin and substance P	-NH_2	LC-ICP-MS and LC-ESI-MS		[173]

1.6 Conclusions

As detailed in this chapter, ICP-MS may serve as a promising alternative detection technique to the current standard methodology, *i.e.* radiodetection, in quantitative drug metabolite profiling, owing to its structure-independent analytical response, high sensitivity and robustness. An overview of the current status of HPLC-ICP-MS in pharmaceutical metabolite profiling studies has been provided. It has been shown that coupling RP-HPLC to ICP-MS is not completely straightforward, as the high organic content of the sample flow must be appropriately taken care of by the modification of the sample introduction system. It has been also demonstrated that quantification issues may arise owing to gradient elution, typically applied during the chromatographic separation. Several successful approaches for the correction of the signal drift, occurring as a result of gradient elution, have been discussed with an indication of the main advantages and disadvantages for each of them. However, it can be concluded that the application of HPLC-ICP-MS is still far from routine in the pharmaceutical industry, partially due to the lack of a simple, straightforward and universally applicable quantification approach which enables GMP-traceable validation. Therefore, a first main conclusion is that further fundamental studies are needed for enabling quantitative ICP-MS detection while using different organic solvents and chromatographic strategies to be able to further extend the applicability of the technique in pharmaceutical R&D. As ICP-MS needs a "visible" hetero-element for detection, the main emphasis of this literature overview was put on the HPLC-ICP-MS determination of hetero-element containing drugs and on derivatization strategies for pharmaceuticals without any such suitable hetero-element.

It can be concluded, that the introduction of tandem ICP-MS/MS has revolutionized the determination of the most important hetero-elements (S, Cl, P) in a pharmaceutical context, although further studies are still necessary for these elements, especially for the determination of Cl in different matrices and for a variety of chromatographic approaches. Also the investigation of the possibilities for ICP-MS detection of F is highly desirable as F is also among the top-5 hetero-elements present in APIs. Due to its higher ionization energy compared to Ar and to the strong spectral interference at m/z 19 (further detailed in Chapter 5), the currently commercially available ICP-MS configurations cannot be considered as powerful tools for F-detection. Therefore the development of novel instrument configurations and methodologies targeting F (*e.g.*, the monitoring of negative ions or the use of a He instead of an Ar plasma) could significantly enhance the applicability of HPLC-ICP-MS in the field of drug discovery and development. In comparison with the number of the applications targeting small molecule drugs, only a few studies aiming at the determination of biopharmaceuticals (especially

oligonucleotides) can be found. Due to the increasing market share of biopharmaceuticals with special emphasis on “biosimilars” (which are the “generic” versions of original biopharmaceuticals), the significance of reliable and robust orthogonal analytical techniques applied for the characterization of the drugs is continuously rising in pharmaceutical R&D. Owing to the simple fact that oligonucleotides contain P, which is sensitively measurable with the state-of-art ICP-MS/MS technology, ICP-MS/MS combined with a variety of chromatographic techniques (*e.g.,* based on reversed phase, ion exchange, HILIC, size exclusion etc.) could potentially offer a valuable and sterling orthogonal analytical approach to the presently applied LC-MS-based methodologies.

With respect to fact that the largest portion of the pharmaceutical drugs do not contain any hetero-elements accessible for ICP-MS, several derivatization strategies for the chemical modification of these drugs, utilizing the reactivity of the most typical functional groups in pharmaceutical substances, have also been discussed. It can be concluded, that intensive research is still strongly needed for the improvement of both derivatization reagents and measurement strategies to be able to establish a well-documented and reliable derivatization database for ICP-MS detection, similar to those already available for, *e.g.*, GC analysis of non-volatile compounds or for amino acid analysis using UV- and/or fluorescence detection.

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CHAPTER 2

Determination of the total drug-related chlorine and bromine contents in human plasma using HPLC-ICP-MS/MS

Adapted from:

B. Klencsár, E. Bolea-Fernandez, M. R. Flórez, L. Balcaen, F. Cuyckens, F. Lynen, F. Vanhaecke, Determination of the total drug-related chlorine and bromine contents in human blood plasma using high performance liquid chromatography – tandem ICP-mass spectrometry (HPLC-ICP-MS/MS), *Journal of Pharmaceutical and Biomedical Analysis*, **124** (2016) 112-119.

2.1 Introduction

During drug discovery, the absorption, distribution, metabolism and excretion (ADME) behaviour of a candidate drug (active pharmaceutical ingredient – API) must be investigated at a sufficiently early stage of development. Prior to and simultaneously with the structural identification and quantitative determination of the individual metabolites, the determination of the total content of drug-related compounds in different biological matrices (*e.g.*, blood, plasma, urine, faeces) provides essential information to make up a mass balance, allowing the calculation of the amount excreted from the body based on the difference between the dose administered and the amount recovered from excreta. It also helps in the validation of the analytical approaches applied for metabolite profiling studies (*e.g.*, to reveal recovery issues) and is required to identify the metabolites that exceed 10% of the total drug-related exposure [1], as these must be subjected to further safety and toxicity studies according to ICH M3 guidelines [2].

As individual standards for metabolites are usually not available in an early stage of drug development, a reliable and routinely applicable analytical technique with structure-independent response is highly desirable in drug safety, toxicity and pharmacology studies. Besides conventional techniques (LC-UV, LC-MS(/MS), CE-MS, GC-MS) [3-7] the standard methodology to quantitatively investigate drug ADME in the pharmaceutical industry is based on radiolabeling [8-11]. In this way, the parent drug and its metabolites containing the radionuclide (usually ^{3}H or ^{14}C) are easily and selectively detected and quantitatively determined based on radioactive decay as this response is structure-independent, unlike with the conventional techniques mentioned above. However, radiolabeling is also characterized by significant drawbacks, *i.e.* the need for synthesis of a radiolabeled version of the API [9, 12] and to handle and use radioactive materials, making these procedures also extremely costly and time-consuming. Ethical issues, such as the use of radioactive substances in human studies (especially if the drug has a long residence time in the body) and/or the handling of radioactive waste cannot be overlooked either.

Therefore, the development of a more suitable alternative approach to radiolabeling is justified for the intention of both metabolite profiling and determination of the total content of drug-related compounds in biological matrices.

High performance liquid chromatography - inductively coupled plasma - mass spectrometry (HPLC-ICP-MS) can be regarded as a promising alternative/complementary technique for/to the more common approaches used in drug ADME studies nowadays [13]. The use of ICP-MS as a detector for drugs and drug-related compounds offers many advantages in this context, *e.g.*,

the element-selective detection, high sensitivity and the fact that the response is independent of the molecular structure [14-17]. However, only a limited fraction of the drugs that are currently commercially available contain an element detectable by ICP-MS. Only a few of them contain a metal, the type of element most easily assayed by ICP-MS [18, 19], but a much larger portion of APIs comprises one or more "hetero-atom(s)". In the drugs approved by the U.S. Food and Drug Administration, S, Cl, F, P and Br – in this order of incidence – are the most abundant hetero-atoms (with Br and Cl being the target analytes of this work) [20]. The determination of these elements by means of ICP-MS is possible, but is typically seriously hampered by (i) their possible endogenous presence at high concentration levels in biological matrices (*e.g.*, Cl, P, S) compared to that of the drug-related compounds of interest and (ii) the occurrence of spectral overlap of the analyte signals with those of interfering ions with the same nominal mass.

Therefore, a preliminary separation step prior to sample introduction into ICP-MS is necessary in most cases. At first instance, solid phase extraction (SPE) followed by offline ICP-MS analysis could be considered as the method of choice when aiming at removing the potentially huge inorganic fraction of the target elements (*e.g.*, approx. 100 mmol L^{-1} Cl^- in blood plasma [21]), thus allowing the accurate measurement of the total content of drug-related compounds in biological matrices. However, a preliminary sample preparation step (*e.g.*, precipitation of blood plasma proteins using acetonitrile and subsequent evaporation of the organic solvent prior to sample loading) cannot be avoided in the case of SPE either. Additionally, this approach requires a significant dilution of the sample (due to the strong matrix effect typically occurring in ICP-MS analysis of biological samples and the need for an aliquot with a volume ≥1 mL for ICP-MS analysis using a standard ICP-MS autosampler), resulting in a degradation of the limits of detection and of quantification (LOD and LOQ). In the specific case of Cl, the use of SPE can also be strongly compromised by Cl contamination originating from the polymer holder or the polymer-based stationary phase generally used for manufacturing SPE cartridges. Contamination issues render the quantification uncertain and non-reproducible among different SPE columns, thus creating difficulties towards method validation and routine application. ICP-MS autosamplers capable of handling <1 mL sample volume and glass SPE holders can theoretically eliminate the issues mentioned above, but compared to SPE, an HPLC-ICP-MS approach has the additional benefits that the separation of the metabolites from the inorganic Cl (for polar compounds) and/or the recovery from the column (for apolar compounds) can be more easily checked [22]. Thus, as the same sample preparation is required prior to SPE and to HPLC and taking into account the additional advantages of HPLC over SPE detailed above,

online HPLC-ICP-MS was preferred over the offline combination of SPE and ICP-MS for the determination of the total content of drug-related compounds in biological matrices.

Unfortunately, F is in principle not detectable with ICP-MS, because its ionization energy is higher than that of the plasma gas Ar. The other elements mentioned above (S, Cl, P and Br) are, but each of them also presents specific challenges for ICP-MS analysis (*e.g.*, high ionization energy, thus a low degree of ionization, and the occurrence of spectral interference) [23], often even more pronounced in organic matrices. This explains the limited use of HPLC-ICP-MS in ADME studies so far. At present, accurate determination of increasingly lower levels of these elements becomes possible owing to the evolution in the field of ICP-MS instrumentation over the last years. In the past decade, collision/reaction cells in ICP-MS systems have been successfully used for the interference-free determination of problematic elements [24-26], and more recently, the introduction of a tandem ICP - mass spectrometer (ICP-MS/MS) with an octopole collision/reaction cell mounted in-between two quadrupole mass analysers [27-30] provides an even more powerful tool to efficiently overcome spectral overlap. The double mass selection using the two quadrupoles (MS/MS mode) allows all matrix elements with a mass-to-charge (*m*/*z*) ratio different from that of the corresponding target analyte to be removed by means of the first quadrupole (Q1), while the second quadrupole (Q2) is usually fixed at the *m*/*z* ratio of the corresponding reaction product ion, thus enabling the interference-free determination of elements otherwise suffering from strong spectral overlap.

Within the context of this study, an HPLC-ICP-MS/MS method has been developed as an alternative approach for the quantitative determination of the total content of drug-related Br- and Cl-compounds in human blood plasma. The method developed was validated and subsequently used for analysis of blood plasma samples from human volunteers that were administered a new API containing both Br and Cl.

2.2 Experimental

2.2.1 Materials and reagents

Diclofenac-sodium (pharmaceutical secondary standard with a certified purity of 99.9%) and 4-bromobenzoic acid (4-BBA) (with a purity of 98%), used as Br- and Cl-containing model compounds for method development, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (both LC-MS grade) were purchased from VWR International (Leuven, Belgium). Formic acid (≥88.0%, *Trace*SELECT®), trifluoroacetic acid (≥99.0%, LC-MS grade) and *ortho*-phosphoric acid (~85.0%, *Trace*SELECT®) were obtained from Sigma-Aldrich. Ultrapure grade water (resistivity ≥ 18.2 MΩ.cm) was obtained from a Milli-Q

Element water purification system (Millipore, Billerica, MA, USA). Sodium hydroxide solution (30 *m*/*m*%, Suprapur®) was obtained from Merck (Darmstadt, Germany). Ammonium chloride (1000 ± 2 mg L^{-1} for Cl) and ammonium bromide (1000 ± 5 mg L^{-1} for Br) elemental stock solutions were purchased from Inorganic Ventures (Christiansburg, VA, USA).

2.2.2 Preparation of stock solutions, calibration standard and quality control (QC) solution

A set of ammonium chloride solutions in a concentration range of 0-1 mg L^{-1} for Cl was prepared by dilution of 1000 mg L^{-1} elemental stock solution in methanol and applied for ICP-MS/MS optimization. Diclofenac sodium was dissolved in Milli-Q water at a concentration of 2.3 g L^{-1} corresponding to a Cl-concentration of 0.5 g L^{-1}. The stock solution of 4-bromobenzoic acid was prepared in methanol at 1.3 g L^{-1} corresponding to a Br-concentration of 0.5 g L^{-1}. The calibration standard solution (STD) was prepared to obtain 1.0 mg L^{-1} Cl and 0.5 mg L^{-1} Br in methanol-water (5:95, v/v). As validation demonstrated their reliability, single-point external or internal standard calibration was applied for quantification (see section 2.3.3). The preparation of the calibration standard solution was duplicated to obtain a QC solution that was injected after three injections of the calibration standard and after every 2 hours and/or at the end of the measurement (when this lasted for < 2 hours). The relative difference between the response factors obtained for these two, identical standard solutions was determined and verified that it was < 5.0%. For evaluation of system suitability, the retention time and symmetry factor (A_s) of diclofenac (according to USP <621>) and the RSD% values for the peak areas of diclofenac and 4-bromobenzoic acid as obtained from three subsequent injections of the calibration standard solution were determined. For quantification, the average of the peak areas of three injections of the calibration standard solution was relied on.

2.2.3 Samples and sample preparation

Human blood plasma samples originating from a clinical study were provided by Janssen R&D (Beerse, Belgium) , but almost no information on the structure of the target drug (or possible metabolites) was made available to the UGent researchers, except for its molecular mass (470.11 g mol^{-1}) and the fact that it contains two Cl and one Br atom(s). Additionally, the dose and frequency of administration (600 mg per week in one administration) were known, and the plasma samples were collected at 0 (immediately after administration), 0.25, 0.5, 1.5, 3, 4, 6, 8, 10 and 24 hours after administration. After collection, the samples were stored at -20°C until analysis. Preceding ICP-MS analysis, 900 µL of acetonitrile was added to 300 µL of human plasma in 15 mL centrifuge tubes (VWR International, Leuven, Belgium) and the samples were

vortexed thoroughly. The suspension was then centrifuged at 4400 rpm for 20 minutes at room temperature with an Eppendorf Centrifuge 5702 (Eppendorf, Hamburg, Germany). 1100 µL of supernatant was pipetted into a 15 mL centrifuge tube and reduced in volume to 250-300 µL by solvent evaporation under N_2 flow. The exact amount of residue was determined based on weighing and by assuming the same density as for water at the corresponding laboratory temperature. The reliability of this estimation was confirmed by the fact that no statistically significant difference was found between the results obtained for the same spiked blank plasma samples with external and internal standard calibration, respectively. The final aliquot obtained after evaporation was filtered with a 0.2 µm pore-size Acrodisc® PVDF syringe filter (Sigma-Aldrich) and injected into the HPLC-ICP-MS/MS system. Due to the limited amount of samples, only 2 parallel sample preparations were carried out at each time-point after administration.

2.2.4 ICP-MS/MS instrumentation

The experiments were carried out using an Agilent 8800 triple quadrupole ICP-MS/MS system (Agilent Technologies, Tokyo, Japan). For coupling with HPLC, the instrument was equipped with a low internal volume PFA-LC nebulizer (Elemental Scientific, Omaha, NE, USA) fitted onto a Peltier-cooled Scott-type spray chamber and a torch with a 1.0 mm ID injector tube (Agilent Technologies, Tokyo, Japan) as a sample introduction system. The analyser unit of the instrument consists of two quadrupole mass analysers (Q1 and Q2) and an octopole collision-reaction cell installed in-between. Br and Cl were determined in MS/MS mode with H_2 as a reaction gas in the cell. In order to guarantee a stable plasma and to avoid carbon build-up on the torch and the cones of the instrument, O_2 was admixed to the Ar gas flow (20% optional gas, containing 20% of O_2 in Ar) and the standard Ni cones were replaced by a Pt sampling (with 15 mm Pt insert) and skimmer cone (Agilent Technologies, Japan), because of the higher inertness of the latter. A more detailed description of the ICP-MS/MS conditions is provided in Table 2.1.

During the optimization of the ICP-MS/MS method (without HPLC), spontaneous nebulization was applied as the Cl background was found to be too high when using peristaltic pump tubing (Tygon R3607, IDEX Health & Science, Wertheim, Germany) with organic solvent (comparable to the intensity observed for a solution containing 1 mg L^{-1} Cl).

Table 2.1: ICP-MS/MS conditions

RF power	1570 W
Carrier gas flow rate	0.33 L min^{-1}
H_2 gas flow rate	3.5 mL min^{-1}
Optional gas (20% O_2 in Ar)	20%
Temperature of spray chamber	-1 ˚C
Monitored transitions / masses	Q1: *m/z* 35 (Cl^+) → Q2: *m/z* 37 (ClH_2^+)
	Q1: *m/z* 79 (Br^+) → Q2: *m/z* 79 (Br^+)
Data collection mode	TRA (Transient signal mode)
Integration time	0.1-0.1 s for *m/z* = 37 and 79

2.2.5 HPLC conditions

The chromatographic separation was performed with a Waters Alliance 2690 HPLC system (Waters Corp. Milford, MA, USA) using a Waters Atlantis T3, 3.0 x 50 mm; 3.5 µm C18 column. The elution system consisted of 0.5% formic acid in water as eluent A and 0.5% formic acid in methanol as eluent B. As the objective was not the full separation and speciation of the drug-related compounds, but the fast quantification of the total organic Br & Cl was aimed at, 1.0 mL min^{-1} flow rate and 50 µL injection volume were employed to improve the LOQ. The separation was carried out at room temperature. The gradient programme consisted of two isocratic steps, *i.e.* eluent A – eluent B (95:5, v/v) was used between 0.0-1.5 min for the elution of endogenous Br and Cl and eluent A – eluent B (15:85, v/v) was applied between 1.6-6.0 min to elute the drug-related compounds, preferably in one peak or peak group. Between 6.1-9.0 min, the column was reconditioned using eluent A – eluent B (95:5, v/v) . The column outlet was directly connected to the PFA nebulizer of the ICP-MS/MS instrument. The sample tray was cooled to 5˚C during the measurements.

2.3 Results and discussion

2.3.1 Method development for the interference-free determination of Br and Cl

As mentioned above, the determination of Cl via ICP-MS is seriously hampered by both the occurrence of significant spectral interference (*e.g.*, $^{16}O^{18}O^1H^+$, $^{16}O^{17}O^1H^1H^+$, $^{34}S^1H^+$ [31]) and high ionization energy (1255.7 kJ·mol^{-1} [32]), which may have a detrimental effect on both the accuracy and sensitivity of Cl determination via ICP-MS. In order to obtain the best experimental conditions, different MS/MS approaches based on the use of different reaction gases and reactions were evaluated. In a first stage, H_2 and O_2 were selected as possible reaction gases to overcome the spectral overlap. It was observed that the reaction efficiency with H_2 was

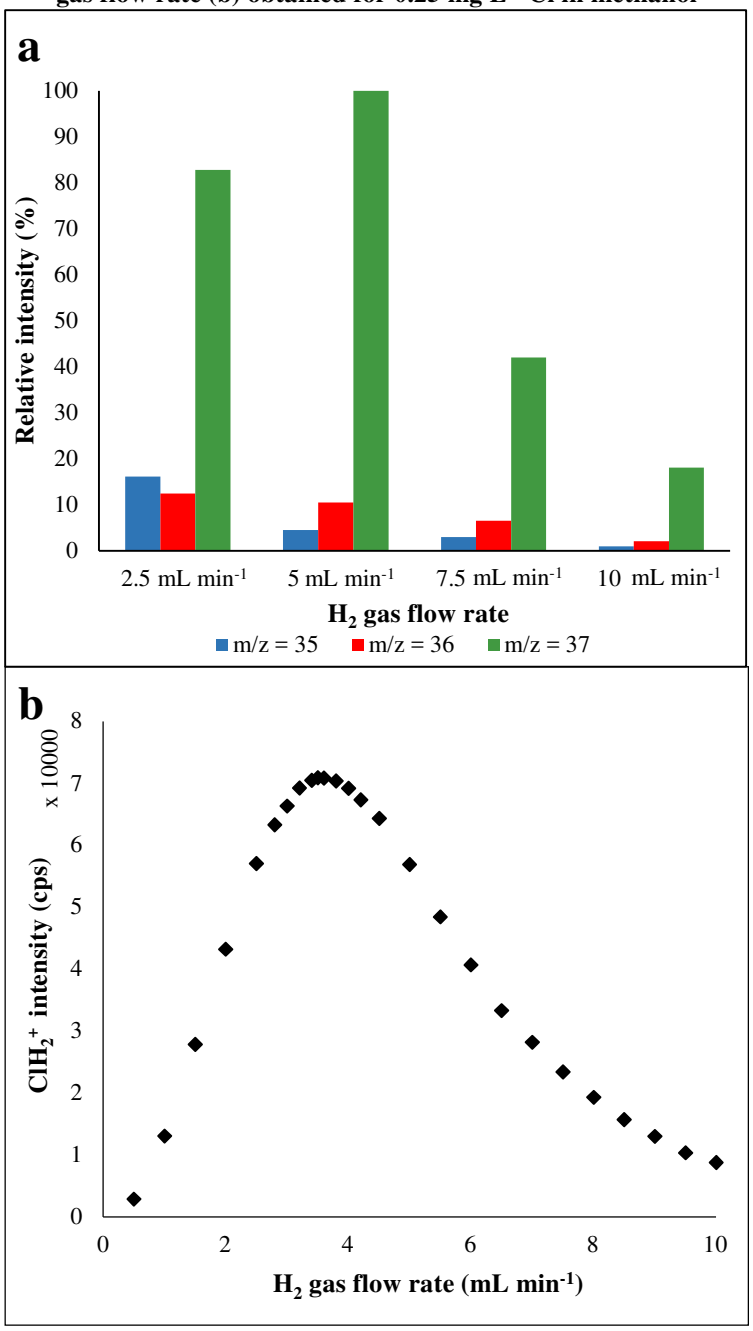
better than that with O_2 (see Table 2.2 for the different reactions and Table 2.3 for the quantitative results). Therefore, addition of H_2 gas in the reaction cell was chosen as the most promising approach for the interference-free determination of Cl in an organic matrix by means of ICP-MS/MS.

Table 2.2: Measurement modes tested for the determination of Cl

Measurement mode	Reaction	Q1 mass (*m* / *z*)	Q2 mass (*m* / *z*)
H_2 mode	$Cl^+ + H_2 \rightarrow ClH_2^+$	35	37
O_2 mode	$Cl^+ + O_2 \rightarrow ClO^+ + O$	35	51
O_2 charge transfer mode	$Cl^+ + O_2 \rightarrow O_2^+ + Cl$	35	32

Via product ion scanning, the different reaction product ions were identified as indicated in Figure 2.1a, which shows the relative intensity for $^{35}Cl^+$ at m/z = 35, $^{35}ClH^+$ at m/z = 36 and $^{35}ClH_2^+$ at m/z = 37 at four different H_2 gas flow rates. It was seen that, independent of the H_2 flow rate, the highest relative intensity was always obtained for $^{35}ClH_2^+$ at m/z = 37. Therefore this reaction product ion was selected for quantification. It must be emphasized that the use of the MS/MS mode is crucial to allow for an interference-free determination of $^{35}Cl^+$ in the H_2 mode. By generating a mass shift of m+2 with the use of H_2 in the reaction cell, it is possible to get rid of the spectral overlap at m/z = 35. However, without the use of MS/MS, the signal of the reaction product ions $^{35}ClH_2^+$ at m/z = 37 would coincide with that of $^{37}Cl^+$, which could also enter the reaction cell and mass spectrometer, making accurate ^{35}Cl determination impossible. The optimal H_2 cell gas flow rate was identified by monitoring the intensity for $^{35}ClH_2^+$ as a function of the H_2 gas flow rate in the range of 0-10 mL min^{-1} while analysing a 0.25 mg L^{-1} Cl solution in methanol (see Figure 2.1b). The highest sensitivity was achieved at 3.5 mL min^{-1} H_2.

Figure 2.1: Relative intensity of reaction products (a) and ClH_2^+ intensity as a function of the H_2 reaction gas flow rate (b) obtained for 0.25 mg L^{-1} Cl in methanol



Once the method was optimized, ammonium chloride solutions (in methanol) were measured at five concentration levels between 0-1 mg L^{-1} Cl for the determination of linearity (sensitivity) and the instrumental LOQ attainable for the approach selected. The LOQ was calculated as 10 times the standard deviation on the Cl intensity obtained for ten successive measurements of methanol (blank), divided by the slope of the calibration curve.

The results are presented in Table 2.3. Next to the results obtained for Cl (main focus of the work), also information on Br has been provided. Although the determination of Br may be

subject to spectral interference as well (*e.g.*, spectral overlap of the signals of $^{79}Br^+$ and $^{38}Ar^{40}Ar^1H^+$ [31, 33]), under the experimental conditions optimized for the determination of Cl, adequate results could also be obtained for Br via on-mass determination of ^{79}Br. A 5-6 fold higher sensitivity was obtained for Br compared to Cl due to both the higher ionization efficiency and the fact that the conversion of Cl in the reaction cell never reaches 100%.

RF power and nebulizer gas flow rate (including 20% of optional gas) were optimized to obtain a maximum intensity for 1 mg L^{-1} Cl and 0.5 mg L^{-1} Br solution in methanol. The temperature of the spray chamber was adjusted to -1 ˚C (in order to decrease the amount of organic solvent entering the ICP, while preventing the freezing of incoming aerosol in the first part of HPLC programme (methanol-water (5:95, v/v) with 0.5% formic acid)).

Table 2.3: Results of ICP-MS/MS optimization

	Cl in H_2 mode	***Cl in O_2 mode***	***Cl in O_2 charge transfer mode***	***Br in H_2 mode***
Intercept	14900	112	146000	5400
Slope	287	4.6	115	1550
R^2	0.9998	0.9991	0.997	0.997
LOQ / µg L^{-1}	4	20	150	1
LOD / µg L^{-1}	1	7	50	0.4

2.3.2 Optimization of HPLC method

For the separation of inorganic Cl and Br forms from the organic (drug-related) compounds and elution of the total drug-related contents of Cl and Br, a fast reversed phase HPLC - ICP-MS/MS method was developed, optimized and validated. In the absence of individual standards and structural information on the drug and its possible metabolites present in human plasma samples, preliminary experiments (not detailed) were needed to gain basic knowledge on the retention features of the compounds of interest (parent drug and possible metabolites) to be able to find appropriate model compounds for method development and validation. Based on screening measurements on a Waters Atlantis T3 (3.0 x 50 mm, 3.5 µm) C18 column and comparison of the chromatograms to that of model compounds, it could be concluded that the molecules of interest show a sufficient retention under acidic conditions with a retention behaviour similar to those of 4-bromobenzoic acid (log*P* = 2.86) and diclofenac (log*P* = 4.06). Therefore, these compounds were chosen as Br- and Cl-containing model compounds for the optimization and method validation.

The chromatographic method was designed to consist of two isocratic steps, one for the elution of all inorganic entities and a second one, for the elution of the drug-related organic compounds,

preferably in one peak or peak group to gain the lowest LOQ values possible and lower the risk of recovery issues due to missing compounds that are < LOQ. As mentioned above, acidic reversed phase LC conditions were applied, thus both eluent A and B were admixed with an acidic eluent additive to gain satisfactory retention and peak shape. 0.1% trifluoroacetic acid, 0.1-0.5% formic acid and 0.01M phosphate buffer (prepared from *ortho*-phosphoric acid solution titrated to $pH = 2.5 \pm 0.1$ with 30 *m*/*m*% sodium hydroxide solution) were tested in this context. To evaluate the chromatograms, the peak width at 5% of peak height and USP tailing factor of diclofenac were monitored. The best peak shapes were observed using either 0.5% formic acid or 0.01M phosphate buffer. However, as the sampling cone was getting contaminated much faster and to a higher extent in the case of phosphate buffer, 0.5% formic acid was selected as the optimal additive. Both acetonitrile and methanol were tested as organic modifiers, but no significant difference was experienced. Therefore, the more cost-effective option and the one giving the lowest carbon deposits on the sampling cone, *i.e.*, methanol, was further employed. Due to a significant difference in the Cl and Br concentrations in methanol and water blanks, the baseline was significantly changing after the sudden switch between eluent A and eluent B. In order to avoid possible interference deriving from this baseline change and to allow for a reliable integration of peaks, the composition of the eluent in the second isocratic part was optimized and a combination of eluent A and eluent B in the ratio 15:85 (v/v) was found to be optimal.

As full separation of organic entities was not aimed at, a high (50 µL) injection volume could be used to improve the LOQs. Self-evidently, this is highly compromising the chromatographic performance indicators, especially the peak symmetry (USP tailing factor of diclofenac is typically 1.7-1.8 under these conditions). Nevertheless, it must be noted that the method is still meeting the current FDA recommendation for the limit of tailing factor ($T \leq 2$) [34].

2.3.3 HPLC-ICP-MS/MS: method validation

For the combination of HPLC and ICP-MS/MS, operated under the conditions described above, different quantification approaches (external (ESTD) and internal standard (ISTD) calibration) were validated using diclofenac and 4-bromobenzoic acid as model compounds. Selectivity, linearity, LOQs for Cl and Br, accuracy and precision were determined as the most important validation parameters.

System suitability was also monitored during the measurement runs. System suitability parameters, requirements and typical values obtained during the research are summarized in Table 2.4.

Table 2.4: System suitability requirements and results

Parameter	Requirement	Typical values
Retention time of diclofenac	3.4 ± 0.2 min	3.40 ± 0.02 min
USP tailing factor of diclofenac*	≤ 2.0	1.7-1.8
RSD% of 3 injections of STD for diclofenac	≤ 5.0 %	0.5-3.0 %
RSD% of 3 injections of STD for 4-bromobenzoic acid	≤ 5.0 %	1.5-4.5 %
STD correlation to QC** for diclofenac	≤ 5.0 %	1.0-3.5 %
STD correlation to QC** for 4-bromobenzoic acid	≤ 5.0 %	1.5-4.5 %

*Calculated from the first STD injection

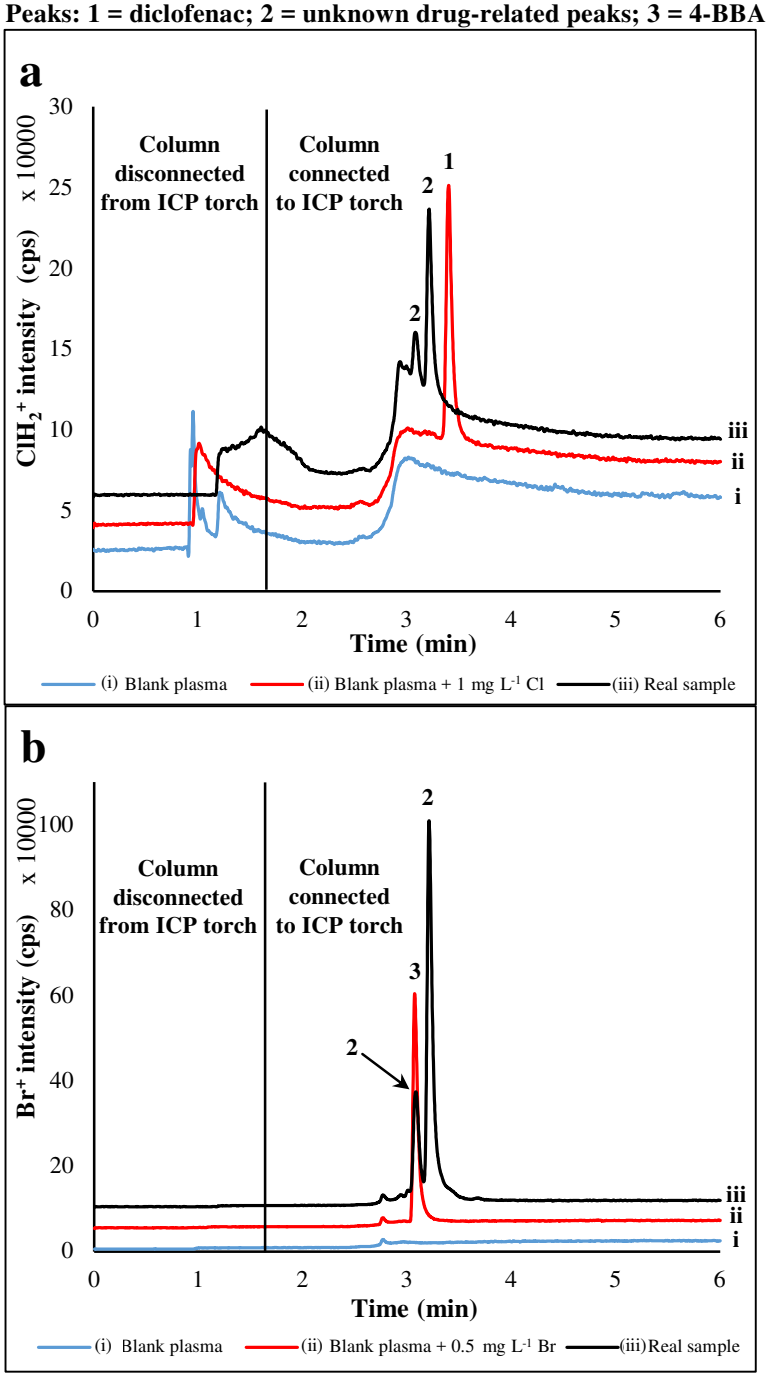
**Relative difference between response factors of average STD and QC injections

The selectivity was studied by comparison of Cl and Br chromatograms of human blank plasma, human blank plasma spiked with the model compounds and a real human plasma sample (containing the drug-related compounds of interest) (Figure 2.2). The chromatograms are shifted on the vertical scale for improved visibility and comparability. It is clearly shown that there are no interfering peaks in chromatograms of the blank plasma either at the position of the model compounds or the target compounds. To avoid the introduction of a huge amount of inorganic salts - present in blood plasma - into the ICP, the HPLC system (column effluent) was disconnected from the ICP sample introduction system during the first 1.2 min of the chromatographic run in case of injection of plasma samples. In this way, contamination and memory effects for Cl in the ICP-MS instrument could be prevented. The time of reconnection can be clearly seen in the baseline in the first isocratic step (between 1.2-1.7 min), but it is apparently not having any influence on the second isocratic step during which the compounds of interest are eluted.

For studying the linearity of the calibration curves, a set of standard solutions at 7 concentration levels was prepared for both Cl (0.05-5.00 mg L^{-1}) and Br (0.01-1.00 mg L^{-1}), in a methanol-water solvent (5:95, v/v). For ISTD calibration, Br at a concentration of 0.5 mg L^{-1} was applied as the ISTD for Cl determination and *vice versa* (*i.e.* Cl at a concentration of 1.0 mg L^{-1} was applied as an ISTD for Br determination). Based on the results (Table 2.5), the linearity with both calibration approaches was considered to be satisfactory for both elements as R^2 was ≥ 0.990 and the 95% confidence interval of the intercept involves *origo*. In agreement with the ICH Q2(R1) guideline on the validation of analytical methods, a more detailed statistical evaluation based on ANOVA was also carried out (presented in the Appendix to Chapter 2) to

assess the applicability of a linear model for the relationship between the peak area and the concentration. Based on the linearity results, it can be concluded that, the simplest ways of quantification, *i.e.* single-point ESTD or ISTD calibration, seem to be promising approaches for the analysis of real samples in the concentration range investigated.

Figure 2.2: ClH_2^+ (a) and Br^+ (b) chromatograms of blank plasma, blank plasma spiked with 1 mg L^{-1} Cl as diclofenac (a) and 0.5 mg L^{-1} Br as 4-BBA (b) and real human blood plasma sample; Peaks: 1 = diclofenac; 2 = unknown drug-related peaks; 3 = 4-BBA



Accuracy and precision were studied by spiking blank human plasma obtained from patients not subjected to any medical treatment, at four concentration levels (0.05, 0.25, 0.50 and 1.00 mg L^{-1}) of Cl as diclofenac and (0.01, 0.25, 0.50 and 1.00 mg L^{-1}) of Br as 4-bromobenzoic acid with three parallel sample preparations at each level. The lowest levels of 0.05 mg L^{-1} and 0.01 mg L^{-1} correspond to the LOQ-values for Cl and Br, respectively, and were calculated as the concentration where a signal-to-noise ratio S/N ≈ 10 was obtained with an RSD ≤ 20% and accuracy between 85-115% in spiked blank plasma. S/N was determined according to USP <621>.

Table 2.5: Results of linearity study

Calibration approach	Parameter	Cl	Br
External standard	Equation of calibration curve	$y = 751024x - 38881$	$y = 4338469x - 12408$
	R^2	0.9996	0.9993
	95% confidence interval of intercept	±42000	±57000
Internal standard	Equation of calibration curve	$y = 0.3333x - 0.0171$	$y = 6.0300x + 0.0079$
	R^2	0.9985	0.9988
	95% confidence interval of intercept	±0.03	±0.10

The recovery for drug-related Cl and Br contents determined by comparing the experimental result to the calculated/real value was used as a measure for accuracy and the RSD% on the results of three parallel sample preparations at each level were given as an indication for precision. As can be seen from Table 2.6, good accuracy (acceptable between 85-115% at all levels, including LOQ) and precision (acceptable if it is ≤20 RSD% at LOQ level and ≤10 RSD% at higher levels) were obtained in all cases.

This validation indicated that the method developed is selective, linear, sufficiently precise (below 5% in most cases) and accurate (within 95-105% in all cases) from the LOQ level for both Cl and Br with either ESTD or ISTD calibration. The validation results found for both Cl and Br are in a good agreement with or even better than those previously published for similar applications. Significantly better LOQs were obtained for both elements than those reported by de Vlieger *et al* [35] for the determination of halogen-containing drug metabolites using high-temperature LC coupled to ICP-MS. Similar results were found as those reported for Br by Bendahl *et al* [36] in terms of linearity, precision and LOQ and by Meermann *et al* [17] in terms of LOQ and precision. The capabilities of the present, ICP-MS/MS-based approach are also

comparable to or better than those attainable via other techniques as continuum source molecular absorption spectrometry, capillary zone electrophoresis, TXRF or radio detection [17, 37].

Table 2.6: Results obtained for the evaluation of accuracy and precision of the HPLC-ICP-MS/MS method (*n* = 3)

Concentration level (mg L^{-1})	Cl				Br			
	ESTD		ISTD		ESTD		ISTD	
	Recovery (%)	Precision (RSD%)	Recovery (%)	Precision (RSD%)	Recovery (%)	Precision (RSD%)	Recovery (%)	Precision (RSD%)
LOQ	100.1	1.3	101.0	4.0	96.6	5.9	101.4	6.1
0.25	97.8	4.6	101.0	2.0	97.2	1.6	99.5	2.0
0.50	99.0	2.9	101.3	2.7	101.1	4.9	102.8	3.8
1.00	96.8	3.7	101.4	1.5	97.2	2.0	97.9	1.6

2.3.4 Analysis of real samples

As the specific drug of interest contains both Cl and Br, only ESTD calibration could be applied for quantification, but the presence of both Cl and Br in the samples provides a way to cross-validate the results obtained for the total drug exposure. The chromatograms obtained for the calibration standard solution (1.0 mg L^{-1} Cl as diclofenac + 0.5 mg L^{-1} Br as 4-bromobenzoic acid) and a sample corresponding to the maximum drug concentration observed (3 hours after administration of the drug) are shown in Fig. 2.3a and b, respectively. The total drug exposure was visualized by plotting the total drug content of plasma, based on both the determination of Cl and Br, together with the corresponding ±3SD values, as a function of time after administration of the drug (Fig. 2.4a). To compare the total drug content based on the two elements, the relative difference between the corresponding values was calculated. Although the trend of total drug content as a function of sampling time based on either Cl or Br (curve with a maximum at 3 hours after administration) is identical, a systematic bias between the values obtained via the respective elements was established: the drug content based on Br is higher by 3-18% than that based on Cl. A statistically significant difference between drug contents obtained based on Cl and Br, respectively, was found at 1% significance level by a paired *t*-test. This seems to be the consequence of the significant difference in sensitivity for Cl and for Br, respectively (5-6 fold higher for Br). Aiming at a determination of the total content of drug-related compounds, every peak observed during the second isocratic part of the method and not present in the chromatogram obtained for the blank plasma, was integrated. Due the 5-

fold better LOQ obtained for Br (compared to that for Cl), there are at least three additional peaks that could be quantified in the Br-chromatograms, but not in the Cl-chromatograms. When the Br-chromatograms were evaluated without the integration of these three small additional peaks, there was a 6-8% decrease in the total drug exposure, improving the relative differences between the results on the basis of Cl and those on the basis of Br to -3 – 11% (Fig. 2.4b). To prove the assumption that the bias observed occurs due to these three minor peaks, a paired *t*-test at 1% significance level was carried out again for the comparison of the original Cl-based values to the biased (re-integrated) Br-based values, which confirmed that there is no statistically significant difference between the results obtained via the two elements in this way.

Figure 2.3: Chromatograms of calibration standard (a) and real human blood plasma sample (b); Peaks: 1 = diclofenac; 2 = 4-BBA; 3 = unknown drug-related peaks

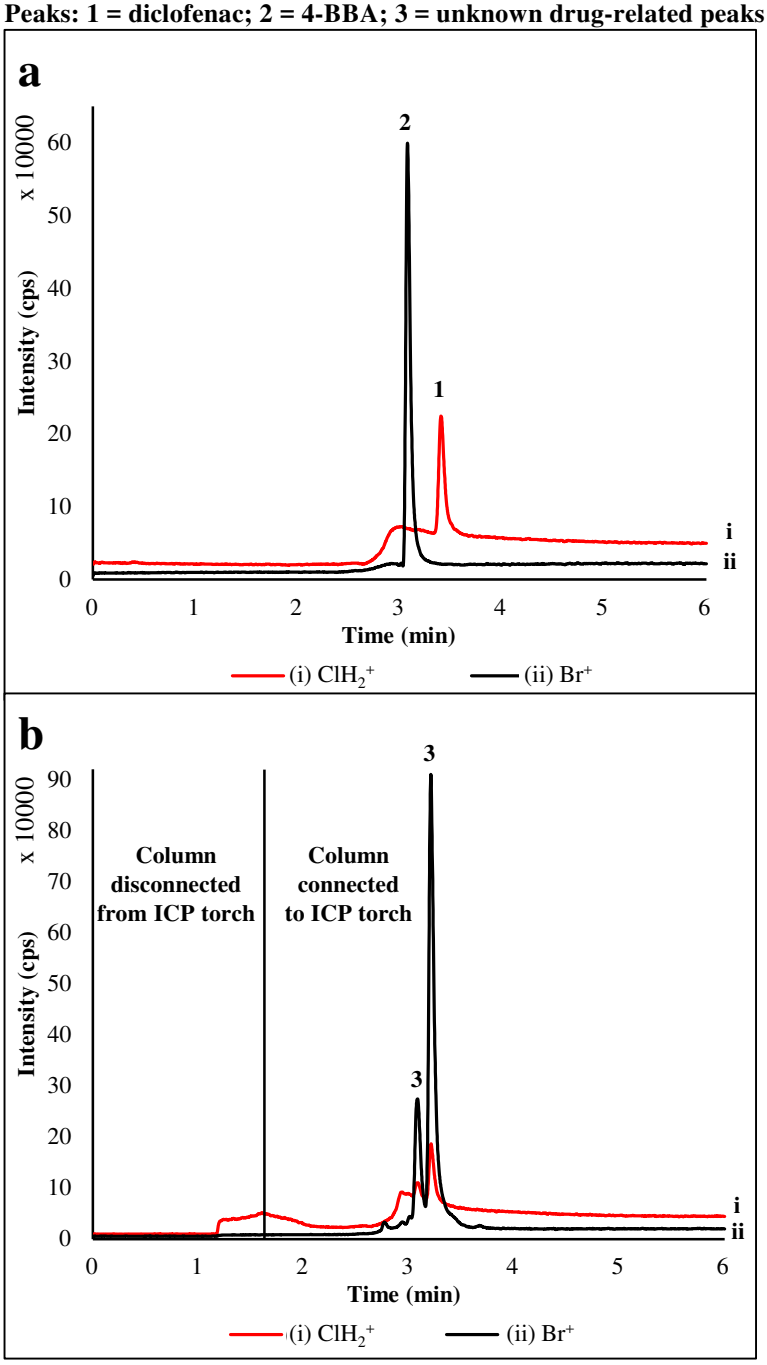
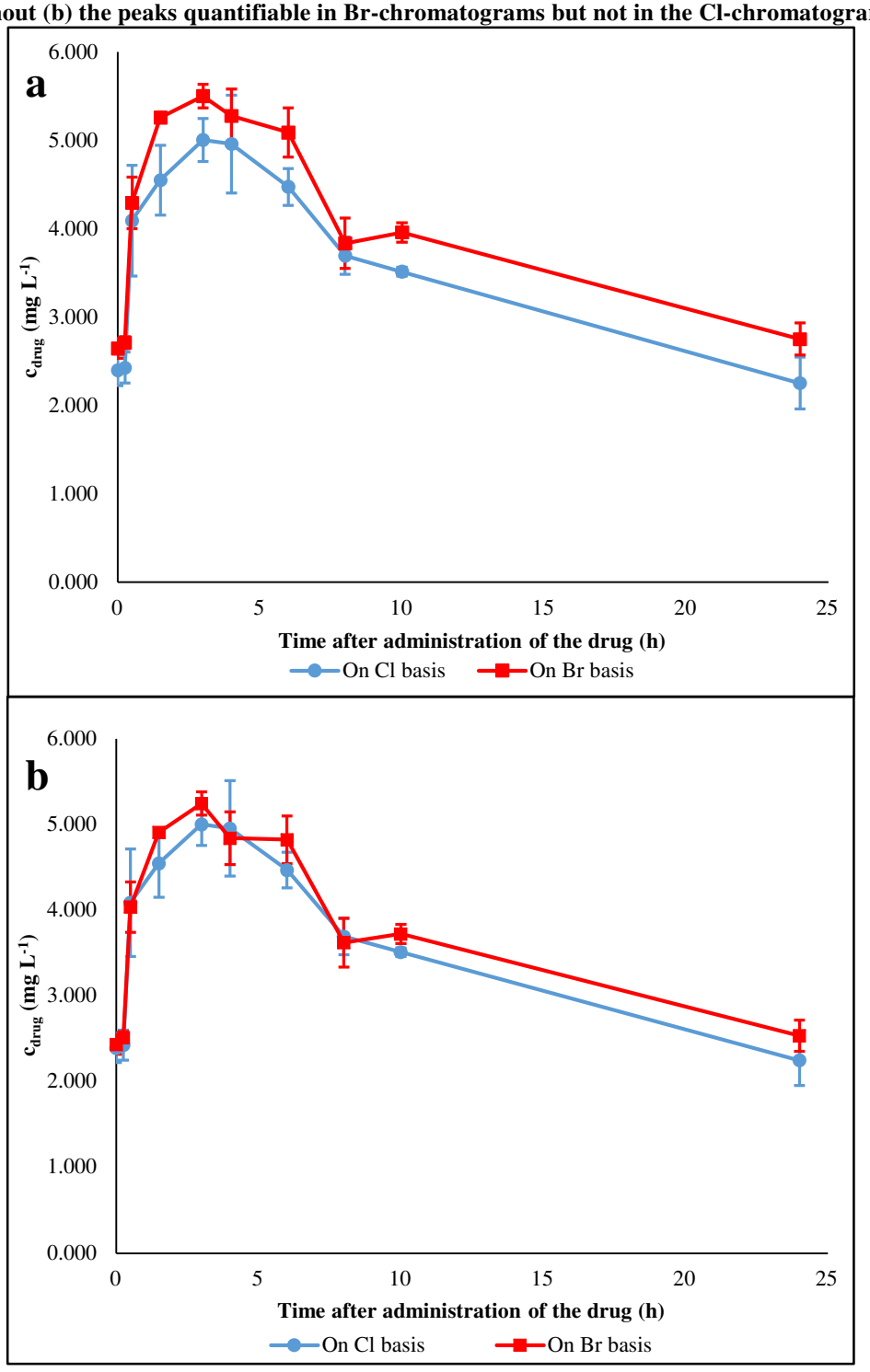


Figure 2.4: Total drug exposure as the concentration of the drug in plasma ± 3SD (*n* = 2) with (a) and without (b) the peaks quantifiable in Br-chromatograms but not in the Cl-chromatograms



2.4 Conclusions

A simple and fast analytical method for the determination of total drug-related Cl- and Br-containing compounds in human blood plasma based on HPLC-ICP-MS/MS has been developed. By means of the state-of-art ICP-tandem mass spectrometer, sensitive and interference-free determination of chlorine was enabled via reaction between Cl^+ and H_2 reaction gas in the collision/reaction cell. The HPLC-ICP-MS/MS method developed is universally applicable for Cl- and Br-containing organic compounds with acidic and neutral character in a broad hydrophobicity range (at least between 2.86 and 4.06, the log*P* values of the model compounds used) covering a large portion of Cl- and Br-containing drugs. This range can be extended/shifted by the modification of the composition of the eluent in the second isocratic part of the gradient programme and subsequent validation. Based on the validation results, it can be stated that the method developed is sufficiently precise and accurate (also at LOQ level) and owing to the excellent linearity and injection precision, the simplest, single-point calibration approach suffices for reliable quantification. As a proof-of-concept, real-life human plasma samples deriving from a clinical study involving a drug with two Cl and one Br atoms were analysed. The total drug exposure was successfully described and agreement was found between the results obtained based on Br and Cl. Therefore, as an overall conclusion it can be stated that a sterling alternative to radioactive counting or to ^{14}C Accelerator Mass Spectrometry measurements was developed for the determination of the total concentration of Cl- and Br-containing drug-related compounds using HPLC-ICP-MS/MS. Despite the possibility of successful application of HPLC-ICP-MS in drug metabolism studies, there are still several challenges that need to be overcome to further broaden the employability of this technique in the field. It was demonstrated that a simple external and/or internal standard calibration approach is viable for the quantification using model compounds containing the same target elements as the molecules of interest (structure potentially unknown). However, it must be noted that an isocratic elution was applied here for the elution of the drug-related entities. The constant solvent composition provides a constant ICP-MS response throughout the chromatographic run. In case of a metabolite profiling study, the application of a gradient elution is, in most cases, inevitable for achieving separation of the target compounds. Gradient elution results in a continuously changing ICP-MS sensitivity, making quantification less straightforward. The possibilities for correction for this changing ICP-MS response during the chromatographic run should be further addressed. Also the ongoing trend towards miniaturization and the introduction of UHPLC raises a challenge as a result of the significant

dead volume of the ICP-MS sample introduction system (*e.g.*, spray chamber), which will probably necessitate re-design efforts.

2.5 References

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Appendix to Chapter 2

The statistical evaluation of the linearity studies performed for Cl and Br during the validation of the HPLC-ICP-MS/MS method (detailed in Section 2.3.3) was carried out with ANOVA at $\alpha = 0.05$ significance level using MS Excel (version 16.0.10228.20080 32-bit). ANOVA is one of the most generally applied statistical tools to test whether a linear association exists between a predictor and the corresponding response via the evaluation of the different sums of squares (SS) defined during linear regression. The total variation of the observations of an experiment is described by the total sum of squares (Total SS) which is further divided to the variation deriving from the regression (regression sum of squares) and to the deviation from the predicted value (based on the fitted model), also referred to as the residual sum of squares. The residual sum of squares (thus the deviation from the predicted model) can originate from (i) pure random error and also from (ii) lack of fit, resulting in a further distinction of residual SS into pure error SS and lack of fit SS. Via the comparison of the residual mean square (residual MS obtained by dividing the residual SS by its degrees of freedom) and the regression mean square (regression MS obtained by dividing the regression SS by its degrees of freedom) by means of an *F*-test, it can be tested whether a linear model needs to be discarded or not at a given significance level. The comparison of the lack of fit mean square and the pure error mean square also allows to test the trueness of linearity at a given significance level [1]. While the first approach is satisfactory for the evaluation of calibration lines where the linear relationship is fundamentally supported (*e.g.* UV detector or quadrupole ICP-MS), the second approach should be used when the linear nature of the detector is also to be studied, requiring replicates at the individual calibration levels [1, 2]. Due to the fundamentally linear behaviour of quadrupole ICP-MS and to the excellent instrument stability (*i.e.* system repeatability), the present linearity studies were carried out with 1 replicate at each concentration level, followed by the evaluation of the linearity based on the comparison of residual SS and regression SS by an *F*-test. The results presented in Table A-2.1 and A-2.2, for Cl and Br, respectively, clearly show that the linear model for the HPLC-ICP-MS/MS response (*i.e.* peak area) as a function of analyte concentration can be accepted at $\alpha = 0.05$ significance level *(i.e.* the value of *significance* $F < 0.05$ indicating the probability of getting such large F statistic if the null hypothesis was true).

Table A-2.1: Statistical evaluation of the linearity study for Cl in the concentration range of 0.05 – 5.00 mg L^{-1} at 7 concentration levels with 1 replicate at each level

Regression Statistics						
Multiple R	0.9998					
R Square	0.9996					
Adjusted R Square	0.9995					
Standard Error	33472					
Observations	7					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	1	1.25E+13	1.25E+13	1.12E+04	1.44E-09	
Residual	5	5.60E+09	1.12E+09			
Total	6	1.25E+13				
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-38881	16233	-2.40	0.06	-80609	2847
Slope	751024	7106	105.69	1.44E-09	732757	769291

Table A-2.2: Statistical evaluation of the linearity study for Br in the concentration range of 0.01 – 1.00 mg L^{-1} at 7 concentration levels with 1 replicate at each level

Regression Statistics						
Multiple R	0.9997					
R Square	0.9993					
Adjusted R Square	0.9992					
Standard Error	45065					
Observations	7					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	1	1.49E+13	1.49E+13	7.32E+03	4.13E-09	
Residual	5	1.02E+10	2.03E+09			
Total	6	1.49E+13				
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-12408	22261	-0.56	0.60	-69631	44816
Slope	4338470	50699	85.57	4.13E-09	4208144	4468796

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CHAPTER 3

Development and validation of a novel quantification approach for gradient elution RP-HPLC-ICP-MS/MS and its application to diclofenac and its related compounds

Adapted from:

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3.1 Introduction

It is well-known that high-performance liquid chromatography coupled to inductively coupled plasma - (tandem) mass spectrometry (HPLC-ICP-MS(/MS)) can serve as a powerful and versatile technique for elemental speciation in environmental and biological samples. So far, most of the studies published focus on metal speciation [1-6], utilizing the excellent capabilities of ICP-MS for metal detection. However, owing to the continuous development of the technique (with special emphasis on quadrupole ICP-MS(/MS) systems equipped with collision/reaction cells [7-10]), also the determination of non-metals (*e.g.*, S, P, Cl, Br, I) with ICP-MS [11-13] is gaining ground. This certainly leads to an increased utility of HPLC-ICP-MS(/MS) in pharmaceutical applications, especially in pharmacology and quantitative drug metabolite profiling.

While the structural identification of the metabolites of a candidate drug is carried out using "traditional" analytical tools, *i.e.* LC-MS(/MS), NMR etc. [14-16], a sensitive and selective analytical technique with structure-independent analytical response is required for quantitative metabolite profiling due to the lack of individual standards for (all of) the metabolites of the candidate drug. Presently, the standard technique is radiolabelling followed by HPLC separation and radiodetection [17], giving rise to obvious difficulties and limitations, such as the handling of radioactive materials and waste, the tremendous cost of the synthesis of a radiolabelled version of the drug and also the ethical issues related to the introduction of radioactive materials into humans [18]. Thus, a sterling alternative technique to radiolabelling is highly desirable.

Although owing to the structure-independent nature of its analytical response, ICP-MS-based detection offers a promising alternative for the detection of drugs and drug-related compounds containing a suitable hetero-element, the application of this technique in this context is far from routine in pharmaceutical research yet due to several difficulties arising when coupling reversed phase HPLC to ICP-MS [19]. As is well-known, reversed phase HPLC applies mixtures of water and organic solvents (typically acetonitrile and/or methanol) for the elution of the target compounds from *apolar* stationary phases (*e.g.*, C18, C8, phenyl). This feature causes a first difficulty towards ICP-MS applications, *i.e.* the presence of a higher concentration of organic solvent in the sample flow, resulting in carbon deposition on the cones and in unstable plasma conditions. By (i) using Pt interface cones instead of the more generally applied Ni cones and (ii) introducing O_2 into the plasma, stable plasma conditions can be maintained and by (iii) using a torch injector tube with a smaller internal diameter and (iv) cooling the spray chamber, the organic load of the plasma can be significantly decreased [19]. Moreover, the necessary

chromatographic separation of highly similar compounds (*e.g.*, drugs and their metabolites) in complex matrices (*e.g.*, blood plasma, urine, faeces extracts) can be achieved by isocratic elution in rare cases only [20]; typically gradient elution is mandatory. This results in a sample flow with continuously changing composition entering into the ICP, thus giving rise to continuously changing plasma conditions and a corresponding varying analytical response (sensitivity) for the target element. This effect must obviously be avoided or at least properly corrected for when aiming at reliable quantification. One option for this correction already described in the literature is quantification via *online* non-specific isotope dilution [18, 21, 22]. Although this approach was demonstrated to be properly reliable, *online* isotope dilution is not readily applied routinely and is not applicable for mono-isotopic elements, such as P or I, or for elements for which interference-free determination with ICP-MS can be achieved for one isotope only. Also application of a post-column compensation (counter) gradient can alleviate the adverse effects of gradient elution [23], but similarly to *online* isotope dilution, it also requires a more complex setup, while doubling the solvent consumption and possibly negatively impacting sensitivity due to dilution and less optimal nebulization. In this work, a technically more simple and cost-effective quantification approach able to compensate for the negative effect of gradient elution was looked for.

Within the context of this study, different quantification approaches for HPLC-ICP-MS/MS have been investigated with attention to the employability of the approach to pharmaceutical research. As a demonstrative example, the Cl-containing drug diclofenac and its related compounds have been applied. Chlorine is one of the most abundant hetero-element present in pharmaceuticals [24] and enables ICP-MS/MS detection. The effect of the concentration of both methanol and acetonitrile on the analytical response for Cl has been systematically evaluated at two different flow rates within the typical flow rate range applied during HPLC analysis. A triple-quadrupole ICP-MS/MS system using H_2 as a reaction gas (as recently published by Nelson *et al* [25] for GC-ICP-MS/MS and Klencsár *et al* [26] for HPLC-ICP-MS/MS applications) was used for the interference-free determination of Cl as $^{35}ClH_2^+$. Diclofenac was synthetically degraded to generate degradation products covering a broad hydrophobicity range, thus also requiring the ICP-MS/MS system to deal with a wide concentration range of organic solvents. 4`-hydroxy-diclofenac was not formed during synthetic degradation and was added to the mixture obtained.

In a final phase of the study, the method developed was applied to human plasma matrix to demonstrate the applicability of the newly developed approach in real-life matrices.

In view of the relatively poor limit of detection (LOD) and limit of quantification (LOQ) values obtainable for Cl with ICP-MS/MS compared to those for metals, an *online* chromatographic pre-concentration procedure has also been developed to further improve the analytical capabilities [27], thus also enabling metabolite profiling of low-dose drugs. The generally applied HPLC injection volume range (2-100 µL) has been extended to 1500 µL by trapping the drug-related compounds on a short reversed phase HPLC column followed by an appropriate washing procedure to get rid of the matrix components prior to the chromatographic separation.

3.2 Experimental

3.2.1 Reagents and materials

Diclofenac-sodium (pharmaceutical secondary standard with a certified purity of 99.9%), 4`-hydroxy-diclofenac (analytical standard with a purity of 99.0%), formic acid (≥88.0%, *Trace*SELECT®), hydrogen peroxide solution (30%, for ultra-trace analysis) and ammonia solution (≥25%, *Trace*SELECT® ultra) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (both LC-MS grade) were purchased from VWR International (Leuven, Belgium). Pro analysis grade nitric acid (65%, Chem-Lab, Zedelgem, Belgium) was further purified through sub-boiling in a PFA equipment. Ultra-pure grade water (resistivity ≥ 18.2 MΩ.cm) was obtained from a Millipore Direct-Q water purification system (MQ-water, Millipore, Billerica, MA, USA). Ammonium chloride (1000 ± 2 mg L^{-1} for Cl) elemental stock solution applied for the optimization of the ICP-MS method was purchased from Inorganic Ventures (Christiansburg, VA, USA).

3.2.2 Preparation of stock solutions and calibration standards

For the optimization of the ICP-MS/MS method, 1 mg L^{-1} Cl standard solution was prepared by dilution of ammonium chloride (1000 ± 2 mg L^{-1} for Cl) elemental stock solution with acetonitrile or methanol. The 5.0 mg L^{-1} standard solutions of inorganic Cl applied for the investigation of the effect of organic solvent concentration on the ICP-MS/MS response for Cl were prepared by diluting the ammonium chloride (1000 ± 2 mg L^{-1} for Cl) elemental stock solution with different MQ-water – organic solvent (acetonitrile or methanol) mixtures. Diclofenac sodium was dissolved in MQ-water at concentrations of 5.1 and 2.3 g L^{-1} for synthetic degradation and for the preparation of standard solutions, respectively, and subsequently stored in the fridge (at 2-8 °C). Stock solution of 4`-hydroxy-diclofenac was prepared in acetonitrile at a concentration level of 2.0 g L^{-1} and subsequently stored at -20 °C.

The standard 5.0 mg L^{-1} Cl solutions of diclofenac sodium applied for the investigation of the effect of the organic solvent concentration on the ICP-MS/MS response for Cl were prepared by diluting the 2.3 g L^{-1} stock solution with different MQ-water – organic solvent (acetonitrile or methanol) mixtures. The standard working solutions containing diclofenac sodium and 4`-hydroxy-diclofenac applied for HPLC-ICP-MS/MS method development, external standard calibration and method validation were diluted from the corresponding stock solutions with MQ-water – acetonitrile 7:3 (v/v). The standard working solutions containing diclofenac sodium and 4`-hydroxy-diclofenac applied for the development and validation of *online* sample pre-concentration were diluted from the corresponding stock solutions with MQ-water – acetonitrile 95:5 (v/v) containing 0.1 % (v/v) formic acid.

3.2.3 Investigation of the effect of organic solvent concentration on the ICP-MS/MS response for Cl

Standard solutions of 5.0 mg L^{-1} Cl (both as inorganic Cl and diclofenac) were prepared in 20, 30, 40, 50, 60, 70, 80 and 90% (v/v) organic solvent (both in acetonitrile and methanol) in MQ-water. 50 µL of the corresponding standard solution was injected 3 times into the HPLC-ICP-MS/MS system, while maintaining an isocratic flow of 20, 30, 40, 50, 60, 70, 80 and 90% (v/v) organic solvent (both acetonitrile and methanol) in MQ-water at a flow rate of 0.5 mL min^{-1} without using a chromatographic column. A 2 minutes equilibration time was respected after every shift to the next organic solvent concentration. The transient signal of $^{35}ClH_2^+$ was monitored as detailed in section 3.2.6. The response factor for Cl was determined as the ratio of the peak area (obtained for each injection and each organic composition) to the concentration (5.0 mg L^{-1}), using both methanol and acetonitrile and for inorganic Cl and organo-Cl (as diclofenac).

3.2.4 Synthetic degradation of diclofenac

A synthetic degradation procedure was developed based on the studies by Panda *et al* [28] and Shaalan *et al* [29] and used to create a variety of diclofenac-related compounds that can be used as a model mixture for HPLC-ICP-MS/MS method development. 0.5 mL of diclofenac sodium stock solution (5.1 g L^{-1}) was mixed with 1.0 mL of methanol, 0.5 mL of 30% H_2O_2 solution and 0.5 mL of 2 M HNO_3 in a 3.5 mL quartz UV-cuvette (Quartz SUPRASIL®, 100-QS, 10 mm, Hellma Analytics, Müllheim, Germany). This solution was then exposed to UV-light (λ = 254 nm; 5 W) for 3 hours. Subsequently, 2 mL of this solution was transferred into a closed 7 mL Savillex Teflon® beaker and heated at 90°C for 30 minutes. After cooling down, the solution

was neutralized using 0.4 mL of 2 M NH_4OH-solution. 0.4 mL of methanol was also added to the solution to ensure the solubility of all degradation products. The degraded diclofenac samples were then stored at -20 °C until analysis.

3.2.5 HPLC conditions

The chromatographic separation was carried out using an Agilent 1260 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with an Agilent 1260 Infinity vacuum degasser, an Agilent 1260 Infinity binary pump, an Agilent 1260 Infinity autosampler, an Agilent 1290 Infinity thermostated column compartment and an Agilent 1290 Infinity series 2 position / 10 port micro-valve. As stationary phases, a Waters XBridge BEH Phenyl (3.0 x 150 mm; 3.5 µm) column and a Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm) column were applied at flow rates of 0.5 and 1.0 mL min^{-1}, respectively. When using the Waters XBridge BEH Phenyl column, the mobile phase consisted of 0.1% (v/v) formic acid in MQ-water as eluent A and of 0.1% (v/v) formic acid in methanol or acetonitrile as eluent B. In the case of the Waters XBridge BEH C18 column, only 0.1% (v/v) formic acid in acetonitrile was applied as an eluent B, based on the results obtained for the comparison of methanol and acetonitrile using the XBridge BEH Phenyl column. Two different gradient methods were developed for the separation of diclofenac, its degradation products and the added 4`-hydroxy-diclofenac, one using methanol and the other using acetonitrile, as detailed in Table 3.1.

Table 3.1: Details of the chromatographic methods applied for the separation of diclofenac and its related compounds

	Without *online* pre-concentration		**With *online* pre-concentration**
Column	Waters XBridge BEH Phenyl (3.0 x 150 mm; 3.5 µm)	Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm)	
Eluent A	0.1 % (v/v) formic acid in MQ-water		
Eluent B	0.1% (v/v) formic acid in methanol	0.1% (v/v) formic acid in acetonitrile	
Gradient	0-25 min: 60 → 0% A (40 → 100% B) 25-26 min: 0% A (100% B)	0-25 min: 70 → 0% A (30 → 100% B) 25-26 min: 0% A (100% B)	0-5 min: 95 → 70% A (5 → 30% B) 5-30 min: 70 → 0% A (30 → 100% B) 30-31 min: 0% A (100% B)
Flow rate	0.5 mL min^{-1}	1.0 mL min^{-1}	
Sample temperature	5 °C		
Column temperature	Room temperature (22-23 °C)		
Injection volume	50 µL		1500 µL
Valve programme	0-3 min: flow into the waste 3-26 min: flow into the ICP-MS/MS		0-5 min: Trapping* 5-31 min: Analysis*

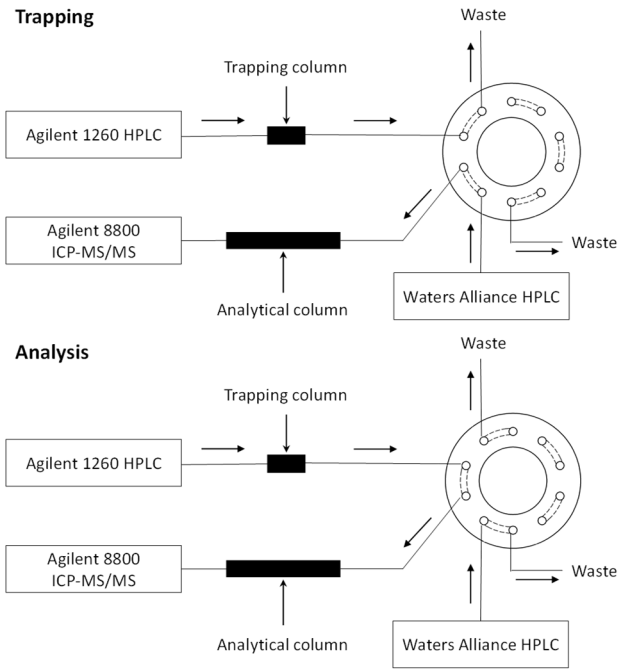
*See the positions in Fig. 3.1.

The chromatographic separations were carried out at room temperature. 50 µL of standard or sample solution was injected. A time-programmable micro-valve was applied to avoid Cl-

contamination of the ICP-MS/MS instrument caused by the high inorganic Cl-content of human plasma by directing the flow into the waste between 0 – 3 min in each chromatographic run involving human plasma.

For *online* sample pre-concentration, the injection volume was extended to 1500 µL by installing an additional 1400 µL stainless steel sample loop (Multidraw Kit, Agilent Technologies, Waldbronn, Germany) into the injection system. The pre-concentration of diclofenac and its related compounds was performed on a Waters XBridge BEH C18 (4.6 x 20 mm; 3.5 µm) intelligent speed column, followed by the chromatographic separation on a Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm) analytical column, as also detailed in Table 3.1. A Waters Alliance 2690 HPLC system (Waters Corp. Milford, MA, USA) was applied as a supplementary pump to maintain a stable, 1.0 mL min^{-1} flow with a composition of 75 % (v/v) eluent A / 25 % (v/v) eluent B through the analytical column, while the drug-related compounds of the sample were being trapped and the flow of the Agilent HPLC-unit was being directed to the waste using the same 2 position / 10 port micro-valve, as described above. A schematic representation of the system applied is shown in Fig. 3.1.

Figure 3.1: Schematic representation of the chromatographic system used for *online* sample pre-concentration



3.2.6 ICP-MS/MS instrumentation

The experiments were done with an Agilent 8800 "triple-quadrupole" ICP-MS/MS system (Agilent Technologies, Tokyo, Japan). The instrument was equipped with a low internal volume PFA-LC nebulizer (Elemental Scientific, Omaha, NE, USA) fitted onto a Peltier-cooled Scott-type spray chamber and a torch with a 1.0 mm ID injector tube (Agilent Technologies, Tokyo, Japan). Cl was determined as $^{35}ClH_2^+$ in MS/MS mode with H_2 as a reaction gas in the cell, as recently published by Klencsár *et al* [26] (detailed in Chapter 2). The method was tuned for maximum sensitivity for $^{35}ClH_2^+$ for acetonitrile and methanol separately. O_2 (introduced as a mixture of 20% of O_2 in Ar) was admixed to the Ar gas flow (mass flow controller setting for optional gas: 20%) to abate the effect of the organic solvent introduced into the instrument, and maintain a stable plasma. Additionally, Pt sampling (with 15 mm Pt insert) and skimmer cones (Agilent Technologies, Japan) were employed instead of the standard Ni cones due the higher inertness of the former ones. A more detailed description of the ICP-MS/MS conditions is provided in Table 3.2.

Table 3.2: ICP-MS/MS instrument settings and data acquisition parameters

RF power	1570 W
Ar carrier gas flow rate	Methanol: 0.33 L min^{-1} Acetonitrile: 0.30 L min^{-1}
H_2 gas flow rate	3.5 mL min^{-1}
Optional gas (20% O_2 in Ar) mass flow controller setting	20%
Temperature of spray chamber	-1 ˚C
Monitored transitions / masses	Q1: *m/z* 35 (Cl^+) → Q2: *m*/*z* 37 (ClH_2^+)
Data collection mode	TRA (Transient signal mode)
Integration time	0.4 s for *m*/*z* = 37

3.2.7 Sample preparation for human plasma

As real-life metabolite profiling is typically carried out in blood plasma, blank human plasma (collected from healthy individuals, not subjected to any medical treatment, then pooled and stored at -20 °C until analysis) was applied as a matrix for method development and validation. A slight modification of the methodology recently described by Klencsár *et al* [26] (detailed in Chapter 2) was applied for sample preparation. Briefly, 1.0 mL of plasma was admixed with 3.0 mL of acetonitrile, then thoroughly vortexed to precipitate the plasma proteins. After centrifugation, the supernatant was removed and reduced in volume under N_2 stream back to

the original volume of the plasma (approx. 1.0 mL). Prior to injection onto the HPLC-ICP-MS/MS system, the residue was centrifuged and the supernatant was acidified by adding 70 µL of 88% formic acid (resulting in approx. 5.7% formic acid in the final sample) to avoid an adverse effect of the sample pH on the chromatographic performance.

For *online* sample pre-concentration purposes, the volume of plasma was doubled, thus 2 mL plasma was mixed with 6 mL acetonitrile for protein precipitation. Thereafter, the total volume of supernatant was evaporated to dryness under N_2 stream, then the residue was reconstituted in 2.0 mL MQ-water – acetonitrile 95:5 (v/v) mixture to have a better control on the exact organic content of the sample injected onto the HPLC-ICP-MS/MS system. The sample was similarly acidified by adding 140 µL of 88% formic acid before injection (resulting in 5.7% formic acid in the final sample).

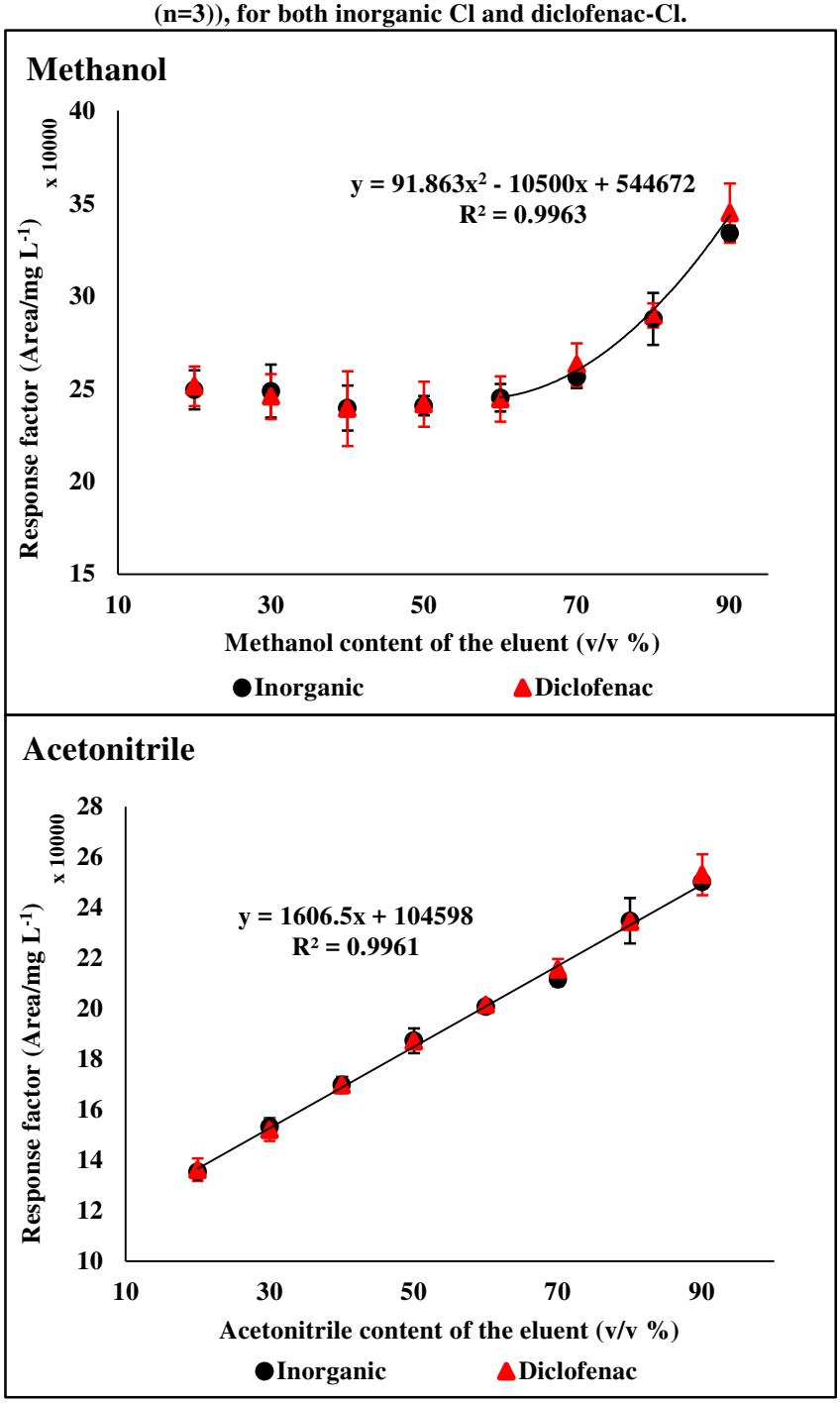
3.3 Results and discussion

3.3.1 Investigation of the effect of the organic solvent concentration on the ICP-MS/MS response for Cl

Although ICP-MS(/MS) is supposed to provide a response which is – for a given element – independent on the molecular structure in which the element is introduced, one of the main challenges of routine application of reversed phase HPLC-ICP-MS(/MS) is the lack of a generally applicable, simple and cost-effective calibration/quantification approach compensating for the effect of gradient elution on the response. Thus, the first step of this study was an exploration and characterization of the effect of both methanol and acetonitrile on the ICP-MS/MS sensitivity for Cl. As detailed previously in section 3.2.3, the ICP-MS/MS response (determined as the ratio of the peak area to the Cl concentration) was investigated for a 20-90 % (v/v) organic solvent content range and for both inorganic Cl and diclofenac-Cl. Two-way ANOVA at a 95% confidence level was applied in the statistical software JMP® (version 12.1.0, SAS Institute Inc., Cary, NC, USA) for the statistical evaluation of the effects of the two independent parameters, *i.e.* chemical form of Cl and organic concentration on the ICP-MS/MS response obtained for Cl. In the case of both methanol and acetonitrile, the response factor was independent of the chemical form, as expected for ICP-MS. However, in both cases, there was a strong dependence on the concentration of the organic solvent, as shown in Fig. 3.2 for both solvents and Cl-forms. Obviously, the dependence of the ICP-MS sensitivity on the eluent composition impedes straightforward quantification, unless clear trends can be established that allow to properly correct for this effect. For both methanol and acetonitrile, the

Cl response factor increases with the increasing organic content, which can be explained by the fact that the ICP-MS/MS settings were optimized for 100% organic solvent (as detailed in section 3.2.6). In the case of methanol, it can be seen from Fig. 3.2. and it was also confirmed by two-way ANOVA, that no statistically significant differences were observed among the response factors in a 20-60 % (v/v) methanol concentration range, while a second order function could be fitted to the values in the 60-90 % (v/v) methanol range. The R^2 for that function was >0.99, thus making its use for correction purposes promising. For acetonitrile, a much more clear and simple trend was observed, *i.e.* the response factor of Cl linearly depends on the acetonitrile content throughout the whole concentration range, with statistically significant differences among the different response factor values. It can be clearly seen, that both the achievable sensitivity (peak area of approx. 350,000 for 1 mg L^{-1} Cl in 90 % (v/v) methanol vs. 250,000 in 90 % (v/v) acetonitrile) and the trend of this sensitivity as a function of the solvent composition are different for methanol and acetonitrile. Although the origin of this different behaviour was not investigated in detail in the context of the present research project, it might be related with the viscosity of methanol – water and acetonitrile – water mixtures as a function of the solvent – water ratio. The viscosity of the sample has a strong impact on the sample transport efficiency of a traditional ICP sample introduction system, *i.e.* pneumatic nebulizer combined with a spray chamber. When plotting the viscosity of methanol – water mixtures as a function of their composition, a curve with a maximum is obtained (maximum value around 50 % (v/v)). In the case of acetonitrile – water mixtures, however, such a curve is rather linear in a broad concentration range (0-80 % (v/v) water in acetonitrile) [30]. Also the total vapour pressures of methanol – water and acetonitrile – water mixtures [31, 32] may be worthwhile to take into consideration, if a detailed investigation of this different behaviour is aimed at. It must be also noted that a clear and mathematically describable trend (function) does not necessarily automatically mean that this function can be applied to the correction of the change of the ICP-MS(/MS) response during a gradient elution due to the fact that this study has been performed by means of flow injection in isocratic conditions without the use of a chromatographic column, allowing longer equilibration times for the ICP-MS/MS instrument when changing between different organic solvent concentrations. Therefore, the employability of this type of correction must be investigated under conditions of an actual gradient elution.

Figure 3.2: Investigation of the effect of the organic solvent content of the eluent (for both methanol and acetonitrile) on the Cl response of ICP-MS/MS (indicated together with their 95% confidence intervals (n=3)), for both inorganic Cl and diclofenac-Cl.



3.3.2 Quantification of synthetically degraded diclofenac samples spiked with 4`-hydroxy-diclofenac: mass balance and accuracy study

Synthetically degraded diclofenac samples spiked with 4`-hydroxy-diclofenac (which is not formed during the degradation procedure described in section 3.2.4) at different concentration levels were applied to test the employability of the quantification approach based on the mathematical function between the ICP-MS/MS response for Cl and the eluent composition under actual gradient elution reversed phase HPLC-ICP-MS/MS conditions. As the total amount of diclofenac used and thus the total amount of Cl in the degraded samples was known, a mass balance study could be performed via analysis of these samples when using either methanol or acetonitrile with 0.1 % (v/v) formic acid as eluent B on the Waters XBridge BEH Phenyl column (3.0 x 150 mm; 3.5 µm) at a flow rate of 0.5 mL min^{-1} (see Table 3.1). Based on the column void time, the gradient delay time of the chromatographic system (determined by injecting 1 mg L^{-1} inorganic Cl and based on the manufacturer specification, respectively) and the retention times for the different degradation products, the solvent composition in which the compound is eluted and introduced into the ICP could be easily calculated for each peak. Thus, after the determination of the peak areas by integrating the chromatograms, the quantification of each peak could be realized based on the Cl-response factors calculated for the solvent compositions corresponding to the position of each peak using the mathematical function between the Cl response factor and eluent composition, deduced from a preliminary flow-injection experiment as described previously (sections 3.2.3 and 3.3.1).

Moreover, spiking the degraded diclofenac samples with the commercially available 4`-hydroxy-diclofenac standard, allowed for a comparison between the newly developed quantification approach (based on the equation describing the Cl-response as a function of the eluent composition) and a more traditional external calibration (using both diclofenac and 4`-hydroxy-diclofenac as standards for the quantification of 4`-hydroxy-diclofenac). For this purpose, the 4`-hydroxy-diclofenac was added to the degraded diclofenac samples at four concentration levels between 0.1 – 3.0 mg L^{-1} Cl and a standard solution containing 1.0 mg L^{-1} Cl as 4`-hydroxy-diclofenac plus 1.0 mg $L^{-1}$ Cl as diclofenac was applied as an external standard. As an indicator for accuracy, recovery values were calculated based on the experimentally obtained and real (spiked) concentration values. The recoveries obtained for 4`-hydroxy-diclofenac and the comparison of external calibration with the quantification based on the Cl response – eluent composition function are detailed in Table 3.3. It is clear that the accuracy for 4`-hydroxy-diclofenac determined via external calibration on the basis of the 4`-hydroxy-diclofenac peak (in the standard chromatogram) is good (recovery between 93-106 %)

at each concentration level for both solvents, thus these values were regarded as reference values at each level for comparison between the results obtained based on the Cl response – eluent composition function and external standard calibration based on the diclofenac peak. It can be clearly seen from the relative recovery values (calculated by comparing the results obtained based on the Cl response – eluent composition function and external standard calibration based on the diclofenac peak to the recovery values obtained with external calibration on the basis of the 4`-hydroxy-diclofenac peak), that a 6-8 % systematic bias is observed in the recovery values when diclofenac is used as an external standard for the determination of 4`-hydroxy-diclofenac. This bias can be considered substantial taking into account that the difference in eluent composition between the time points of 4`-hydroxy-diclofenac and diclofenac elution is only approx. 8% organic solvent (in case of both solvents). Or in other words, less than 10% difference in the eluent composition causes 6-8% bias, if traditional external calibration is applied for quantification based on one of the compounds. With quantification based on the function established between the Cl-response and eluent content (measured on the same day), this bias is decreased below 1.5%. This means that the mathematical function, derived from a flow injection experiment prior to the sample analysis is able to successfully correct for the biasing effect of the gradient elution. Owing to the excellent stability of the ICP-MS/MS instrument, no internal standard was needed for the correction of a sensitivity shift of the instrument.

Fig. 3.3 shows the overlaid chromatograms of (i) a blank, (ii) a standard solution (used for external calibration) and (iii) a degraded diclofenac sample (with 10 mg L^{-1} nominal total Cl concentration) spiked with 1.0 mg L^{-1} Cl as 4`-hydroxy-diclofenac using both acetonitrile and methanol with 0.1% (v/v) formic acid as eluent B. It can be clearly seen, that good chromatographic separation was achieved with both solvents, but the Cl-background obtained using methanol was much higher than that using acetonitrile, thus the obtainable signal-to-noise ratio is significantly better in acetonitrile, thus enabling the quantification of peaks corresponding to lower concentrations as well.

Table 3.3: Comparison of different quantification approaches for the determination of 4`-hydroxy-diclofenac in synthetically degraded diclofenac samples analysed using a Waters XBridge BEH Phenyl column (3.0 x 150 mm; 3.5 µm) with both methanol and acetonitrile as organic solvents

Accuracy using methanol as organic solvent					
	Recovery (%)			Relative recovery (%)*	
Concentration (mg L^{-1} Cl)	ESTD** (4`-hydroxy-diclofenac)	ESTD** (diclofenac)	Response vs. eluent composition function	ESTD** (diclofenac)	Response vs. eluent composition function
0.1	106.0	97.8	104.5	92.3	98.6
0.5	100.0	92.3	98.6		
1.0	101.8	93.9	100.4		
3.0	96.4	89.0	95.1		
Accuracy using acetonitrile as organic solvent					
	Recovery (%)			Relative recovery (%)*	
Concentration (mg L^{-1} Cl)	ESTD** (4`-hydroxy-diclofenac)	ESTD** (diclofenac)	Response vs. eluent composition function	ESTD** (diclofenac)	Response vs. eluent composition function
0.1	96.3	90.9	96.7	94.3	100.4
0.5	93.0	87.7	93.4		
1.0	94.8	89.4	95.2		
3.0	97.8	92.2	98.1		

*Recovery values relative to the reference recovery values obtained via ESTD calibration based on the standard peak of 4`-hydroxy-diclofenac

** External standard calibration based on either 4`-hydroxy-diclofenac or diclofenac

As mentioned above, also a mass balance was established for the degraded diclofenac samples on the basis of the quantification using the function of Cl response *versus* eluent composition, as detailed in Table 3.4. The total Cl content was determined by summing the Cl-concentrations corresponding to each peak above LOQ level (see next section). In the case of acetonitrile, the recovery was excellent (> 96 %), while with methanol a slightly worse recovery (84 %) was obtained. Nevertheless, also the latter value can be considered acceptable when taking into account the higher LOQ in methanol (as a result of the lower signal-to-background ratio), the complex and high portion of degradation and the general considerations on pharmaceutical forced degradation studies, according to which a mass balance of >80% may be considered acceptable, especially when a degradation of a large fraction of the API is observed [33-36].

Figure 3.3: Chlorine chromatograms for (i) a blank, (ii) a standard solution containing 1 mg L^{-1} Cl as 4`-hydroxy-diclofenac plus 1.0 mg $L^{-1}$ Cl as diclofenac and (iii) a solution of synthetically degraded diclofenac (nominal total Cl concentration = 10 mg $L^{-1}$) spiked with 1 mg $L^{-1}$ Cl as 4`-hydroxy-diclofenac using methanol (upper pane) and acetonitrile (lower pane) with 0.1 % (v/v) formic acid as eluent B separated on a Waters XBridge BEH Phenyl (3.0 x 150 mm; 3.5 µm) column
Peaks: 1: 4`-hydroxy-diclofenac; 2: diclofenac, rest of the peaks: degradation products with unknown chemical structure

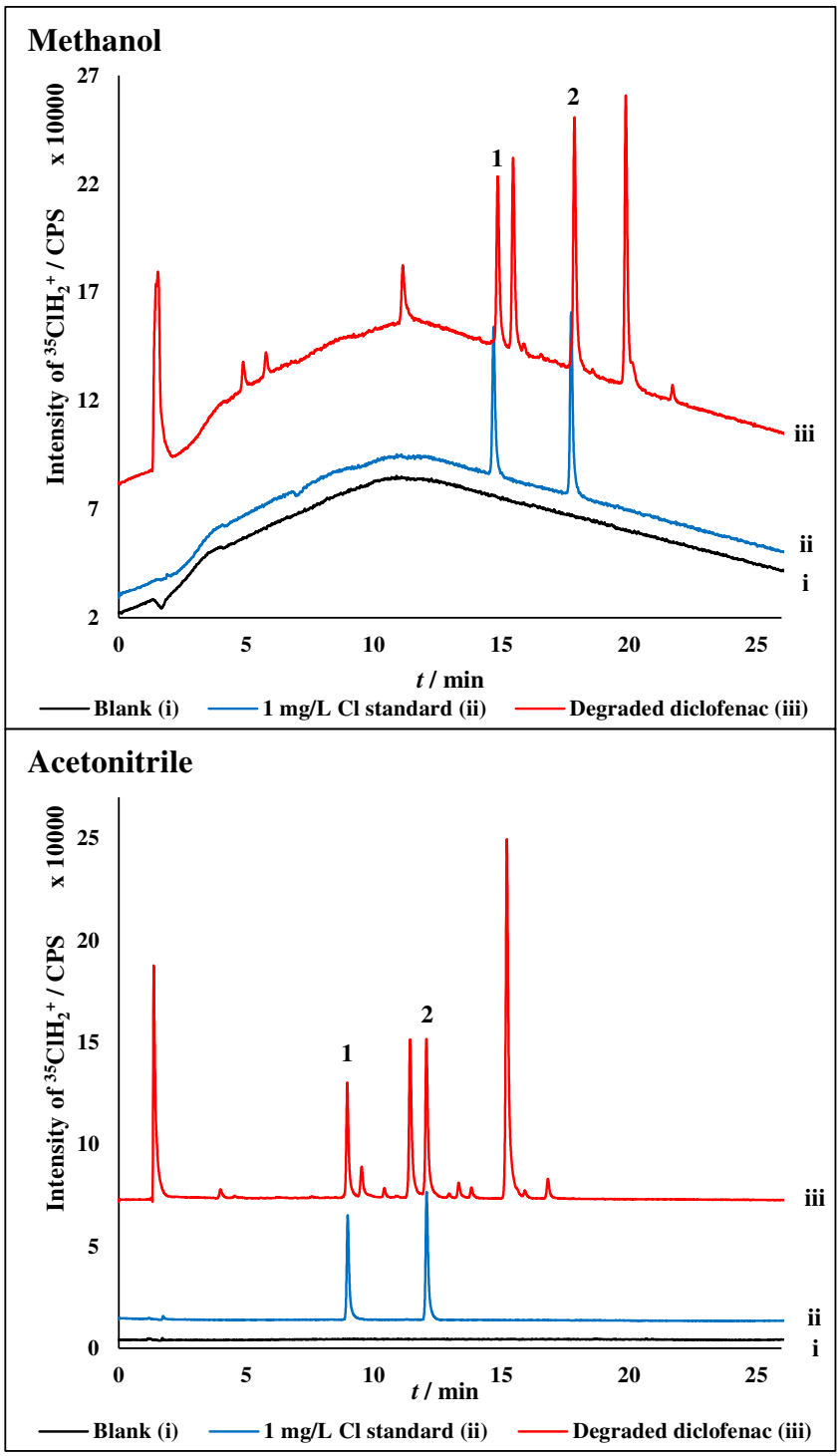


Table 3.4: Mass balance for synthetically degraded diclofenac for methanol- and acetonitrile-based chromatographic methods on a Waters XBridge BEH Phenyl column (3.0 x 150 mm; 3.5 µm)

Methanol			Acetonitrile		
t_R / min	v/v % of methanol*	Cl concentration (mg L^{-1})	t_R / min	v/v % of acetonitrile*	Cl concentration (mg L^{-1})
1.5	40	2.46	1.4	30	2.94
4.9	48	0.22	4.0	37	0.10
5.8	50	0.17	9.6	53	0.31
11.1	63	0.50	10.4	55	0.08
15.5	73	1.13	11.4	58	1.39
15.9	75	0.08	12.1	60	1.45
17.9	79	1.41	13.0	62	0.04
19.9	84	1.35	13.3	64	0.11
20.1	85	0.18	13.8	65	0.07
21.7	88	0.08	15.2	69	2.96
			15.9	71	0.06
			16.8	73	0.15
Total Cl content (mg L^{-1})		**7.58**	**Total Cl content (mg L^{-1})**		**9.68**
Recovery (%)		**84.1**	**Recovery (%)**		**96.6**

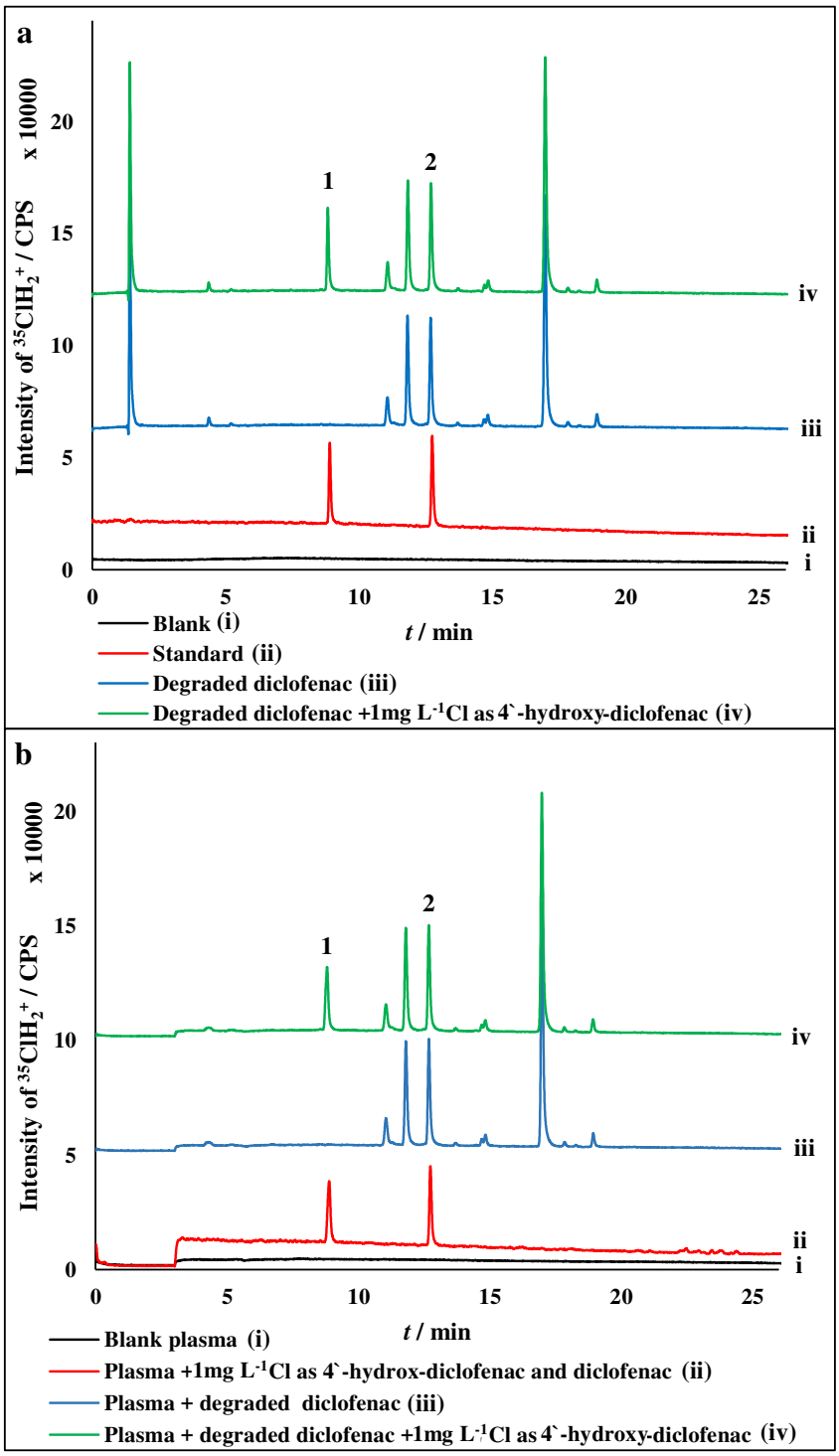
*The eluent composition in which the compound with the indicated retention time is eluted

3.3.3 Investigation of the matrix effect of human plasma and method validation

So far, the calibration approach compensating for the effect of gradient elution had been evaluated for synthetically degraded and spiked diclofenac samples only. In a next step, this quantification approach was also tested and validated in the presence of human plasma, one of the main matrices typically to be analysed in drug metabolite profiling studies. Based on the experience obtained for the synthetically degraded diclofenac samples, only acetonitrile with 0.1% (v/v) formic acid was further used as eluent B due to its significantly lower Cl-background compared to methanol. When applying a Waters XBridge BEH Phenyl column (3.0 x 150 mm; 3.5 µm), an unsatisfactory peak shape (USP tailing factor > 2.0) was obtained for 4`-hydroxy-diclofenac in the presence of human plasma. Therefore, a Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm) column was further used with a flow rate of 1.0 mL min^{-1} and with the same gradient programme (see Table 3.1). The instructions of the ICH Q2(R1) guideline on analytical validation (step 4 version, November 2005) were respected throughout the whole validation. Selectivity was studied by comparing the chromatograms of (i) a blank, (ii) a standard solution containing 1.0 mg L^{-1} Cl as 4`-hydroxy-diclofenac plus 1.0 mg $L^{-1}$ Cl as diclofenac, (iii) a synthetically degraded diclofenac sample with a nominal total Cl content of 10 mg $L^{-1}$, (iv) a synthetically degraded diclofenac sample (with a nominal total Cl content of 10 mg $L^{-1}$) spiked with 1.0 mg $L^{-1}$ Cl as 4`-hydroxy-diclofenac in the absence (a) and presence (b) of human

plasma, as also shown in Fig. 3.4. It can be seen that no interfering peaks were observed in blank human plasma at the positions of diclofenac and its related compounds.

Figure 3.4: chlorine chromatograms for (i) a blank , (ii) 1 mg L^{-1} Cl as 4`-hydroxy-diclofenac plus 1.0 mg $L^{-1}$ Cl as diclofenac, (iii) 10 mg $L^{-1}$ Cl as synthetically degraded diclofenac and (iv) 10 mg $L^{-1}$ Cl as synthetically degraded diclofenac spiked with 1 mg $L^{-1}$ Cl as 4`-hydroxy-diclofenac with (b) and without (a) the presence of human plasma matrix separated on a Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm) column using 0.1 % (v/v) formic acid in acetonitrile as eluent B
Peaks: 1: 4`-hydroxy-diclofenac; 2: diclofenac, rest of the peaks: degradation products with unknown chemical structure



Accuracy and precision were investigated by spiking blank human plasma with 4`-hydroxy-diclofenac and diclofenac at three concentration levels (between 0.5 – 3.0 mg L^{-1} Cl) with three replicates at each level. Recoveries for both compounds were determined in the same way as detailed in the previous section using the novel quantification approach. As for the precision, both intra-day (repeatability) and inter-day precision were investigated and expressed as the RSD% of the three results obtained at each level. The function between the Cl response and eluent composition was determined in the same way as previously described (section 3.2.3), but a 1.0 mL min^{-1} flow rate was obviously applied in this case due to modification of the chromatographic conditions. Taking the requirements typically established towards the analytical methods applied for metabolite profiling studies into account, a recovery between 80-120% and a precision below 15 RSD% can be considered sufficient. The results are summarized in Table 3.5. Excellent accuracy and precision was obtained with recovery values between 90-100% and RSD% values below 4% at each level for both compounds.

Table 3.5: Accuracy and precision of the results for 4`-hydroxy-diclofenac and diclofenac in the presence of human plasma matrix using a Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm) column (*n* = 3)

Cl concentration as 4`-hydroxy-diclofenac and diclofenac (mg L^{-1})	Recovery (%)		Intra-day precision (RSD%)		Inter-day precision (RSD%)	
	4`-hydroxy-diclofenac	Diclofenac	4`-hydroxy-diclofenac	Diclofenac	4`-hydroxy-diclofenac	Diclofenac
0.5	92.4	97.5	2.7	3.2	3.1	2.5
1.0	95.0	97.2	1.8	1.8	1.9	2.3
3.0	91.9	91.8	0.2	0.3	0.3	0.6

In addition to the accuracy and precision for 4`-hydroxy-diclofenac and diclofenac, blank human plasma was also spiked with synthetically degraded diclofenac (at a nominal total Cl concentration of 10 mg L^{-1}) and a mass balance was also established in the presence of the human plasma matrix. The results have been summarized in Table 3.6. It can be seen that, similarly to the previous mass balance study using acetonitrile (Table 3.4), the recovery for the total Cl content is excellent, both in the absence and presence of a human plasma matrix (93 and 92%, respectively). This is further confirmed by comparing the results with and without the presence of plasma for each peak separately. The relative difference observed was < 12% in each case, with higher values closer to the LOQ level. Therefore, it can be concluded that the human plasma matrix has no adverse effect on the quantification approach developed.

As a basic validation parameter, also the linearity of the method was tested by injecting diclofenac standard solutions at five concentration levels between 0.05 – 5.0 mg L^{-1} Cl. Excellent linearity was found with an $R^2 > 0.99$ and a 95% confidence interval of the intercept

including *origo*. In agreement with the ICH Q2(R1) guideline, a more detailed statistical evaluation based on ANOVA was also carried out (presented in the Appendix to Chapter 3) to assess the applicability of a linear model for the relationship between the peak area and the concentration. The limit of quantification (LOQ) was determined for both the Waters XBridge BEH Phenyl and the C18 column (at flow rates of 0.5 and 1.0 mL min^{-1}, respectively) following the Signal-to-Noise (S/N) approach detailed in the ICH Q2(R1) guideline (Part II, section 7.2). The LOQ was given as the concentration corresponding to an S/N ≈ 10, where S/N was determined according to USP <621>. The LOQ values were 0.03 mg L^{-1} Cl as diclofenac on the Waters XBridge BEH Phenyl column and 0.05 mg L^{-1} Cl as diclofenac on the Waters XBridge BEH C18 column.

As a conclusion of the validation work, it can be stated that a sufficiently sensitive, accurate and precise methodology has been developed for the quantification of diclofenac and its related compounds in human plasma by using RP-HPLC-ICP-MS/MS with gradient elution. It should be noted however, that the novel quantification approach relies on a response vs eluent composition function established at the beginning of the analysis, therefore an excellent instrument stability is an essential pre-requisite for the applicability of the present strategy and should be carefully validated taking the chromatographic run time and the amount of samples into account in case of a real-life application (*e.g.,* analysis of samples from a clinical study).

Table 3.6: Comparison of mass balance studies performed in the absence and presence of human plasma using a Waters XBridge BEH C18 (4.6 x 150 mm; 3.5 µm) column

t_R / min	v/v % of acetonitrile*	Cl concentration without plasma (mg L^{-1})	Cl concentration with plasma (mg L^{-1})	Relative difference (%)**
1.4	30	2.10	2.10	0.0
4.4	38	0.10	0.10	-2.3
11.1	57	0.43	0.44	3.3
11.8	59	1.29	1.30	1.5
12.7	62	1.25	1.27	1.0
14.7	67	0.07	0.07	-9.3
14.8	68	0.15	0.14	-5.6
17.0	74	2.89	2.94	1.5
17.8	76	0.05	0.06	12.3
18.9	79	0.15	0.16	0.2
Total Cl content (mg L^{-1})		**8.49**	**8.57**	
Recovery (%)		**92.2**	**93.1**	

*The eluent composition in which the compound with the indicated retention time is eluted
**Compared to the values obtained in the absence of human plasma matrix

3.3.4 Improvement of LOQ via online sample pre-concentration

Due to the relatively poor ICP-MS/MS sensitivity obtainable for Cl compared to that for metallic elements and even some other non-metals [11, 26, 37], an improvement in the LOQ for Cl with reversed phase HPLC-ICP-MS/MS is highly desirable to be able to provide a sterling alternative for the metabolite profiling of low-dose drugs as well. A simple and convenient sample pre-concentration procedure, not requiring any additional sample pre-treatment, was developed by trapping the drug-related compounds of human plasma on a Waters XBridge BEH C18 (4.6 x 20 mm; 3.5 µm) intelligent speed column prior to the analytical separation and ICP-MS/MS detection, as shown in Fig. 3.1. A gradient trapping step was applied in the first 5 minutes of the chromatographic programme (see Table 3.1) to minimize peak broadening and keeping the target compounds focused on the trapping column, while the interfering matrix components (including inorganic Cl) are washed from the column and directed into the waste. A supporting flow was maintained on the analytical column while the drug-related compounds were being trapped to keep it equilibrated and avoid a pressure shock which could occur when changing from trapping to analysis position without a supplementary flow. Due to the large injection volume, the risk for a possibly adverse chromatographic effect deriving from the non-optimal solvent composition of the sample is significantly higher than in the case of general chromatographic methods with a typical injection volume in the range of 2-100 µL. Therefore, special attention needs to be paid to control both the pH and organic composition of the sample injected into such system. In this specific case, the sample preparation was slightly modified (see section 3.2.7) by evaporating the supernatant collected after protein precipitation, to dryness followed by reconstitution in MQ-water – acetonitrile 95:5 (v/v) mixture. The sample was then acidified with 88% formic acid to ensure a suitable pH, matching with the eluent system. The comparison of the HPLC-ICP-MS/MS chromatograms obtained with 50 µL injection (without sample pre-concentration) and 1500 µL injection (with sample pre-concentration) for standard solutions and spiked human plasma are shown in Fig. 3.5. It can be clearly seen that the sample trapping and the increment of the injection volume from 50 to 1500 µL did not induce a significant peak broadening or impairment of the peak shape (typical USP tailing factor: 1.6 for both peaks in both cases). This observations holds with and without the presence of plasma matrix and for both 4`-hydroxy-diclofenac and diclofenac.

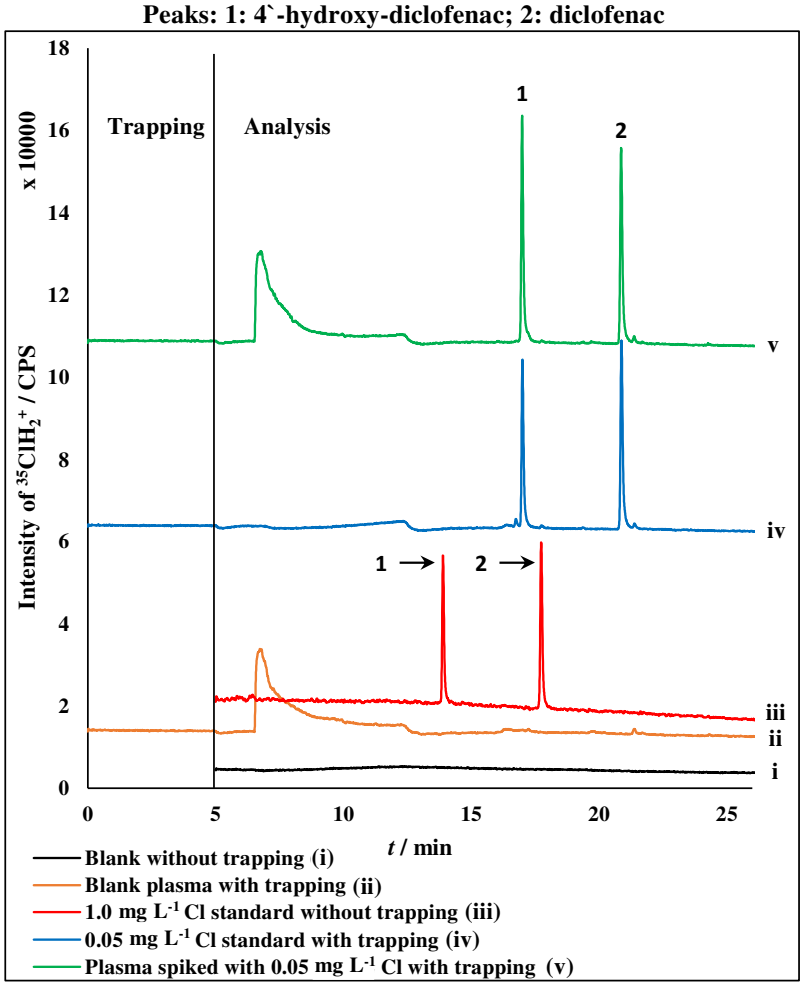
As a first step of the method validation, blank human plasma was spiked with 0.5 mg L^{-1} Cl as 4`-hydroxy-diclofenac plus 0.5 mg L^{-1} Cl as diclofenac to investigate the sample uptake capability of the trapping column. 100; 500; 1000 and 1500 µL of this sample was injected and

the peak areas were determined for both 4`-hydroxy-diclofenac and diclofenac. The peak area of both compounds increased linearly with the injection volume, thus the maximum value (*i.e.* 1500 µL) could be applied as an injection volume. For validation of the method, also the LOQ, linearity and accuracy were characterized. The LOQ was determined in the same way as detailed in the previous section, and was found to be 0.002 mg L^{-1} Cl as diclofenac, which is a 25-fold improvement compared to the methodology without sample pre-concentration. The linearity was investigated by injecting diclofenac standard solutions at five concentration levels between 0.005 – 0.06 mg L^{-1} Cl and found to be excellent ($R^2 > 0.99$ with a 95% confidence interval of intercept including *origo* (see a more detailed statistical evaluation in the Appendix to Chapter 3). The accuracy was investigated by spiking blank human plasma with 4`-hydroxy-diclofenac and diclofenac at three concentration levels between 0.005 – 0.05 mg L^{-1} Cl, as detailed in Table 3.7. Excellent recovery values between 94-98% were obtained for both compounds and at each level (considered acceptable between 80-120%). As for quantification, the same methodology as described before (*i.e.* the injection of 50 µL of 5.0 mg L^{-1} Cl as diclofenac standard solution) was used to determine the Cl response as a function of the eluent composition. The response factor was then converted to 1500 µL injection volume and used as a basis for quantification.

Table 3.7: Recoveries obtained for 4`-hydroxy-diclofenac and diclofenac in the presence of human plasma matrix, when using a simple sample pre-concentration procedure.

Cl concentration as 4`-hydroxy-diclofenac and diclofenac (µg L^{-1})	Recovery (%)	
	4`-hydroxy-diclofenac	Diclofenac
5	95.7	96.7
30	97.8	95.7
50	97.4	93.9

Figure 3.5: chlorine chromatograms for (i) a blank without trapping (50 µL injection); (ii) blank human plasma with trapping (1500 µL injection); (iii) 1.0 mg L^{-1} Cl standard as 4`-hydroxy-diclofenac plus 1.0 mg $L^{-1}$ Cl as diclofenac without trapping (50 µL injection); (iv) 0.05 mg $L^{-1}$ Cl standard as 4`-hydroxy-diclofenac plus 0.05 mg L^{-1} Cl as diclofenac with trapping (1500 µL injection); (v) human plasma spiked with approx. 0.05 mg L^{-1} Cl as 4`-hydroxy-diclofenac plus 0.05 mg L^{-1} Cl as diclofenac with trapping (1500 µL injection)
Peaks: 1: 4`-hydroxy-diclofenac; 2: diclofenac



3.4 Conclusions

A simple and straightforward quantification approach was developed for the determination of diclofenac and its related compounds including its major metabolite, 4`-hydroxy-diclofenac, based on Cl detection using reversed phase HPLC-ICP-MS/MS. It was proved that using a simple and fast flow injection experiment prior to the sample analysis, a mathematical function describing the relationship between the ICP-MS/MS response for Cl and the eluent composition can be found for both methanol and acetonitrile, the most commonly used organic solvents in reversed phase HPLC. Using synthetically degraded diclofenac spiked with 4`-hydroxy-diclofenac as a model mixture, it was also demonstrated that this mathematical function can be successfully applied to quantitative analysis of samples of Cl-containing compounds, by means

of HPLC-ICP-MS/MS. Excellent accuracy was observed for 4`-hydroxy-diclofenac, with recoveries between 93-105 % at the different concentration levels investigated in each solvent applied as eluent B during the chromatographic separation. A mass balance for the total Cl-content was also demonstrated using both methanol and acetonitrile. As real-life metabolite profiling must be performed in different biological matrices, human plasma was applied as a model matrix during analytical validation. Excellent accuracy (recoveries between 90-100 %) and precision (<4 RSD%) was found for both diclofenac and 4`-hydroxy-diclofenac in human plasma.

In summary, the applicability of the novel quantification approach developed was successfully tested and validated for the two most commonly used solvents (methanol and acetonitrile) applied in reversed phase HPLC at two different flow rates (0.5 and 1.0 mL min^{-1}).

Due to the limitations related to the ICP-MS/MS determination of Cl, also a simple and cost-effective *online* sample pre-concentration procedure without the need of extra steps during the sample preparation was developed by trapping the drug-related compounds from a larger volume of human plasma to improve the attainable LOQ. Owing to the excellent peak shape and minimal peak broadening when applying 1500 µL injection volume instead of 50 µL, a 25-fold reduction of LOQ was obtained achieving 0.002 mg L^{-1} Cl as diclofenac instead of 0.05 mg L^{-1}. This broadens the application range also to metabolite profiling of low-dose Cl-containing pharmaceutical drugs. As an overall conclusion, it can be stated that a simple and cost-effective novel quantification approach was developed for the speciation of Cl-containing compounds with gradient reversed phase HPLC-ICP-MS/MS, which is not significantly more labour-intensive or time-consuming than a simple external- or internal standard calibration typically applied in HPLC analysis, while it eliminates the disadvantages of the quantification approaches (isotope dilution, compensation gradient) suggested so far in gradient reversed phase HPLC-ICP-MS(/MS) analysis.

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Appendix to Chapter 3

The statistical evaluation of the linearity studies performed for Cl during the validation of the HPLC-ICP-MS/MS method (detailed in Section 3.3.3 without sample pre-concentration and in Section 3.3.4 with sample pre-concentration) was carried out with ANOVA at $\alpha = 0.05$ significance level using MS Excel (version 16.0.10228.20080 32-bit) following an identical approach as detailed in the Appendix to Chapter 2. Similarly to Chapter 2, the results presented in Table A-3.1 and A-3.2, without- and with sample pre-concentration, respectively, clearly show that a linear model for the HPLC-ICP-MS/MS response (*i.e.* peak area) as a function of analyte concentration can be accepted at $\alpha = 0.05$ significance level.

Table A-3.1: Statistical evaluation of the linearity study for Cl in the concentration range of 0.05 – 5.00 mg L^{-1} at 5 concentration levels with 1 replicate at each level without sample pre-concentration

Regression Statistics						
Multiple R	0.9999					
R Square	0.9999					
Adjusted R Square	0.9998					
Standard Error	2871					
Observations	5					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	1	1.85E+11	1.85E+11	2.24E+04	6.56E-07	
Residual	3	2.47E+07	8.24E+06			
Total	4	1.85E+11				
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	293	1785	0.16	0.88	-5388	5974
Slope	100924	674	149.79	6.56E-07	98780	103068

Table A-3.2: Statistical evaluation of the linearity study for Cl in the concentration range of 0.005 – 0.06 mg L^{-1} at 5 concentration levels with 1 replicate at each level with sample pre-concentration

Regression Statistics						
Multiple R	1.0000					
R Square	0.9999					
Adjusted R Square	0.9999					
Standard Error	538					
Observations	5					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	1	1.05E+10	1.05E+10	3.62E+04	3.20E-07	
Residual	3	8.69E+05	2.90E+05			
Total	4	1.05E+10				
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-394	400	-0.99	0.40	-1666	878
Slope	2246957	11812	190.23	3.20E-07	2209366	2284547

CHAPTER 4

Comparative evaluation of ICP sample introduction systems to be used in the metabolite profiling of chlorine-containing pharmaceuticals via HPLC-ICP-MS

Adapted from:

B. Klencsár, C. Sánchez, L. Balcaen, J. Todolí, F. Lynen and F. Vanhaecke, Comparative evaluation of ICP sample introduction systems to be used in the metabolite profiling of chlorine-containing pharmaceuticals via HPLC-ICP-MS, *Journal of Pharmaceutical and Biomedical Analysis*, **153** (2018) 135-144.

4.1 Introduction

Inductively coupled plasma - (tandem) mass spectrometry (ICP-MS(/MS)) is a well-known analytical technique for quantitative trace elemental analysis. Coupled with an adequate separation technique [1, 2], *e.g.,* (ultra)high-performance liquid chromatography ((U)HPLC) [3-8], gas chromatography (GC) [9, 10], capillary electrophoresis (CE) [11-13], ICP-MS(/MS) is also deployed in speciation studies, as a sensitive and element-specific detector. Due to its – at first sight – straightforward compatibility with (U)HPLC, (U)HPLC-ICP-MS(/MS) also shows great potential in drug metabolite profiling, as has recently been reviewed by Klencsár *et al* [2]. For a detectable hetero-element, ICP-MS(/MS) provides an analytical response that is independent of the chemical structure of the molecule in which the element is present, thus rendering the technique suitable for quantitative analysis even without access to individual standards for each analyte of interest. This is an important advantage over other MS-based detection techniques, such as electrospray ionization-MS (ESI-MS). As ICP-MS is originally designed for the analysis of aqueous samples, several challenges accompany the hyphenation of reversed phase (RP) HPLC and ICP-MS, such as the handling of organic solvents and quantification issues owing to the use of gradient elution. Carbon depositions on the cones and torch can be avoided and stable plasma conditions can be maintained *via* the introduction of O_2 into the plasma when organic solvents are analysed. As with gradient elution, the matrix composition of the mobile phase is continuously changing, its use brings about continuously changing plasma conditions and thus, a continuously varying sensitivity for the target element throughout the chromatographic run. Of course, such changes in sensitivity must be avoided or need to be properly corrected for [2, 6, 8, 14]. Strategies addressing this problem include quantification using online species-unspecific isotope dilution [6, 14, 15], the use of a compensation gradient [16-18] or mathematical correction [8, 19, 20]. The difficulties caused by spectral interferences, which are particularly pronounced in the case of light hetero-elements (*e.g.,* S, Cl, P) typically present in pharmaceuticals [2, 21], cannot be overlooked either. Spectral interferences can be overcome by the application of higher mass resolution (HR) in sector field ICP-MS instruments [22] or by using kinetic energy discrimination (KED) and/or chemical resolution in the collision/reaction cell of quadrupole-based ICP-MS units [23, 24]. The introduction of tandem ICP-MS instrumentation has rendered the latter approach much more powerful.

However, besides the capabilities of HPLC-ICP-MS(/MS) for sensitive detection and accurate quantification, demonstrated in multiple works already [3-8], also the chromatographic characteristics of these methods are of the utmost importance. Therefore, especially in the

context of pharmaceutical analysis, where GMP validation is needed and strictly regulated by the Authorities, the chromatographic characteristics must be paid adequate attention to. It is, *e.g.*, self-evident, that additional dead volumes should be minimized in the HPLC-ICP-MS coupling to avoid peak broadening to the highest possible extent, rendering micro-flow LC nebulizers with lower ID preferable over the traditional nebulizers in this context. Also the volume and geometry of the spray chamber can have a substantial impact on the peak broadening and peak shape, and thus indirectly on the accuracy, limit of detection (LOD) and limit of quantification (LOQ) achievable. An appropriate temperature control of the spray chamber (if control is possible) can also have an important effect on the figures of merit.

Taking the strict Authority requirements for the different system suitability parameters (*e.g.,* USP tailing factor should be ≤ 2 according to U.S. FDA [25]) and the fact that the chromatographic performance indicators also fundamentally determine the suitability and validatability of an HPLC-ICP-MS method into account, a systematic comparative study of different ICP sample introduction systems was carried through. The most critical chromatographic parameters were characterized using diclofenac and its major metabolite, 4`-hydroxy-diclofenac, as model compounds.

A Scott-type double-pass spray chamber, a cyclonic spray chamber, a POINT® sample introduction kit, recently introduced by Meinhard for the analysis of highly volatile organic solvents, and a total sample consumption system called High-Temperature Torch-Integrated Sample Introduction System (hTISIS) developed by Todolí *et al* [26-28] were systematically evaluated for their use in HPLC-ICP-MS/MS using different gradient conditions. The figures of merit thus obtained were compared to the corresponding data of the HPLC-UV chromatograms, which were regarded as points of reference. The target compounds were monitored based on the Cl-atom they contain, using a state-of-art ICP-MS/MS system, as recently published by Klencsár *et al* [7, 8] (detailed in Chapters 2 and 3).

4.2 Experimental

4.2.1 Materials, stock and standard solutions

Diclofenac sodium (pharmaceutical secondary standard with a purity of 99.9%), 4`-hydroxy-diclofenac (analytical standard with a purity of ≥99.0%) and formic acid (purity ≥88.0%, *Trace*SELECT®) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (LC-MS grade) was purchased from VWR International (Leuven, Belgium). Ultra-pure grade water (resistivity ≥ 18.2 MΩ.cm) was obtained from a Millipore Direct-Q water purification system (MQ-water, Millipore, Billerica, MA, USA). Ammonium chloride (1000 ± 2 mg L^{-1} for Cl)

elemental stock solution applied for the optimization of the ICP-MS method was purchased from Inorganic Ventures (Christiansburg, VA, USA).

For the optimization of the ICP-MS/MS method, 1 mg L^{-1} Cl standard solution was prepared by dilution of ammonium chloride elemental stock solution with acetonitrile. Stock solution of diclofenac sodium was prepared by dissolving 21.1 mg of diclofenac sodium in 10 mL MQ-water and subsequently stored in the fridge at 2-8 °C. Stock solution of 4`-hyrdoxy-diclofenac was prepared by dissolving 1.15 mg of 4`-hydroxy-diclofenac in 500 µL acetonitrile and stored in the freezer at -20 °C. The standard solution containing the model compounds and used for the evaluation of different HPLC-ICP-MS/MS strategies was prepared by appropriate dilution of diclofenac and 4`-hydroxy-diclofenac stock solutions with acetonitrile – MQ-water 3-7 (v/v) mixture to obtain concentrations of 5.0 mg $L^{-1}$ Cl as diclofenac plus 5.0 mg $L^{-1}$ Cl as 4`-hydroxy-diclofenac.

4.2.2 ICP-MS/MS instrumentation

An Agilent 8800 "triple-quadrupole" ICP-MS/MS system (Agilent Technologies, Tokyo, Japan) was applied for the Cl-selective detection of the target compounds as $^{35}ClH_2^+$ in MS/MS mode with H_2 as a reaction gas in the octopole collision/reaction cell, as recently published by Klencsár *et al* [7]. The method was tuned for maximum sensitivity for $^{35}ClH_2^+$ separately for the different sample introduction systems using a 1 mg L^{-1} Cl standard solution in acetonitrile, as mentioned above.

The instrument was equipped with a torch with a 1.0 mm ID injector tube (Agilent Technologies, Tokyo, Japan). Four different sample introduction systems were tested for HPLC-ICP-MS/MS purposes. A low internal volume PFA-LC nebulizer (Elemental Scientific, Omaha, NE, USA) was combined with (i) a Peltier-cooled Scott-type spray chamber (Agilent Technologies, Tokyo, Japan), (ii) a PC^3 Peltier-cooled cyclonic spray chamber (Elemental Scientific, Omaha, NE, USA) and (iii) a High-Temperature Torch-Integrated Sample Introduction System (hTISIS), developed by Todolí *et al* [28-30]. The 9 mL single-pass spray chamber of hTISIS typically works at a higher temperature, depending on the solvent from room temperature to 400 ºC, to ensure quantitative sample transport into the plasma. Therefore, only low sample flow rates can be used to avoid overloading of the ICP. The hTISIS was operated in continuous sample aspiration mode and the spray chamber was heated to 150 °C during the analysis.

Additionally, also a (iv) POINT® sample introduction kit (Meinhard, Golden, CO, USA) was tested. The POINT® kit is a miniaturized sample introduction system designed for the analysis

of highly volatile organic solvents at low flow rates, typically < 200 µL min^{-1}. It consists of a Meinhard High Efficiency Nebulizer (HEN-90-A0.2) and a quartz dual-stage micro spray chamber. Unlike the aforementioned spray chambers, it does not have an active cooling system with the possibility of temperature control, but instead, the self-cooling of the micro spray chamber owing to the endothermic evaporation of the organic solvents during the aerosol formation is utilized. This makes the system considerably simpler and more cost-effective than the Peltier-cooled spray chambers, while similarly decreasing the organic load of the plasma owing to the "spontaneous" cooling. However, it must be also noted, that the temperature of the micro spray chamber and aerosol formation strongly depends on the solvent composition introduced, which also has a direct effect on the material transport efficiency.

A detailed description of the optimal ICP-MS/MS conditions with each sample introduction system is provided in Table 4.1.

The effect of spray chamber temperature was studied by comparing the figures of merit obtained at -1, 10 and 20 °C and without temperature control for the Peltier-cooled Scott-type spray chamber. The PC^3 Peltier-cooled cyclonic spray chamber could only be tested at -1 °C and without temperature control due to the limited temperature setting possibilities.

O_2 (introduced as an optional gas and consisting of 20% of O_2 in Ar) was admixed to the Ar plasma gas flow to deal with the organic solvent introduced into plasma. Due to the more corrosive nature of an O_2-containing plasma, Pt sampling (with 15 mm Pt insert) and skimmer cones (Agilent Technologies, Japan) were employed instead of the standard Ni cones.

4.2.3 HPLC conditions

An Agilent 1260 Infinity HPLC system (Agilent Technologies, Waldbronn, Germany), equipped with an Agilent 1260 Infinity vacuum degasser, an Agilent 1260 Infinity binary pump, an Agilent 1260 Infinity autosampler, an Agilent 1290 Infinity thermostated column compartment and an Agilent 1260 Infinity multiple wavelength UV-detector was used. The chromatographic separation was carried out on Waters XBridge BEH C18 columns (Waters Corp. Milford, MA, USA) filled with a 3.5 µm particle size stationary phase using two different column dimensions: 4.6 x 150 mm and 1.0 x 150 mm. Taking the acidic character of the target analytes into account, 0.5 % (v/v) formic acid in MQ-water and 0.5 % (v/v) formic acid in acetonitrile were employed as eluent A and B, respectively. For the chromatographic column with an ID of 4.6 mm, the mobile phase flow rate was set at 1.0 mL min^{-1} and it was used in combination with the Peltier-cooled Scott-type spray chamber and the PC^3 Peltier-cooled cyclonic spray chamber. For the column with an ID of 1.0 mm, the mobile phase flow rate was

set at 0.1 mL min^{-1} and this column was used for the evaluation of the POINT® and the hTISIS sample introduction systems. Additionally, it was also used in combination with the Peltier-cooled Scott-type spray chamber at -1 °C for the sake of comparison. Five gradient programmes with the same starting and final eluent compositions, but with different gradient slopes were tested for each condition: 70 → 0% eluent A (30 → 100% eluent B): 0 – 10/15/20/25/30 min. The injection volumes were 1 and 21 µL for the columns with 1.0 and 4.6 mm ID, respectively. The chromatographic separation was carried out at room temperature. A detailed description of the different conditions is presented in Table 4.1.

Table 4.1: Optimal HPLC-ICP-MS/MS conditions tested with the different sample introduction systems

Sample introduction system	**PFA-LC nebulizer + Scott-type SC***	**PFA-LC nebulizer + Cyclonic SC* (PC^3 inlet)**	**POINT®**	**PFA-LC nebulizer + hTISIS**
Temperature setting	-1 °C		UCT**	150 °C
Carrier gas flow rate	0.30 L min^{-1}	0.28 L min^{-1}	0.30 L min^{-1}	
Optional gas flow (mass flow controller setting)	20%			32%
H_2 gas flow rate	3.5 mL min^{-1}			
RF power	1570 W			
Monitored transitions/masses	Q1, *m/z* 35 (Cl^+) → Q2, *m/z* 37 (ClH_2^+)			
Data collection mode	TRA (Transient Signal Mode)			
Integration time	0.4 s			
Column ID	1.0 mm	4.6 mm	1.0 mm	
Flow rate	0.1 mL min^{-1}	1.0 mL min^{-1}	0.1 mL min^{-1}	
Injection volume	1 µL	21 µL	1 µL	

*SC = Spray Chamber

** UCT = Uncontrolled Temperature

Besides the comparison of the different ICP-MS sample introduction systems to one another (at the two flow rates separately), also HPLC-UV chromatograms at a wavelength of 278 nm were recorded at both flow rates (and using both column types) using each gradient elution program to obtain a "point of reference" for each critical chromatographic parameter investigated. The baseline peak width (w_b), USP tailing factor (A_s), and USP signal-to-noise ratio (USP S/N) were determined according to USP <621> [31] and the peak capacity (P) was calculated according to Eq. 4.1. [32, 33], as key performance indicators.

$$\text{Eq. 4.1: } P = 1 + {}^{t_g}/_{\overline{w_b}},$$

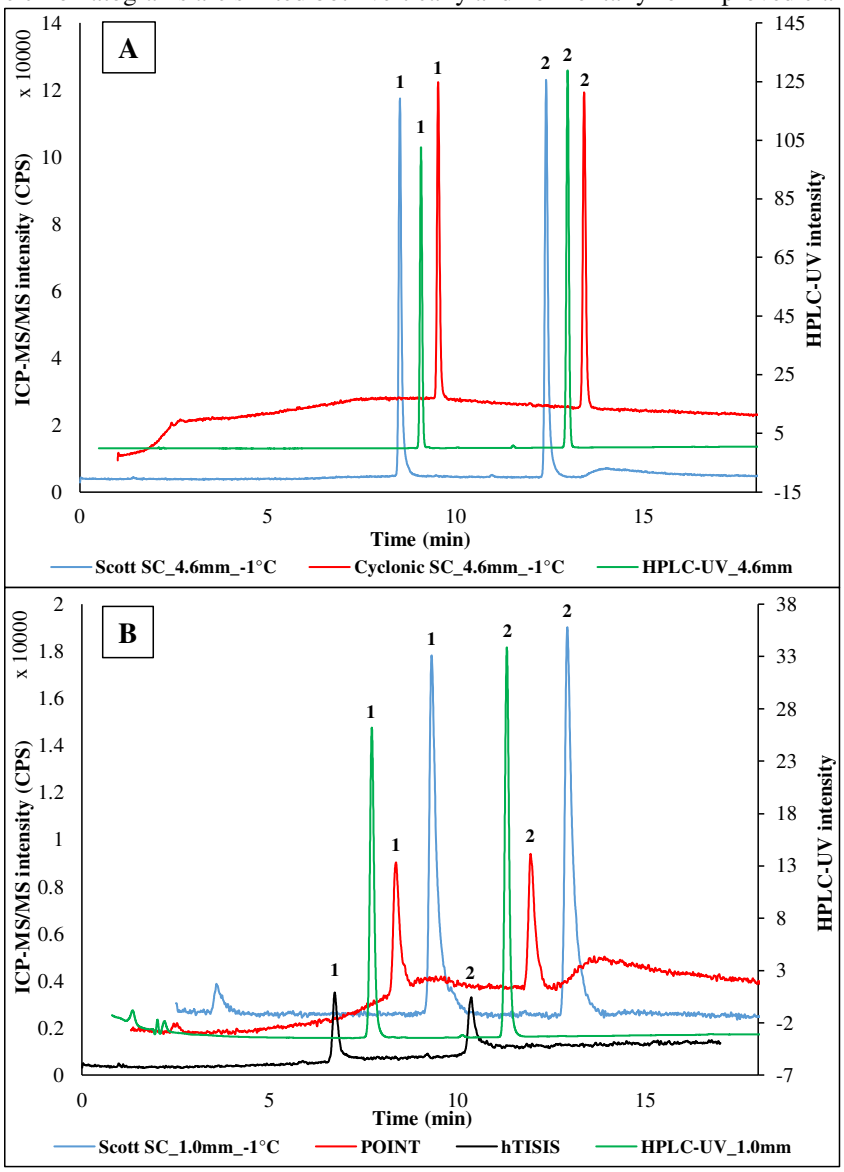
where t_g is the gradient time (min) and $\overline{w_b}$ is the average baseline peak width (min)

The generally applied “tangent method” was followed for the determination of w_b, *i.e.* w_b was determined by drawing tangent lines onto the front and back slopes of the chromatographic peak and the baseline peak width is defined as the distance between the intersections of the tangent lines with the baseline.

4.3 Results and discussion

Representative chromatograms obtained with the different sample introduction systems using a gradient length of 25 min are shown in Fig.4.1A and B for 1.0 and 0.1 mL min^{-1}, respectively.

Figure 4.1.: Representative chromatograms obtained with the different sample introduction systems at 1.0 (A) and 0.1 mL min^{-1} (B) using a gradient with a duration of 25 min
Peaks: 1: 4`-hydroxy-diclofenac, 2: diclofenac
(The chromatograms are shifted both vertically and horizontally for improved clarity)



4.3.1 Baseline peak width (w_b) and peak capacity (P)

The baseline peak widths and peak capacity values obtained under the different conditions tested are presented in Figs. 4.2 and 4.3, respectively. Peak capacity defines the maximum number of peaks which can be theoretically separated on a column under given chromatographic conditions. Therefore, it can also be considered as one of the most critical key performance indicators for gradient separations allowing straightforward comparison between columns, systems and methods [32, 33]. As *P* is estimated from the baseline peak width, it is reasonable to discuss these two parameters together. Although the theoretically achievable peak capacity and baseline peak width should be the same on both columns (providing they have the same packing density), independent of the detection mode applied (since they are filled with the same stationary phase and have the same length), substantial differences can be observed among the different detectors (UV and ICP-MS with different sample introduction systems). For both HPLC-UV and HPLC-ICP-MS/MS, considerably more peak broadening (thus lower peak capacity) can be observed with the 1.0 mm ID column (at a flow rate of 0.1 mL min^{-1}) than with the 4.6 mm ID column (at 1.0 mL min^{-1}), which is a direct consequence of the application of the same traditional HPLC instrument for the two column types with the same length but 21-fold difference in their volumes. Even though the HPLC instrument was operated in low delay volume configuration in the former case and normal delay volume configuration in the latter, and the linear velocity of the eluent is even higher on the 1.0 mm ID column at 0.1 mL min^{-1} compared to the 4.6 mm ID column at 1.0 mL min^{-1}, the extra volumes of the system (*e.g.,* ID and length of tubing, injector volume, connections etc.) could not be proportionally decreased together with the column volume. Thus, while increasing the linear velocity of the eluent flow through the column, it became drastically lower in other parts of the system at 0.1 mL min^{-1} flow rate, giving rise to extra longitudinal diffusion, thus extra peak broadening. Nevertheless, the comparison of the HPLC-ICP-MS/MS results with the corresponding HPLC-UV data (separately for 0.1 and 1.0 mL min^{-1} flow rate) is possible for the different sample introduction systems. Larger peak broadening (thus lower peak capacity) is observed for HPLC-ICP-MS/MS than for HPLC-UV for all of the ICP-MS sample introduction systems and at both flow rates. As tubing with the same length and ID was applied between the column outlet and detector entrance (UV cell or ICP nebulizer), this difference in the performance indicators is due to the detector only. When comparing the results obtained with the Scott-type spray chamber to the corresponding UV data, it must be noted that the difference is more pronounced at a low flow rate. While the additional peak broadening is limited to 7-28% only at 1.0 mL min^{-1}, 34-65% larger w_b values were obtained at 0.1 mL min^{-1} in comparison with the

corresponding UV data. It is also worthwhile to mention that independently of the flow rate, the negative effect of the Scott-type spray chamber becomes less pronounced when slower gradients are applied, as while 28 and 65% larger w_b values are observed for the fastest gradient programme at 1.0 and 0.1 mL min^{-1}, respectively, these increments decrease monotonously to 7 and 34%, respectively, for the slowest elution profile. Similar trends can also be observed in the case of the cyclonic spray chamber with typically slightly smaller peak broadening (thus slightly larger peak capacity) compared to the Scott-type spray chamber, thus values closer to the corresponding HPLC-UV data (at 1.0 mL min^{-1}). However, as the difference between the two spray chambers is generally not more than 2-8%, it can be considered negligible.

For the POINT® and hTISIS (used at 0.1 mL min^{-1}) sample introduction systems, it can be seen that typically slightly better values are obtained in comparison with the Scott-type spray chamber under the same conditions. Comparing these two systems, it is interesting to see that while the behaviour of peak broadening obtained with the hTISIS follows the expectations – slightly broader peaks at higher retention times – it remains rather stable in the case of the POINT® system. As a result, their performance can be regarded as similar at slow gradient programmes, but the hTISIS seems to be able to handle steep gradients more successfully.

Figure 4.2.: The baseline peak width (w_b) obtained for 4`-hydroxy-diclofenac and diclofenac using different gradient conditions for HPLC-UV and for HPLC-ICP-MS/MS with different sample introduction systems

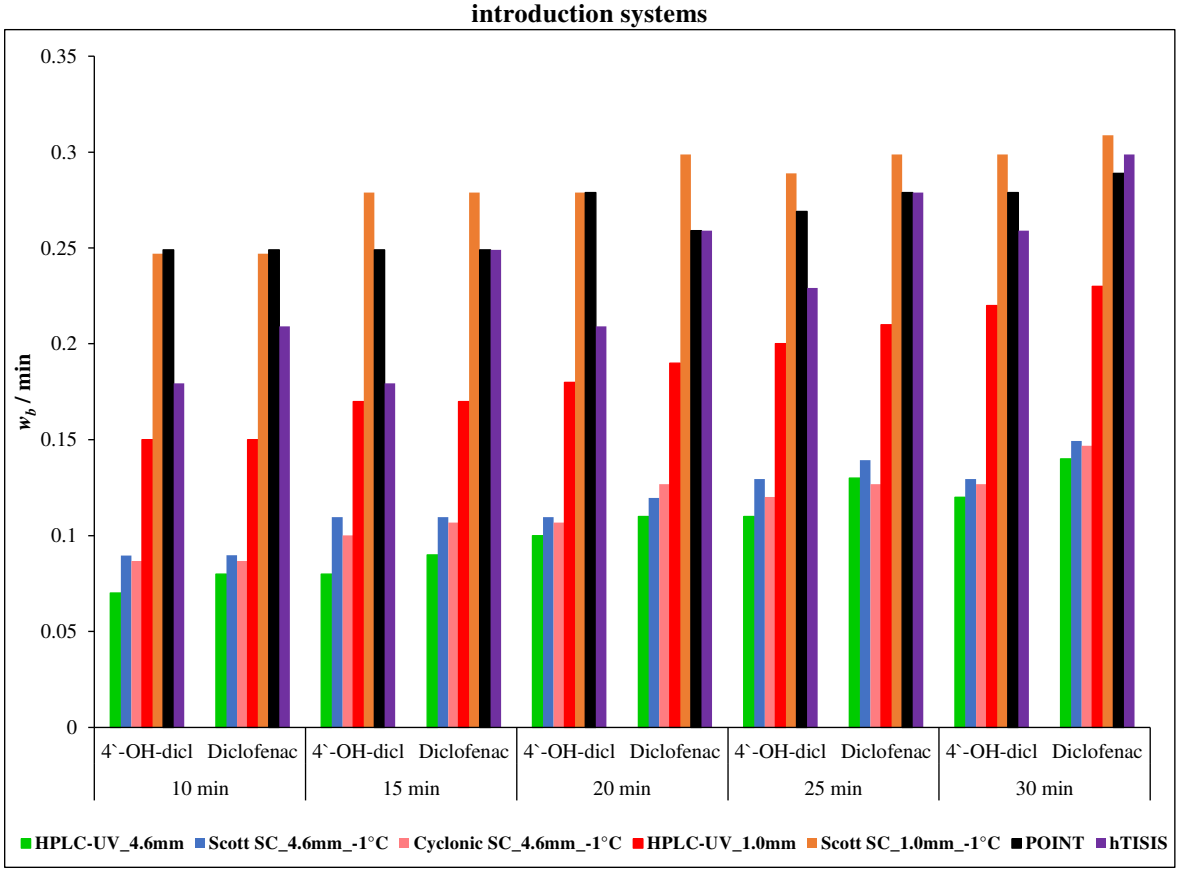
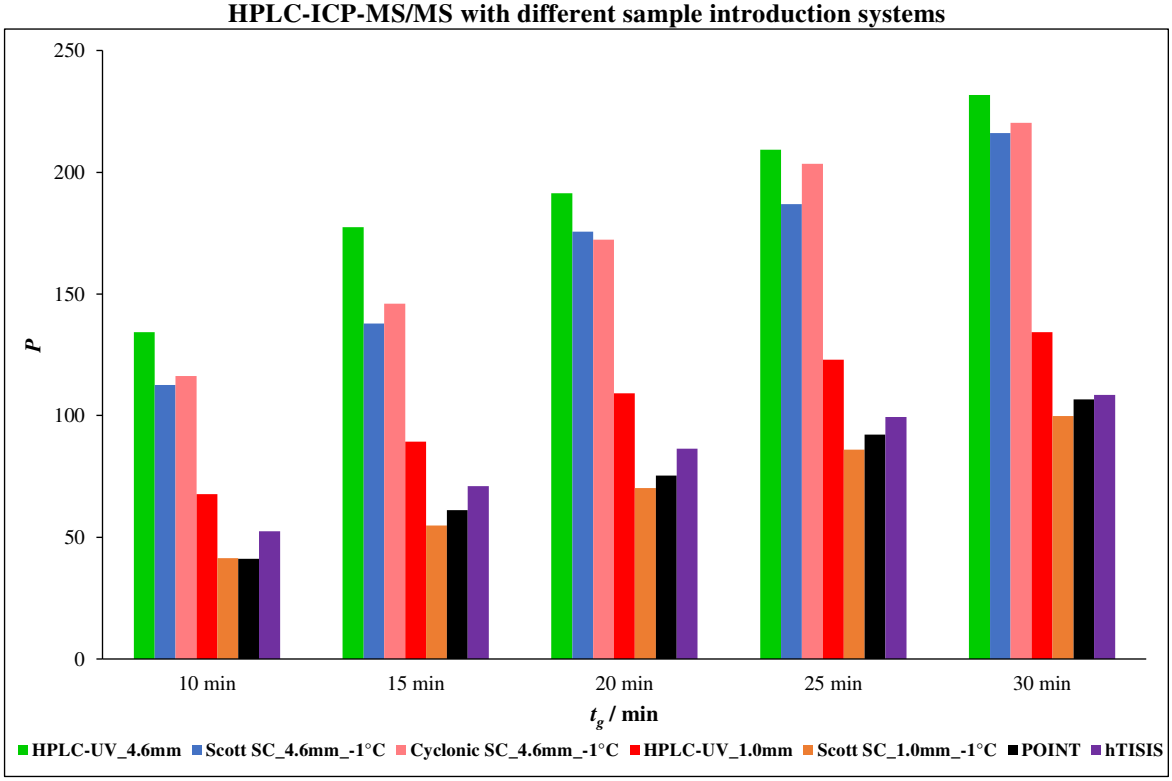


Figure 4.3.: The peak capacity (P) obtained for the different gradient conditions for HPLC-UV and for HPLC-ICP-MS/MS with different sample introduction systems

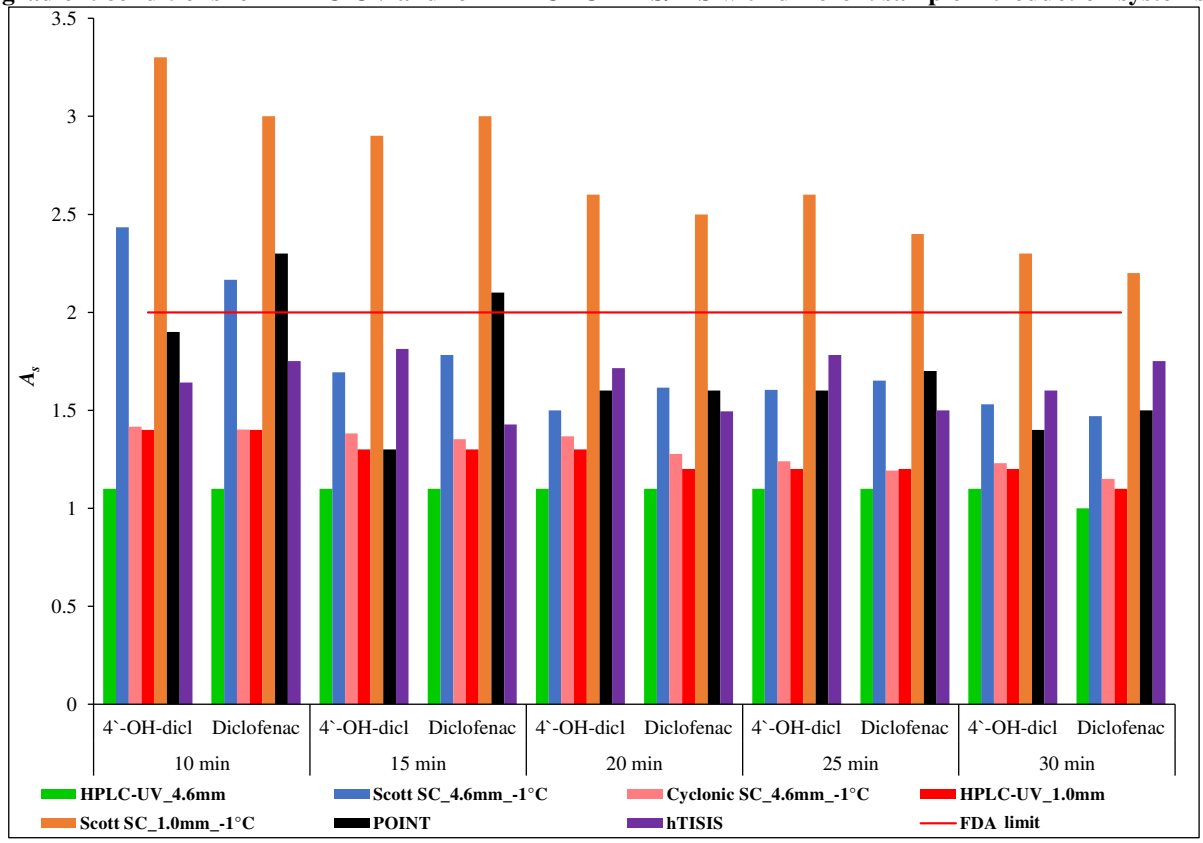


4.3.2 USP tailing factor (A_s)

Next to peak broadening, also peak asymmetry must be taken into consideration during chromatographic method development and validation due to its major impact on the uncertainty of peak integration, thus on the accuracy of the method. In fact, the USP tailing factor can be regarded as the most strictly regulated among the parameters investigated: a sufficiently low tailing factor is a mandatory part of system suitability requirements and the FDA provides a clear recommendation, *i.e.* $A_s \leq 2$ [25], while, for the other parameters, the limit values established typically depend on the experience gained during method development. Fig. 4.4. shows the USP tailing factor values obtained for the different sample introduction systems and HPLC-UV at both flow rates. It can be concluded again, that HPLC-UV presents excellent results with slightly worse values at 0.1 mL min^{-1} (but still $A_s \leq 1.4$) due to the reasons detailed above. Unlike for UV detection, major issues are revealed for some ICP sample introduction systems. Looking at the results obtained at 1.0 mL min^{-1}, it can be seen that the extent of tailing strongly depends on the gradient slope, providing unsatisfactory values (A_s = 2.4 and 2.2 for 4`-hydroxy-diclofenac and diclofenac, respectively) for the steepest gradient programme, when the Scott-type spray chamber is employed. When comparing the Scott-type and cyclonic spray chambers, it is clear that the latter performs considerably better with A_s values between 1.1 and 1.4 and with much less dependence on the gradient slope. Similar observations were found by Todolí *et al* [28, 34] for peak tailing while investigating different sample introduction systems including a cyclonic and also a Scott-type spray chamber for air segmented micro-sample introduction. The peak tailing in the different spray chambers can be explained by the dispersion of the aerosol on its path towards the plasma and re-nebulization processes (*i.e.* a vaporization process from the spray chamber walls that can cause memory effect), which typically become more pronounced in case of spray chambers with a more complex geometry, as is the case for the Scott-type spray chamber compared to the cyclonic one. This feature makes the cyclonic spray chamber preferable for HPLC-ICP-MS purposes. When comparing the results obtained at 0.1 mL min^{-1} (*i.e.* the POINT®, hTISIS and Scott-type spray chamber), the most conspicuous observation is that with the Scott-type spray chamber, the FDA recommendation is not met in any case using the present chromatographic system, as the USP tailing factor varies between 2.2 and 3.3. Only the hTISIS provides satisfactory results under all circumstances (A_s = 1.4 – 1.8), while the POINT® exceeds the FDA recommendation for diclofenac (A_s = 2.1 – 2.3) in the case of the faster gradient programmes. As the hTISIS consists of a single-pass spray chamber, its good performance, which is similar to that of the cyclonic spray chamber, can be explained by its geometrical simplicity. It should be also noted however, that hTISIS provides also the

lowest sensitivity (also demonstrated by the USP S/N results discussed in section 4.3.3), thus, an artificial effect due to the appreciably lower sensitivity compared to, *e.g.*, Scott-type spray chamber under the same conditions (see Fig. 4.5), cannot be excluded either. It can be concluded, that the traditional cyclonic spray chamber seems to be the best choice at higher flow rates, while the hTISIS shows great potential at low flow rates, possibly safely meeting the FDA recommendation in both cases for all gradient programmes tested.

Figure 4.4.: The USP tailing factor (A_s) obtained for 4`-hydroxy-diclofenac and diclofenac using different gradient conditions for HPLC-UV and for HPLC-ICP-MS/MS with different sample introduction systems

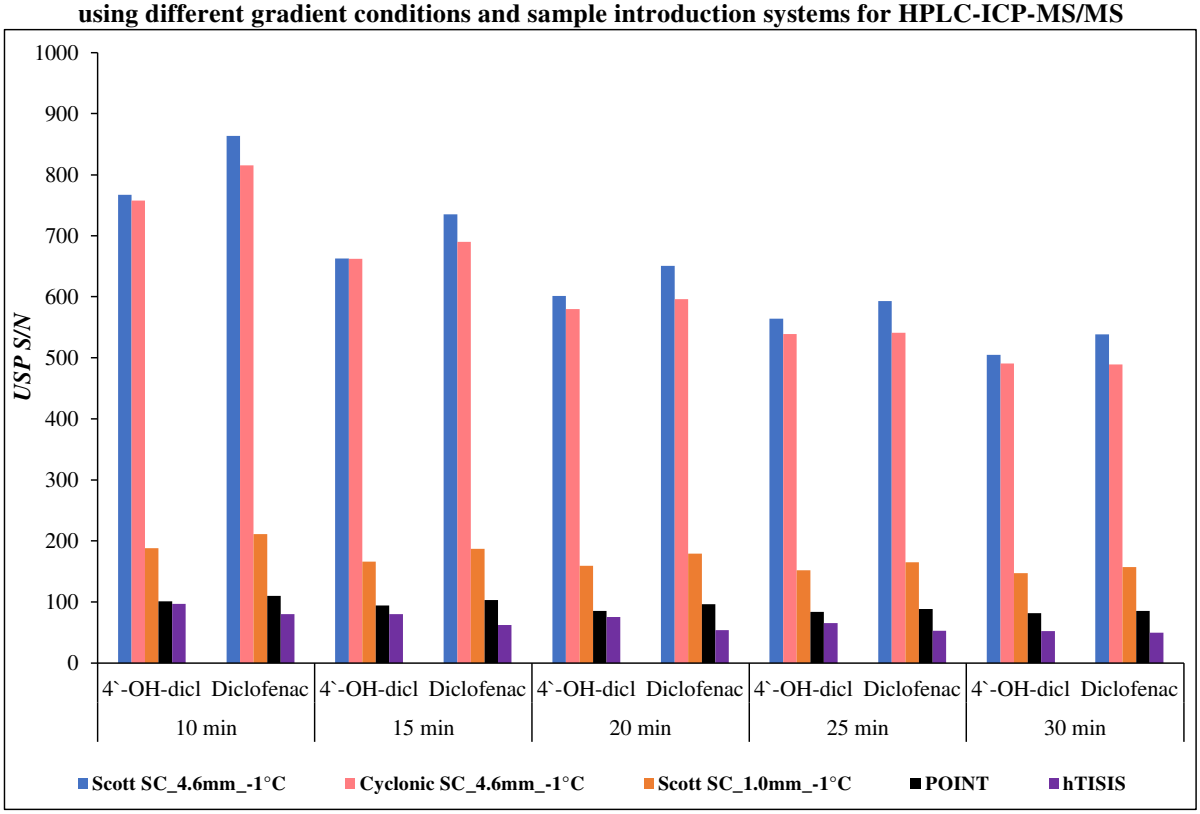


4.3.3 USP signal-to-noise ratio (USP S/N)

Also the signal-to-noise ratio for a peak can be considered one of the most crucial chromatographic parameters, as it has a direct impact on the LOD and LOQ values achievable. Besides the baseline noise, the USP S/N is governed by both the sensitivity of the detector and the magnitude of peak broadening, thus by the peak height corresponding to a given peak area. Fig. 4.5 shows the USP S/N values obtained for both peaks using the different ICP sample introduction systems. As mentioned previously, USP S/N was determined according to USP <621> by applying a distance of 5-times the peak width at its half-height situated symmetrically around the peak of interest (thus USP S/N was calculated from the baseline next to the peak on

both sites). Looking at the results obtained at 0.1 mL min^{-1} (and using 1 µL injection volume), the POINT® and hTISIS are characterized by better peak shape than and similar peak broadening as the Scott-type spray chamber (see Fig. 4.2 and 4.4), but the USP S/N obtained with these sample introduction systems is typically well below that observed with the Scott-type spray chamber under the same conditions. Therefore, it can be concluded that hTISIS is still promising at a low flow rate, however a substantial loss in USP S/N, thus a deterioration in LOD and LOQ should be taken in account in comparison with the more traditional sample introduction system (spray chamber combined with a nebulizer) when Cl-speciation is aimed at. The performance of the cyclonic spray chamber at 1.0 mL min^{-1} is roughly the same as or only slightly worse than (the higher sample introduction efficiency leads to a higher Cl-background due to contamination of the solvent) that of the Scott-type spray chamber in the context of USP S/N under the same conditions, thus the conclusion drawn in the previous section, *i.e.* the cyclonic spray chamber seems to be preferable for HPLC-ICP-MS purposes at higher flow rates, is confirmed.

Figure 4.5.: The USP signal-to-noise ratio (USP S/N) obtained for 4`-hydroxy-diclofenac and diclofenac using different gradient conditions and sample introduction systems for HPLC-ICP-MS/MS



4.3.4 Investigation of the effect of the temperature of the spray chamber

As the temperature within a spray chamber has a direct effect on the aerosol (final droplet size distribution) and thus, the material transport efficiency into the plasma, also the effect of the spray chamber temperature was investigated with both the Scott-type and cyclonic spray chambers. Each of the aforementioned chromatographic parameters was determined at -1, 10, 20 °C and without temperature control using the Scott-type spray chamber and at -1 °C and without temperature control using the cyclonic spray chamber at a flow rate of 1.0 mL min^{-1} (using the chromatographic column with 4.6 mm ID and an injection volume of 21 µL). As can be seen in Fig. 4.6 and 4.7, the spray chamber temperature does not have a visible effect on the baseline peak width or peak capacity. For the USP tailing factor (Fig. 4.8), a moderate effect can be observed in case of the Scott-type spray chamber; *i.e.* A_s seems to slightly decrease (to values below 2 under each gradient condition) when the temperature is elevated to 20 °C or not controlled at all. No similar clear effect of the temperature is experienced in case of the cyclonic spray chamber when comparing -1 °C to the situation without temperature control. In case of both the Scott-type and cyclonic spray chamber, the temperature strongly affects the USP S/N, as shown in Fig. 4.9. The Scott-type spray chamber provides a nearly double signal-to-noise ratio when cooled to a sub-zero temperature, while the increment upon cooling is approx. 30-40% in the case of the cyclonic spray chamber . The negative effect of a higher spray chamber temperature can be attributed to an elevated Cl-background and thus, increased baseline noise value at higher temperatures due to the more extensive formation of solvent vapour (and Cl contamination of the solvent) and its transport into the plasma. Comparing the ratio of USP S/N values obtained at -1 °C and at uncontrolled temperature for both spray chambers, it can be clearly seen that the cyclonic spray chamber is much less sensitive to a change in spray chamber temperature than the Scott-type spray chamber, thus contributing to an enhanced robustness of an HPLC-ICP-MS method. It can be concluded from these experiments that the proper control of temperature plays also an important role in the optimization of an HPLC-ICP-MS method with cooling to sub-zero degrees having a positive effect on the LOD and LOQ values achievable.

Figure 4.6.: The baseline peak width (w_b) obtained for 4`-hydroxy-diclofenac and diclofenac at different spray chamber temperatures using different gradient conditions for HPLC-ICP-MS/MS

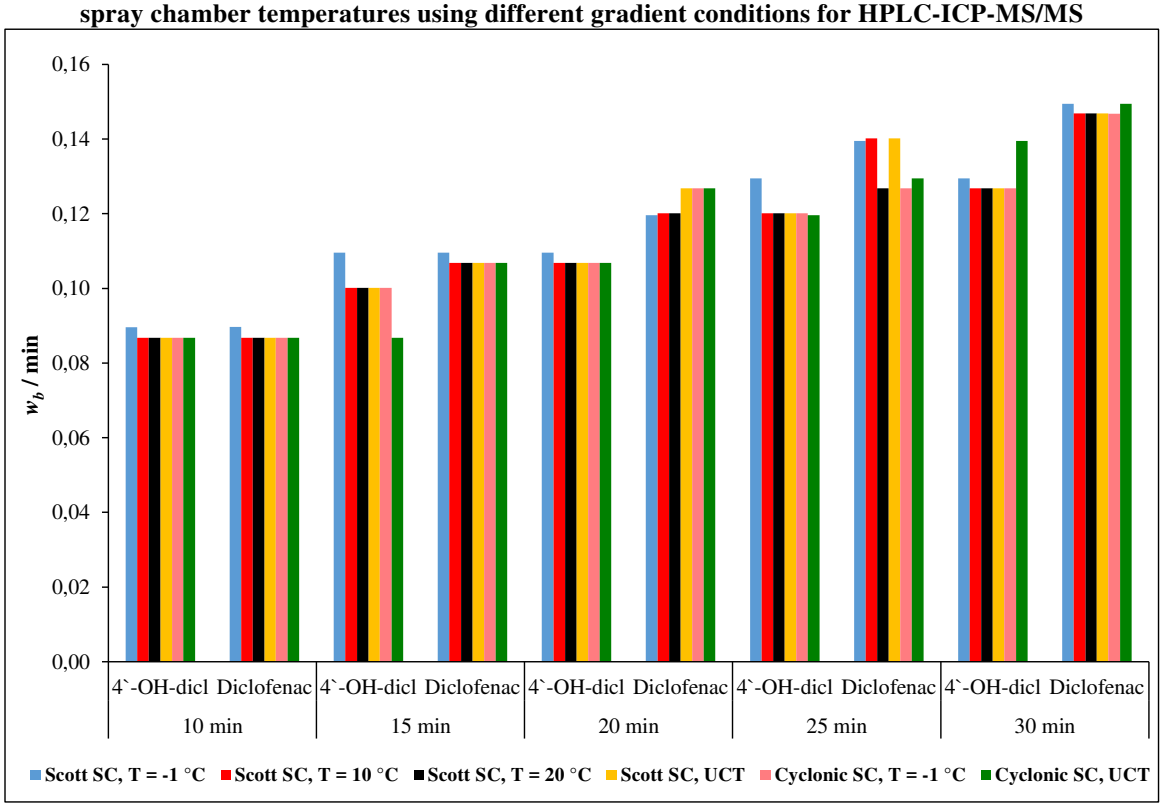


Figure 4.7.: The peak capacity (P) obtained at different spray chamber temperatures using different gradient conditions for HPLC-ICP-MS/MS

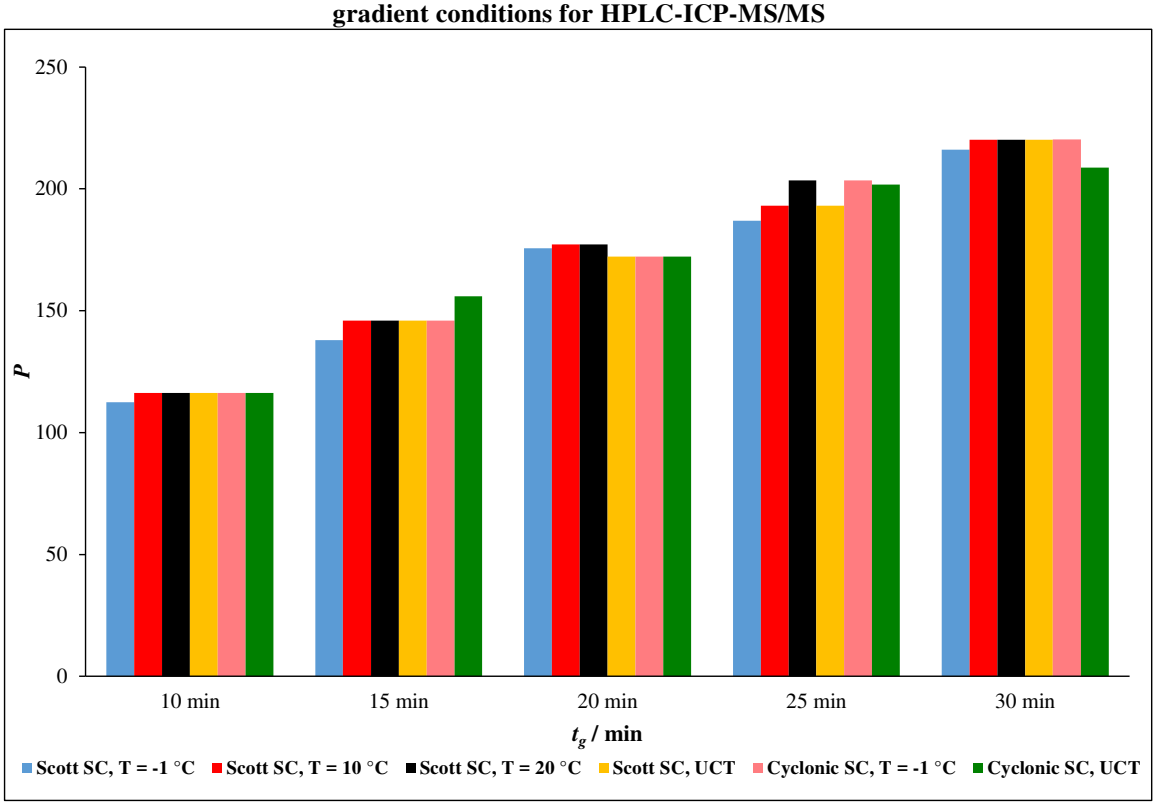


Figure 4.8.: The USP tailing factor (A_s) obtained for 4`-hydroxy-diclofenac and diclofenac at different spray chamber temperatures using different gradient conditions for HPLC-ICP-MS/MS

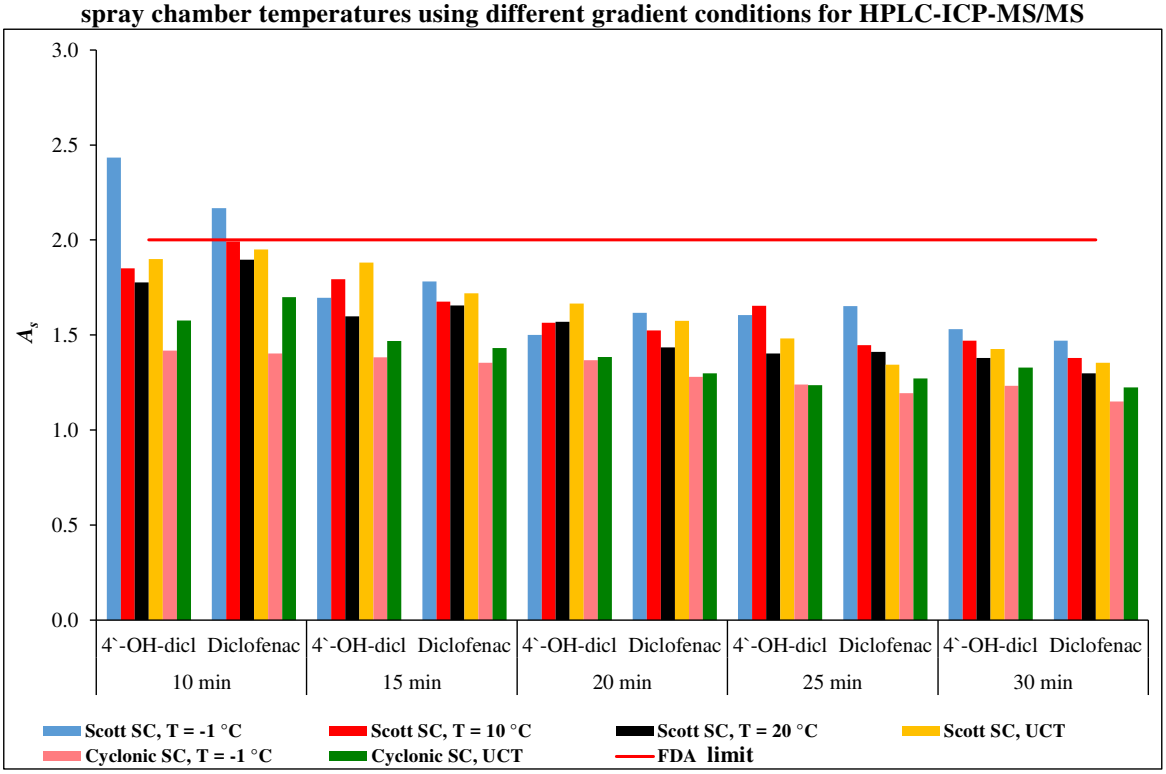
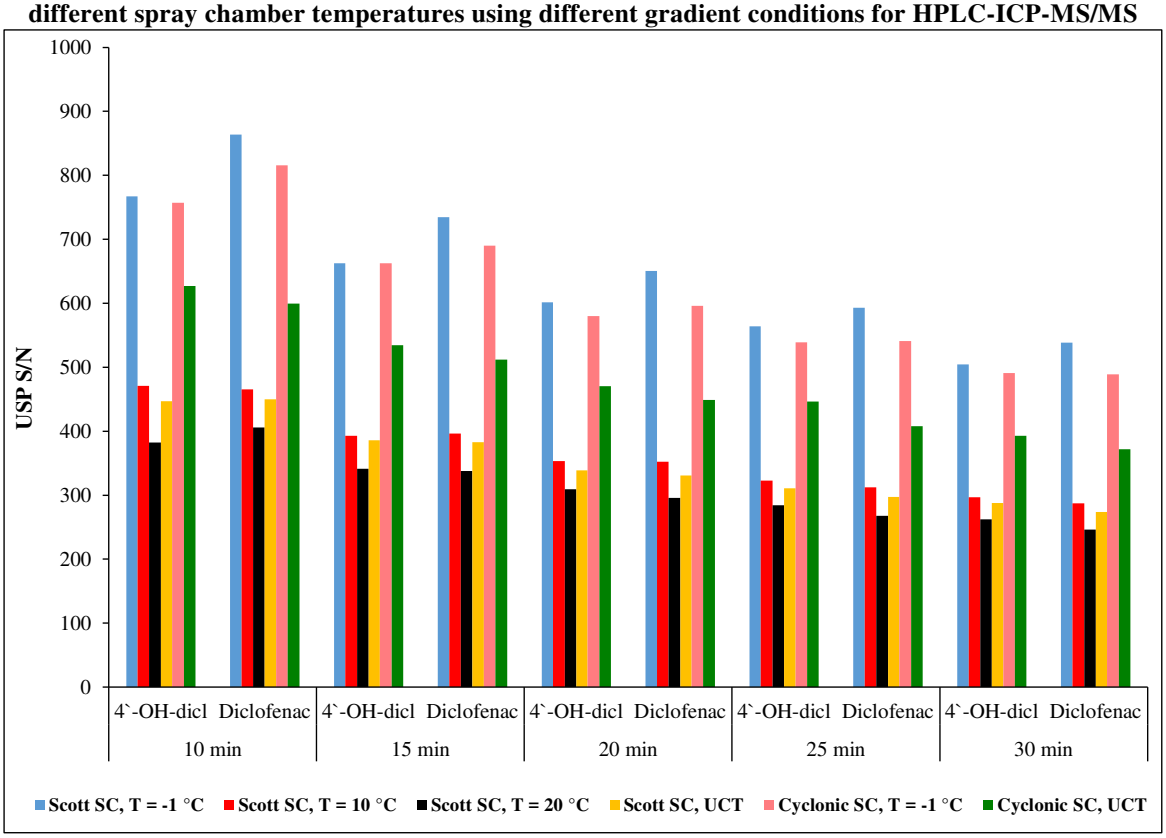


Figure 4.9.: The USP signal-to-noise ratio (USP S/N) obtained for 4`-hydroxy-diclofenac and diclofenac at different spray chamber temperatures using different gradient conditions for HPLC-ICP-MS/MS



4.4 Conclusions

A systematic evaluation of four different ICP sample introduction systems using five different gradient methods in the context of HPLC-ICP-MS-based metabolite profiling of Cl-containing drugs revealed major differences among the different sample introduction systems. First of all, it must be concluded that with none of the ICP sample introduction systems, HPLC-ICP-MS could provide the same chromatographic performance as did HPLC-UV. Due to its simpler geometrical design, the Peltier-cooled cyclonic spray chamber was found to be the best suited sample introduction system at 1.0 mL min^{-1} for HPLC-ICP-MS purposes owing to its considerably better performance in terms of the USP tailing factor (A_s = 1.1 – 1.4 vs. 1.5 – 2.4 obtained with the Peltier-cooled Scott-type spray chamber). For the three other performance indicators, its results are similar to those obtained with the Peltier-cooled Scott-type spray chamber. At a low flow rate (0.1 mL min^{-1}), appreciably worse chromatographic performance was observed both with HPLC-UV and HPLC-ICP-MS, regardless of the sample introduction system used. This was attributed to application of the same HPLC instrument for both 4.6 and 1.0 mm ID columns. The alternative sample introduction systems (POINT® and hTISIS) designed for low flow work provided better chromatographic performance at 0.1 mL min^{-1}, especially in terms of peak shape in comparison with the Scott-type spray chamber. However, although the use of hTISIS allowed the FDA recommendation for USP tailing factor to be met independently of the gradient programme used, the sensitivity for Cl achieved with both the POINT® and hTISIS were much lower than that with the Scott-type spray chamber under the same conditions, thus strongly compromising the USP S/N and the LOD and LOQ values achievable.

Therefore, as an overall conclusion, it can be stated that optimal conditions with high potential for GMP validatability were found for traditional HPLC conditions at a flow rate of 1.0 mL min^{-1} using a Peltier-cooled cyclonic spray chamber for sample introduction. Low flow systems require further development before they can provide the same performance. Based on the results obtained in the course of this study, the combination of a pneumatic micro-flow LC nebulizer and a cyclonic micro spray chamber can be recommended for micro-flow (U)HPLC-ICP-MS(/MS).

The investigation of the effect of spray chamber temperature revealed its utmost importance in HPLC-ICP-MS method development. It was found that for both the Scott-type and cyclonic spray chamber, a sub-zero temperature setting has a strongly beneficial effect on the Cl background and thus, the baseline noise, thus on the USP S/N which has a direct effect on the LOD and LOQ values achievable. The cyclonic spray chamber seems to be more robust towards

the effect of temperature, while providing only a slightly worse USP S/N at -1 °C compared to the Scott-type spray chamber, thus confirming the main conclusion, *i.e.* the application of a Peltier-cooled cyclonic spray chamber seems to be preferable with traditional HPLC conditions for the speciation of Cl-containing pharmaceuticals.

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CHAPTER 5

Determination of F using ICP-MS/MS

5.1 Introduction

Although fluorine-containing organic compounds typically do not occur in nature, they are commonly and extensively produced by the chemical industry for various purposes (*e.g.*, for use as refrigerants, surfactants, lubricants and heat-resistant and chemically resistant materials) due to their interesting, somewhat unusual chemical properties. [1-3]. Due to the fact that these compounds are usually not biodegradable, their close monitoring in biological and environmental samples has become of great importance. Thus, targeted methods, mostly based on HPLC-ESI-MS, have been developed for the determination of the most widely used organofluorine compounds, enabling their sensitive and accurate quantification. Besides the chemical industry, F-containing organic compounds also play an important role in a pharmaceutical context. Approximately 20% of the new pharmaceuticals entering the market contain (a) F atom(s) [1] and, as also mentioned previously, F is among the top-5 hetero-elements present in FDA-approved APIs [4]. Therefore, a F-selective analytical technique with a response independent of the chemical structure of the analyte of interest would be very valuable for the quantitative metabolite profiling of F-containing pharmaceuticals, especially also taking into account that, unlike for, *e.g.*, Cl, no endogenous forms of F would compromise such approach.

However, not even ICP-MS can be used as a powerful technique for the analysis of F as its ionization energy (17.4 eV) is higher than that of Ar (15.7 eV) [3], resulting in a very poor ionization efficiency in the plasma, while accurate F-determination is further compromised by strong spectral overlap as a result of the occurrence of polyatomic ions, such as $^{16}O^{1}H_3^+$,$^{17}O^{1}H_2^+$ and $^{18}O^{1}H^+$ [2]. A successful approach for F determination is based on high-resolution continuum source molecular absorption spectrometry (HR-CS-MAS), utilizing the pronounced tendency of the F atom to form diatomic molecules with different elements, as recently reviewed comprehensively by Butcher [5]. MAS is based on the spectroscopic monitoring of diatomic molecules, which are providing considerably more complex spectra than atoms do, as the wavelength at which absorption occurs (in the UV-visible region) is not only governed by the change in electronic energy, but also by the change in vibrational energy level and by that in rotational energy level. Therefore, the analytical capabilities of the technique have significantly improved by the introduction of high-resolution continuum source atomic absorption spectrometry (HR-CS-AAS), as this instrumentation is equipped with a high-intensity universal source, a high resolution spectrometer and a charge transfer device allowing a section of the spectrum to be documented in detail. As mentioned above, F tends to form highly stable diatomic molecules with several elements, such as Al [6], Ga [7-9], Ca [10], Sr

[11] and Ba [12], thus enabling F-determination with a structure-independent analytical response [9]. Although examples of F-speciation using CS-MAS can be found in the literature [13], a major disadvantage of the technique is that its online combination with HPLC is not possible [2], thus strongly hindering its applicability for pharmaceutical metabolite profiling.

A combination of ICP-MS/MS and the basic idea of MAS (*i.e.* the utilization of the formation of diatomic molecules) has been recently suggested for the determination of F using ICP-MS/MS by Yamada [14] and Jamari *et al* [2]. The online addition of Ba solution to F-containing samples enables the formation of BaF^+ in the ICP according to the mechanisms presented in Eqs. 5.1 and 5.2 [2].

Eq. 5.1: $^{138}Ba^+ + {}^{19}F^0 \rightarrow {}^{138}Ba^{19}F^+$

Eq. 5.2: $^{138}Ba^{2+} + {}^{19}F^- \rightarrow {}^{138}Ba^{19}F^+$

As it eliminates the issue related to the very low efficiency of F^+ formation in the Ar plasma, this strategy appears promising, although it must also be noted that monitoring of $^{138}Ba^{19}F^+$ at *m*/*z* 157 is strongly compromised by spectral overlap due to the occurrence of other Ba-containing polyatomic ions, such as $^{138}Ba^{18}O^1H^+$, $^{138}Ba^{16}O^1H_3^+$ and $^{138}Ba^{17}O^2H^+$ [2]. Therefore, besides a stable and reproducible formation of the $^{138}Ba^{19}F^+$ diatomic ion, this approach also requires a powerful strategy for the removal of the aforementioned spectral interferences. Yamada [14] suggested the application of an Agilent 8800 ICP-MS/MS system using O_2 as a reaction gas in its collision/reaction cell. According to this study, O_2 reacts with the Ba-containing interfering ions ($^{138}Ba^{18}O^1H^+$, $^{138}Ba^{16}O^1H_3^+$ and $^{138}Ba^{17}O^2H^+$) more effectively than with $^{138}Ba^{19}F^+$ in high-energy mode (provided by a low octopole bias setting), enabling the interference-free determination of the BaF^+ adduct ion. After further optimization of the different parameters with respect to the type of reaction gas, reaction gas flow rate, RF power, sampling position, nebulizer gas flow rate, Ba concentration and acquisition time, Jamari *et al* [2] successfully applied the novel methodology for the speciation of fluoride (F^-) and fluoroacetate (FAA^-) using the combination of anion exchange chromatography and ICP-MS/MS.

Within the context of this chapter, this novel approach for F-determination was evaluated with respect to the feasibility of carrying out F-speciation using RP-HPLC-ICP-MS/MS for the purpose of quantitative metabolite profiling of F-containing pharmaceutical drugs.

5.2 Experimental

5.2.1 Reagents and materials

Trifluoroacetic acid (TFA, >99.0%, eluent additive for LC-MS) and barium acetate (BaAc, 99.999%, trace metal analysis grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Al, Ga, Ca, Sb, Sc, Bi and U element standard stock solutions (1 g L^{-1} each) were obtained from Inorganic Ventures (Christiansburg, VA, USA). Pro analysis grade nitric acid (65%, Chem-Lab, Zedelgem, Belgium) was further purified through sub-boiling in PFA equipment. Ultra-pure water (MQ water, resistivity ≥ 18.2 MΩ.cm) was obtained from a Millipore Direct-Q water purification system (Millipore, Billerica, MA, USA).

5.2.2 Preparation of stock solutions and working solutions

Fluorine stock solution was prepared by the dissolution of 13.4 µL of TFA in 10.0 mL MQ water to achieve a F concentration of 1 g L^{-1}. Ba stock solution was prepared at a concentration of 10 g L^{-1} Ba by dissolving 930 mg of BaAc in 50 mL of 0.3 M HNO_3 (in MQ-water). Ba-containing blank solutions were obtained from the Ba stock solution by dilution with MQ water to achieve Ba concentrations of 20, 50, 100, 200 and 500 mg L^{-1}. F-containing working solutions were obtained by dilution of the corresponding stock solutions to obtain mixtures of 10 mg L^{-1} F and 20, 50, 100, 200 and 500 mg L^{-1} of Ba. Blank solutions containing either Al, Ga, Ca, Sb, Bi or U were prepared from the corresponding element standard stock solutions to obtain a concentration of 50 mg L^{-1} in 0.1 M HNO_3. 10 mg L^{-1} F solutions containing either Al Ga, Ca, Sb, Bi or U at a concentration of 50 mg L^{-1} were obtained by dilution of the appropriate stock solutions using 0.1 M HNO_3 as a diluent.

5.2.3 Instrumentation and parameters investigated

An Agilent 8800 “triple-quadrupole” ICP-MS/MS system (Agilent Technologies, Tokyo, Japan), equipped with a low internal volume PFA-LC nebulizer (Elemental Scientific, Omaha, NE, USA) fitted onto a Peltier-cooled Scott-type spray chamber and a torch with a 1.0 mm ID injector tube (Agilent Technologies, Tokyo, Japan) was applied for the determination of F. Pt sampling (with 15 mm Pt insert) and skimmer cones (Agilent Technologies, Japan) were employed instead of the standard Ni cones since the final aim of the study was the application of the novel approach for the speciation of organofluorine compounds by using RP-HPLC-ICP-MS/MS. Besides the no-gas mode, also O_2 was tested as a reaction gas. O_2 was introduced at different gas flow rates in the reaction/collision cell and different octopole bias settings were tested for their ability of eliminating the spectral interferences compromising the accurate

determination of BaF^+ at *m*/*z* 157. The effects of the Ba concentration (in a range of 20 – 500 mg L^{-1}) and of the nebulizer and make-up gas flow rates on the sensitivity and signal-to-background ratio obtained for BaF^+ at *m*/*z* 157 were also investigated. The range studied for the different ICP-MS/MS parameters is provided in Table 5.1. Next to Ba, also other elements (Al, Ga, Ca, Sb, Sc, Bi and U) were tested for their affinity to form polyatomic ions with F as possible alternatives for Ba.

Table 5.1: Parameters and ICP-MS/MS settings investigated during the optimization

RF power	1570 W
Ar carrier gas flow rate	0.4 – 1.3 L min^{-1}
Make-up gas flow rate	0.0 – 0.6 L min^{-1}
Reaction gas flow rate	O_2: 0 – 1 mL min^{-1}
Spray chamber temperature	1 ˚C
Monitored transitions / masses	Q1: *m/z* 157 (BaF^+; $Ba^{18}OH^+$) → Q2: *m*/*z* 157 (BaF^+; $Ba^{18}OH^+$)
Octopole bias	-5 – -80 V
Energy discrimination	-10 mV
Integration time	1 s

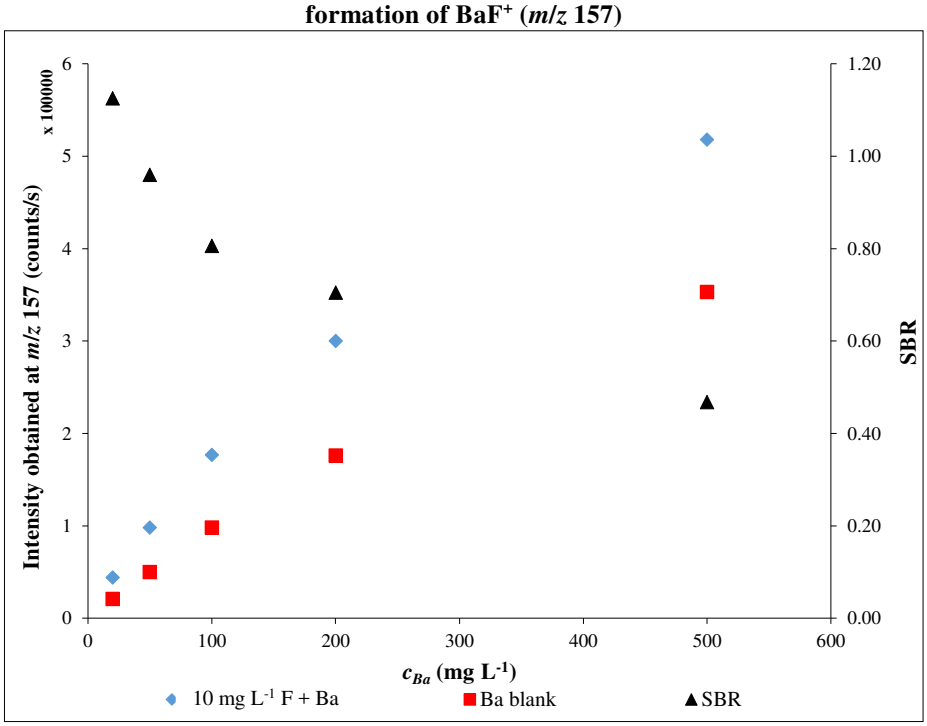
5.3 Results and discussion

5.3.1 Investigation of the effect of the Ba concentration

As the approach tested is based on the formation of the BaF^+ diatomic ion in the plasma, one of the most crucial parameters to optimize is the Ba concentration within the sample flow. This parameter was tested between 20-500 mg L^{-1} of Ba introduced simultaneously with 10 mg L^{-1} of F (as TFA). Also the corresponding blank, with the same Ba concentration, but without F was analysed. The measurements were carried out in no-gas mode with an octopole bias setting of -5.0 V, using a carrier gas flow rate of 1.0 L min^{-1} (without make-up gas) as to obtain information on the background level (*i.e.* extent of spectral interference) as a function of the Ba concentration. Fig. 5.1 shows the intensities obtained for both the blanks and 10 mg L^{-1} F standard solutions (both containing Ba at the same concentration) together with the corresponding signal-to-background ratios. It is clear that both the background values (*i.e.* the interfering Ba-containing ions; from ~21,000 to ~350,000 counts/s) and signal intensities obtained for 10 mg L^{-1} F (from ~23,000 to ~165,000 counts/s) increase with the Ba concentration. Unfortunately, the background is increasing more than the signal obtained for F, which has a negative effect on the SBR values, *i.e.* they are monotonously decreasing as a function of the Ba concentration. Therefore, it can be concluded from these experiments that

the removal of spectral interferences is essential for the success of this approach, especially taking into account that the SBR for 10 mg L^{-1} F hardly exceeds 1 in the optimal case (*i.e.* 20 mg L^{-1} Ba), thus only permitting very poor LOD and LOQ values .

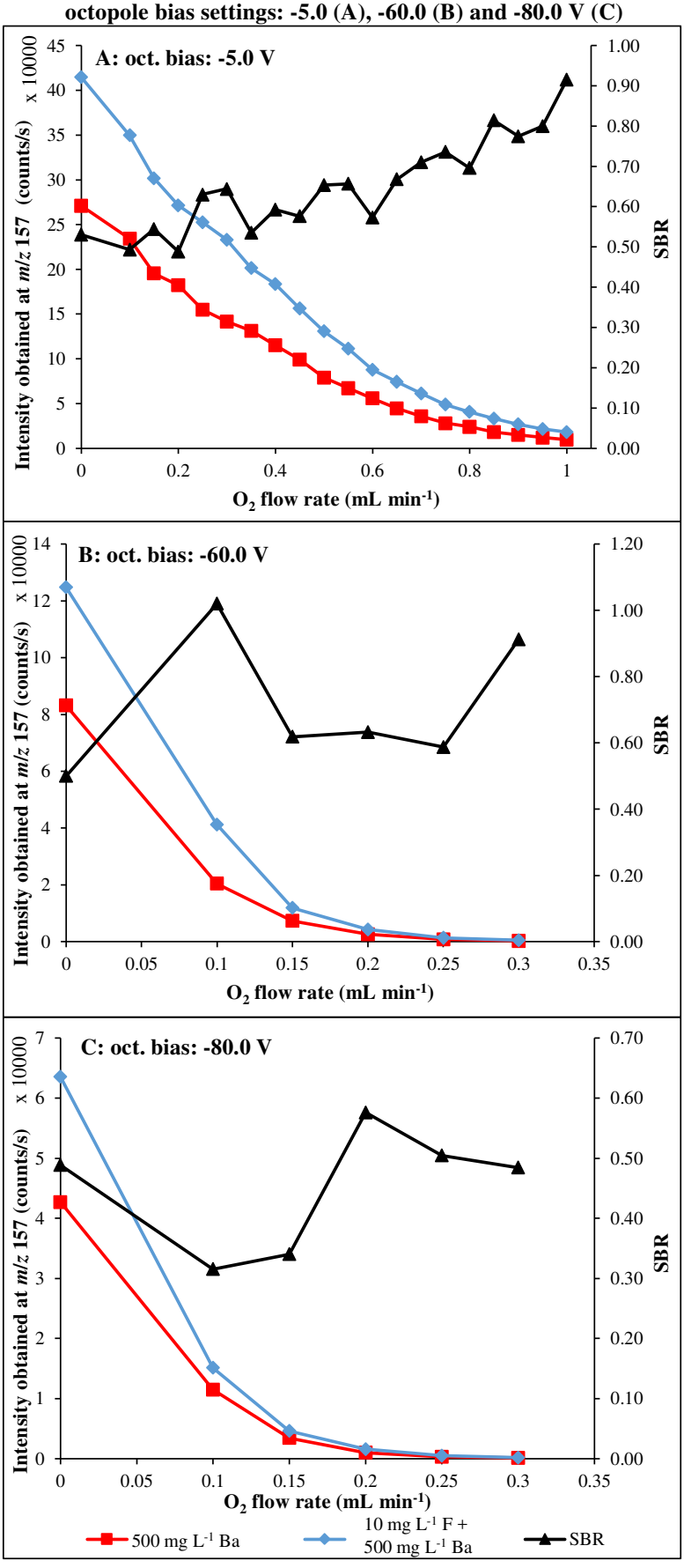
Figure 5.1: Investigation of the effect of Ba concentration of the sample on the background and the formation of BaF^+ (*m/z* 157)



5.3.2 Possibilities for the elimination of Ba-related spectral interferences: application of reaction gas and different octopole bias settings

Owing to the fact that both the target ion ($^{138}Ba^{19}F^+$) and the ions causing spectral interference ($^{138}Ba^{18}O^1H^+$, $^{138}Ba^{16}O^1H_3^+$ and $^{138}Ba^{17}O^2H^+$) are polyatomic adducts, the removal of these interferences is especially challenging. In other words, a selective approach is needed, which is able to eliminate the unwanted polyatomic ions, while keeping the also polyatomic target ion intact. Yamada [14] compared the application of O_2 and NH_3 as reaction gases for this purpose, while Jamari *et al* [2] studied the applicability of O_2 and H_2. O_2 was found to react more effectively with the interferences than with BaF^+ in high-energy mode (octopole bias < -50 V), suggesting a significant difference between the stability of BaF^+ and that of the other Ba-containing polyatomic ions. Therefore, in the present study, the application of O_2 was studied at different gas flow rates and at different octopole bias settings. Figure 5.2 shows the results obtained with the application of O_2 at three different octopole bias settings (-5.0, -60.0 and -80.0 V).

Figure 5.2: Attempt at removal of spectral interferences at *m/z* 157 using O_2 as a reaction gas at different octopole bias settings: -5.0 (A), -60.0 (B) and -80.0 V (C)

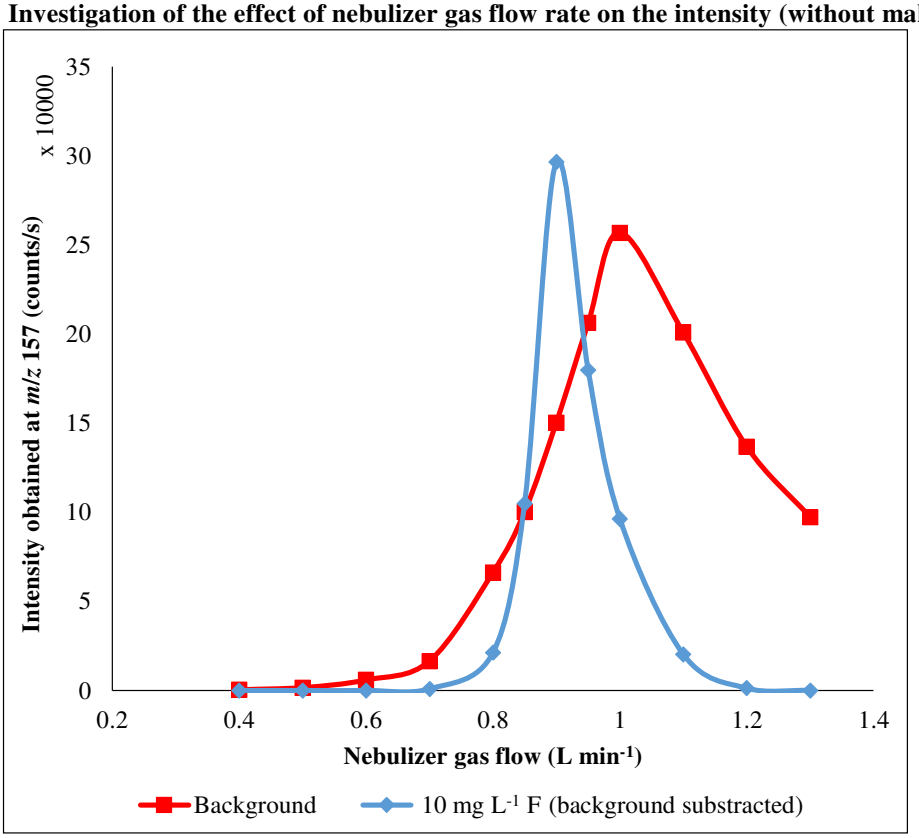


These experiments were carried out using a standard solution containing 10 mg L^{-1} of F and 500 mg L^{-1} Ba as under these circumstances the highest BaF^+ signal intensity was obtained. Also the corresponding blank (same concentration of Ba, but no F) was analyzed. As can be clearly seen from Fig. 5.2, both the reduction of octopole bias voltage and the increment of the O_2 gas flow rate cause a significant loss in the signal intensity obtained for both the background (500 mg L^{-1} Ba) and the analyte signal (10 mg L^{-1} F plus 500 mg L^{-1} Ba). In fact, no more signal could be detected in high-energy mode (at octopole bias -60.0 and -80.0V) when O_2 was introduced into the collision/reaction cell at a flow rate exceeding 0.30 mL min^{-1}. Fig. 5.2A demonstrates a slightly increasing trend in the SBR (from ~0.50 to ~0.95) as a function of the O_2 flow rate, supporting the observation of Yamada [14] that O_2 seems to react slightly more efficiently with the interfering ions than with BaF^+, although it is important to note that the SBR at 10 mg L^{-1} F is still not higher than 1. Regarding the octopole bias, our observation was not similar to that of Yamada [14], thus no increment in the SBR could be observed when using the high-energy mode instead of the -5.0 V octopole bias setting. It is also important to note that, although 20 mg L^{-1} Ba provides the best SBR in no gas mode (see Fig. 5.1), a reduction of the Ba concentration to that level is not advisable when O_2 is applied in the collision/reaction cell (not even at an octopole bias setting of -5.0 V) due to the very strong decrease in signal intensity observed upon the use of O_2, as can be clearly seen from Fig. 5.2. Therefore, in conclusion, no improvement in terms of SBR (or in the LOD and LOQ values attainable) could be achieved by the application of O_2 as a reaction gas and different octopole bias settings compared to the no-gas mode.

5.3.3 Investigation of the effect of nebulizer and make-up gas flow rates

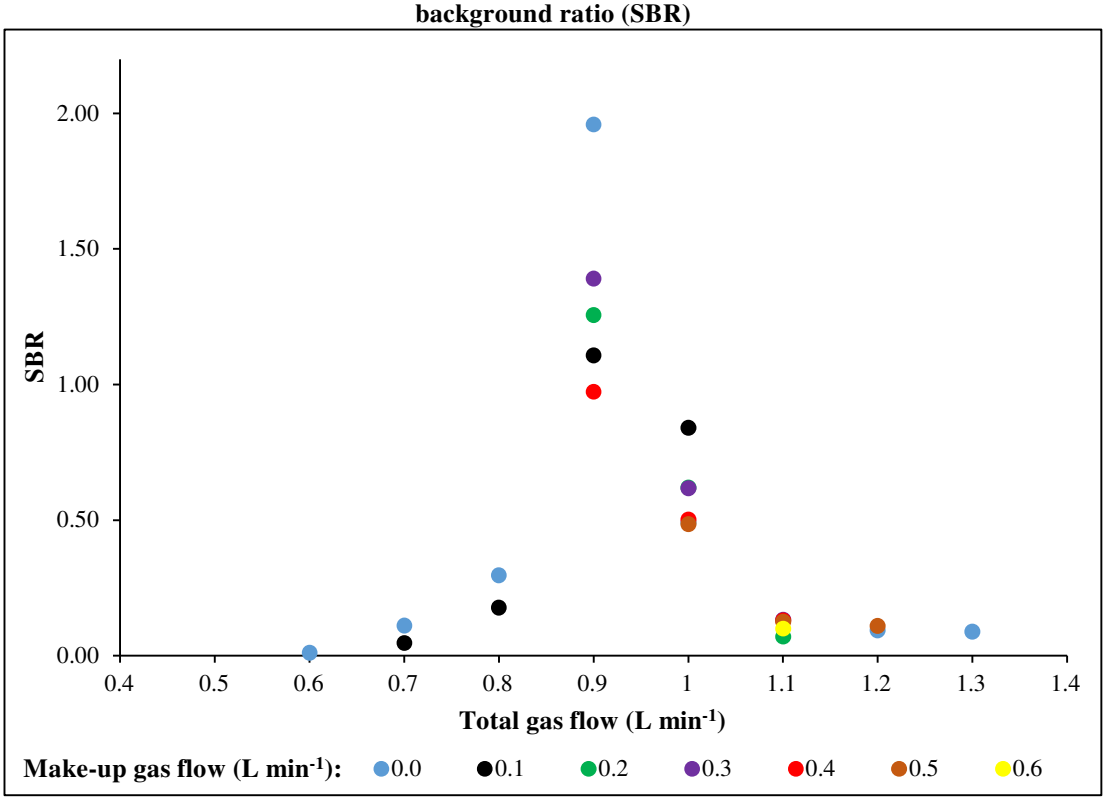
Proper selection of both the nebulizer and make-up gas flow rate was found critical by Jamari *et al* [2] during the optimization of the ICP-MS/MS method aiming at F determination. Therefore, these parameters were also investigated in the present work by monitoring the signal intensity obtained for a 500 mg L^{-1} Ba blank and a 10 mg L^{-1} F plus 500 mg L^{-1} Ba standard solution in no-gas mode (using an octopole bias setting of -5.0 V) at different nebulizer and make-up gas flow rates. Figure 5.3 shows the signal intensity obtained at *m*/*z* 157 as a function of the nebulizer gas flow rate (without make-up gas). Similarly to Jamari *et al* [2], the gas flow rates corresponding to the maximum intensity were different for BaF^+ (0.9 L min^{-1}) and for the Ba-containing interfering ions (1.0 L min^{-1}).

Figure 5.3: Investigation of the effect of nebulizer gas flow rate on the intensity (without make-up gas)



The combined effect of nebulizer and make-up gas flow rate is demonstrated in Figure 5.4 by indicating the signal-to-background ratios obtained for BaF^+ under different conditions. The nebulizer gas flow rate was investigated in a range of 0.6 – 1.3 L min^{-1}, while the make-up gas flow rate was varied between 0.0 – 0.6 L min^{-1} in multiple combinations to reveal the possible interactions between the two parameters. Similarly to the previous results (Fig. 5.3), the highest SBR could be obtained at a total gas flow rate of 0.9 L min^{-1}, indicating that the most critical factor influencing the sensitivity (and SBR) attainable is the total sample gas flow rate entering the plasma. However, significant differences in SBR values (between 1.0 and 2.0 at 0.9 L min^{-1} total gas flow) were observed depending on the ratio of make-up to nebulizer gas flow rate, resulting in the same total gas flow rate. The highest signal-to-background ratio (SBR ≈ 2.0) was obtained without make-up gas at a nebulizer gas flow rate of 0.9 L min^{-1}, while a beneficial effect of using make-up gas (at a flow rate of 0.1-0.3 L min^{-1}) on the SBR values was observed at a total gas flow rate of 1.0 L min^{-1}. Therefore, no straightforward and clear effect of the application of make-up gas was observed during these experiments. In fact, the best SBR result was achieved without using any make-up gas. It must however be noted once again, that although the optimization of gas flows resulted in some improvement, the highest SBR achieved so far for 10 mg L^{-1} F is ~2.0, indicating very poor LOD and LOQ values attainable.

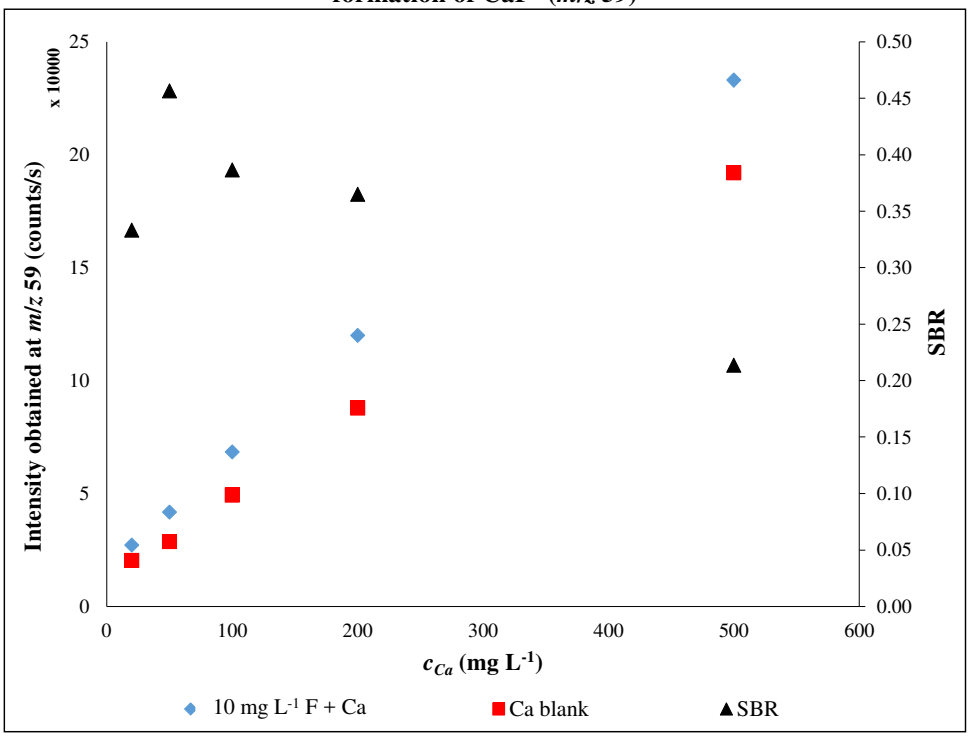
Figure 5.4: Investigation of the effects of nebulizer and make-up gas flow rates on the signal-to-background ratio (SBR)



5.3.4 Test of the affinity of different elements to form polyatomic ions with F in the ICP

As mentioned above, several elements show affinity to form polyatomic molecules with F. Therefore, also other elements than Ba (Al, Ga, Ca, Sb, Sc, Bi and U) were investigated for their potential applicability in F-determination using ICP-MS/MS. 10 mg L^{-1} F standard solutions containing the corresponding element at a concentration of 50 mg L^{-1} were analysed together with the corresponding blank solutions (containing the element of interest at a concentration of 50 mg L^{-1}) to reveal their affinity to form polyatomic ions with F in the plasma. Relying on the experience obtained with Ba, the analysis was performed in no-gas mode with an octople bias setting of -5.0 V and using a nebulizer gas flow of 0.9 L min^{-1} (without make-up gas). Besides that of the diatomic ion, also the *m*/*z* values corresponding to polyatomic adduct ions (XF_n^+) were monitored, with X representing the element of interest and n = 1-6. Only Ca showed a similar affinity to form CaF^+ (monitored at *m*/*z* 59) as Ba to form BaF^+. Therefore, a similar optimization to that of Ba was carried out to investigate the feasibility of the application of Ca in F-determination. The investigation of the effect of Ca concentration on the intensity obtained at *m*/*z* 59 and the corresponding SBR ratios can be seen in Figure 5.5.

Figure 5.5: Investigation of the effect of Ca concentration of the sample on the background and the formation of CaF^+ (*m/z* 59)



Similarly to Ba, the highest sensitivity for F (unfortunately, together with the highest background intensity) can be observed at 500 mg L^{-1} Ca, while the maximum SBR was obtained at 50 mg L^{-1} Ca without removal of the spectral interferences. Following the same strategy as during the optimization with Ba, 500 mg L^{-1} Ca was applied with 10 mg L^{-1} F to investigate the possibilities for the elimination of the strong spectral interference. During the investigation of the effect of the nebulizer gas flow rate on the intensity achieved for F and its background, a very similar situation was observed to that with Ba, *i.e.* the maximum intensities for CaF^+ and the interfering ions were obtained at different nebulizer gas flow rates, *i.e.* at 0.95 and 1.10 L min^{-1}, respectively. In an attempt to remove the spectral interference, the application of O_2 was tested in the collision/reaction cell at different flow rates using two different octopole bias settings (-5.0 and -60.0 V). Once again, a very similar behaviour to that obtained with Ba was observed, but with the introduction of O_2 and a more negative octopole bias setting having an even stronger negative effect on the signal intensity. Due to the completely identical trends in comparison with those demonstrated in Figures 5.2 and 5.3 for Ba, the results are not shown separately for Ca. It can be concluded again, that no significant increment in the SBR could be achieved by applying O_2 reaction gas and/or the high-energy mode (low octopole bias setting). The highest SBR values were around 0.8 for 10 mg L^{-1} F, which demonstrates even poorer LOD and LOQ values than in the case of Ba.

5.4 Conclusions

Within the context of this study, a novel approach published by Yamada [14] and Jamari *et al* [2] for the determination of F with ICP-MS/MS was tested using an Agilent 8800 ICP-MS/MS system, aiming at the use of the novel approach for the quantitative metabolite profiling of F-containing pharmaceuticals. Slightly worse analytical performance was observed compared to the studies mentioned above, with partially contradictory results. Ba was added to the samples to form BaF^+ in the plasma, which could be monitored at *m*/*z* 157. However, the quantification of F in the form of BaF^+ was strongly compromised by interference from other Ba-containing polyatomic ions, as also presented by Jamari *et al* [2]. The possibility for the elimination of these interferences was studied by monitoring the intensity and signal-to-background ratio achievable for 10 mg L^{-1} F standard solutions in comparison with the corresponding blanks. In an attempt to selectively remove the aforementioned polyatomic interfering ions, O_2 was tested as a reaction gas at different octopole bias settings. A minor improvement in the SBR could be achieved by optimization of the nebulizer gas flow rate, application of O_2 and the reduction of the Ba concentration of the sample to 20 mg L^{-1}. However, the use of O_2 reaction gas cannot be combined successfully with the reduction of Ba concentration as the use of O_2 suppresses the signal intensity. Moreover, the improvement in SBR achieved by changing these parameters did not exceed a factor of 2. Although a similar trend was found as that published by Yamada [14], *i.e.* O_2 reacts slightly more effectively with the interfering ions than with BaF^+, the reduction in signal intensity, it gives rise to, was found to be too strong in view of its very moderate beneficial effect, jeopardizing its applicability for effective removal of the spectral interferences using the present instrumental set-up. Also use of the high-energy mode in the collision/reaction cell, thus with < -50.0 V octopole bias setting, did not improve the conditions. The affinity of other elements to form polyatomic ions with F in the plasma was also tested. Ca shows a behaviour that is very similar to that of Ba. However, the analytical performance observed was even worse, with a maximum SBR of ~0.8. Taking into account that only a very low SBR could be achieved for 10 mg L^{-1} F using either Ba or Ca during the optimization, corresponding to very poor LOD and LOQ values, the present approach was found unsatisfactory for the metabolite profiling of F-containing pharmaceuticals.

As a sufficiently sensitive ICP-based F-selective detection technique would undoubtedly be very beneficial for pharmaceutical development, other approaches deserve to be the subject of further research. Use of a He plasma instead of an Ar one provides a higher ionization efficiency for elements characterized by a high ionization energy as a result of the higher ionization energy of He compared to Ar, as already discussed in several studies [15-17]. Mass analysis of the

negative ions produced in the ICP ion source could also be potentially utilized in this context. While some early-generation ICP-MS instruments could be operated in either positive or negative ion mode [18, 19], present-day commercially available units no longer offer this possibility.

5.5 References

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SUMMARY AND CONCLUSIONS

The aim of this PhD research was an assessment and an extension of the potential of RP-HPLC-ICP-MS(/MS) in the field of quantitative drug metabolite profiling. For this purpose, several issues originally compromising this strategy have been addressed. As an introduction, a comprehensive review on the current status of HPLC-ICP-MS in this field was provided, focusing on compatibility and quantification issues. Also the capabilities of ICP-MS for the determination of non-metal hetero-elements typically present in pharmaceuticals was discussed into detail. Finally, also derivatization approaches targeting drug molecules originally not containing any ICP-MS detectable hetero-element were briefly overviewed. Based on this literature study, it became clear that the application of HPLC-ICP-MS in pharmaceutical R&D is far from routine yet, partially due to the lack of a simple and universally applicable quantification strategy. The lack of such quantification strategy is partially due to the mostly necessary use of gradient elution in RP-HPLC and to the spectral interferences hampering accurate ICP-MS determination of the most typical hetero-elements (*i.e.* S, Cl, F, P, Br and I) present in APIs. While in the literature, several papers describe the successful determination and speciation of S-, P-, Br- and I-containing compounds, for which the analytical figures of merit were significantly improved upon the commercial introduction of a tandem ICP-mass spectrometer (ICP-MS/MS) or "triple quadupole" (ICP-QQQ) system by Agilent Technologies, relatively few studies address the speciation and metabolite profiling of Cl- and F-containing drugs. Therefore, in this PhD research project, specific attention was devoted to the aforementioned compatibility and quantification issues, together with the possibilities for Cl-determination using a state-of-art ICP-(QQQ)MS/MS system.

As a first step, a fast, accurate and precise method was developed for the separation of endogenic and exogenic (or drug-related) Cl and Br, and for the determination of the total drug-related Cl and Br content in human plasma based on isocratic RP-HPLC-ICP-MS/MS, as described in **Chapter 2**. The novel approach was proved to be a suitable alternative to the currently applied standard methodology (*i.e.* radiolabelling followed by radio-HPLC), while eliminating the disadvantages of the latter. Different strategies for the interference-free determination of Cl in organic media using ICP-MS/MS were evaluated and the use of H_2 as

reaction gas in the collision/reaction cell with the monitoring of the $^{35}ClH_2^+$ reaction product at a mass-to-charge ratio of 37 was found to be the best approach. The method was found to be sufficiently precise (repeatability < 10% RSD) and accurate (recovery between 95 and 105%), while providing significantly lower LOQ values (0.05 and 0.01 mg/L for Cl and Br in blood plasma, respectively) than those obtained with a vented cell and on-mass monitoring. Quantification was accomplished via either external or internal standard calibration, providing reliable results for both elements. After thorough analytical validation, human plasma samples from a clinical study involving a newly developed Cl- and Br-containing API were analysed as a proof-of-concept. The results obtained permitted an adequate description of the variation in the total drug concentration in blood plasma as a function of time. Cross-validation was achieved by comparing the results obtained on Cl- and on Br-basis.

As the separation of drug metabolites can often not be accomplished under isocratic conditions, the quantification issue arising during the application of gradient elution was addressed in a next step, as an essential pre-requisite to fulfil the aim (*i.e.* to extend the applicability of RP-HPLC-ICP-MS(/MS) in pharmaceutical analysis), as described in **Chapter 3**. A novel quantification approach based on mathematical correction was developed and subsequently successfully used for the compensation of the effect of gradient elution on the instrumental response during RP-HPLC-ICP-MS/MS analysis using diclofenac and its related compounds, including its major metabolite *i.e.* 4`-hydroxy-diclofenac, as model compounds present in human plasma matrix. The ICP-MS/MS strategy for Cl described in Chapter 2 was used for the interference-free determination of Cl in this context. The effect of the eluent composition on the instrumental response for Cl was thoroughly investigated for the most commonly used organic solvents in RP-HPLC, *i.e.* methanol and acetonitrile. A mathematical function describing the effect of the eluent composition on the sensitivity for Cl, enabled adequate correction for the otherwise detrimental effect of the gradient elution for both solvents. Validation using synthetically degraded diclofenac samples, spiked with its major metabolite 4`-hydroxy-diclofenac, demonstrated appropriate accuracy (recovery for 4`-hydroxy-diclofenac between 95 and 105 %) and a recovery > 90% and > 80% for total Cl when using acetonitrile and methanol, respectively. When applied to spiked human plasma (one of the most important matrices in drug metabolism studies) samples, a satisfactory accuracy (recovery of 92-98%) and precision (<4% RSD) were established for both diclofenac and 4`-hydroxy-diclofenac. The LOQ for Cl (as diclofenac) using the novel method was found to be 0.05 mg L^{-1}, similar to the value obtained in the work aiming at the determination of the total drug-related Cl-content (Chapter 2). This value could be significantly improved (to 0.002 mg L^{-1})

via *online* sample pre-concentration using a trapping chromatographic column and a time-programmable 10 ports / 2 positions microvalve.

When pharmaceutical analysis is aimed at, GMP validation of an analytical approach can be an important aspect of the method development. Strict regulations may apply for the chromatographic methods used in the pharmaceutical industry, therefore **Chapter 4** was dedicated to the chromatographic evaluation of different ICP-MS sample introduction systems used in the context of HPLC-ICP-MS analysis. Five different gradient programmes were tested and the baseline peak width (w_b), peak capacity (P), USP tailing factor (A_s) and USP signal-to-noise ratio (USP S/N) were determined as major indicators of the chromatographic performance. The values obtained were compared to the corresponding FDA recommendations (if applicable). Four different ICP-MS sample introductions systems, including two units typically working at higher flow rates (~1.0 mL min^{-1}) and another two systems working at lower flow rates (~0.1 mL min^{-1}), were investigated. Optimal conditions with potential for application under GMP conditions were found at a mobile phase flow rate of 1.0 mL min^{-1} by using a pneumatic micro-flow LC nebulizer mounted onto a Peltier-cooled cyclonic spray chamber cooled to -1 °C for sample introduction. Under these conditions, HPLC-ICP-MS provided a chromatographic performance similar to that of HPLC-UV. The peak shape (USP tailing factor = 1.1 – 1.4) was considerably improved compared to that obtained with the Peltier-cooled Scott-type spray chamber. As mentioned above, two alternative sample introduction systems – a POINT® and a High-Temperature Torch-Integrated Sample Introduction System (hTISIS) – were also tested at a flow rate of 0.1 mL min^{-1} using a chromatographic column with 1.0 mm ID. Although an improvement of the peak shape in comparison with that obtained with the traditional Scott-type spray chamber was achieved with these systems, the limits of detection and of quantification attainable were strongly compromised due to the significantly lower sensitivity observed for Cl. In addition to a comparison of the aforementioned sample introduction systems, also the effect of spray chamber temperature was evaluated and it was demonstrated that proper temperature control plays an essential role in the optimization of HPLC-ICP-MS methods.

As mentioned above, also F is among the top-5 hetero-elements present in APIs, thus a suitable analytical technique offering F-selective detection that can be combined with RP-HPLC would be of great value for the quantitative metabolite profiling of F-containing pharmaceuticals. Recognizing this potential, ICP-MS/MS was tested as a possible F-selective detector during the present research work, aiming at the extension of the application field of RP-HPLC-ICP-MS/MS in a pharmaceutical context. However, F is an especially difficult element for ICP-MS

as its ionization potential is higher than that of Ar and strong spectral interference further complicates F assay. Nevertheless, a novel strategy for F-determination using ICP-(QQQ)MS/MS based on an in-plasma reaction with Ba was recently published and this approach was tested in the context of this PhD research (**Chapter 5**) for its suitability for metabolite profiling studies. An analytical performance similar to or only slightly worse than that presented in the studies taken was observed in this study. However, the removal of other Ba-containing polyatomic ions, leading to strong spectral interference at the target *m*/*z* value of BaF^+ could not be successfully accomplished, resulting in very poor LOD and LOQ values attainable. Therefore, the application of this approach using the present instrumentation was not found satisfactory for real-life metabolite profiling based on F-detection.

As a major conclusion of this PhD, it can be stated that HPLC-ICP-MS(/MS) shows great potential in the field of pharmaceutical metabolite profiling. Several successful applications in which the general requirements of the pharmaceutical authorities were always adhered to have been published, demonstrating the compatibility of RP-HPLC and ICP-MS(/MS). A simple and easy-to-validate quantification strategy was developed, which can eliminate the major disadvantages and limitations of, *e.g.*, online ID. Interference-free determination of Cl was successfully accomplished using ICP-MS/MS. As a result, at the time of writing this PhD dissertation, reliable, robust and sufficiently sensitive HPLC-ICP-MS(/MS) strategies have been described in the literature for 5 out of the 6 hetero-elements most abundant in pharmaceuticals. Respecting the possible need for GMP validation when HPLC-ICP-MS(/MS) is applied in the pharmaceutical industry, also a chromatographic evaluation of the technique was carried through, demonstrating that smooth GMP validation is possible if the right sample introduction system is chosen and the operating parameters are properly optimized.

Although the potential use of HPLC-ICP-MS(/MS) for quantitative metabolite profiling is not questionable anymore, there are still several aspects that require further research. As mentioned in the introduction, the largest portion of APIs does not contain any hetero-element which can serve as a basis for ICP-MS detection, thus the development and optimization of derivatization strategies utilizing the reactivity of the functional groups typically present in pharmaceuticals seems to be inevitable to further extend the application range of this technique. It is also worthwhile to mention that the majority of the applications described in the literature focuses on the metabolite profiling of “traditional” small-molecule pharmaceuticals. However, next to these drugs, biopharmaceuticals play an increasingly important role in drug development, and these novel drugs require a more versatile and sophisticated analytical toolbox. The continuously expanding field of “biosimilars” further confirms this conclusion, as in generic

drug development, it is far more complex to characterize and compare a biomolecule to the original version of the drug than it is in the case of small molecules. Therefore, an increased number of orthogonal analytical approaches becomes necessary to convince the Authorities of the bioequivalence, safety and efficacy of these new "biosimilar" products. HPLC-ICP-MS/MS may also serve as a useful tool in this field since many of these biomolecules contain P and/or S, enabling direct, straightforward and sensitive quantification based on these elements. Moreover, ICP-MS/MS can be used as a sensitive and element-selective detector allowing accurate quantification for a variety of chromatographic approaches, *e.g.*, reversed phase, normal phase, ion exchange and size exclusion LC, thus allowing orthogonal separations to be exploited.

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