# Liquid chromatography coupled with tandem mass spectrometry for the quantitative analysis of anticancer drugs in biological matrices

# Liquid chromatography coupled with tandem mass spectrometry for the quantitative analysis of anticancer drugs in biological matrices

Vloeistofchromatografie gekoppeld met tandem massaspectrometrie voor de kwantitatieve analyse van cytostatica in biologische matrices

(met een samenvatting in het nederlands)

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Voor jou

## Contents

## Preface

The efficacy and toxicity of many anticancer drugs are related to parent drug and/or metabolite concentrations in body fluids and tissues. Therefore, during both pre-clinical and clinical drug development, profound knowledge of the pharmacokinetic properties (absorption-distribution-metabolism-elimination) of the drug is essential. Furthermore, since anticancer agents often have narrow therapeutic ranges and inter- and intra-patient variabilities in pharmacokinetic properties are high, drug monitoring in routine clinical practice may be required for safe and efficacious therapeutic use [1]. For many years, liquid chromatography (LC) with predominantly ultraviolet (UV), fluorescence, or electrochemical detection has been employed for this purpose. UV detection, however, is not very sensitive and suffers from low specificity, while fluorescence and electrochemical detection, although more sensitive and specific than UV detection, are limited to compounds with fluorescent or electro-active groups or do otherwise require derivatization.

Mass spectrometry (MS) and off-line LC-MS for qualitative analysis, as well as on-line gas chromatography-MS for quantitative detection, have been common practice for many years. On-line LC-MS for quantitation purposes, on the other hand, was complicated until the introduction of atmospheric pressure ionization (API) techniques (e.g. atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI)) in the 1980s. In recent years LC-MS interfaces have further improved, mainly because techniques such as ionspray (ISP; pneumatically assisted ESI), turbo-ionspray (TISP; pneumatically assisted ESI with an additional hot drying gas perpendicular to the spray), and heated nebulizer (HN)-APCI (pneumatically assisted APCI) became available that facilitate the evaporation of LC solvents [2]. Simultaneously, mass analyzers have improved. For quantitative assays employing MS detection triple quadrupole (TQ) systems are most commonly used. When a TQ is operated in the multiple reaction monitoring (MRM) mode the analyte is identified and detected not only by means of its molecular ion but also by means of a typical fragment ion, obtaining higher sensitivity and superior selectivity than for any other MS system. As a result of these developments LC-MS has now become an accepted, wide spread, and commonly used, bench-top technique especially in pharmaceutical analyses.

In this thesis the development, validation, and implementation into clinical and pre-clinical studies of LC-TISP-TQ-MS/MS methods for the quantitative analyses of anticancer drugs is described. First, in chapter 1, a selected overview of publications describing the quantitative bioanalysis of anticancer agents using LC-MS is given. Chapters 2 to 5 are focussed on marine derived anticancer agents (chapter 2), a matrix metalloproteinase inhibitor (chapter 3), tubulin inhibitors (chapter 4) and a topoisomerase I inhibitor (chapter 5). Finally, in chapter 6, internal standards for quantitative LC-MS/MS assays are discussed.

1. Van den Bongard HJGD, Mathôt RAA, Beijnen JH, Schellens JHM. Pharmacokinetically guided dosing of chemotherapeutic agents. Clin Pharmacokinet 2000; 39: 345.
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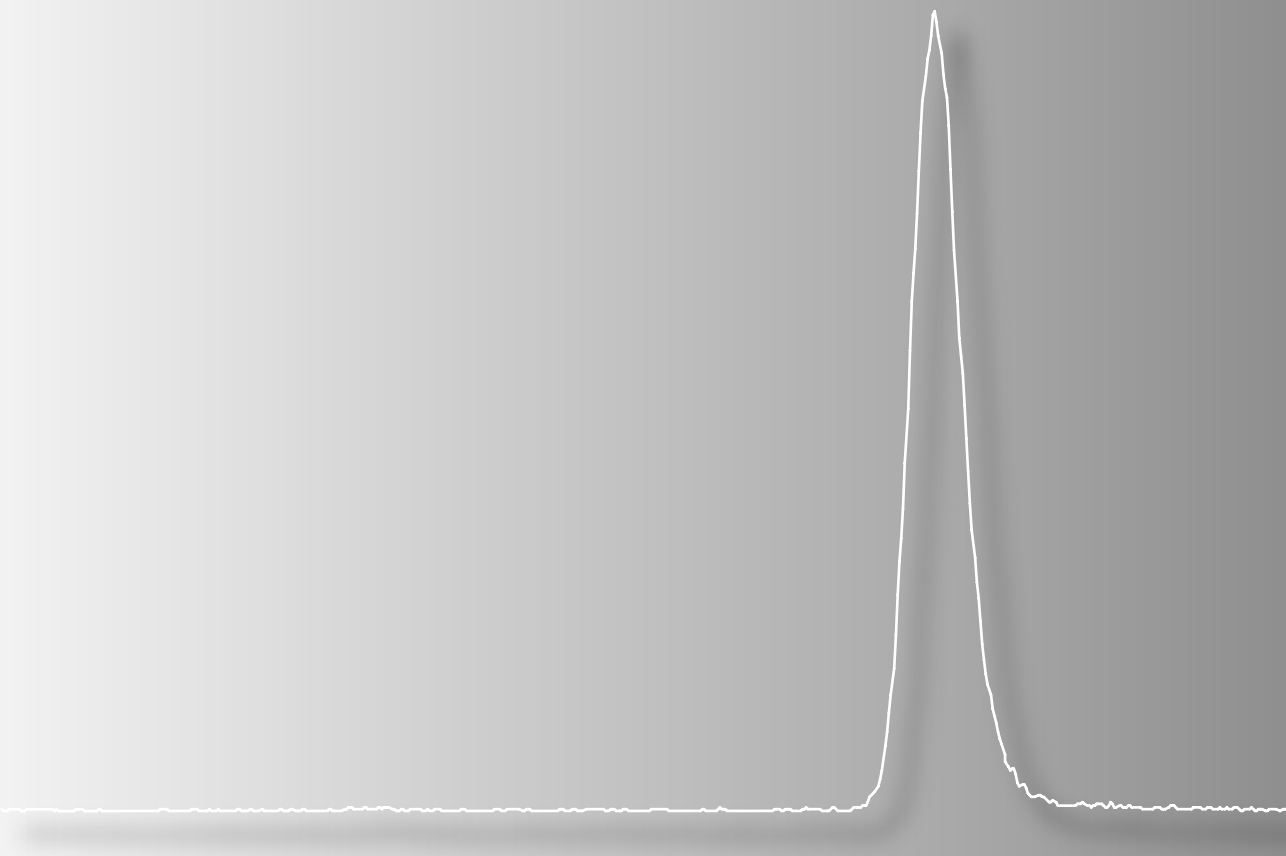
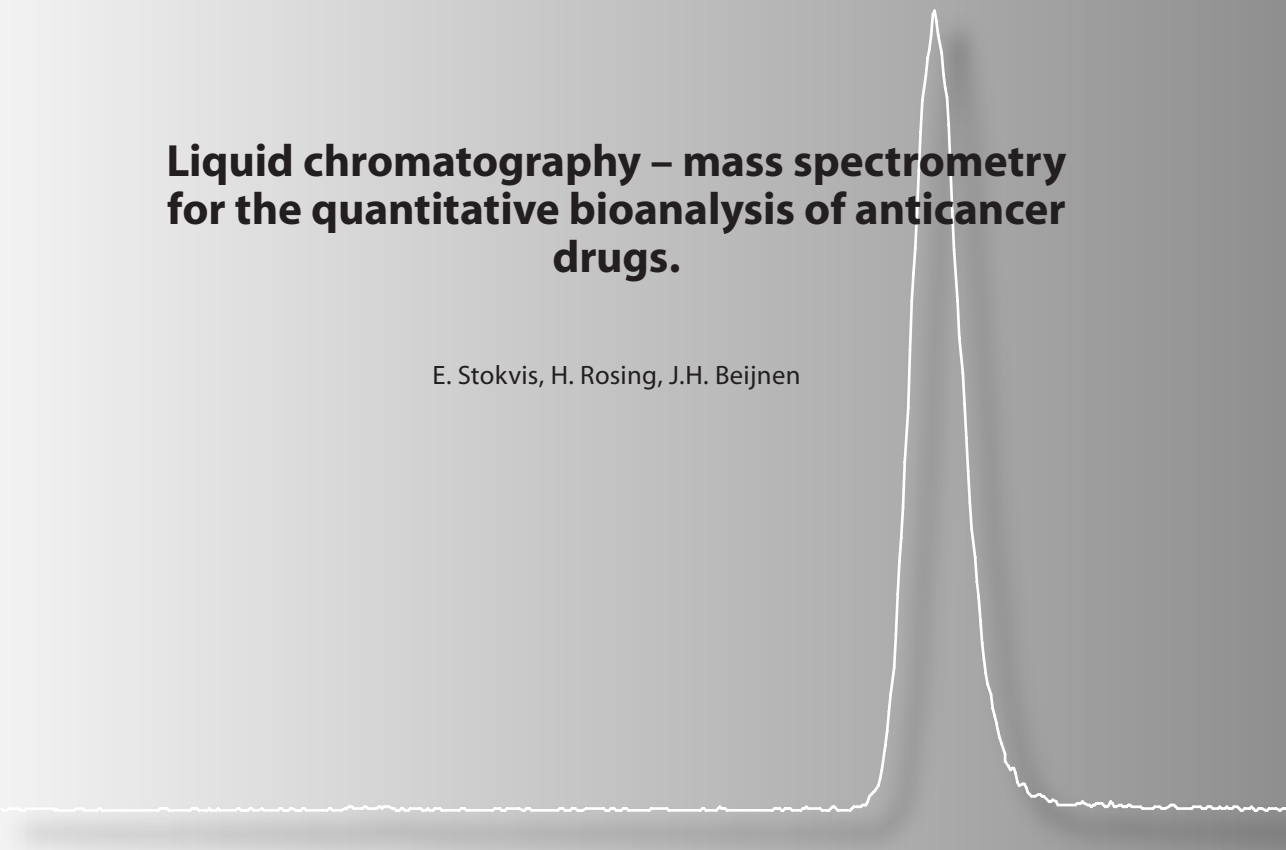
# Chapter 1



## Introduction

# 1.1



## Liquid chromatography – mass spectrometry for the quantitative bioanalysis of anticancer drugs.

E. Stokvis, H. Rosing, J.H. Beijnen

*Submitted*

## Abstract

The monitoring of anticancer drugs in biological fluids and tissues is important during both pre-clinical and clinical development and often in routine clinical use. Traditionally, liquid chromatography (LC) in combination with ultraviolet (UV), fluorescence, or electrochemical detection is employed for this purpose. The successful hyphenation of LC and mass spectrometry (MS), however, has dramatically changed this. MS detection provides better sensitivity and selectivity than UV detection and, in addition, is applicable to a significantly larger group of compounds than fluorescence or electrochemical detection. Therefore, LC-MS has now become the method of first choice for the quantitative bioanalysis of many anticancer agents. There are still, however, a lot of new developments to be expected in this area, such as the introduction of more sensitive and robust mass spectrometers, high-throughput analyses, and further optimization of the coupled LC systems. Many articles have appeared in this field in recent years and are reviewed here. We conclude that LC-MS is an extremely powerful tool for the quantitative analysis of anticancer drugs in biological samples.

## Introduction

The efficacy and toxicity of many anticancer drugs are related to parent drug and/or metabolite concentrations in body fluids and tissues. Therefore, during both pre-clinical and clinical drug development, profound knowledge of the pharmacokinetic properties (absorption-distribution-metabolism-elimination; ADME) of the drug is essential. Furthermore, since anticancer agents often have narrow therapeutic ranges and inter- and intra-patient variability in pharmacokinetic properties are high, drug monitoring in routine clinical use may be required for safe and efficacious therapeutic use [1]. Traditionally, liquid chromatography (LC) has been employed for this purpose. Since most anticancer agents are non-volatile, thermolabile, and polar compounds, they are usually not amendable to gas chromatography (GC). LC-detection involves predominantly ultraviolet (UV), fluorescence, or electrochemical detection. UV detection, however, is not very sensitive and suffers from low specificity, while fluorescence and electrochemical detection, although more sensitive and specific than UV detection, are limited to compounds with fluorescent or electro-active groups or do otherwise require derivatization.

Mass spectrometry (MS), off-line LC-MS for qualitative analysis, as well as on-line GC-MS for quantitative detection, have been common practice for many years. On-line LC-MS for quantitation purposes, on the other hand, was complicated until the introduction of atmospheric pressure ionization (API) techniques (e.g. atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI)) in the 1980s. In recent years LC-MS interfaces have further improved, mainly because techniques such as ionspray (ISP; pneumatically assisted ESI), turbo-ionspray (TISP; pneumatically assisted ESI with an additional hot drying gas perpendicular to the spray), and heated nebulizer (HN)-APCI (pneumatically assisted APCI) became available that facilitate the evaporation of LC solvents. LC-MS

has now become an accepted, wide spread, and commonly used, bench-top technique especially in pharmaceutical analyses.
As a result of the exciting developments and strategies in the field of LC-MS, the number of publications on the quantitative bioanalysis of anticancer drugs using LC-MS has increased tremendously over the last few years. The papers have appeared in a large range of scientific journals covering the many disciplines this research comprises, including medicine, pharmacy, and chemistry. This article gives a selected overview of publications describing the quantitative bioanalysis of anticancer agents using LC-MS, which is one of our own most important research topics.

## Bioanalytical LC-MS/MS assays

Bioanalytical, quantitative LC-MS assays normally consist of three components: sample pretreatment, chromatography and detection. All influence the accuracy, precision, selectivity, and sensitivity of the analytical method.

### Detection

A mass spectrometer is a selective and sensitive LC detector, suitable for the detection of a large range of compounds, provided that the compound can be ionized. Its sensitivity made MS a clear candidate for quantitative analysis, although MS is not quantitative by nature. The MS response depends on a compound's molecular structure and may vary due to several instrument related parameters and experimental conditions. These variations can be corrected by using an internal standard. The most appropriate internal standards for MS are stable isotopically labeled (SIL) internal standards or structural analogues of the analyte, since these compounds are expected to possess similar ionization efficiencies as the analyte. The selectivity of MS detection allows for a less elaborate chromatographic separation and sample pretreatment than for instance UV. Unfortunately, matrix constituents, although not detected, can still interfere with the analysis of the analyte by means of ion suppression. Therefore, sample pretreatment and chromatographic separation for bioanalytical LC-MS assays are usually aimed at the separation of the analytes from the bulk of endogenous compounds.
For quantitative assays employing MS detection triple quadrupole (TQ) systems are most commonly used. When a TQ is operated in the multiple reaction monitoring (MRM) mode the analyte is identified and detected not only by means of its molecular ion but also by means of a typical fragment ion, obtaining higher sensitivity and superior selectivity than for any other MS system. TISP and HN-APCI are normally used as LC-MS interfaces. Both ionization methods can manage LC eluent flow rates up to 1 mL/min without the need for a prior eluent split.

### Chromatography

LC is one of the most widely used bioanalytical chromatographic techniques. A variety of mobile and stationary phases are commercially available to analyze a large range of

compounds. This versatility is often not found in GC, since the mobile phase cannot be varied. In contrary to most LC-UV assays, in bioanalytical LC-MS/MS chromatography is mainly used to separate the analytes from matrix components and not from other analytes, internal standards, or potential metabolites. Co-eluting peaks do not interfere with a correct analysis of the analyte due to the selectivity of the MS. Large levels of matrix components, however, may cause ion suppression and therefore need to be separated from the analyte. Short LC columns are usually sufficient to obtain this separation and thus run times can be reduced substantially.

MS detection is not compatible with each solvent or eluent additive that is commonly used in assays employing LC separation. Normal phase or ion-exchange chromatography for instance can usually not be employed in combination with MS, since their eluents (organic solvents, salts and other additives) are not compatible with the MS. Furthermore, mobile phase additives such as phosphates are undesirable since they contaminate the MS and volatile strong acids such as trifluoroactic acid (TFA), commonly used as ion-pairing agent for chromatography of peptides and proteins, reduce the MS signal dramatically. In LC-MS, reversed phase chromatography is almost exclusively used in combination with eluents that consist of water, methanol, or acetonitrile. Ammonium acetate, ammonium formate, acetic acid, and formic acid are the most commonly used additives for positive ionization LC-MS/MS [2]. Ammonium hydroxide may be used for assays employing negative ionization. Additives are known to improve chromatography, but are also important to stimulate ionization.

Although current interfaces, such as TISP and HN-APCI, are capable of processing traditional LC flow rates of ~1 mL/min, lower flow rates are more suitable for LC-MS assays. The LC system is miniaturized by using analytical columns with smaller internal diameters (ID; 2.1, 2.0 and 1.0 mm) and lower flow rates (<200 µL/min). Since the MS is a concentration sensitive detector the resulting decrease in the amount of sample introduced into the MS does not necessarily influence the sensitivity of the assay. Advantages of miniaturized LC systems are less contamination of the LC-MS interface, less consumption of eluent, and high sensitivity due to less diffusion of the sample during chromatography.

**Sample pretreatment**

In most cases biological samples cannot be assayed directly, but require a pretreatment to dispose the sample of endogenous compounds, such as proteins, carbohydrates, salts, and lipids. In most biological fluids and tissues these compounds are present in large amounts and may interfere with the analysis. In the bioanalysis of pharmaceutical compounds solid phase extraction (SPE) is a commonly used technique for sample pretreatment. SPE is a chromatographic procedure, based on the same principles as LC. Due to the wide range of cartridges and solvents that can be employed, SPE is a versatile technique. However, SPE is also a labor-intensive and often complex procedure. Liquid-liquid extraction (LLE) is especially suited for lipophilic compounds since the analyte transfers from the usually aqueous matrix to an apolar organic phase. This procedure is followed by evaporation of the organic phase, since these solvents cannot be directly injected onto the LC-MS system. Solvents such as hexane or ethers, often used in LLE procedures, are rapidly evaporated

and, by limiting the volume of the solvent used to redissolve the residue, the sample may be concentrated. Protein precipitation is the simplest means of sample pretreatment as it involves only the addition of a precipitating solvent (for instance methanol, acetonitrile, or a perchloric acid solution) and subsequent homogenizing and centrifugation. The clear supernatant may be injected on to the LC-MS system. Protein precipitation, however, is not always sufficient since it leaves many matrix constituents in the sample that may interfere with the assay. Due to the selectivity of MS detectors it was believed that sample pretreatment for LC-MS/MS assays was redundant. Although sample pretreatment for LC-MS/MS assays does not need to be as elaborate as for other LC based assays especially those utilizing UV detection, it remains pivotal to remove matrix components that may contaminate the system or cause ion suppression when high sensitivity is desired. Some matrices do not require an elaborate sample pretreatment since they consist mainly of water, such as cerebrospinal fluid (CSF), tear fluid, and even urine. Nevertheless, the extent of sample pretreatment needed will become evident while setting up the assay.
With decreasing LC run times sample pretreatment has become the rate-limiting step in bioanalytical assays. In order to reduce the time to prepare a sample several forms of automation have emerged in the field of bioanalysis. Automated sample pretreatment or on-line sample pretreatment may be suitable means of time reduction for sample preparation as long as the procedure ensures an adequately removal of interfering matrix components.

**Validation**
Following development of a bioanalytical LC-MS/MS assay and before implementation into clinical pharmacological studies and routine use, it needs to be validated. Validation is essential to ensure the accuracy and precision of the acquired data [3]. In 2001, the Food and Drug Administration (FDA) published guidelines for the validation of bioanalytical assays, which are considered to be the standard for validation parameter assessments and requirements [4]. The guidelines generally apply to bioanalytical procedures such as GC and LC based assays (including GC-MS, LC-MS, GC-MS/MS, and LC-MS/MS), but also immunological and microbiological procedures for the quantitation of drugs and/or metabolites. The guidelines provide general recommendations for the correct assessment of linearity, accuracy and precision, selectivity and specificity, stability, and recovery of the assay. They describe which parameters should be assessed, how they should be assessed, and the requirements that should be met. In addition, the guidelines specifically describe the need to ensure the lack of matrix effects for LC-MS/MS assays [4].

## Alkylating agents

Alkylating anti-cancer agents (Table 1) contain reactive alkyl moieties that can covalently bind to vital cellular components. Particularly DNA cross-linking, by which a link between two complementary DNA strains is formed, interferes with cell proliferation. The alkylating agents are well-known for their reactivity and instability, posing a challenge for

their analysis. The nitrogen mustards are the largest class of alkylating agents.

**Nitrogen Mustards**

Cyclophosphamide and its isomer ifosfamide are oxazaphosphorines or cyclic phosphoramide mustards used in the therapy of various types of cancer. Both compounds are non-cytotoxic prodrugs, that require a bio-activation step to yield the true alkylating agent phosphoramide mustard. Cyclophosphamide is initially metabolized by cytochrome P450 enzymes to form 4-hydroxycyclophosphamide. Subsequently, chemical degradation of aldocyclophosphamide, the tautomer of 4-hydroxycyclophosphamide, results in the formation of phosphoramide mustard and acrolein (Figure 1). Ifosfamide is metabolized in a similar way as cyclophosphamide. It is desirable to determine both pro-drug and phosphoramide mustard concentrations, however, since phosphoramide mustard is located mainly inside cells, 4-hydroxycyclophosphamide or 4-hydroxyifosfamide concentrations are often determined instead. Measuring the 4-hydroxy compounds in plasma, however, is difficult, since they are very unstable, and therefore have to be trapped by derivatization

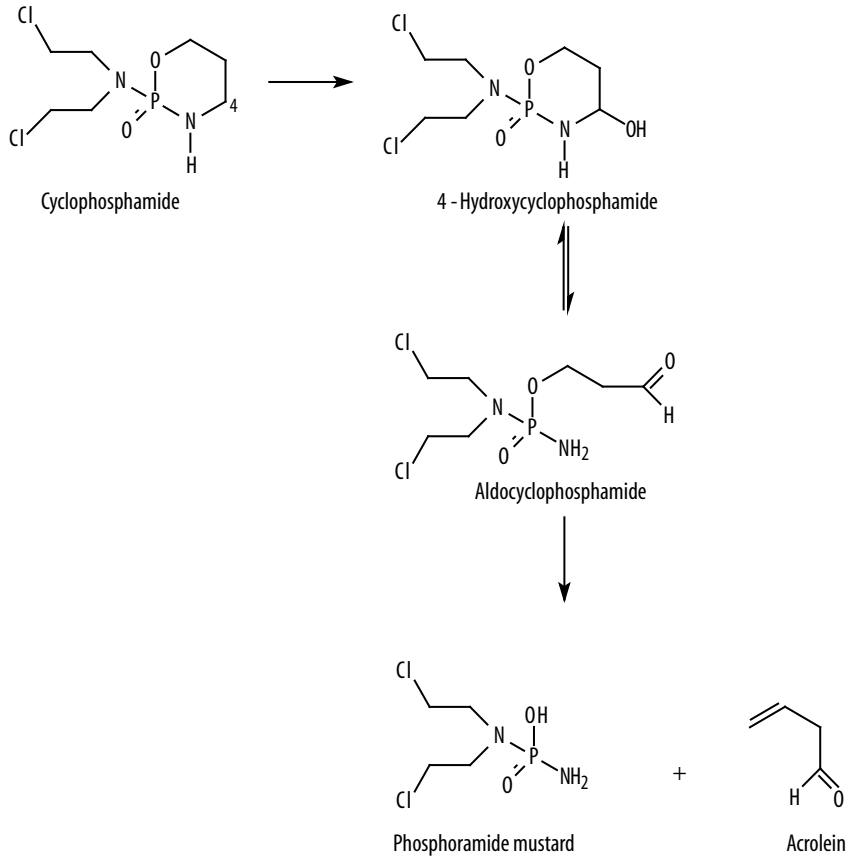



**Figure 1.** Metabolic pathway of the alkylating agent cyclophosphamide.

**Table 1.** Selected overview of LC-MS methods for alkylating agents in various biological matrices

| Compound | Matrix | Sample (µL) | Sample Pretreatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. Quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cyclophosphamide | Human urine | 5000 | LLE | I: M-AA pH4 | ESI (+) | MRM | None | 0.2 | 10 | No | Monitoring hospital personnel | [14] |
| Ifosfamide | Human urine | 5000 | LLE | I: M-AA pH4 | ESI (+) | MRM | None | 0.2 | 10 | No | Monitoring hospital personnel | [14] |
| Cyclophosphamide | Human plasma | 450 | SPE | I: M-AA,HA | ESI (+) | SIM | An. | 50 | 20 | Yes | | [7] |
| Cyclophosphamide | Human plasma | 100 | PP | G: A-AH | ESI (+) | MRM | An. | 200 | 9 | Yes | | [8] |
| Thiotepa | Human plasma | 100 | PP | G: A-AH | ESI (+) | MRM | An. | 5 | 9 | Yes | | [8] |
| Cyclophosphamide | Mouse plasma | 250 | PP | I: A-W-F | ESI (+) | MRM | An. | 3.1 | 2.5 | Yes | | [6] |
| Cyclophosphamide | Mouse spleen | 25 mg | PP | I: A-W-F | ESI (+) | MRM | An. | 1250 ng/g | 2.5 | Yes | | [6] |
| Phosphoramide mustard | Human plasma | 500 | PP | I: M-AA pH4 | ESI (+) | SIM | SIL | 660 | 7 | - | Metabolite of cyclophosphamide | [18] |
| Chlorambucil | Human plasma | 200 | Aut. SPE | I: A-W-F | ESI (+) | MRM | SIL | 4 | 2.5 | Yes | | [20] |
| Chlorambucil | Human serum | 200 | Aut. SPE | I: A-W-F | ESI (+) | MRM | SIL | 4 | 2.5 | Yes | | [20] |
| Melphalan | Human plasma | 200 | Aut. SPE | I: A-W-F | ESI (+) | MRM | SIL | 2 | 2.5 | No | | [21] |
| Melphalan | Human serum | 200 | Aut. SPE | I: A-W-F | ESI (+) | MRM | SIL | 2 | 2.5 | No | | [21] |
| Thiotepa-mercapturate | Human urine | 90 | None | I: A-AA pH4.8 | ESI (+) | Q1 | Other | 1000 | 14 | - | Metabolite of thiotepa | [24] |
| Busulfan | Human plasma | 200 | LLE | I: M-AA | ESI (+) | SIM | SIL | 5 | 10 | No | | [26] |
| Busulfan | Human plasma | 200 | LLE | G: A-AA,HA | ESI (+) | SIM | SIL | 5 | 11 | No | | [27] |
| MTIC | Rat plasma | 70 | PP | I: M-AA,F | ESI (+) | MRM | An. | 10 | 4.5 | No | | [28] |
| MTIC | Dog plasma | 70 | PP | I: M-AA,F | ESI (+) | MRM | An. | 10 | 4.5 | No | | [28] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; AF Ammonium formate in water; AH Ammonium hydroxide in water; An. Structural analogue; Aut. Automated; ESI Electrospray ionization; F Formic acid; G Gradient elution; HA Acetic acid; I Isocratic elution; IS Internal standard; LC Liquid Chromatography; LLOQ Lower limit of quantitation; LLE Liquid-liquid extraction; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal standard; SIM Selective ion monitoring mode; SPE Solid phase extraction; W Water.

to stop the rapid chemical and enzymatic degradation ($t_{1/2}$ = 6 min) [5]. Derivatization with reagents such as methyl-hydroxylamine [6,7], semicarbazide [8], *p*-nitrophenylhydrazine [9], potassium cyanide [10], and 4-aminophenol/hydroxyl-aminehydrochloride [11] have been described. Derivatization with semicarbazide or methyl-hydroxylamine is relatively straightforward compared to the more complex procedures using the other derivatization reagents [6]. Derivatization with methyl–hydroxylamine yields the stable methyloxime of the 4-hydroxy metabolite [6,7], and requires an incubation time of 5 minutes at 50 °C. On the other hand, derivatization with semicarbazide, yielding 4-hydroxycyclophosphamide-semicarbazide [8], requires incubation of the sample for approximately 2 hours at 35 °C.

Due to the instability of oxazaphosphorines for high GC temperatures and the lack of chromophores for LC-UV analysis, LC-MS was considered more appropriate even before this hyphenation was accomplished on-line [12,13]. On-line LC-MS has been successfully applied to the quantitative analysis of oxazaphosphorines in human urine [14], human plasma [7,8] and mouse plasma and spleen tissue [6]. Cyclophosphamide and ifosfamide were determined in low concentrations in urine of hospital personnel involved in the preparation and administration of antineoplastic drugs. The assay also included methotrexate and platinum compounds, but no metabolites. LLE with ethylacetate was the preferred technique to obtain the high sensitivity needed for drug determination after occupational exposure [14].

SPE on $C_{18}$ cartridges was used for the extraction of cyclophosphamide and its metabolites 4-hydroxycyclophosphamide, 4-ketocyclophosphamide, carboxyphosphoramide, and 3-dechloroethylifosfamide from human plasma [7]. SPE extraction is a labor-intensive procedure, but it allowed the determination of all metabolites of cyclophosphamide. Fast and simple protein precipitation with methanol-acetonitrile, however, has also resulted in high recoveries for cyclophosphamide and 4-hydroxycyclophosphamide from human plasma (89-100%) [8]. In addition, the use of protein precipitation with acetonitrile to extract cyclophosphamide and 4-hydroxycyclophosphamide from mouse plasma and spleen tissue homogenate yielded the highest sensitivity for cyclophosphamide in plasma reported so far (3.1 ng/mL from 100 µL of plasma) [6]. The latter assays, however, did not include as many metabolites.

The assay for the determination of cyclophosphamide and 4-hydroxycyclophosphamide from human plasma described by the Jonge at al. also included the alkylating ethyleneimine thiotepa and its metabolite tepa [8]. The simultaneous analysis of these compounds was necessary for fast dose-adjustment within 24 hours in a high-dose chemotherapy study to prevent drug concentration related toxicity or treatment failure. Interestingly, the assay employs an alkaline mobile phase containing ammonium hydroxide combined with LC-MS/MS in the positive ion mode, while the other assays employ acidic eluents containing ammonium acetate and acetic acid, or formic acid. The generation of positive ions from an eluent containing ammonium hydroxide probably results from gas-phase ion-molecule reactions between ammonium cations and the analyte molecule or collision-induced dissociation of ammonium adducts of the analyte yielding in both cases the protonated analyte and an ammonia molecule [15]. This analytical approach has demonstrated to be very appropriate for the quantitative analysis of other basic anticancer

drugs [16,17].
Kalhorn et al. describe the analysis of three metabolites of cyclophosphamide, phosphoramide mustard, 3-hydroxypropylphosphoramide mustard, and carboxy-ethylphosphoramide mustard using LC-MS. Cyclophosphamide itself, as well as metabolites 4-oxocyclophosphamide and deschloroethylcyclophosphamide were determined using GC-MS [18]. Protein precipitation with methanol-acetonitrile was used for the extraction of the analytes intended for LC-MS analysis from plasma. For phosphoramide mustard a quadruply SIL internal standard was available but its analysis was complicated because the parent ion mass overlaps with the mass of the analyte's $^{37}Cl_2$ containing isotope. This problem was solved by monitoring the internal standard's $^{35}Cl^{37}Cl$ isotope (60% intensity compared to the $^{35}Cl_2$ isotope). Mutual interference in both channels was less than 1%. The triple quadrupole MS, although capable of MRM analysis, was used in the less sensitive and specific selective ion monitoring (SIM) mode. The authors describe that the SIM mode was appropriate to obtain the required sensitivity for support of clinical studies involving high-dose cyclophosphamide treatment, and, in addition, using SIM mode, a method was created that is adaptable to as many laboratories as possible [18].
Chlorambucil, an aromatic nitrogen mustard derivative, is mostly used in the treatment of lymphatic disorders. The drug is rapidly and extensively metabolized to form the cytotoxic metabolite phenyl acetic acid mustard. Several analytical methods employing LC separation followed by UV detection have been reported, as was reviewed by Paci et al. [19]. Davies et al. exclusively described an LC-MS/MS method for the monitoring of chlorambucil and its metabolite in both human serum and plasma [20]. This method is more rapid and at least ten-fold more sensitive than LC methods employing UV detection and has been used successfully for pharmacokinetic support in biocomparability studies with chlorambucil. This assay was later adjusted for the bioanalysis of another aromatic mustard, melphalan, in human plasma and serum [21]. In contrast with chlorambucil, melphalan does not generate active metabolites but undergoes rapid chemical degradation. In order to adapt the chlorambucil assay for the analysis of melphalan, only small adjustments were needed, such as a different proportion of acetonitrile and water in the eluent and a different analytical column [21].

**Others**

The ethyleneimine thiotepa has been applied in cancer therapy for more than 40 years [22]. Its major metabolite tepa is rapidly generated in the liver and has antineoplastic activity. Bioanalysis of thiotepa and tepa has been described using GC-nitrogen phosphorous detection (NPD) [23]. De Jonge and co-workers describe the first LC-MS/MS assay for the quantitative analysis of thiotepa and tepa [8]. This assay also includes cyclophosphamide and 4-hydroxycyclophosphamide and was discussed in the previous paragraph.
Recently, a new metabolite of thiotepa with alkylating capacity was identified in urine, thiotepa-mercapturate [24]. Due to the metabolite's polarity assays based on GC were not appropriate. For the determination of thiotepa-mercapturate in human urine an assay using LC with MS detection was described by van Maanen et al. [24]. Single quadrupole MS scanning in the positive ion mode was used. For thiotepa-mercapturate (MW 352 Da) a

mass range from m/z 350 to 360 was scanned. To 90 µL of urine 10 µL of a solution of the internal standard sulphadiazine was added and 10 µL aliquots of this mixture were directly injected onto the analytical column. Urine samples of patients treated with thiotepa were processed and 6.2-16.4% of the dose was excreted in urine as thiotepa-mercapturate. Despite the structural resemblance of tepa with thiotepa, tepa-mercapturate could not be detected in patient's urine [24].

The alkyl sulfonate busulfan (1,4-butanediol dimethanesulfonate) is a bifunctional agent, used since the 1950s at low doses (µg/kg ranges) for the treatment of chronic myelogenous leukemia or at high doses (mg/kg ranges) concomitantly with cyclophosphamide followed by bone marrow transplants. When administered at high doses with cyclophosphamide, the major dose limiting toxicity is hepatic veno-occlusive disease, which is believed to be related to drug levels in plasma. As a result of this and the high inter- and intra-individual variability in busulfan plasma concentrations after oral administration, therapeutic drug monitoring is considered essential for individual optimization of busulfan therapy. Busulfan is a very polar compound with poor UV absorbancy. For both LC-UV and LC-fluorescence assays derivatization is required to increase sensitivity. Derivatization was also needed for GC based assays. Already in the early days of LC-MS hyphenation its potentials for the determination of busulfan were recognized. LC-MS analysis of busulfan in serum and CSF of children using a particle beam interface and electron impact (EI) ion source is described by Pichini and co-workers [25]. Several typical ions observed in the EI mass spectrum of busulfan were monitored. Two LLE procedures using dichloromethane were performed on 500 µL serum and 1000 µL of CSF yielding LLOQs of 100 ng/mL busulfan in both matrices [25]. With current LC-MS systems sensitivity for busulfan has increased. Two LC-TISP-TQ-MS methods for busulfan in human plasma have been published [26,27]. Quernin et al. describe an LLE procedure with ethyl acetate followed by isocratic reversed phase chromatography, while Mürdter et al. report an extraction using dietlylether and subsequent gradient elution from a reversed phase analytical column. Both methods employed eluents containing ammonium acetate and ammonium adducts of busulfan were monitored in MRM rather than the more commonly observed protonated molecular ions. Similar results were obtained for sensitivity and run time using the two methods [26,27].

MTIC (5-(3-*N*-methyltriazen-1-yl)-imidazole-4-carboxamide) is a metabolite of temozolomide formed by hydrolysis. The antitumor activity of temozolomide is believed to result from the potent alkylating ability of MTIC. MTIC is a highly unstable compound. Chowdhurry et al. were the first researchers to publish a bioanalytical assay for the determination of MTIC in rat and dog plasma. Stability experiments indicated very poor benchtop, freeze-thaw, in process, and autosampler storage stability. Therefore, samples were processed one at a time using a simple protein precipitation procedure with ice-cold reagents and were then analyzed immediately. Validation data on accuracy and precision demonstrated that the assay's performance was excellent. The method was successfully applied to preclinical toxicokinetic and drug safety studies following oral dosing of temozolomide [28].

**Table 2.** Selected overview of LC-MS methods for anti-metabolites in various biological matrices

| Compound | Matrix | Sample (µL) | Sample Pretreatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. Quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Methotrexate | Human plasma | 200 | PP + LLE 96 well | I: A-W-F | ESI (+) | MRM | SIL | 0.1 | 1.2 | Yes | Additional column switching | [29] |
| Methotrexate | Human plasma | 20 | SPE 384 well | I: A-W-F | ESI (+) | MRM | SIL | 5 | 1.9 | Yes | | [30] |
| Methotrexate | Human urine | 20 | SPE 384 well | I: A-W-F | ESI (+) | MRM | SIL | 1000 | 1.9 | Yes | | [30] |
| Methotrexate | Human urine | 5000 | SPE | I: M-AA pH4 | ESI (+) | MRM | An. | 500 | 8 | No | Monitoring hospital personnel | [14] |
| 5-Fluorouracil | Human plasma | 500 | LLE | I: A-AA pH4.5 | APCI (-) | MRM | SIL | 1 | 3.5 | No | Derivatization required | [31] |
| 5-Fluorouracil | Human plasma | 500 | PP + SPE | I: M-AF | ESI (-) | MRM | SIL | 3 | ~4 | Yes | Metabolite of capecitabine | [32] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; AF Ammonium formate in water; An. Structural analogue; APCI Atmospheric pressure chemical ionization; ESI Electrospray based ionization; F Formic acid; I Isocratic elution; IS Internal standard; LC Liquid chromatography; LLE Liquid-liquid extraction; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal standard; SPE Solid phase extraction; W Water.

## Anti-metabolites

### Folate Antagonists

The folic acid antagonist methotrexate (amethopterin, Table 2) has been in use since the 1950s in the therapy of solid tumors and leukaemias. After cellular uptake methotrexate is poly-glutamated and inhibits DNA and RNA synthesis, which eventually causes cell death. It is a very cytotoxic agent. Methotrexate is a polar compound and its main metabolite 7-hydroxy-methotrexate although expected to be even more polar than methotrexate, is known to be less polar due to an intra-molecular hydrogen bonding between the hydroxyl and one of the carboxylic acid groups (Figure 2).

Mass spectrometric analysis of methotrexate was not possible until the development of modern techniques such as electrospray ionization, due to the polarity and consequent low volatility and thermal instability of the drug. So far, three assays for the LC-MS/MS analysis of methotrexate have been published [14,29,30]. A very rapid and sensitive assay for the determination of methotrexate and 7-hydroxymethotrexate in human plasma is described by Steinborner and Henion [29]. Sample processing is performed by means of protein precipitation with acetonitrile followed by LLE with chloroform in 96-well plates, and all steps were automated. Aliquots of 80 µL of the aqueous layer were injected on to a trapping column, followed by a transfer of the analytes to a narrow bore (1 mm I.D.) analytical column by column switching. Excellent sensitivities were obtained within 1.2 min per sample [29]. Later, researchers from the same group described the potential of using SPE in a 384-well format as a means of sample pretreatment for this assay. Both LLOQ and cycle time, however, are higher for the latter assay [30].

An assay used for the monitoring of hospital personnel potentially exposed to cytotoxic drugs has also been developed for methotrexate [14]. The method utilizes large volumes of urine and the LLOQ determined for methotrexate was relatively high (500 ng/mL). The assay also included the monitoring of cyclophosphamide, ifosfamide and platinum and has been described in the previous chapter [14].

### Others

The pyrimidine antagonist 5-fluorouracil is one of the most commonly used anticancer agents. Bioanalysis of the drug is complex, since it is hydrophilic, which makes it cumbersome to separate it from endogenous substances. Another problem associated with the analysis of 5-fluorouracil is a rapid build up of less polar endogenous compounds on the analytical column because of the low percentage of modifier normally present in eluents

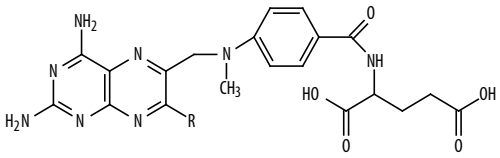



**Figure 2.** Structures of methotrexate (R=$CH_3$) and its metabolite 7-hydroxymethotrexate (R=OH).

used for the chromatographic separation of the drug. In addition, because the drug has a very low molecular weight, selective MS detection is problematic as well [31].
Wang et al. describe the quantitative analysis of 5-fluorouracil using LC-MS/MS [31]. In positive ion mode ESI or APCI the drug gave practically no response, probably because it is a weak acid. In negative APCI mode it yielded a good response, however target LLOQs were not achieved due to a high chemical background noise probably caused by 5-fluorouracil sticking in the APCI source. This problem as well as difficulties associated with chromatography of 5-fluorouracil were solved by using a derivatization step. Derivatization was performed with 4-bromo-methyl-7-methoxycoumarin to yield a di-derivatized product with a much higher molecular weight and lipophilicity (Figure 3). Derivatized 5-fluorouracil was eluted from the reversed phase column using an eluent containing 65% organic modifier and the observed background noise was low enough to achieve an LLOQ of 1 ng/mL from 500 µL of plasma [31].
5-Fluorouracil can also be chromatographed without prior derivatization as demonstrated by Reigner et al. Capecitabine and its metabolites, including 5-fluorouracil have been analyzed using LC-UV and two LC-MS/MS methods [32]. The use of three methods was needed because of the wide range of polarities for capecitabine and its metabolites. Capecitabine is a prodrug, which eventually forms 5-fluorouracil at the tumor site by a tumor associated angiogenic factor, and thereby the exposure to 5-fluorouracil is mainly limited to the tumor site. Following extraction of plasma samples with acetonitrile, the supernatant was loaded onto $C_{18}$ SPE cartridges from which elution with methanol yielded fraction A containing capecitabine, which was analyzed by LC-UV. Elution with an ammonium acetate solution yielded fraction B, containing 5-fluorouracil and its metabolite dihydro-5-fluorouracil. The compounds were analyzed without prior derivatization using an eluent of methanol - 5 mM ammonium formate in water (15:85, v/v) with LC-MS/MS in the negative ion mode. Metabolite $\alpha$-fluoro-ß-alanine was derivatized under basic conditions. The derivatization agent is described as diphenylfranone. The compound's IUPAC name or structure is, however, not specified. Derivatized $\alpha$-fluoro-ß-alanine was analyzed using an eluent of acetonitrile – 5 mM ammonium formate (30:70, v/v). All other conditions were similar for the two LC-MS/MS methods [32]. The authors have demonstrated that derivatization is no longer required for the LC-MS/MS analysis of 5-fluorouracil. However, this assay is threefold less sensitive than the method employing derivatization of the drug and, in addition, for the analysis of metabolites or prodrug of 5-fluorouracil, multiple preparation steps and analyses are required.

## Antimitotic agents

Antimitotic agents (Table 3) bind to tubulin and thereby inhibit cell division. This class of anticancer agents predominantly consists of the *vinca* alkaloids and the taxanes, although other compounds with antimitotic features have been introduced recently.

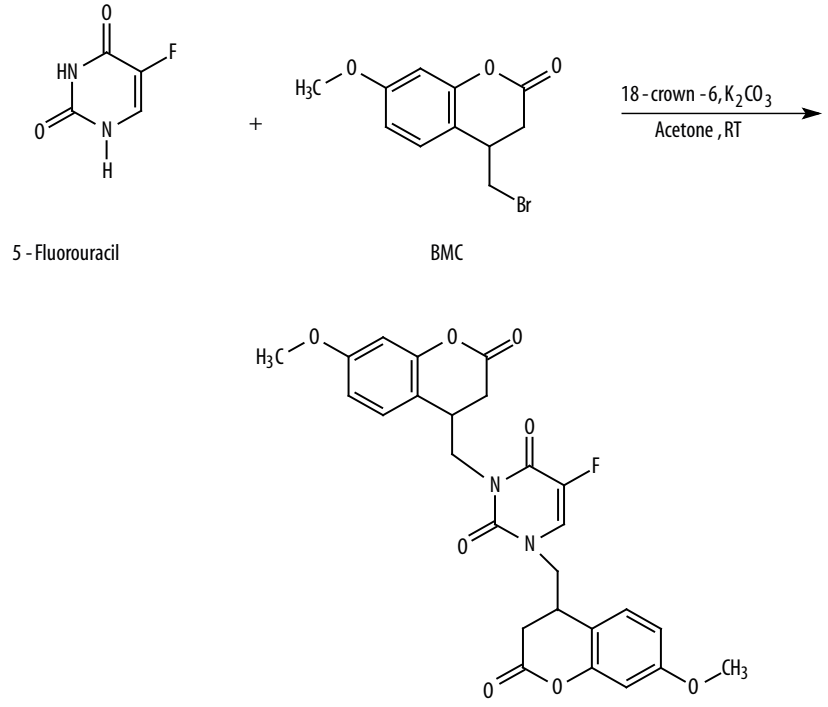



**Figure 3.** Derivatization of 5-fluorouracil with 4-bromo-methyl-7-methoxycoumarin (BMC).

**Vinca Alkaloids**

The *vinca* alkaloids vincristine and vinblastine are isolated from the plant *Vinca rosea L*, while vinorelbine and vindesine are semi-synthetic derivatives of vinblastine. Most methods reported for the bioanalysis of the *vinca* alkaloids describe the use of radioimmuno assays or LC methods with UV, fluorescence or electrochemical detection (references in [33]).

Ramírez et al. describe in their paper that LC-MS/MS was needed in order to determine vincristine, vinblastine, and the vinblastine metabolite desacetylvinblastine in human plasma with high selectivity and sensitivity [33]. They present three separate methods, one for each analyte, that are similar apart from the LC eluent and compound related MS parameters. Low LLOQs were obtained for all three compounds, however, rather large sample volumes (up to 2.0 mL) were used [33].

In 2001, the bioanalysis of vinorelbine in human serum [34], blood, plasma, urine, and faeces [35] was reported. Both methods apply reversed phase chromatography followed by tandem MS detection in the MRM mode using vinblastine as internal standard. For the extraction of vinorelbine from 500 µL of serum SPE is used and gradient elution using a mixture of acetonitrile and ammonium formate in water (pH 3). Run time was 15 min and an LLOQ of 0.5 ng/mL was obtained [34]. Van Heugen et al. obtained a lower LLOQ for vinorelbine from human plasma using the same amount of sample [35]. In addition, this

**Table 3.** Selected overview of LC-MS methods for antimitotitc agents in various biological matrices

| Compound | Matrix | Sample (µL) | Sample preparation | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. Quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vinblastine | Human plasma | 1500 | LLE | I: A-AA | APCI (+) | SIM | An. | 0.51 | 4 | Yes | | [33] |
| Vincristine | Human plasma | 2000 | LLE | I: M-AA | APCI (+) | SIM | An. | 0.3 | 3 | No | | [33] |
| Vinorelbine | Human serum | 500 | SPE | G: A-AF pH 3 | ESI (+) | MRM | An. | 0.5 | 15 | Yes | | [34] |
| Vinorelbine | Human plasma | 500 | PP | I: A-AA-F pH 3 | ESI (+) | MRM | An. | 0.25 | 20 | Yes | | [35] |
| Vinorelbine | Human blood | 500 | PP | I: A-AA-F pH 3 | ESI (+) | MRM | An. | 0.5 | 20 | Yes | | [35] |
| Vinorelbine | Human urine | 1000 | - | I: A-AA-F pH 3 | ESI (+) | MRM | An. | 2.5 | 20 | Yes | | [35] |
| Vinorelbine | Human faeces | 100 mg | SLE | I: A-AA-F pH 3 | ESI (+) | MRM | An. | 2 | 20 | Yes | | [35] |
| Paclitaxel | Human plasma | 500 | Aut. SPE | I: A-W-F | ESI (+) | MRM | An. | 5 | 8 | No | | [38] |
| Paclitaxel | Dog plasma | 100 | LLE | I: A-W-F | ESI (+) | MRM | An. | 2 | 6 | No | LC-UV: 10 ng/mL 500 µL, SPE, 15 min | [45] |
| Paclitaxel | Human plasma | 400 | LLE | I: A-W-HA | ESI (+) | MRM | SIL | 0.1 | 3.5 | Yes | | [39] |
| Paclitaxel | Dog plasma | 400 | LLE | I: A-W-HA | ESI (+) | MRM | SIL | 0.1 | 3.5 | Yes | | [39] |
| Paclitaxel | Human plasma | 100 | Aut. LLE | I: A-AA pH 5 | ESI (+) | MRM | An. | 1 | 4.5 | No | | [43] |
| Paclitaxel | Human serum | 100 | On-line SPE | - | APCI (-) | MRM | None | 1 | 1.33 | No | On-line SPE-MS, no LC | [44] |
| Paclitaxel | Mouse plasma | 50 | SPE | I: A-W-F | ESI (+) | SIM | Other | 36 | 8 | No | | [46] |
| Paclitaxel | Mouse brain | 50 | SPE | I: A-W-F | ESI (+) | SIM | Other | 54 | 8 | No | | [46] |
| Paclitaxel | Human plasma | 1000 | SPE | I: M-W-F | ESI (+) | SIM | An. | 0.853 | 7 | No | | [40] |
| Docetaxel | Human plasma | 1000 | SPE | I: M-W-F | ESI (+) | SIM | An. | 0.242 | 7 | No | | [40] |
| Docetaxel | Human plasma | 50 | LLE | I: A-W-F | ESI (+) | MRM | An. | 5 | 5 | No | | [41] |
| Docetaxel | Human tear fluid | NS | - | G: M-AA pH 8.5 | ESI (-) | MRM | None | 10 ng | 5 | No | | [42] |
| Docetaxel | Human plasma | 1000 | SPE | G: M-AA pH 8.5 | ESI (-) | MRM | None | NS | 5 | No | | [42] |
| BAY59-8862 | Mouse plasma | 200 | SPE | I: A-AA pH 5 | ESI (+) | MRM | An. | 50 | 12 | No | Metabolites identified | [47] |
| BAY59-8862 | Mouse liver | 2.5 mg | PP | I: A-AA pH 5 | ESI (+) | MRM | An. | 50 ng/g | 12 | No | Metabolites identified | [47] |
| Dolastatin-10 | Human plasma | 1000 | LLE | G: A-W | ESI (+) | NS | An. | 0.005 | 4.2 | Yes | | [48] |

**Table 3.** Selected overview of LC-MS methods for antimitotitc agents in various biological matrices - continued

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TZT-1027 | Human plasma | 1000 | SPE | I: A-W-TFA | APCI (+) | SIM | SIL | 0.25 | 5 | No | [49] |
| TZT-1027 | Dog plasma | 1000 | SPE | I: A-W-TFA | APCI (+) | SIM | SIL | 0.25 | 5 | No | [49] |
| TZT-1027 | Rat plasma | 1000 | SPE | I: A-W-TFA | APCI (+) | SIM | SIL | 0.25 | 5 | No | [49] |
| TZT-1027 | Monkey plasma | 500 | SPE | I: A-W-TFA | APCI (+) | SIM | SIL | 0.5 | 5 | No | [49] |
| TZT-1027 | Mouse plasma | 100 | SPE | I: A-W-TFA | APCI (+) | SIM | SIL | 2.5 | 5 | No | [49] |
| LY355703 | Dog plasma | 100 | SPE | I: A-W-2P | APCI (+) | MRM | An. | 2.1 | 2.5 | No | [50] |
| LY355703 | Mouse plasma | 100 | SPE | I: A-W-2P | APCI (+) | MRM | An. | 2.1 | 2.5 | No | [50] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; AF Ammonium formate in water; An. Structural analogue; APCI Atmospheric pressure chemical ionization; ESI Electrospray ionization; F Formic acid; G Gradient elution; HA Acetic acid; I Isocratic elution; IS Internal standard; LC Liquid Chromatography; LLE Liquid-liquid extraction; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified ; MRM Multiple reaction monitoring mode; MS Mass Spectrometry; NS Not Specified; 2P 2-Propanol; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal standard; SIM Selective ion monitoring mode; SLE Solid-liquid extraction; SPE Solid phase extraction; UV Ultraviolet; W Water.

assay used protein precipitation and isocratic elution which are more straightforward and less time-consuming than SPE and gradient elution described above [34], although the run time was longer [35].

**Taxanes**

The tubulin inhibitor paclitaxel (Taxol®) is derived from the Pacific yew tree *Taxus brevolia*. It is presently used in therapy for breast, lung, and ovarian cancer and is being investigated for other therapeutic indications. Paclitaxel has three known but less active metabolites 6-α-hydroxy paclitaxel, 3'-*p*-hydroxy paclitaxel, and 6-α-3'-*p*-dihydroxy paclitaxel. Docetaxel (Taxotere®) is a semisynthetic and more potent analogue of paclitaxel. For docetaxel, hydroxylation also constitutes the major metabolic pathway.

Many assays for the quantitative determination of paclitaxel and docetaxel have been described using LC-UV (e.g. [36,37]). However, they all suffer from relatively low sensitivity (typically LLOQ > 5 ng/mL; 1,000 µL sample volume). With current developments of the taxanes there is a need for more sensitive assays and MS has become the detection method of choice. LC-MS/MS assays have been described for the quantitative determination of paclitaxel and/or docetaxel in human plasma [38-43], human serum [44], human tear fluid [42], dog plasma [39,45], and mouse plasma and brain tissue [46]. The first LC-MS/MS assay for the quantitative determination of a taxane was published by Sottani and co-workers for paclitaxel in human plasma. Apart from a two- to four-fold increase in sensitivity, the LC run time could be decreased compared to the LC-UV assays, due to the superior specificity of MS [38]. Baldrey and co-workers report similar observations. They have compared LC-UV and LC-MS/MS for the determination of paclitaxel in dog plasma. The selectivity of the MS detector allowed for a more than 50% reduction of the LC run time and, in addition a 25-fold increase in sensitivity [45].

For the extraction of the analytes and internal standards from the matrix either SPE [38,40,42,44,46] or LLE methods are used [39,41,43,45]. LLE was performed using either tert-butylmethylether [39,41,43] or di-ethylether [45]. LLE extraction is more rapid and straightforward than SPE procedures and good results for sensitivity of the taxanes are obtained after LLE procedures. In fact, the highest sensitivity reported so far for paclitaxel was after extraction of the drug from human and dog plasma by LLE with tert-butylmethylether (0.1 ng/mL from 400 µL plasma volumes) [39]. In addition, this article by Alexander et al. demonstrated that the extraction procedure was also very appropriate for the more polar hydroxylated metabolites of paclitaxel. 6-α-Hydroxy paclitaxel and 3'-*p*-hydroxy paclitaxel were quantified with the same high sensitivity as paclitaxel. 6-α-3'-*p*-dihydroxy paclitaxel was not determined. This the first assay describing the use of a SIL internal standard for paclitaxel, $^{13}C_6$-paclitaxel, while traditionally docetaxel has been used for this purpose. Excellent results on accuracy and precision were obtained during the validation according to FDA regulations as presented in 2001 [4,39].

Since the taxanes are weak basic drugs, positive ionization in the MS source is very suitable. There are two articles, on the other hand, describing negative ionization for a taxane [42,44], however, both without explaining the reason for it, in stead of the more commonly used positive ionization. Schellen et al. published an assay for the determina-

tion of paclitaxel using on-line SPE-negative ion APCI-MS/MS, without the use of additional LC separation [44]. The assay's sensitivity is comparable to the sensitivity of other assays, but it is the most rapid with a total cycle time of only 1.33 min. A dual Prospekt system (Spark Holland) was used for on-line parallel SPE and several solvents were employed for solvation, equilibration, sample application, and clean-up. Good performance was obtained for this assay, even without the use of an internal standard [44]. Based on a hypothesis that docetaxel may be secreted in tear fluid after intravenous infusion, Esmaeli and co-workers developed an assay for the determination of docetaxel in this unusual matrix [42]. Tear fluid samples were assayed without sample pretreatment, and plasma samples, also included in the assay, were processed using SPE. Collected tear volumes typically ranged from 15 to 30 µL, and an LLOQ of 10 ng was reported; the injection volume was not specified. The triple quadrupole MS was used in the negative ion mode. Docetaxel was found in tear fluid of patients and the concentrations ranged from 14.1 to 69.4% of the docetaxel plasma concentrations [42].
An assay for the determination of the taxane derivative, BAY59-8862, using LC-MS/MS has been published [47]. BAY59-8862 was determined in mouse plasma and liver. Apart from using the MS in the MRM mode for quantitative analysis in murine samples, it was also used in the full scan mode for the characterization of the drug's metabolic profile in rat bile samples. Several mono- and dihydroxylated metabolites were identified [47].

**Others**

The antimitotic and anticancer properties of dolastatin-10 have been identified decades ago, but only a few years ago a phase I trial of the drug has commenced [48]. Dolastatin-10 is a unique, linear, lipophilic pentapeptide isolated from the sea hare *Dolabella auricularia,* consisting of several unusual amino acids, such as dolavaline and dolaisoleucine (Figure 4). Since the starting doses of dolastatin-10 in the phase I trial were in the low mg/m$^2$ range a sensitive method was needed. A quantitative assay for dolastatin-10 in human plasma and for the identification, structural elucidation, and quantification of metabolites of dolastatin-10 was developed [48]. Unfortunately, the authors do not describe the type of MS or the mode used for their quantitative and qualitative work. A very low LLOQ of 5 pg/mL was obtained for dolastatin-10 from 1,000 µL of plasma sample. Following IV administration of the drug the N-demethylated metabolite could be identified [48]. Later, Ochiai et al. published a sensitive LC-APCI-MS assay for the determination of the dolastatin-10 derivative TZT-1027 in human, dog, rat, mouse, and monkey plasma [49].
Berna et al. have exclusively described an assay for the determination of cryptophycin 52 (LY355703) in dog and mouse plasma by LC-MS/MS. An LLOQ of 2.1 ng/mL was obtained from 100 µL samples volumes of either dog or mouse plasma, with a signal to noise ratio of ~40. They indicated that an LLOQ of 0.25 ng/mL could be obtained if higher sensitivity is desired [50].

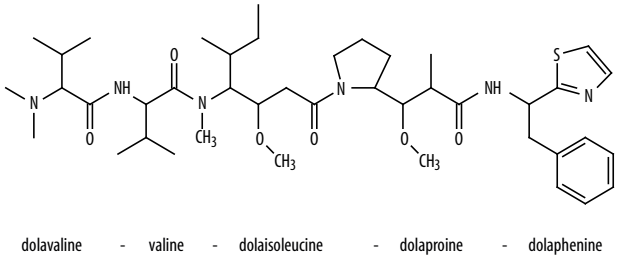



**Figure 4.** Structure of dolastatin-10.

## Antitumor antibiotics

Due to the many structural differences between antitumor antibiotics, a mutual mechanism of antitumor action cannot be assigned. However, the final result is mostly DNA damage. These agents (Table 4) are frequently used in the treatment of several forms of cancer, often in combination therapy with an alkylating agent.

### Anthracycline Derivatives

Examples of anthracycline derivatives are epirubicin, doxorubicin, daunorubicin, idarubicin, mitoxantron, and the still investigational agent nemorubicin. Several assays employing LC with fluorescence detection have been described as was reviewed in [51]. These methods were very suitable for support of pharmacokinetic studies. As a result only two papers have appeared so far on the bioanalysis of an anthracycline using LC-MS. The first appeared in 2000 and describes the simultaneous determination of epirubicin, doxorubicin, daunorubicin, and idarubicin, and the active metabolites of the last thee, doxorubicinol, daunorubicinol, and idarubicinol in human serum. The assay originally included pirarubicin, but it was excluded because of a rapid and significant conversion into doxorubicin. The analytes were chromatographically separated using a microbore analytical column with an internal diameter of 1 mm. The associated mobile phase flow rate was 50 µL/min. A disadvantage of the presented assay is its long run-time of 25 min [52].
Two years later an assay for the determination of the investigational doxorubicin analogue nemorubicin and its 13-OH metabolite in human plasma was published. This assay, although it also has a relatively long run time (14 min) is very sensitive with an LLOQ of 0.1 ng/mL from 500 µL of plasma sample. This low LLOQ was needed as the drug is administered in very low doses [53]. In neither of the articles, aglycon metabolites of the anthracyclines were quantified.

### Others

The quinocarmycin analogue 7-cyanoquinocarcinol and the cyclic depsipeptide FR901228 both suffer from the lack of a good chromophore thereby making them not amendable to LC-UV analysis [54,55]. LC-MS was successfully applied for the bioanalysis of these

**Table 4.** Selected overview of LC-MS methods for antitumor antibiotics in various biological matrices

| Compound | Matrix | Sample (µL) | Sample preparation | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. Quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Epirubicin | Human serum | 500 | Aut. SPE | I: A-AF pH3 | ESI (+) | MRM | An. | 2.5 | 25 | No | | [52] |
| Doxorubicin | Human serum | 500 | Aut. SPE | I: A-AF pH3 | ESI (+) | MRM | An. | 2.5 | 25 | Yes | | [52] |
| Daunorubicin | Human serum | 500 | Aut. SPE | I: A-AF pH3 | ESI (+) | MRM | An. | 5 | 25 | Yes | | [52] |
| Idarubicin | Human serum | 500 | Aut. SPE | I: A-AF pH3 | ESI (+) | MRM | An. | 5 | 25 | Yes | | [52] |
| Nemorubicin | Human plasma | 500 | SPE 96 well | I: A-AF pH4.15 | ESI (+) | MRM | An. | 0.1 | 14 | Yes | | [53] |
| 7-cyanoquinocarcinol | Human plasma | 500 | SPE | I: A-AF pH3 | ESI (+) | MRM | Other | 0.25 | 4 | Yes | | [54] |
| FR901228 | Rat plasma | 100 | LLE | I: A-AA-TFA | ESI (+) | MRM | An. | 10 | 1.8 | No | | [55] |
| FR901228 | Human plasma | 500 | LLE | I: A-M-AA | ESI (+) | MRM | An. | 0.1 | 4 | No | | [58] |
| FR901228 | Rat plasma | 100 | LLE | | | MRM | An. | 0.5 | 4 | No | | [58] |
| Actinomycin D | Human plasma | 500 | PP | G: M-HA-AH | ESI (+) | SIM | An. | 1 | 14 | No | | [59] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; AF Ammonium formate in water; AH Ammonium hydroxide in water; An. Structural analogue; Aut. Automated; ESI Electrospray ionisation; G Gradient elution; HA Acetic acid; I Isocratic elution; IS Internal standard; LC Liquid chromatography; LLE Liquid-liquid extraction; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; PP Protein precipitation; Ref. References; SIM Selective ion monitoring mode; SPE Solid phase extraction; TFA Trifluoracetic acid.

compounds. 7-Cyanoquinocarcinol and its metabolite or degradation product quinocarmycin were determined in human plasma. The sensitivity of the assay was adequate and the LC run time was only 4 minutes [54]. FR901228 possesses potent antitumor activities against several human tumor cell lines. An LC-MS/MS assay for the quantitative analysis of this drug in rat plasma is described [55]. The mobile phase contained 0.1% TFA, a common additive to obtain good chromatographic performance for peptides and proteins. TFA, however, may cause significant signal suppression when used with ESI-MS [56,57], although the authors do not report adverse effects of the use of TFA [55]. Using this assay, an LLOQ of 10 ng/mL was obtained from 100 µL rat plasma volumes. Run time was only 1.8 min. Later, the same research group described a more sensitive assay for the determination of FR901228 in rat and human plasma. The former method was not sensitive enough for an adequate pharmacokinetic monitoring of the drug. In this improved assay, the eluent did not contain TFA but 12 mM ammonium acetate. The LLOQ for FR901228 could thereby be improved and was found to be 0.1 ng/mL from 500 µL of human plasma and 0.5 ng/mL from 100 µL of rat plasma. The assay was successfully applied to early clinical studies [58].
Previous assays for actinomycin D include thin layer chromatography (TLC), enzyme-linked immunosorbent assays (ELISA), and radioimmunoassays. The first LC-MS based assay for the determination of this drug was reported by Veal et al. [59]. Although a triple quadrupole MS was available for MRM analysis, it was operated in the SIM mode, since suitable product ions with improved sensitivity over SIM were not discovered. Gradient elution was performed using a mobile phase consisting of methanol and an acetate buffer, which was prepared by adjusting a solution of 1% acetic acid to pH 4 with ammonia. The assay was sensitive with an LLOQ of 1 ng/mL using sample volumes of 500 µL and it was successfully applied to the pharmacokinetic analysis of actinomycin D in pediatric patients [59].

## Topo-isomerase inhibitors

The nuclear enzymes topo-isomerase I and II are involved in the cleavage and regulation of single stranded DNA (topo-isomerase I) or double stranded DNA (topo-isomerase II) during the cell cycle. By inhibiting these enzymes, this class of anticancer agents (Table 5) damages DNA and causes cell death.

### Topoisomerase I Inhibitors

In the 1970s preliminary clinical trials with the water-insoluble natural alkaloid camptothecin demonstrated promising antitumor activity. Unfortunately, severe side effects were also observed. When it was discovered that the antitumor activity of camptothecin resulted from its ability to inhibit topo-isomerase I inhibitors, its structure was modified to create analogues with better water solubility and less severe side effects.
Camptothecin and its analogues contain a lactone group (Figure 5) which is believed to be essential for inhibition of the DNA-enzyme complex. This group, however, is very unstable and undergoes reversible hydrolysis to a hydroxycarboxylate form, which is

devoid of topo-isomerase I inhibitory activity. The hydrolysis rate is dependent on for instance pH, ionic strength, and protein concentration. At physiological pH an equilibrium between the two forms exists. At pH values lower than 4, the drug is present exclusively in its lactone form. Several analytical methods have been described for the determination of the lactone form alone, for the simultaneous determination of the lactone and carboxylate form and for the analysis of total (lactone and hydroxycarboxylate) concentrations of camptothecin analogues as reviewed by Oguma, Palumbo et al., and Zufía et al. [60-62]. There is some debate whether it is necessary to analyze the different forms of these drugs [63-65]. In order to accomplish the separate analysis of the lactone in plasma, the equilibrium should be stabilized by immediate cooling of the samples and performing rapid protein precipitation [66].

The bioanalysis of campthotecin analogues is usually performed using LC with fluorescence detection [60-62]. Fluorescence detection is suitable to obtain very low LLOQs for the camptothecin analogues (range 0.05-10 ng/mL). The use of MS detection does not necessarily increase the sensitivity of the assays. In fact, LLOQs are often higher for LC-MS analyses than for LC fluorescence analyses as was reported by both Ragot et al. and Sai et al [63,67]. The semi-synthetic and water-soluble derivative of camptothecin, irinotecan, also known as CPT-11, is converted into its 100 to 1,000-fold more active metabolite SN-38 after enzymatic cleavage by carboxylesterases (Figure 6). Therefore, most assays for the determination of irinotecan also include the quantitation of SN-38. Ragot et al. describe an LC-MS assay for the determination of total irinotecan and SN-38 levels from human serum. The lactone – carboxylate equilibrium was completely shifted to the lactone form by acidifying the supernatant with sodium citrate buffer (pH 2) after protein precipitation with acetonitrile. Residual carboxylate forms were not detected. Expected irinotecan levels were 100 times higher than SN-38 levels and since it was not possible to obtain both a high sensitivity for SN-38 and a large dynamic range for irinotecan (10-5,000 ng/mL), the two compounds were assayed separately. For irinotecan an LLOQ of 10 ng/mL was obtained from 200 µL serum volumes, for SN-38 this was 0.5 ng/mL [63]. LLOQs ranging from 0.5 to 5 ng/mL have been reported for irinotecan and SN-38 after LC analysis with fluorescence detection (reviewed in [60]). In addition, using fluorescence detection, two separate assays for the quantitation of irinotecan and SN-38 were not necessary.

Sai et al. presented an assay for the quantitative analysis of total irinotecan, SN-38, and two additional metabolites (SN-38 glucoronide and 7-ethyl-10-[4-N-(5-aminopentanoic

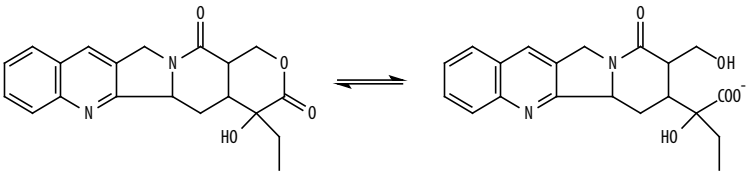



**Figure 5.** Structure of camptothecin and the equilibrium between the lactone and hydroxycarboxylate form of the molecule.

**Table 5.** Selected overview of LC-MS methods for topo-isomerase inhibitors in various biological matrices

| Compound | Matrix | Sample (µL) | Sample pre-treatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Irinotecan | Human serum | 200 | PP | G: A-AF pH3 | ESI (+) | SIM | An. | 10 | 10 | Yes | | [63] |
| Irinotecan | Human plasma | 200 | PP | I: A-M-AA pH4.5 | ESI (+) | SIM | An. | 10 | 20 | Yes | Fluorescence 5 ng/mL | [67] |
| DX-8951 | Human plasma | 300 | SPE | G: M-W-TFA | APCI (+) | MRM | An. | 0.1 | 10 | Yes | | [68] |
| DX-8951 | Human urine | 300 | SPE | G: M-W-TFA | APCI (+) | MRM | An. | 1 | 10 | Yes | | [68] |
| 4-piperidinopiperidine | Human plasma | 1000 | SPE | I: A-AA pH4 | ESI (+) | MRM | An. | 2.5 | NS | No | Metabolite of irinotecan | [69] |
| Alkoxypyridoquinoline | Bovine serum | 1000 | LLE | I: A-AF pH3 | APCI (+) | SIM | None | 0.5 | 8 | No | | [70] |
| Thiopyridoquinoline | Bovine serum | 1000 | LLE | I: A-AF pH3 | APCI (+) | SIM | None | 0.5 | 8 | No | | [70] |
| Aminopyridoquinoline | Bovine serum | 1000 | LLE | I: A-AF pH3 | APCI (+) | SIM | None | 0.5 | 8 | No | | [70] |
| Indolocarbazole I | Human plasma | 250 | SLE | I: M-AH | ESI (-) | MRM | An. | 0.05 | 2.5 | No | | [71] |
| Etoposide | Human plasma | 100 | LLE | I: A-W-HA | ESI (+) | SIM | None | 58.9 | 10 | No | | [74] |
| Etoposide | Human serum | 200 | LLE | I: A-W-HA | ESI (+) | SIM | None | 73.6 | 10 | No | | [74] |
| Etoposide | Human plasma | 50 | PP | I: A-AF-F | ESI (+) | MRM | An. | 200 | 10 | Yes | | [75] |
| Etoposide | Human ultrafiltrate | 200 | PP | I: A-AF-F | ESI (+) | MRM | An. | 25 | 10 | Yes | | [75] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; AF Ammonium formate in water; AH Ammonium hydroxide in water; An. Structural analogue; APCI Atmospheric pressure chemical ionization; ESI Electrospray ionization; F Formic acid; G Gradient elution; HA Acetic acid; I Isocratic elution; IS Internal standard; LC Liquid Chromatography; LLOQ Lower limit of quantitation; LLE Liquid-liquid extraction; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; NS Not specified; PP Protein precipitation; Ref. References; SIM Selective ion monitoring mode; SLE Solid liquid extraction; SPE Solid phase extraction; TFA Trifluoroacetic acid; W Water.

acid)-1-piperidino]carbonyloxycamptothecin (APC)) using LC separation with parallel MS and fluorescence detection [67]. The carboxylate form of irinotecan was completely converted into the lactone form by performing protein precipitation using a mixture of methanol and 5% perchloric acid in water (1:1, v/v). For the compounds included in this assay fluorescence detection appeared to be on average two times more sensitive than MS detection. For irinotecan LLOQs were 10 and 5 ng/ml and for SN-38 2 and 1 ng/mL using MS and fluorescence detection, respectively. Camptothecin analogues may have a higher fluorescent response than ionization efficiency. In addition, assay performance was better for data generated using fluorescence detection than for data generated using MS detection when quality control samples with the same concentration for both methods were compared. However, for the determination of APC, which is expected to be a major product in clinical samples, selective MS detection is preferred as other metabolites may overlap its peak in the LC-fluorescence assay [67]. For the determination of the new camptothecin derivative DX-8951 and its 4-hydroxylmethyl metabolite (UM-1) in human plasma and urine, MS detection resulted in higher sensitivity (0.1 ng/mL) than fluorescence detection (0.2 ng/mL). The metabolite, that could not be analyzed using LC-fluorescence techniques due to interfering plasma components could be successfully quantified using LC-MS/MS with an LLOQ of 1 ng/mL [68]. To our knowledge LC-MS/MS assays have not been reported for the quantitative analysis of camptothecin, topotecan, or other camptothecin derivatives.

Dodds and co-workers developed an assay exclusively for the quantitative analysis of

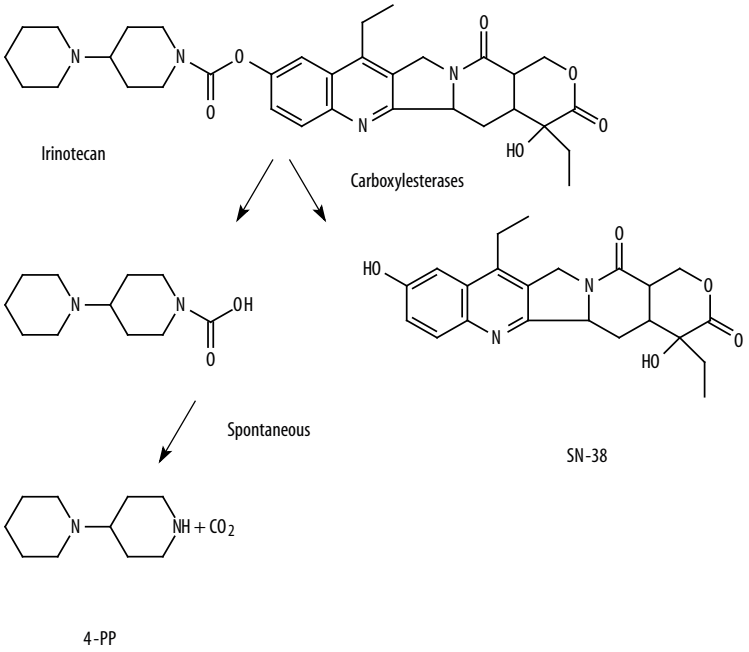



**Figure 6.** Formation of SN-38 and 4-piperidinopiperidine (4-PP) from irinotecan.

the irinotecan metabolite 4-piperidinopiperidine (4-PP). When SN-38 is formed from irinotecan, the by-product (4-piperidino-N-piperidinecarboxylic acid) is spontaneously converted into 4-PP (Figure 6). 4-PP does not contain the lactone ring and has shown to induce apoptosis in a lymphoma cell line. Its antineoplastic features appear to result from a mechanism other than topoisomerase I inhibition. The method has been used successfully to examine the pharmacology of 4-PP in patients treated with irinotecan [69].
Topoisomerase I inhibitors are not necessarily camptothecin analogues. Picó and coworkers have reported on the quantitative analysis of three pyridoquinoline derivatives in bovine serum using LC-HN-APCI-MS in the SIM mode [70] and Wang et al. describe the rapid and sensitive determination of an indolocarbazole in human plasma using LC-MS/MS in negative ion mode [71]. The pyridoquinoline derivatives amino-, thio-, and alkoxypyridoquinoline were originally under development as antimalarial compounds and their antitumor, topoisomerase I inhibitory activity has been identified in vitro [70]. An assay with poor chromatography, resulting in bad peak shapes is described, using rather large sample volumes. However, linearity, inter-assay precision, and selectivity were good [70]. For the extraction of indolocarbazole derivative I from plasma, solid-liquid extraction on diatomaceous earth plates was used for sample pretreatment. Diatomaceous earth consists of siliceous fossil remains of unicellular alga (diatoms) and is used as filter agent and all-purpose adsorbent, as well as for specialized applications, such as catalyst carriers. Sample volumes of 250 µL mixed with 25 µL of a 20 ng/mL analogous internal standard solution were pipetted onto the plates. After 5 min on the plates, the analyte and the analogous internal standard were eluted with 1.6 mL of 9% isopropyl alcohol in methyl-*tert-butyl* ether. Following evaporation of the elution solvent, the residue was redissolved in 150 µL of methanol and water (50:50, v/v). The MS was operated in the negative ion mode as it was approximately nine times more sensitive than the positive ion mode. This was mainly due to the fact that multiple major product ions were formed in positive MS/MS while in negative mode one fragment ion was predominant. The LC eluent consisted of methanol - 6.7 mM ammonium hydroxide in water (70:30, v/v). The pH of the aqueous phase was approximately 10. Run time was only 2.5 min and excellent data were obtained during the validation of the assay [71].

**Topoisomerase II Inhibitors**

The topoisomerase II inhibitor etoposide was introduced into clinical trials in the early 1970s and is currently one of the most commonly used antineoplastic agents. Traditionally, LC with UV or electrochemical detection was used for its bioanalysis, however specificity was often insufficient [72]. Already in 1985 the first LC-MS bioanalytical assay for etoposide was published. The drug was analyzed using LC followed by $^{252}Cf$ plasma desorption – time-of-flight (TOF) MS using a rotating disc interface. Using teniposide as an internal standard a dynamic range from 1-100 µg/mL etoposide in human plasma and urine could be established, which is less sensitive than especially electrochemical detection. This assay was successfully applied in an etoposide pharmacokinetics study [72,73].
Since the implementation of hyphenated LC-MS in routine bioanalysis two assays for the

quantitative analysis of etoposide have been described. One describes LC-ESI-MS after LLE for the detection of etoposide in human serum and plasma [74]. The other publication describes protein precipitation of plasma samples and similar dilution of plasma ultrafiltrate samples, followed by LC-ESI-MS/MS [75]. The first assay was approximately two times more sensitive for the determination of the drug in plasma even though the SIM mode was used, while for the latter method the MRM mode was used.

## Platinum anticancer drugs

The platinum complex drug cisplatin is one of the most commonly used anticancer drugs. After intracellular activation, it can form covalent links with DNA and thereby interferes with DNA replication. Although few other anticancer agents are as effective as cisplatin, it also causes severe toxicity and induces platinum drug resistance. Therefore, it has been attempted to develop less toxic analogues of cisplatin, such as carboplatin and oxaliplatin, and agents that can circumvent resistance, such as ZD0473.

Since platinum is not an endogenous metal it is possible to determine the platinum anticancer agents (Table 6) very selectively and sensitively in biological fluids or tissues by atomizing the sample and specifically analyzing the platinum atom. For this purpose atomic absorption spectrometry (AAS) techniques and, since its introduction in the early 1990s, inductively coupled plasma (ICP)-MS are used. LC separation prior to either of the techniques is often not necessary, since the analyte does not need to be separated from matrix components. As a result, analysis time can be very short. Still, LC is sometimes used prior to AAS or ICP-MS e.g. for discrimination between parent drug and metabolites.

Some researchers prefer not to atomize the compound but to detect the intact drug as this may yield more adequate pharmacokinetic profiles. An assay for the quantitative

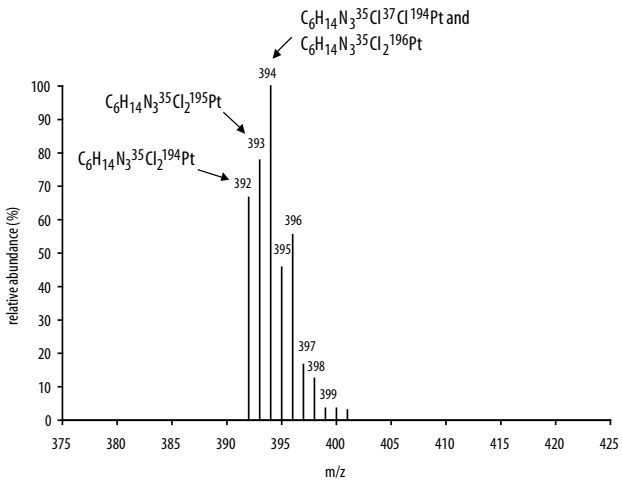



**Figure 7**. Q1 Mass Spectrum of ZD0437 [76].

**Table 6.** Selected overview of LC-MS methods for platinum anticancer agents in various biological matrices

| Compound | Matrix | Sample (µL) | Sample pretreatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ZD0473 | Human ultrafiltrate | 100 | - | I: M-AA-HA | ESI (+) | MRM | SIL | 10 | 15 | No | | [76] |
| ZD0473 | Human urine | 100 | - | I: M-AA-HA | ESI (+) | MRM | SIL | 200 | 15 | No | | [77] |
| ZD0473 | Dog ultrafiltrate | 100 | - | I: M-AA-F | ESI (+) | MRM | SIL | 5 | 7 | No | | [78] |
| ZD0473 | Dog ultrafiltrate | 100 | - | I: M-AA-F | ICP (+) | Scan | SIL | 0.1 | 7 | No | | [78] |
| JM216 | Human plasma | 100 | PP | G: M-W-OPA | ICP (+) | Scan | None | 2 | 20 | Yes | | [79] |
| Carboplatin | Rat ultrafiltrate | 50 | SPE | I:A-W-HA | ESI (+) | SIM | None | 70 | 5 | No | | [80] |
| Carboplatin | Rat tumor | 200 | SPE | I:A-W-HA | ESI (+) | SIM | None | 34 | 5 | No | | [80] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; ESI Electrospray ionization; F Formic acid; G Gradient elution; HA Acetic acid; I Isocratic elution; ICP Inductively coupled plasma; IS Internal standard; LC Liquid Chromatography; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; OPA Orthophosphoric acid; PP Protein precipitation; Ref. References; SIM Selective ion monitoring mode; SPE Solid phase extraction; W Water.

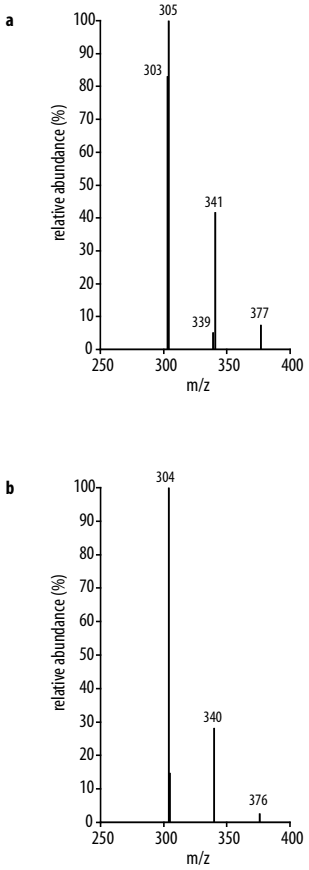



**Figure 8.** Product ion mass spectrum of ZD0437 from m/z 394 (a) and m/z 393 (b) [76].

analysis of intact ZD0473 (cis-amminedichloro(2-methylpyridine)platinum(II)) in human plasma ultrafiltrate and urine using LC-TISP-MS/MS has been described [76,77]. TISP-MS for the analysis of platinum and chloride containing species is challenging since there are three major isotopes of platinum ($^{194}$Pt, $^{195}$Pt, and $^{196}$Pt) and two of chlorine ($^{35}$Cl and $^{37}$Cl). Molecular species are complex and sensitivity may be limited by segregation of the MS signal into a number of different isotopes (Figure 7). The most intense molecular ion peak at m/z 394 corresponds to a combination of the $^{35}Cl_2{}^{196}$Pt and $^{35}$Cl$^{37}$Cl$^{194}$Pt species. The product ion spectrum of m/z 394, however, is complex because of the presence of $^{35}$Cl and $^{196}$Pt or $^{37}$Cl and $^{194}$Pt containing species (Figure 8a). Therefore, for MRM the most intensive transition from m/z 393 (containing $^{35}CL_2{}^{195}$Pt) to m/z 304 was used (Figure 8b). LLOQs of 10 ng/mL and 200 ng/mL were obtained from 100 µL sample volumes for human ultrafiltrate and urine, respectively [76,77]. ICP-MS allows for a simultaneous determination of the isotopes and, in addition, is able to combine the separate isotope signals to one. A disadvantage of ICP ionization is its intolerance for organic solvents as they suppress the

**Table 7.** Selected overview of LC-MS methods for matrix metalloproteinase inhibitors in various biological matrices

| Compound | Matrix | Sample (µL) | Sample pretreatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Marimastat | Human plasma | NS | LLE | I: M-W | APCI (?) | SIM | An. | 5 | NS | No | | [82] |
| AG3340 | Mouse plasma | NS | LLE | I: A-W-HA | ESI (+) | MRM | SIL | 0.1 | NS | No | | [83] |
| Compound I | Rat plasma | 100 | Aut. SPE 96 well | G:A-W-F | ESI (+) | MRM | SIL | 0.6 | 3 | No | | [84] |
| Compound II | Rat cartilage | 5 mg | Aut. SPE 96 well | G:A-W-F | ESI (+) | MRM | SIL | 12 ng/g | 3 | No | | [84] |
| Compound III | Rat plasma | 100 | Aut. LLE 96 well | G:A-BCB pH7.4 | ESI (-) | MRM | An. | 0.3 | 2.5 | No | | [85] |
| Compound IV | Rat plasma | 100 | Aut. LLE 96 well | G:A-BCB pH7.4 | ESI (-) | MRM | An. | 0.6 | 2.5 | No | | [85] |
| COL-3 | Human plasma | 250 | PP | G: A-O pH 2.2 | APCI (+) | SIM | An. | 30 | 30 | No | | [86] |
| COL-3 | Human plasma | 250 | LLE | I: A-W-F | ESI (+) | MRM | An. | 30 | 5.5 | No | | [87] |
| ABT-518 | Human plasma | 500 | SPE | I: M-AH | ESI (+) | MRM | An. | 10 | 8 | Yes | | [16] |
| PNU-248686A | Human plasma | 50 | PP 96 well | I: A-AF pH 5 | ESI (-) | MRM | None | 5 | 2.5 | No | | [88] |

Abbreviations: A Acetonitrile; AF Ammonium formate in water; AH Ammonium hydroxide in water; An. Structural analogue; APCI Atmospheric pressure chemical ionisation; Aut. Automated; BCB Bicarbonate buffer in water; ESI Electrospray ionisation; F Formic acid; G Gradient elution; HA Acetic acid; I Isocratic elution; IS Internal standard; LC Liquid chromatography; LLE Liquid-liquid extraction; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; NS Not specified; O Oxalic acid in water; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal; SIM Selective ion monitoring mode; SPE Solid phase extraction.

analyte signals, but this can be overcome by using an appropriate nebulizer. The assay described above for the determination of ZD0473 in human ultrafiltrate and urine has been applied to dog plasma ultrafiltrate and its performance has been compared with LC coupled with ICP-MS [78]. By using a more sensitive TISP-MS/MS than in the original article [76,77] the LLOQ was now 5 ng/mL from 100 µL of ultrafiltrate. The ICP-MS, however, was more sensitive, because of its ability to combine the signals of the separate isotope peaks. By using LC separation prior to ICP-MS detection, this assay could also specifically detect the parent drug. An LLOQ of 0.1 ng/mL from 100 µL of dog plasma ultrafiltrate was obtained [78].

LC-ICP-MS was applied to the quantitative analysis of the oral platinum anticancer drug JM-216 in human plasma. Analysis of JM216 is challenging because of the low concentrations and extensive biotransformation of the drug. LC separation prior to ICP-MS allowed the quantitative analysis of unchanged drug and biotransformation products. This assay replaced a less sensitive and more time consuming off-line LC-AAS assay [79].

Only recently, the first and so far only paper describing the quantitative bioanalysis of carboplatin using LC-MS has appeared. Earlier, intact carboplatin was determined using relatively insensitive LC-UV assays. LLOQs were 34 and 70 ng/mL from tumor and plasma ultrafiltrate, respectively [80].

## Metalloproteinase inhibitors

Matrix metalloproteinases (MMPs; Table 7) are a family of enzymes involved in the degradation of extracellular matrix proteins in processes of tissue formation and remodeling [81]. Overexpression of MMPs, and in particular MMP-2 (gelatinase A) and MMP-9 (gelatinase-B) has been demonstrated in many invasive tumors. MMP inhibitors have antineoplastic abilities.

One of the first MMP inhibitors to be tested was marimastat, which is an inhibitor of all MMP enzymes. Millar et al. published the results of a study with marimastat in healthy volunteers. In their publication they describe the LC-MS assay for human plasma. An LLOQ of 5 ng/mL was obtained. Unfortunately, the sample volume, LC run time and polarity of the APCI are not specified in the manuscript [82]. Shalinsky et al. present data that suggest that the antitumor efficacy of MMP inhibitor AG3340 is associated with the maintenance of a minimum effective plasma concentration. An assay using positive ion ESI–MS/MS in the MRM mode yielding an LLOQ of 0.5 ng/mL is described. Sample volume and run time were not specified [83].

Interestingly, the analysis of MMPIs sometimes involves unusual matrices and analytical conditions. Peng et al. describe the quantitative analysis of two hydroxamic acid-based MMP inhibitors, referred to by the authors as compound I and II. Compound I was determined in rat plasma, compound II in rat cartilage tissue. MMP inhibitors are not only tested for their antineoplastic abilities but also for the treatment of arthritic disorders, as these disorders are also associated with the extracellular matrix. Both for the preparation of calibration standards and QC samples and the processing of animal samples, approximately

5 mg of wet cartilage was grinded with 1.5 mL of methanol. The supernatant was used for further experiments [84]. Later, the same group published a rapid and sensitive assay for two carboxylic acid based MMP inhibitors, referred to as compound III and IV. The mobile phase used for chromatographic separation of the compounds contained a 10 mM bicarbonate buffer (pH 7.4), an uncommon additive for bioanalysis with LC-MS [85].
COL-3 is a tetracycline analogue that completely inhibits MMP-2 and MMP-9. Tetracyclines are compounds that are difficult to analyze chromatographically due to problems with solubility, peak shape, and chelation with matrix metal ions [86]. The latter two problems were solved by adding oxalic acid to the eluent, which was apparently not detrimental to the MS source. Protein precipitation of the human plasma samples as sample pretreatment provided the best sensitivity for COL-3, although the extracts were not completely clean after this procedure. Late eluting matrix components suppressed the signal of the following sample and therefore gradient elution and a run time of at least 30 min were needed [86]. Later, it was reported that LLE is more suitable for obtaining high recoveries of COL-3 than protein precipitation, provided that the sample is acidified prior to LLE to approximately pH 1 with hydrochloric acid, to release COL-3 from chlelated complexes. Isocratic elution with a mixture of acetonitrile, water, and formic acid in combination with a YMCbasic analytical column (Waters) yielded good peak shapes and allowed a reduction of the LC run time to 5.5 min [87]. YMCbasic column material provides an alternative bonding approach to reduce peak tailing of basic pharmaceuticals without the need for ion pair reagents or amine modifiers.
For the determination of the specific MMP-2 and MMP-9 inhibitor ABT-518 and the screening of six potential metabolites in human plasma, an LC-MS/MS assay using an alkaline mobile phase containing ammonium hydroxide in combination with positive ion TISP has been described [16]. This approach has demonstrated to yield better sensitivities and peak shapes for the analytes than other mobile phase additives, such as ammonium acetate, ammonium formate, formic acid, acetic acid, etc. The generation of positive ions from an eluent containing ammonium hydroxide probably results from gas-phase ion-molecule reactions between ammonium cations and the analyte molecule or collision-induced dissociation of ammonium adducts of the analyte yielding in both cases the protonated analyte and an ammonia molecule [15]. This analytical system may be suitable for other basic drugs [16].
For the determination of MMP-2 and MMP-9 inhibitor PNU-248686A in human plasma a negative ion TISP-MS/MS method is described [88]. Protein precipitation in a 96-well format was described for rapid sample pretreatment by addition of the precipitation solution (methanol) to the plasma samples in the plates. Interestingly, after shaking and centrifugation of the plates, the supernatant was directly injected onto the system, without transfer of the supernatant to a clean plate, which has been described for the other 96 well protein precipitation procedures for the determination of methotrexate [29] and imatinib [89], respectively.

## Anti-hormones

Anti-hormones (hormone antagonists; Table 8) block the action of a hormone on receptor sites. They are receiving increasing attention in a variety of treatments. The anti-estrogen or selective estrogen receptor modulator (SERM) tamoxifen is used in the treatment of breast cancer. Idoxifene, a halogenated tamoxifen derivative, has a greater affinity for the estrogen receptor and is less uterotrophic than tamoxifen. Other SERMs include raloxifene and nafoxidine.

A high-throughput assay for the determination of tamoxifen, idoxifene, raloxifene, nafoxidine and the tamoxifen metabolite, 4-hydroxytamoxifen in human plasma, capable of analyzing 2,000 samples per day, has been described [90]. Ultra-fast chromatography was performed on a Luna $C_{18}$(2) column (30 x 1.0 mm ID, 3 µm) and the mobile phase consisting of methanol, acetonitrile, and 3 mM ammonium acetate in water (pH 4.6) was pumped through the column with a very high flow rate of 0.5 mL/min, considering the ID of the column. A column temperature of 80 °C significantly reduced the operating pressure of the system and was needed to elute idoxifene within 30 s. Validation results, however, were not within FDA guidelines on bioanalytical method validation (accuracy >±20% at LLOQ level, precision >20% at LLOQ level and >15% at other levels), but were considered sufficient for drug discovery purposes [90]. Later, the same authors published an even faster assay for the determination of idoxifene and its pyrrolidinone metabolite in human plasma. The flow rate was 0.7 mL/min employing the same Luna column as for the assay described above. Run time was reduced to 15 s. The authors comment that these high-throughput strategies are best suited for those applications where less stringent method validation criteria are acceptable and where low detection limits can be sacrificed for

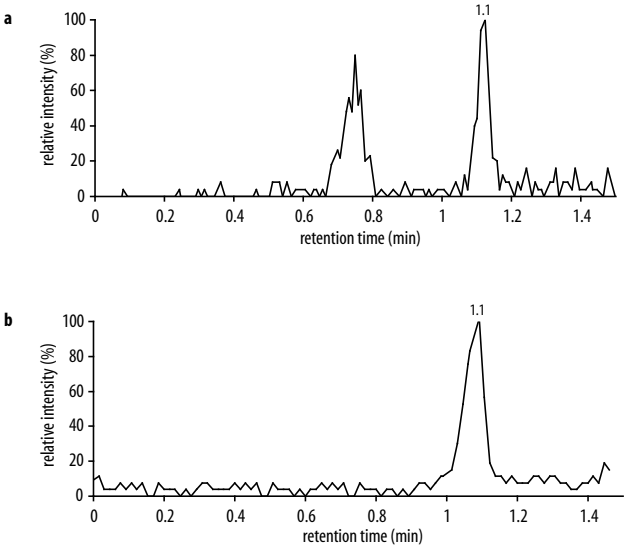



**Figure 9.** Representative chromatograms of idoxifene spiked in human plasma at the LLOQ obtained with LC-reflectron TOF-MS (a; 5 ng/mL) and LC-MRM TQ-MS (b; 0.5 ng/mL) [92].

**Table 8.** Selected overview of LC-MS methods for anti-hormones in various biological matrices

| Compound | Matrix | Sample (µL) | Sample pre-treatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tamoxifen | Human plasma | 100 | LLE 96 well | I: M-A-AA pH4.6 | ESI (+) | MRM | An. | 1.4 | 0.5 | Yes | | [90] |
| Idoxifene | Human plasma | 100 | LLE 96 well | I: M-A-AA pH4.6 | ESI (+) | MRM | SIL | 14 | 0.5 | No | | [90] |
| Raloxifene | Human plasma | 100 | LLE 96 well | I: M-A-AA pH4.6 | ESI (+) | MRM | An. | 7 | 0.5 | No | | [90] |
| Nafoxidine | Human plasma | 100 | LLE 96 well | I: M-A-AA pH4.6 | ESI (+) | MRM | An. | 2.8 | 0.5 | No | | [90] |
| Idoxifene | Human plasma | 100 | LLE 96 well | I: A-W-F | ESI (+) | MRM | SIL | 10 | 0.25 | Yes | | [91] |
| Idoxifene | Human plasma | 100 | LLE 96 well | G: A-AA-F | ESI (+) | MRM | SIL | 0.5 | 1.5 | No | | [92] |
| Idoxifene | Human plasma | 100 | LLE 96 well | G: A-AA-F | ESI (+) | TOF | SIL | 5 | 1.5 | No | | [92] |
| 4-hydroxytamoxifen | Mouse plasma | 25 | PP | G: A-W-F | ESI (+) | MRM | None | 0.1 | 20 | - | Metabolite of tamoxifen | [94] |
| Exemestane | Human plasma | 1000 | SPE + LLE | I: A-AA pH4.5 | TS (+) | SIM | An. | 1 | 16 | No | | [95] |
| Exemestane | Human plasma | 500 | SPE 96 well | I: A | APCI (+) | MRM | SIL | 0.05 | 4 | No | | [96] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; An. Structural analogue; APCI Atmospheric pressure chemical ionisation; ESI Electrospray ionisation; F Formic acid; G Gradient elution; I Isocratic elution; IS Internal standard; LC Liquid chromatography; LLE Liquid-liquid extraction; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal; SIM Selective ion monitoring mode; SPE Solid phase extraction; TOF Time-of-flight; TS Thermospray ionisation; W Water.

time-saving benefits, such as during the first stages of drug development [91].
TQ mass spectrometers have been used almost exclusively for the quantitative bioanalysis of anticancer agents. In the MRM mode the sensitivity and selectivity of this system is unrivalled. On the other hand, TOF mass spectrometers and especially linear TOF systems are the most sensitive full-scan mass spectrometers. Zhang and Henion have compared reflectron TOF-MS and TQ-MS in the MRM mode for the quantitative determination of idoxifene in human plasma [92]. The results demonstrated that the sensitivity of the TQ-MS in MRM mode is far superior to the reflectron TOF-MS. The obtained LLOQs were 0.5 and 5 ng/mL, respectively. Nevertheless, linear TOF-MS may be up to ten times more sensitive than reflection TOF-MS [93], and thereby comparable with TQ MRM analysis. In addition, TOF-MS provides simultaneous qualitative information [92]. Representative chromatograms of idoxifene at LLOQ level obtained using TOF-MS and TQ-MS are presented in Figure 9a and 9b, respectively.
Sensitivity was more important than high-throughput for the determination of tamoxifen metabolite 4-hydroxytamoxifen in murine plasma after low dose administration of the drug to mice. Sensitivity was increased by miniaturizing the LC system using a capillary column with an internal diameter of 0.3 mm and a very low flow rate of 0.01 mL/min. From only 25 µL of sample, an LLOQ of 0.1 ng/mL was obtained. Run time, however, was 20 min and no internal standard was used [94].
Exemestane is a selective aromatase inhibitor, thereby preventing the formation of estrogens. An existing assay using thermospray-MS was not sensitive enough to determine low concentrations in plasma from patients receiving the drug. An LLOQ of 1 ng/mL from 1,000 µL plasma volumes was obtained. In addition, run time was 16 min [95]. A very sensitive assay for the quantitative analysis of exemestane in human plasma has been described with an LLOQ of 0.05 ng/mL from 500 µL sample volumes to replace the earlier assay [96]. Apart from an increase in sensitivity the run times was reduced to 4 min. Interestingly, the assay employs a mobile phase of 100% acetonitrile. Normally, a mobile phase consisting exclusively of organic modifier is not suitable for LC separation, since compounds are often not retained on the analytical column. However, the authors do not report any negative effects [96].

## Signal transduction modulators

This class of drugs (Table 9) interferes with specific pathways of the signal transduction, involved in cell proliferation, differentiation, and communication.
Alkylphosphocholines disrupt lipid-mediated signal transduction pathways necessary for tumor cell growth and survival. Similar to the lipids they are derived from, this class of drugs contains a positively charged quaternary amine and a negatively charged phosphate moiety (Figure 10). As a result, chromatography of these compounds is challenging. Alkylphosphocholine perifosine has been quantified in human plasma with LC-MS/MS using the lead compound of this class of drugs, miltefosine, as internal standard [97]. The assay could also be used *vice versa* to determine miltefosine and using perifosine

**Table 9.** Selected overview of LC-MS methods for signal transduction modulators in various biological matrices

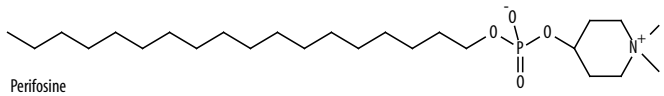
| Compound | Matrix | Sample (µL) | Sample pre-treatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Perifosine | Human plasma | 250 | Aut. SPE | I: A-M-AA pH6.5 | ESI (+) | MRM | An. | 4 | 5 | No | | [97] |
| Perifosine | Human plasma | 100 | PP | I: A-AF pH 8 | ESI (+) | SIM | An. | 5 | 5 | No | | [98] |
| Imatinib | Human plasma | 200 | PP 96 well | I: M-AA | APCI (+) | MRM | SIL | 4 | 2.5 | Yes | | [89] |
| Imatinib | Monkey plasma | 250 | SPE 96 well | I: M-AA | APCI (+) | MRM | SIL | 1 | 2.5 | Yes | | [99] |
| Compound I | Human plasma | 1000 | SPE | I: A-M-W-TFA | APCI (+) | MRM | An. | 0.5 | 4.5 | No | | [100] |
| Compound I | Human urine | 1000 | SPE | I: A-M-W-TFA | APCI (+) | MRM | An. | 2.5 | 4.5 | No | | [100] |
| ES-285 | Human plasma | 100 | PP | I: M-AF pH4 | ESI (+) | MRM | SIL | 10 | 8 | No | | [101] |
| ES-285 | Mouse plasma | 100 | PP | I: M-AF pH4 | ESI (+) | MRM | SIL | 10 | 8 | No | | [101] |
| ES-285 | Rat plasma | 100 | PP | I: M-AF pH4 | ESI (+) | MRM | SIL | 10 | 8 | No | | [101] |
| ES-285 | Dog plasma | 100 | PP | I: M-AF pH4 | ESI (+) | MRM | SIL | 10 | 8 | No | | [101] |
| OSI-774 | Human plasma | 100 | PP | I: A-W-F | ESI (+) | MRM | An. | 10 | 2.5 | Yes | | [102] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; AF Ammonium formate in water; An. Structural analogue; APCI Atmospheric pressure chemical ionisation; Aut. Automated; ESI Electrospray ionisation; F Formic acid; I Isocratic elution; IS Internal standard; LC Liquid chromatography; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal; SIM Selective ion monitoring mode; SPE Solid phase extraction; TFA Trifluoroacetic acid; W Water.

as an internal standard. Before this publication no LC based assay had been reported for perifosine, due to the lack of chromophores and electroactive groups in the molecule. The LC-MS/MS assay was rapid, sensitive, and thoroughly validated. Interestingly, the assay employed normal phase chromatography on a silica column, using a reversed phase eluent consisting of acetonitrile, methanol and 8 mM ammonium acetate. Unfortunately, peak tailing was observed as well as potential column degradation after ~150 sample injections [97]. Other researchers attempted to improve the chromatographic properties of the assay. Reversed phase chromatography was performed on a Develosil C30-UG column with an eluent consisting of acetonitrile and 9 mM ammonium formate (pH 8), resulting in improved peaks shapes. The MS was used in the positive ion MRM mode. The obtained LLOQ was comparable to the earlier assay, but a smaller volume of plasma was processed and sample pretreatment involved a simple protein precipitation procedure. Validation results were excellent [98].

Signal transduction inhibitor 571 (STI571) or imatinib (Gleevec) is a revolutionary drug for the treatment of chronic myeloid leukemia. Imatinib is a protein-tyrosine kinase inhibitor, which functions at the molecular level with high specificity. Bakthiar and coworkers describe an LC-MS/MS assay for the quantitative determination of imatinib and its main metabolite, desmethyl imatinib, in human plasma [89] and monkey plasma [99]. In the first assay 200 µL sample volumes were subjected to protein precipitation in a 96 well format, resulting in an LLOQ of 4 ng/mL [89]. For the second assay a higher sensitivity was optioned, since this assay was used for toxicokinetic screening with lower doses of imatinib. SPE in a 96 well format was performed on 250 µL monkey plasma volumes, yielding an LLOQ of 1 ng/mL [99].

In the cell cycle farnesylation of Ras proteins is essential for an uninterrupted growth signal. Mutant Ras proteins are associated with many types of human tumors. Farnesyl transferase inhibitors are investigated as potential anticancer agents. To our knowledge, so far only one publication describing the quantitative bioanalysis of a farnesyl transferase inhibitor using LC with MS detection has appeared. The imidazole analogue, 4-[1-(4-cyanobenzyl)-5-imidazolylmethyl]-1-(3-chlorophenyl) piperazinone (compound I), has been quantified in human plasma and urine. During development TFA appeared to be more appropriate as a mobile phase additive than formic acid for a short run time, high



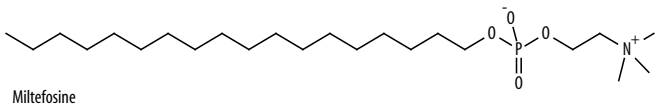




**Figure 10.** Structures of perifosine and miltefosine.

**Table 10.** Selected overview of LC-MS methods for apoptosis inducing agents in various biological matrices

| Compound | Matrix | Sample (µL) | Sample preparation | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Betulinic acid | Mouse blood | 1000 | PP | I: A-W | ESI (-) | SIM | None | 100 | 10 | No | Post-column mixing | [103] |
| Betulinic acid | Mouse tumor | 1000 | PP | I: A-W | ESI (-) | SIM | None | 100 | 10 | No | Post-column mixing | [103] |
| Betulinic acid | Mouse tissue | 1000 | PP | I: A-W | ESI (-) | SIM | None | 100 | 10 | No | Post-column mixing | [103] |
| Betulinic acid | Mouse plasma | 50 | PP | I: A-W | ESI (-) | SIM | An. | 0.2 | 17 | No | | [104] |
| Betulinic acid | Rat plasma | 50 | PP | I: A-W | ESI (-) | SIM | An. | 0.2 | 17 | No | | [104] |
| Betulinic acid | Dog plasma | 50 | PP | I: A-W | ESI (-) | SIM | An. | 0.2 | 17 | No | | [104] |
| EB 1089 | Human serum | 1000 | PP + Aut. SPE | G: M-AA | ESI (+) | MRM | SIL | 0.01 | 6 | No | | [105] |
| EB 1089 | Pig serum | 1000 | PP + Aut. SPE | G: M-AA | ESI (+) | MRM | SIL | 0.01 | 6 | No | | [105] |
| Aplidin | Human plasma | 100 | PP + LLE | G: A-W-F | ESI (+) | MRM | An. | 5 | 10 | No | | [109] |
| Aplidin | Human urine | 500 | PP + LLE | G: A-W-F | ESI (+) | MRM | An. | 1.25 | 10 | No | | [109] |
| Aplidin | Human plasma | 100 | LLE | I: A-AA-F | ESI (+) | MRM | An. | 0.05 | 3 | No | | [110] |
| Acetyldinaline | Human plasma | 500 | Aut. SPE | I.A-M-AA pH5.8 | ESI (+) | MRM | An. | 1 | 3 | No | | [111] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; An. Structural analogue; Aut. Automated; ESI Electrospray ionisation; F Formic acid; G Gradient; I Isocratic elution; IS Internal standard; LC Liquid chromatography; LLE Liquid-liquid extraction; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal; SIM Selective ion monitoring mode; SPE Solid phase extraction; W Water.

sensitivity, and good peak shape. The authors conclude that this method may have utility for other imidazole-based farnesyl transferase inhibitors as well [100].
ES-285 (spisulosine; 2-amino-3-hydroxy-octadecane) is believed to decrease the activity of Rho proteins. Rho proteins are members of the Ras family, and involved in cellular events that play an important role in cancer invasion and metastasis. The quantitative analysis of ES-285 in human, mouse, rat, and dog plasma using LC-MS/MS has been published [101]. In the MRM mode a fairly unusual mass transition was monitored from the molecular ion to a fragment ion corresponding to the elimination of water. The monitoring of this particular transition, although not considered very robust, appeared to be very suitable as demonstrated by the excellent results of a thorough validation according to the current FDA guidelines [101].
OSI-774 (erlonitib, Tarceva™) is an epidermal growth factor receptor inhibitor which has completed phase III evaluation as first line treatment in combination with chemotherapy but is still evaluated either as monotherapy or in combination with chemotherapy, radiation, or hormone therapy. Zhao et al. reported on the quantitative bio-analysis of OSI-774 and its hydroxy-metabolite OSI-420 in human plasma [102]. Using the MS/MS in the MRM mode an LLOQ of 10 ng/mL could be obtained from 100 µl of plasma. The LLOQ for the metabolite was 1 ng/mL. The LLOQ for OSI-774 was sufficiently low, since in earlier studies the lowest concentration determined for OSI-774 was 283 ng/mL [102].

## Apoptosis inducing agents

The agents described in this chapter (Table 10) induce programmed cell death, in stead of cell growth arrest which is the usual mechanism of action of cytostatic drugs.
Betulinic acid is a product from natural sources such as the bark of white birch trees, and is tested in pre-clinical trials for the treatment of melanoma. Since betulinic acid does not contain a strong UV chromophore, LC-UV was not suited for detection of trace levels of the drug in biological samples. An LC-MS assay for quantitative analysis of betulinic acid in mouse blood, tumor, and liver, lung, or kidney tissue is described. After protein precipitation with acetonitrile-ethanol (1:1, v/v) and following LC on a $C_{18}$ column with an eluent consisting of 80% acetonitrile the eluent was mixed post-column with methanol, water, and triethylamine to enhance negative ionization in the ESI source. An LLOQ of 100 ng/mL was obtained from 1,000 µL sample volumes, which was sufficient for the determination of the distribution of betulinic acid in mice. With signal to noise of >50 at the LLOQ level, however, the sensitivity of the assay could have been improved. The drug was identified and quantified in all matrices, with the highest concentration in tumor tissue [103]. Later, the same researchers applied this assay for the determination of betulinic acid in mouse, rat and dog plasma. Unlike the original assay, which does not employ an internal standard an analogous internal standard was chosen, which, however, did not seem to improve assay performance. Chromatographic separation of the analyte and internal standard was necessary, since they are isomers. Post-column mixing of the eluent is no longer used, even though the eluent contained no additives to enhance negative ionization. Excellent

sensitivities, however, were obtained with an LLOQ of 0.2 ng/mL from only 50 µL of plasma [104].
EB 1089 or seocalcital is an analogue of 1α,25-dihydroxyvitamin $D_3$, the physiological active form of vitamin $D_3$. 1α,25-dihydroxyvitamin $D_3$ plays a crucial role in cell growth regulation and calcium homeostasis. Its potential use in clinical cancer treatment is limited by its effect on calcium homeostasis, with the risk of inducing hypercalcemia and soft tissue calcifications. EB 1089 has a strong antiproliferative effect, but reduced effects on calcium metabolism and is currently under investigation in clinical phase II trials. Since EB 1089 is administered in very low doses (1 to 25 µg orally) LC with MS/MS detection in the MRM mode was investigated for ultra-sensitive detection. From 1,000 µL serum volumes an LLOQ of 0.01 ng/mL was obtained for EB 1089 in both human and pig serum on an API 3000 MS (Sciex). The assay was thoroughly validated and is currently employed for pharmacokinetic studies in humans and toxicokinetic studies in pigs [105].
Aplidin induces apoptosis in human cancer cells, however it is not clear via which pathway yet [106,107]. The depsipeptide aplidin is isolated from a marine tunicate and contains a proline moiety resulting in a cis- and trans-isomer of aplidin. At low temperatures the cis-trans equilibrium is slow, resulting in two separate chromatographic peaks, while at higher temperatures, the equilibrium is rapid, so that the two peaks merge to one [108]. Celli et al. chose to add up the responses of the two peaks [109] while Yin and co-workers increased the temperature of the chromatographic column to 60 °C to induce peak overlap [110]. The first assay yielded an LLOQ of 5 ng/mL from 100 µL of human plasma and 1.25 ng/mL from 500 µL of human urine [109]. The second assay was 100-fold more sensitive with an LLOQ of 0.05 ng/mL from 100 µL plasma volumes, which may result from the peak overlap approach but also from the use of a more sensitive MS (API 4000 vs. API 365 (Sciex)) [110].
Penn et al. describe the combination of automated SPE with LC-MS/MS in the MRM mode for a rapid and sensitive detection of acetyldinaline (CI-994) using a structural analogue as internal standard. An LLOQ of 1 ng/mL was obtained for the drug from 500 µL plasma volumes within 3 min. After an extensive validation the assay was successfully applied to the pharmacokinetic support of a phase I trial with the drug [111].

## Others

There are several anticancer agents with mechanisms of action that differ from the usual classes of anticancer agents, or of which the primary mechanism of action has not yet been elucidated (Table 11).
The marine anticancer agent ET-743 (trabectedin, Yondelis) is currently investigated in phase II clinical trials [112]. The drug is believed to exert its antitumor activity, among others, by interacting with minor groove DNA guanine and interfering with the nucleotide excision repair pathways of cancer cells. An assay for the quantitative determination of ET-743 in human plasma using LC-MS/MS was developed to replace an existing LC-UV assay that was not sensitive enough to quantify the trace amounts of ET-743 in plasma following intravenous infusion of the drug in the low µg/$m^2$ range. The LC-MS/MS assay

(LLOQ 0.01 ng/mL) was 100-fold more sensitive than the LC-UV assay (LLOQ 1 ng/mL), and it was very appropriate for the pharmacokinetic support of clinical trials as demonstrated in Figure 11. It is obvious that without the LC-MS/MS assay a lot of pharmacokinetic data would not have become available. The internal standard was a structural analogue corresponding to the N-dealkylation of ET-743. Although this reaction may be a part of the metabolic pathway of ET-743, this potential metabolite was not identified in plasma from patients treated with ET-743, which justifies its use [113].

Kahalalide F and thiocoraline are depsipeptide marine anticancer agents of which kahalalide F is currently under investigation in clinical trials. Chromatography of kahalalide F was challenging, since ion-pairing agent TFA, traditionally used for the chromatography of peptides and proteins caused too much suppression of the analyte signal in TISP-MS. Other acids, such as formic acid, acetic acid, and propionic acid did improve the kahalalide F MS response, but caused unacceptable chromatography. An alkaline eluent containing ammonium hydroxide yielded excellent chromatographic performance as well as MS response in the positive ion mode. Ammonium hydroxide may thus be an appropriate replacement for TFA in the LC-MS analysis of other peptides and proteins [17]. Formic acid was a suitable mobile phase additive for the LC-MS/MS analysis of the thiocoraline. Peak shapes and sensitivity were excellent [114].

TNP-470 [O-(chloroacetylcarbamoyl)fumagillol] is a synthetic derivative of fumagillin and has demonstrated anti-angiogenesis activity. This agent is subject to extensive metabolism, with two major metabolites, AGM-1883 and MII, and at least four minor metabolites. An assay for the quantitative analysis of TNP-470 and its two major metabolites in human plasma was developed using the MS in the SIM mode. MRM was investigated but resulted in a decrease of sensitivity compared to SIM, probably due to the formation of multiple fragment ions. Sample pretreatment using SPE allowed and LLOQ of 0.63 ng/mL from 500 µL of plasma, while protein precipitation resulted in an LLOQ of 2.5 ng/mL from the same

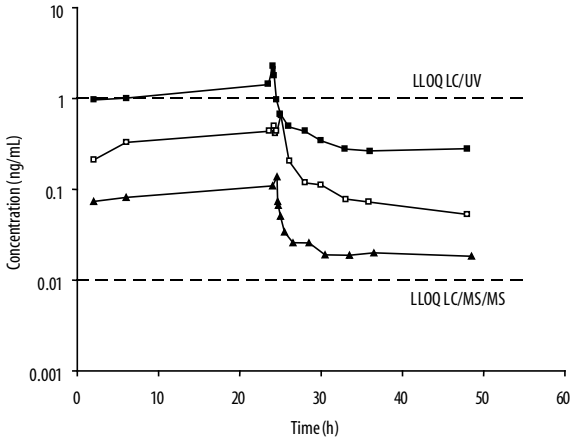



**Figure 11.** Plasma concentration vs. time profile of ET-743 in patients treated with 100 (▲), 600 (□), and 1,200 (■) µg/m$^2$. The dotted lines represent the LLOQ of the LC-UV method (1 ng/mL) and the LC-MS/MS method (0.01 ng/mL) [113].

**Table 11.** Selected overview of LC-MS methods for rest-group anticancer agents in various biological matrices

| Compound | Matrix | Sample (µL) | Sample pre-treatment | LC | Interface | MS mode | IS | LLOQ (ng/mL) | Run time (min) | Met. quan. | Remarks | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ET-743 | Human plasma | 500 | SPE | I: M-AA-F | ESI (+) | MRM | An. | 0.01 | 6 | No | | [113] |
| Kahalalide F | Human plasma | 500 | SPE | I: A-AH | ESI (+) | MRM | An. | 1 | 8 | No | | [17] |
| Thiocoraline | Mouse plasma | 400 | PP + SPE | I: M-W-F | ESI (+) | MRM | An. | 0.1 | 3 | | | [114] |
| TNP-470 | Human plasma | 500 | PP | I: A-AA | APCI (+) | SIM | SIL | 2.5 | 10 | Yes | | [115] |
| TNP-470 | Human plasma | 500 | SPE | I: A-AA | APCI (+) | SIM | SIL | 0.63 | 10 | Yes | | [115] |
| TNP-470 | Human plasma | 1000 | LLE | I: A-AA | APCI (+) | MRM | SIL | 0.25 | 5 | Yes | | [116] |
| 3´-C-ethynylcytidine | Human plasma | 200 | SPE | I: M-AA | ESI (+) | MRM | SIL | 1 | 7 | No | | [117] |
| 3´-C-ethynylcytidine | Human urine | 50 | SPE | I: M-AA | ESI (+) | MRM | SIL | 10 | 7 | No | | [117] |
| Methoxyamine | Human plasma | 150 | On-line SPE | I: A-W | ESI (+) | MRM | SIL | 1 | 5 | No | | [118] |
| Methoxyamine | Mouse plasma | 150 | On-line SPE | I: A-W | ESI (+) | MRM | SIL | 1 | 5 | No | | [118] |

Abbreviations: A Acetonitrile; AA Ammonium acetate in water; AH Ammonium hydroxide in water; An. Structural analogue; APCI Atmospheric pressure chemical ionisation; Aut. Automated; ESI Electrospray ionisation; F Formic acid; I Isocratic elution; IS Internal standard; LC Liquid chromatography; LLE Liquid-liquid extraction; LLOQ Lower limit of quantitation; M Methanol; Met. Quan. Metabolites quantified; MRM Multiple reaction monitoring mode; MS Mass spectrometry; PP Protein precipitation; Ref. References; SIL Stable isotopically labeled internal; SIM Selective ion monitoring mode; SPE Solid phase extraction; W Water.

amount of sample [115]. Ong et al. demonstrated, however, that SPE produced a high variability in the obtained results, especially for the metabolites. LLE appeared to be more robust for the extraction of TNP-470 and metabolites from human plasma. In addition, it was shown that when blood is collected it should be acidified immediately to prevent degradation of TNP-470. An LLOQ of 0.25 ng/mL was obtained from 1,000 µL of plasma [116].

3´-C-ethynylcytidine, a novel cytidine analogue, inhibits RNA synthesis by blocking RNA polymerases I, II, and III. A sensitive and selective assay for the quantitative determination of this compound in human plasma and urine has been developed using SPE-LC-MS/MS. A stable isotopically labeled internal standard was available and validation results were excellent [117].

The anticancer agent methoxyamine is a biochemical inhibitor of the base excision repair pathway and appears to synergize with alkylating antitumor agents. Methoxyamine is a small basic molecule which is positively charged at physiological pH, thus complicating chromatography. Methoxyamine has a molecular weight of 47.06 Da, which it is too low for LC-MS analysis. Furthermore, the molecule does not contain a chromophore. Methoxyamine was derivatized with 4-(N,N-diethylamino)benzaldehyde in the presence of formic acid at 80 °C for one hour resulting in methoxyiminato-4-(N,N-diethylamino)benzaldehyde (MW 206 Da) and a reaction yield of 80% or higher. The high temperature was necessary to recover methoxyamine from protein-binding. After derivatization sample clean-up was performed on-line using an Oasis HLB extraction column. The assay was used to analyze plasma samples from a pharmacokinetic study in mice [118].

## Conclusions and perspectives

The successful hyphenation of LC and MS has had an enormous impact on the field of quantitative analysis of anticancer agents from biological samples. MS detection provides better sensitivity and selectivity than UV detection and, in addition, is applicable to a significantly larger group of compounds than fluorescence or electrochemical detection. Most publications discussed in this paper appeared in the last couple of years. Developments in the field of hyphenated LC and MS for quantitative detection are still to be expected, such as more sensitive and robust mass spectrometers and the further exploration of the potential of MS detection for high-throughput analyses, such as infusion nanoelectrospray chips [119]. Furthermore, quantitative isotope measurements using accelerated MS may be implemented more widely. This technique allows the very sensitive detection of rare and long-lived isotopes from very small sample volumes, with a minimum of radioactivity [120]. It may be an important tool for the determination of Phase I ADME profiles of new compounds, and the analysis of very small amounts of analyte in small or complex samples. In addition, new MS techniques are emerging to fulfill the ever increasing demands of researchers, such as the Q-Trap™. This MS provides the same sensitivity for quantitative analysis as TQ machines in MRM mode, but also high sensitivity full scans for qualitative analysis [121].

## Abbreviations

| | |
|---|---|
| AAS | Atomic Absorption Spectroscopy |
| ADME | Absorption-Distribution-Metabolism-Elimination |
| APCI | Atmospheric Pressure Chemical Ionization |
| API | Atmospheric Pressure Ionization |
| BMC | 4-Bromo-methyl-7-methoxycoumarin |
| CSF | Cerebrospinal Fluid |
| Da | Dalton |
| DNA | Deoxyribose Nucleic Acid |
| EI | Electron Impact |
| ELISA | Enzyme-linked Immunosorbent Assays |
| ESI | Electrospray Ionization |
| FDA | Food and Drug Administration |
| GC | Gas Chromatography |
| HN | Heated Nebulizer |
| ICP | Inductively Coupled Plasma |
| ID | Internal Diameter |
| ISP | Ionspray |
| IUPAC | The International Union of Pure and Applied Chemistry |
| LC | Liquid Chromatography |
| LLE | Liquid-liquid Extraction |
| LLOQ | Lower Limit of Quantitation |
| MMP | Matrix Metalloproteinase |
| MMPI | Matrix Metalloproteinase Inhibitor |
| MRM | Multiple Reaction Monitoring |
| MS | Mass Spectrometry |
| NPD | Nitrogen Phosphorous Detection |
| 4-PP | 4-Piperidinopiperidine |
| RNA | Ribose Nucleic Acid |
| SERM | Selective Estrogen Receptor Modulator |
| SIL | Stable Isotopically Labeled |
| SIM | Selective Ion Monitoring |
| SLE | Solid-liquid Extraction |
| SPE | Solid Phase Extraction |
| TFA | Tri-fluoroacetic Acid |
| TISP | Turbo Ionspray |
| TLC | Thin Layer Chromatography |
| TOF | Time-of-Flight |
| TQ | Triple Quadrupole |
| UV | Ultraviolet |

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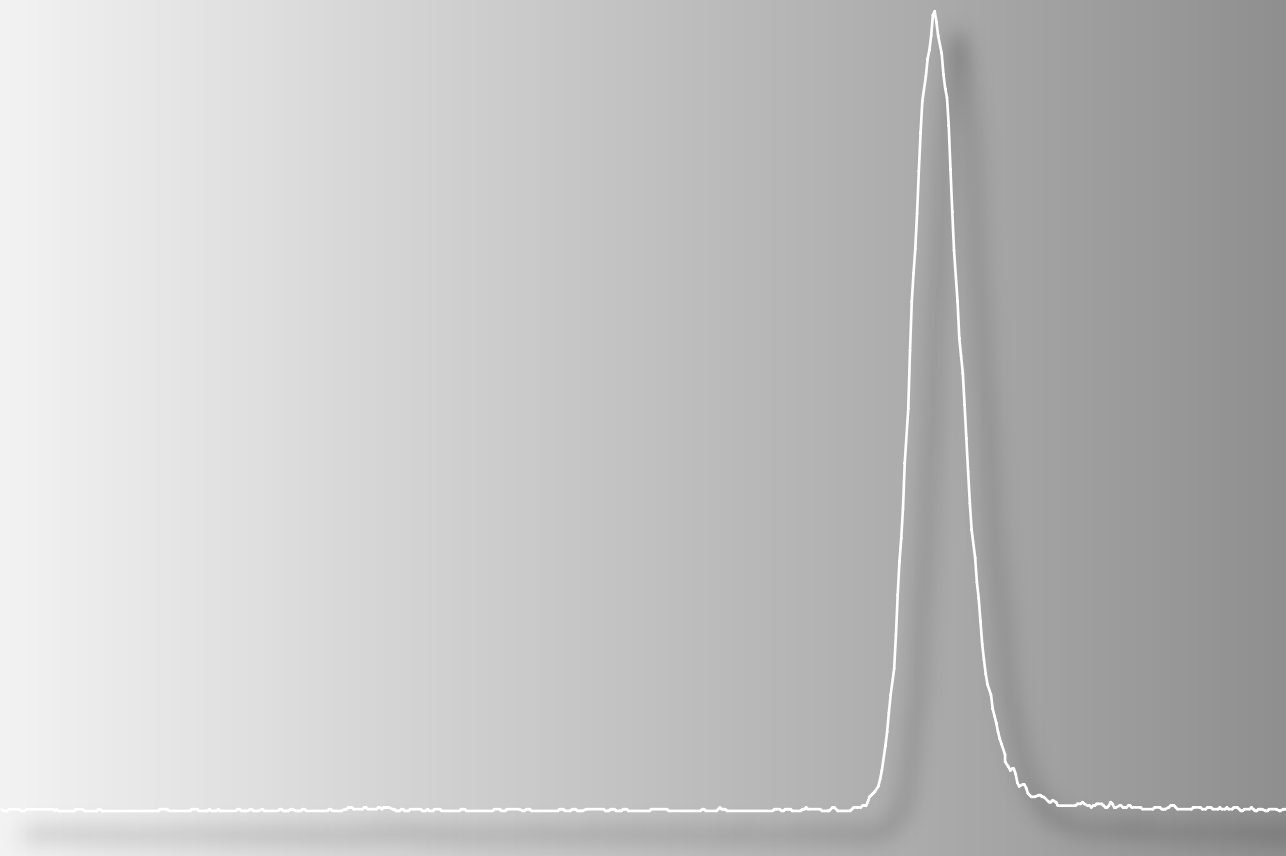
# Chapter 2

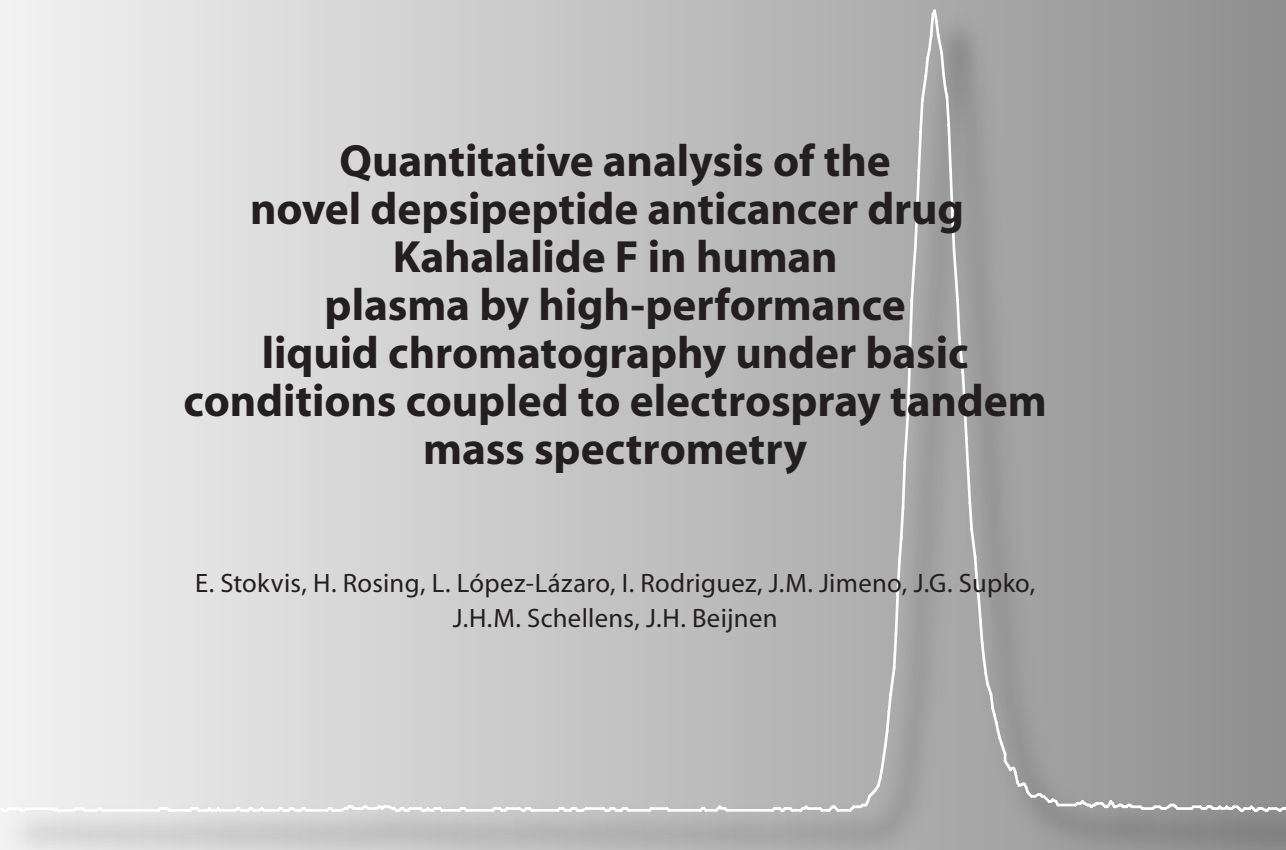


## Marine anticancer agents

# 2.1

## Quantitative analysis of the novel depsipeptide anticancer drug Kahalalide F in human plasma by high-performance liquid chromatography under basic conditions coupled to electrospray tandem mass spectrometry

E. Stokvis, H. Rosing, L. López-Lázaro, I. Rodriguez, J.M. Jimeno, J.G. Supko, J.H.M. Schellens, J.H. Beijnen



J Mass Spectrom 2002; 37: 992

## Abstract

Kahalalide F (KF) is a novel cyclic depsipeptide anticancer drug, which has shown anticancer activity both *in vitro* and *in vivo* especially against human prostate cancer cell lines. To characterize the pharmacokinetics of KF during a Phase I clinical trial in patients with androgen refractory prostate cancer, a method was developed and validated for the quantitative analysis of KF in human plasma using high-performance liquid chromatography (HPLC) coupled to positive electrospray tandem mass spectrometry (ESI-MS/MS). Microbore reversed-phase liquid chromatography (LC) performed with mobile phases containing trifluoroacetic acid, an additive commonly used for separating peptides, resulted in a substantial suppression of the signal for KF on ESI-MS/MS. An alternative approach employing a basic mobile phase provided an excellent response for KF when detected in the positive ion mode. Plasma samples were prepared for LC-MS/MS by solid phase extraction on C18 cartridges. The LC separation was performed on a Zorbax Extend C18 column (150 x 2.1 mm ID, particle size 5 μm) with acetonitrile – 10 mM ammonium hydroxide in water (85:15, v/v) as the mobile phase, at a flow-rate of 0.20 mL/min. A butyric acid analogue of KF was used as the internal standard. The lower limit of quantitation (LLOQ) using a 500 μL sample volume was 1 ng/mL and the linear dynamic range extended to 1,000 ng/mL. Inter-assay accuracy of the assay was -15.1% at the LLOQ and between –2.68 and –9.05% for quality control solutions ranging in concentration from 2.24 to 715 ng/mL. Inter-assay precision was 9.91% or better at these concentrations. The analyte was stable in plasma under all relevant conditions evaluated and for a period of 16 hours after reconstituting plasma extracts for LC analysis at ambient temperature.

## Introduction

Kahalalide F (KF; Figure 1) is a depsipeptide originally isolated from the marine mollusc *Elysia rufescens*, an organism living in the seas near Hawaii [1]. KF is a tridecapeptide with a molecular weight of 1478 composed of a macrolidic region and a linear region with a short chain fatty acid conjugated to the *N*-terminus. It represents the largest and most biologically active peptide that has been isolated from *E. rufescens,* showing *in vitro* and *in vivo* antiproliferative activity against cell lines established from a variety of human solid tumors, particularly prostate cancer. KF appears to target lysosomes as its most likely mechanism of action, among other effects [1,2]. A clinically acceptable formulation for the drug was developed as a lyophilized product for intravenous use in order to support the phase I clinical trials program, undertaken by the Netherlands Cancer Institute and several Spanish centers [3].

The marine derived anticancer drugs, including KF, are known for their high potencies [4]. Since these compounds are typically administered at very low doses, considerable demands are placed upon the sensitivity of analytical methods used to characterize their pharmacokinetic behavior in humans. Analytical methods with lower limits of quantitation (LLOQs) in the nanogram or picogram per millilitre range are often required, rendering

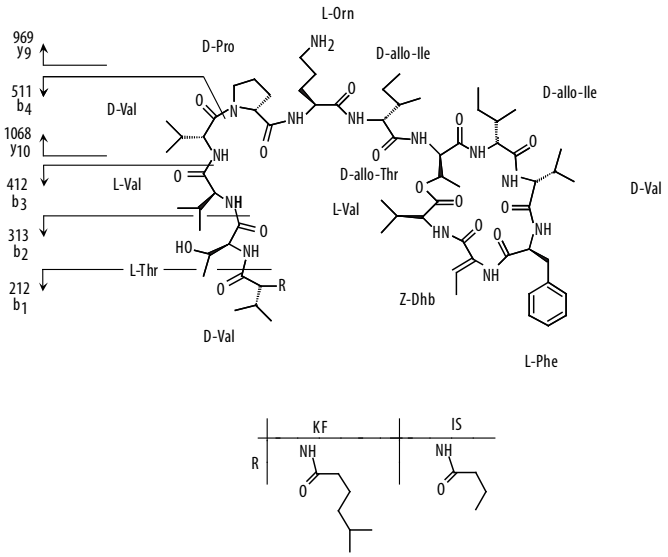



**Figure 1.** Structures of KF and the internal standard. Proposed fragmentation patterns of KF are indicated.

conventional methods of detection following liquid chromatography (LC) unsuitable [5,6]. Because of its sensitivity and selectivity, mass spectrometry (MS) is the detection method of choice for high-performance liquid chromatography (HPLC) in the field of pharmacokinetics [7-12] and has been for more than ten years [13].
This is the first report describing the development of a bioanalytical method for the quantitative analysis of KF in human plasma using reversed-phase HPLC with tandem mass spectrometric detection, its validation, and implementation in the clinical phase I trial.

## Experimental

### Chemicals

KF reference standard (lot 300) and its butyrate derivate (lot 12; Figure 1), which was used as the internal standard (IS), were obtained from Pharma Mar S.A. (Madrid, Spain). HPLC grade acetonitrile and methanol were purchased from Biosolve Ltd. (Amsterdam, The Netherlands). Analytical grade ammonium hydroxide (25%, w/v) and hydrochloric acid (37%, w/v) were obtained from Merck (Darmstadt, Germany). Double distilled water was used throughout the analyses. Drug free plasma was obtained from the Central Laboratory for Blood Transfusion (Amsterdam, The Netherlands).

### Instrumentation

Chromatographic separations of KF and the IS were carried out using an 1100 Series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) consisting of a binary pump, auto sampler, mobile phase degasser, and column oven. A mobile phase of acetonitrile - aqueous 10 mM ammonium hydroxide (85:15, v/v) was delivered at a flow rate of 0.2

mL/min through a Zorbax Extend C18 column (150 x 2.1 mm ID, particle size 5 μm; Agilent Technologies) protected with a 5 μm inline filter (Upchurch Scientific, Inc, Oak Harbor, WA, USA), and thermostatted to 30°C. The eluent was split 1:4 before entering a PE Sciex API 2000 triple quadrupole MS equipped with an electrospray source (ESI; Sciex, Thornhill, ON, Canada). The injection volume was 10 μL. Multiple reaction monitoring (MRM) chromato-

**Table 1.** Settings of the API 2000

| **Parameter** | **Setting** | |
|---|---|---|
| Duration | 8 min. | |
| Ionspray voltage (positive ion mode) | +5500V | |
| Curtain gas ($N_2$) | 15 psi | |
| Temperature | 350 °C | |
| Nebulizer gas (compressed air) | 35 psi | |
| Turbo gas ($N_2$) | 65 psi | |
| Interface heater | On | |
| Collision Activated Dissociation gas ($N_2$) | 3 psi | |
| Deflector | -200 V | |
| Channel Electron Multiplier | 2400 V | |
| Ion Energy Q1 | 0.6 V | |
| Ion Energy Q3 | 2.5 V | |
| | **Kahalalide F** | **Internal standard** |
| Q1 mass (amu) | 740 | 719 |
| Q3 mass (amu) | 212 | 370 |
| Dwell time | 150 ms | 150 ms |
| Declustering Potential | 71 V | 51 V |
| Focussing Potential | 310 V | 350 V |
| Entrance Potential | -9 V | -12 V |
| Collision Cell Entrance Potential | 30 V | 32 V |
| Collision Energy | 49 V | 29 V |
| Collision Cell Exit Potential | 4 V | 8 V |

grams were used for quantification using Analyst™ software version 1.1 (Sciex). The ESI-MS/MS operating parameters used in this study are listed in Table I.

**Preparation of calibration standards and quality control samples**

A KF stock solution was prepared in methanol at a concentration of 100 μg/mL. This solution was diluted further with methanol to obtain working solutions with concentrations of 0.1, 1, and 10 μg/mL, respectively. The working solutions were spiked into plasma to provide quality control samples at three concentration levels (2.24, 446, and 715 ng/mL) and an additional solution around the LLOQ level (0.894 ng/mL). An independently prepared stock solution was diluted with methanol to obtain working solutions with concentrations ranging from 20 ng/mL to 20 μg/mL. These working solutions were used to prepare calibration standards. A fixed amount of each working solution (25 μL) was added to 500 μL plasma to yield calibration standards with concentrations ranging from

1-1,000 ng/mL. A stock solution of the IS (100 µg/mL in methanol) was diluted further with methanol to yield a working solution with a concentration of 1 µg/mL. All solutions were stored at nominally –20 °C.

**Sample preparation**

Bond Elut C18 solid phase extraction (SPE) cartridges (1 mL/100 mg, Varian, Harbour City, CA, USA) were activated with 1.0 mL of methanol and washed with 2.0 mL of water. A 500 µL aliquot of plasma, to which 25 µL of the IS working solution was added, was loaded onto the cartridge at a flow rate of approximately 1 mL/min. After successively washing with 2.0 mL of water and 2.0 mL of 80% methanol, KF and the IS were eluted with 1 mL of 0.1 N hydrochloric acid in methanol. After evaporation of the solvent, the residue was redissolved in 400 µL acetonitrile - 10 mM aqueous ammonium hydroxide (50:50, v/v) by vortexing the solutions for 15 minutes, then passed through a 0.45 µm Micro-spin filter (Alltech Associates. Inc., Deerfield, IL, USA). The volume of the final solution injected onto the analytical column was 10 µL.

**Validation procedures**

*Linearity*

Seven plasma calibration standards, with KF concentrations ranging from 0.927 to 927 ng/mL, were prepared and analyzed in duplicate in three separate analytical runs. Calibration curves were calculated by least-squares linear regression using a weighting factor of $1/x^2$ (the reciprocal of the squared concentration). From a constructed calibration curve standard concentrations were back-calculated. Deviations of the mean calculated concentrations over three runs should be within ±15% from nominal concentrations for at least two-third of the non-zero calibration standards, except at the LLOQ where a deviation of ±20% was permitted [14].

*Accuracy and precision*

Accuracy and precision of the assay were established by analyzing quality control samples of KF. Five replicates of each sample were analyzed together with a complete set of calibration standards, in 3 analytical runs. Intra-assay accuracy was determined as the percent difference between the mean concentration per analytical run and the nominal concentration, inter-assay accuracy as the percent difference between the mean concentration after three analytical runs and the nominal concentration. The coefficient of variation provided the measure of intra- and inter-assay precision. Accuracy should be within 15% except at the LLOQ concentration, where it should be within ±20%. Precisions should be less than 15% except at the LLOQ concentration, where it should be less than 20% [14].

*Specificity and selectivity*

Sets of drug-free plasma from six individual donors were prepared for analysis and analyzed to determine whether endogenous matrix constituents interfere with the analyte or IS. Interference may occur when co-eluting endogenous compounds produce ions at the same m/z values that are used to monitor the analyte and IS. Peak areas of en-

dogenous compounds co-eluting with the analyte should not exceed 20% of the analyte peak area at the LLOQ [15].

*Recovery*
Absolute recovery of KF from plasma was determined by comparing the chromatographic peak area of the drug for plasma quality control samples at the three concentration levels to a corresponding set of solutions containing 100% of the theoretical concentration. Recovery need not be 100%, but should be consistent, precise and reproducible [14].

*Stability*
The stability of KF was investigated at various concentrations during all steps of the analysis, which includes the stability in the stock and working solutions, in plasma during storage, processing, and after three freeze (-20 °C) - thaw cycles, in elution solvent, in the dry extract, and in the final extract. KF is considered stable in the biological matrix when 80-120% of the initial concentration is found. In stock and working solutions KF is considered stable when 90-110% of the initial concentration is found.

**Clinical studies**
During the initial phase I trial undertaken by the Netherlands Cancer Institute patients received a 1 h intravenous infusion daily for five consecutive days, every three weeks (one cycle). This trial complies with the requirements of the "Declaration of Helsinki" and is performed under a protocol which has been reviewed and approved by the appropriate Ethics Committee. Blood samples were collected in heparinized tubes at several time points and immediately centrifuged (4 °C, 10 min at approximately 1,200 g). Plasma was removed and stored in polypropylene tubes at –20 °C until analysis. Pharmacokinetic parameters were obtained using a model independent approach. For each patient the maximum drug concentration ($C_{max}$) and the time to maximum drug concentration were generated directly from the experimental data. From a concentration versus time plot the total area under the curve ($AUC_{0\rightarrow\infty}$) was calculated using the trapezoidal rule with extrapolation to infinity using terminal rate constant k ($C_{last}/k$, where $C_{last}$ is the last determined concentration). The elimination half-life ($t_{½}$) was calculated as (ln 2)/k and the total plasma clearance ($Cl_{tot}$) as the dose divided by the AUC. The volume of distribution was determined using the equation $V=Cl_{tot}/k$.

## Results and Discussion

During optimization of the mass spectrometric parameters, the most intense peak in the Q1 spectrum of KF corresponded to the doubly protonated molecular ion at m/z 740 (Figure 2). The peak at m/z 1479 corresponds to the singly charged molecular ion. Furthermore, doubly charged sodium and potassium adducts as well as fragment ions were observed. MS/MS experiments were performed to determine the optimal fragmentation reaction for MRM analysis. In Figure 3, the peak corresponding to the molecular ion

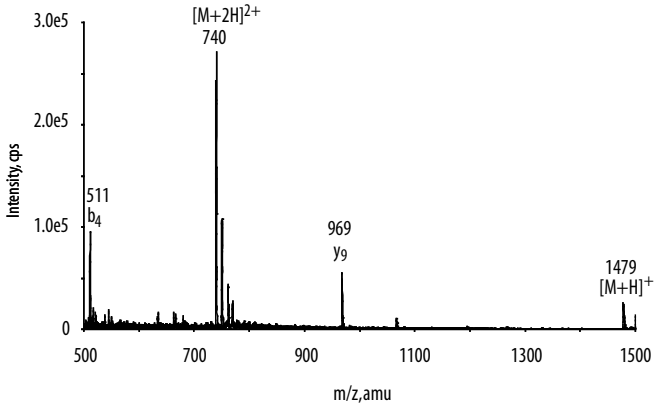



**Figure 2.** Q1 mass spectrum of KF.

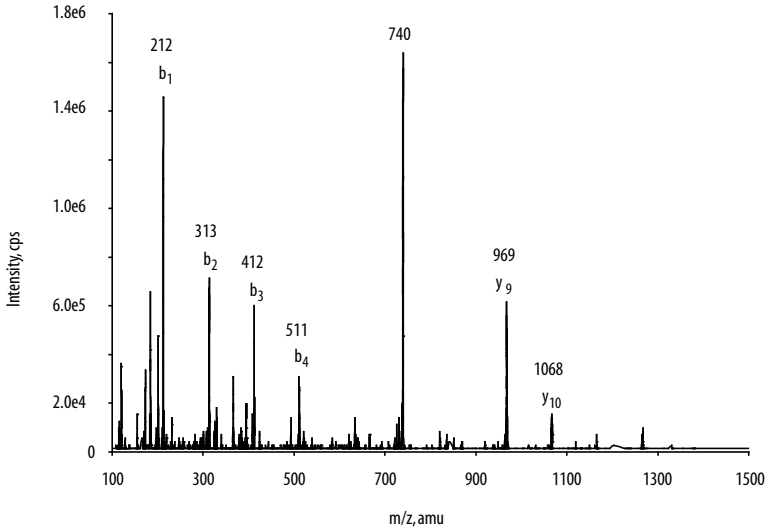



**Figure 3.** Tandem mass spectrum of KF (selected ion m/z 740).

(m/z 740) is present as the base peak in the spectrum. When a doubly charged molecule is induced to dissociate, singly charged fragments can form, some of which may have a higher m/z value than the molecular ion. The fragment at m/z 212 is consistent with D-valine-(5-methyl-hexanoic acid), a $b_1$-ion (Roepstorff nomenclature for peptide fragmentation), resulting from cleavage of the amide bond between the D-valine and L-threonine residues in the linear region of the molecule, yielding a singly charged product ion, as illustrated in Figure 1. The fragments at m/z 313, 412, and 511 correspond to a $b_2$, $b_3$, and $b_4$-ion respectively, as depicted in Figure 1. The y-ions at m/z 969 and 1068 in the spectrum correspond to the same fragmentation reactions as the b-ions at m/z 511 and 412, respectively, however with the charge retained on the C-terminus. The most sensitive mass

transition for MRM analysis consisted of m/z 740 for the parent ion and m/z 212 for the product ion, after optimalization of MS/MS parameters.

Trifluoroacetic acid (TFA) is commonly used as a mobile phase modifier for the LC separation of peptides and proteins, because it produces excellent chromatographic peak-shape [16,17]. Unfortunately, volatile strong acids such as TFA cause significant signal suppression when used with electrospray ionization mass spectrometry, typically resulting in a >10-fold reduction in sensitivity [16]. Methods have been reported to enhance response, such as reducing the TFA concentration, replacing TFA with other aliphatic acids such as formic acid, acetic acid, or propionic acid, or post-column addition of a mixture of propionic acid and isopropanol (75:25, v/v) after a chromatographic separation in the presence of TFA in the eluent [16,17]. The effects on the sensitivity for detecting KF using several of these methods were explored. Results are presented in Figure 4. Relative to an eluent containing 0.04% TFA, decreasing the TFA concentration 10 and 100 fold to 0.004% and 0.0004% TFA results in a signal gain of 1.8 and 2.7, respectively. Replacing TFA with 0.04% formic acid, acetic acid, or propionic acid, results in a signal gain as well. However, although these changes in eluent composition have a positive effect on the sensitivity, the chromatographic behavior deteriorates, probably resulting from the poor ion-pairing characteristics of the eluent [18]. Post-column addition of a mixture of isopropanol and propionic acid does not interfere with chromatography and in some cases the signal increases 10-50 fold [16,17]. However, for KF a decrease in signal was observed when this mixture was added post-column.

An alternative approach that we explored was to perform the HPLC separation of KF under basic conditions using an eluent composed of aqueous ammonium hydroxide and acetonitrile together with a column containing a base-stable stationary phase. This

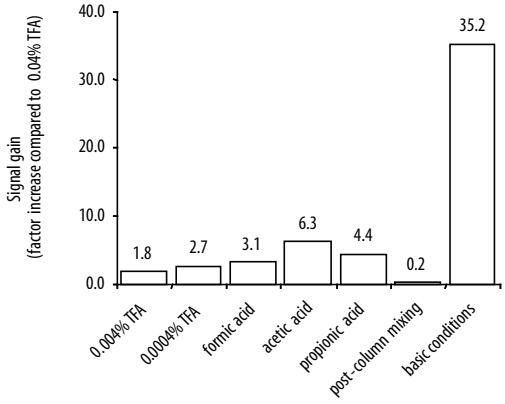



**Figure 4.** Comparison of signal gain for different methods providing alternatives for the use of TFA in the eluent. The factor of increase is determined with respect to signal obtained using 0.04% TFA in the eluent. The TFA concentration was decreased to 0.004% and 0.0004%. 0.04% TFA was replaced by 0.04% formic acid, 0.04% acetic acid, or 0.04% propionic acid. After chromatographic separation utilizing 0.04% TFA in the eluent, a mixture of propionic acid – isopropanol (75: 25, v/v) was mixed with the eluent post-column. Elution was carried out under basic conditions using a mixture of acetonitrile and 10 mM ammonium hydroxide in water (85:15, v/v).

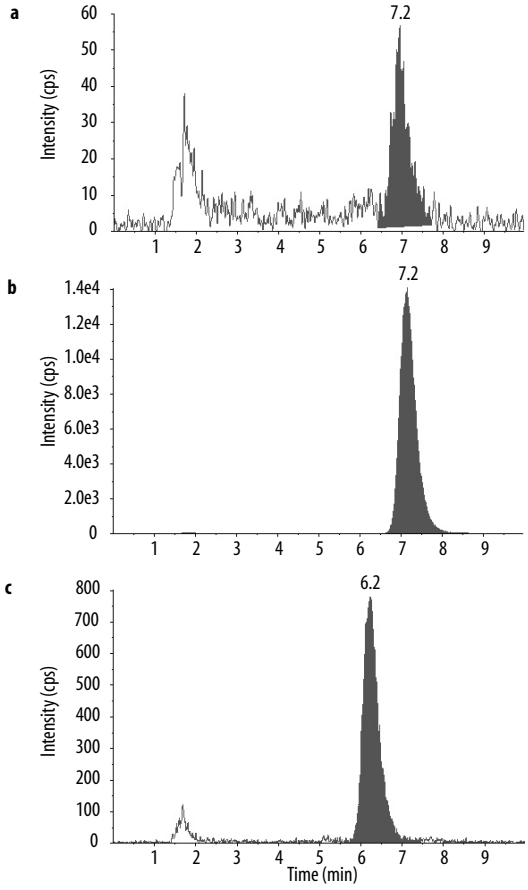



**Figure 5.** Representative MRM chromatograms of KF and IS. (a) Control blank human plasma spiked at the LLQ level of 1 ng/mL for KF. (b) Control human blank plasma spiked at a concentration in the middle of the dynamic range (500 ng/mL). (c) The butyric acid analogue of KF.

**Table 2.** Calibration concentrations back-calculated from the nominal concentrations.

| Run number | Concentration (ng/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.927 | 4.64 | 9.27 | 46.4 | 92.7 | 464 | 927 |
| 1 | 0.957 | 4.48 | 9.44 | 44.7 | 105 | 420 | 847 |
| | 0.909 | 4.51 | 8.81 | 48.3 | 106 | 460 | 913 |
| 2 | 0.922 | 4.70 | 9.39 | 49.8 | 95.2 | 432 | 879 |
| | 0.908 | 5.01 | 9.80 | 47.2 | 84.5 | 472 | 870 |
| 3 | 0.981 | 4.92 | 10.3 | 50.8 | 102 | 434 | 878 |
| | 0.849 | 4.67 | 9.30 | 44.7 | 90.4 | 441 | 816 |
| Mean | 0.921 | 4.72 | 9.51 | 47.6 | 97.2 | 443 | 867 |
| C.V.(%) | 4.95 | 4.53 | 5.28 | 5.36 | 8.89 | 4.36 | 3.79 |
| Dev. (%) | -0.647 | 1.62 | 2.55 | 2.55 | 4.84 | -4.49 | -6.45 |

Dev. Deviation; C.V. Coefficient of variation.

approach resulted in excellent chromatography for KF and a 35-fold improvement in response for the compound as compared to the mobile phase with 0.04% TFA (Figure 4). Although ammonium hydroxide is traditionally used in electrospray negative-ion detection [19,20], the most intense peak in the spectrum of KF was still the doubly positively charged molecular ion at m/z 740. Protonation of the analyte in the mobile phase is not expected because the pH of the aqueous component is approximately 10. The protonation of peptides and other compounds during electrospray (ESI) mass spectrometry performed with a basic eluent has been described [21-24]. In addition, excess protonation has been observed from solutions at near neutral pH, where a more intensive signal for the protonated analyte was observed than could be explained by the $pK_a$ of the analyte and the pH of the eluent [24]. Several mechanisms for generating excess protons have been proposed. For example protons can be generated through solvent oxidation in positive ESI. The resulting decrease in pH has been confirmed using laser-induced fluorescence [25]. Gatlin and Turecek suggested that such effects may be amplified by excess protons that are confined within a thin surface layer of the droplet, so that the local acidity can be 3-4 orders of magnitude higher than that of the bulk solution [26]. These theories seem plausible to account for the excess of protonated analytes observed in unbuffered solutions at near pH neutral; however, in strongly basic solutions, these generated protons will readily react with the excess base although not significantly affecting the pH. In the case of adjusting the pH with ammonium hydroxide, the ammonium ion becomes the predominant cation in the gas phase, rather than protons. It is likely that the strong signal for a doubly protonated molecular ion for KF under these conditions results from gas-phase ion-molecule reactions between ammonium cations and the analyte molecule or collision induced dissociation of ammonium adducts of the analyte. In both cases the protonated analyte and an ammonia molecule are formed. Zhou and Cook state that for either process to be thermodynamically favourable, the analyte´s basic sites, proline and ornithine, although they do not have a higher proton affinity than ammonia in solution, should have a higher proton affinity in the gas-phase. This theory was confirmed for caffeine [24].

Plasma samples were prepared for chromatographic analysis by SPE. This sample pretreatment was favored above protein precipitation, which is simpler and less time consuming. However, SPE allowed KF to be concentrated in the final solution and produced cleaner extracts. Extraction on C18 (100 mg/1 mL) cartridges produced the highest recovery values. Extraction from the cartridges however needed a strong eluent, since both methanol and acetonitrile were not strong enough to elute KF from the SPE columns. Elution with 0.1 N hydrochloric acid in methanol resulted in high, reproducible recoveries (91.1 ± 0.5%). After evaporation of the eluent the residue was redissolved in a more aqueous solution than the eluent in order to concentrate the sample on the top of the analytical column. Representative chromatograms of KF at the LLOQ level (1 ng/mL) and at a median concentration (500 ng/mL) in the validated range and the IS are shown in Figure 5.

**Validation**

Calibration standards were analyzed in duplicate in three analytical runs. Concentra-

**Table 3.** Assay performance data.

| Nominal concentration (ng/mL) | Measured concentration (ng/mL) | Inter-assay accuracy (%) | Inter-assay precision (%) | Number of analytical runs | Number of replicates |
|---|---|---|---|---|---|
| 0.894 | 0.759 | -15.1 | 9.15 | 3 | 15 |
| 2.24 | 2.04 | -9.05 | 6.75 | 3 | 15 |
| 446 | 417 | -6.41 | 9.48 | 3 | 15 |
| 715 | 696 | -2.68 | 9.91 | 3 | 15 |

tions were back-calculated from the nominal concentrations (Table 2). Deviations from mean calculated concentrations over three runs were between –6.45 and 4.84% and the precision ranged from 3.79 to 8.89% for all concentrations. Correlation coefficients of the standard curves were 0.99 or better.

Inter-assay performance data are presented in Table 3. Inter-assay accuracy was within ±15.1% for the LLOQ and within ±9.05% for the other concentrations. Intra-assay accuracy was between –18.8 and –11.4% for the LLOQ and between –12.7 and 8.11% for the other concentrations (data not shown). Inter-assay precision was less than 9.91% for all tested concentrations. Intra-assay precision did not exceed 12.6% (data not shown).

MRM chromatograms of six batches of control human plasma samples did not contain peaks corresponding to endogenous compounds co-eluting with the analyte or the IS, demonstrating the selectivity and specificity of the method.

In Table 4 the results of stability experiments are summarized. KF is stable for at least 10.5 months in the stock solutions, for at least 7.5 months in the working solutions at –20 °C and for at least 24 h at ambient temperatures. Stability in plasma is established after three freeze (-20 °C) – thaw cycles, during at least 24 h at ambient temperatures, and 12 months of storage at –20 °C. During sample pretreatment KF was stable in the elution solvent for at least 24 h at ambient temperatures. Furthermore dry extracts of KF can be kept at 4 °C for at least 22 days. The stability of KF in the autosampler however, is limited. Stability in the final extract is guaranteed for a maximum of 16 h at ambient temperatures, which is sufficient to permit overnight analysis.

**Clinical studies**

The method was applied to support a clinical Phase I trial of KF. To show the applicability of the method a concentration *vs.* time profile of KF of day 1 in a patient treated with 425 μg/m$^2$ KF per day is presented in Figure 6. Maximum concentrations of KF were reached at the end of the infusion. Post infusion the drug concentration declines rapidly. Because of the very low doses that are administered in this study and the rapid elimination of KF from the body KF concentrations were determined in plasma samples up to 2.5 h after the end of the infusion. Relevant pharmacokinetic parameters of KF were calculated and are listed in Table 5.

Possible metabolic reactions of KF have recently been investigated *in vitro* using different enzymatic systems. No indications of potential metabolites were observed after incubating KF with microsomes, human plasma, and uridine 5′-diphosphoglucuronyl transferase

**Table 4.** Stability data

| Conditions | Matrix | Initial conc (ng/mL) | Found conc (ng/mL) | Dev. (%) | C.V. (%) | Number of replicates |
|---|---|---|---|---|---|---|
| -20 °C, 10.5 months | Methanol (stock solution) | $1.01*10^5$ | $1.06*10^5$ | 4.9 | 3.9 | 3 |
| -20 °C, 7.5 months | Methanol (working solutions) | 101 | 100 | -1.0 | 4.6 | 3 |
| | | $2.02*10^3$ | $2.02*10^3$ | 0.0 | 2.7 | 3 |
| | | $2.02*10^4$ | $2.01*10^4$ | -0.5 | 2.1 | 3 |
| Ambient, 24 h | Methanol (working solutions) | 20.2 | 21.2 | 4.9 | - | 1 |
| | | 101 | 94.1 | -6.8 | - | 1 |
| | | 202 | 216 | 6.9 | - | 1 |
| | | $1.01*10^3$ | $1.00*10^3$ | -1.0 | - | 1 |
| | | $2.02*10^3$ | $2.08*10^3$ | 3.0 | - | 1 |
| | | $1.01*10^4$ | $1.03*10^4$ | 2.0 | - | 1 |
| | | $2.02*10^4$ | $1.96*10^4$ | -3.0 | - | 1 |
| 3 freeze (-20 °C) - thaw cylces | Plasma | 2.24 | 2.15 | -4.0 | 5.4 | 3 |
| | | 446 | 483 | 8.3 | 17.4 | 3 |
| | | 715 | 720 | 0.7 | 6.1 | 3 |
| Ambient, 24 h | Plasma | 2.24 | 2.30 | 2.7 | 1.0 | 3 |
| | | 446 | 488 | 9.4 | 2.1 | 3 |
| | | 715 | 673 | -5.9 | 0.1 | 3 |
| -20 °C, 12 months | Plasma | 2.24 | 2.08 | -7.1 | 17.2 | 3 |
| | | 446 | 431 | -3.4 | 2.6 | 3 |
| | | 715 | 647 | -9.5 | 5.8 | 3 |
| Ambient, 24 h | Elution solvent[1] | 2.24 | 2.30 | 2.7 | 6.5 | 3 |
| | | 446 | 437 | -2.0 | 5.5 | 3 |
| | | 715 | 690 | -3.5 | 7.0 | 3 |
| 4 °C, 22 days | Dry extract | 2.24 | 2.47 | 10.3 | 9.8 | 3 |
| | | 446 | 413 | -7.4 | 1.5 | 3 |
| | | 715 | 682 | -4.6 | 4.6 | 3 |
| Autosampler, ambient, 16 h | Reconstitution solvent[2] | 2.24 | 2.36 | 5.3 | - | 1 |
| | | 446 | 476 | 6.7 | - | 1 |
| | | 715 | 654 | -8.5 | - | 1 |
| Autosampler, ambient, 24 h | Reconstitution solvent[2] | 2.24 | 1.53 | -31.7 | 1.7 | 3 |
| | | 446 | 372 | -16.6 | 2.8 | 3 |
| | | 715 | 541 | -24.3 | 5.9 | 3 |
| Autosampler, 4 °C, 16 h | Reconstitution solvent[2] | 2.24 | 2.51 | 12.1 | - | 1 |
| | | 446 | 4.83 | 8.3 | - | 1 |
| | | 715 | 6.94 | -2.9 | - | 1 |

[1]Elution solvent is 0.1 N hydrochloric acid in methanol; [2]Reconstitution solvent is 10 mM ammonium hydroxide in water – methanol (50:50, v/v); conc concentration, Dev. deviation, C.V. coefficient of variation.

**Table 5.** Pharmacokinetic parameters of KF calculated for day 1 in a patient who received a dose of 425 μg/m$^2$.

| Pharmacokinetic parameter | Value |
|---|---|
| Dose (μg/m$^2$) | 425 |
| Total dose (μg) | 833 |
| $T_{max}$ (h) | 0.67 |
| $C_{max}$ (ng/mL) | 61.0 |
| $AUC_{0\rightarrow\infty}$ (h·ng/mL) | 71.9 |
| Half-life $t_{½}$ (h) | 0.53 |
| Clearance (L/h/m$^2$) | 5.91 |
| Distribution volume (L/m$^2$) | 4.49 |

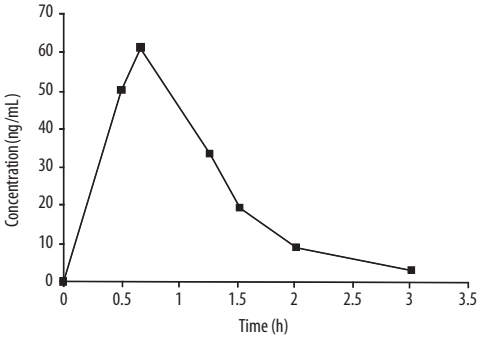



**Figure 6.** Concentration vs. time profile for KF on day 1 in a patient treated with a dose of 425 μg/m2 in a one hour infusion.

(from rat liver) [27]. However a degradation product of KF, referred to as KG was observed under neutral, but especially basic conditions. KG is an acidic analogue of KF in which the lactone in the macrolidic region of the molecule has been hydrolysed [27,28]. The analysis of plasma samples from patients receiving KF in full scan mode with the first quadrupole did not detect any peaks corresponding to metabolites of the drug or KG.

## Conclusions

An LC-ESI-MS/MS assay for the quantitation of the novel anticancer drug KF in human plasma involving SPE to prepare samples for analysis and chromatography performed under basic conditions with positive ion detection mode, has been developed and validated. Concentrations of KF within a range of 1 – 1,000 ng/mL were determined using a 500 μL sample volume.

Results of the validation experiments showed that the method was accurate, reproducible, and selective according to currently recommended guidelines. KF was found to be stable under all conditions evaluated. Upon extraction from plasma, KF is sufficiently stable in solution to permit the overnight analysis of study specimens using an autosampler. This

assay is being successfully applied to the analysis of pharmacokinetic samples acquired during the course of a Phase I clinical trial of the drug in cancer patients. Subsequent studies are being directed to investigate the metabolism of KF.

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# 2.2

## Switching from an analogous to a stable isotopically labeled internal standard for the LC-MS/MS quantitation of the novel anticancer drug Kahalalide F significantly improves assay performance.

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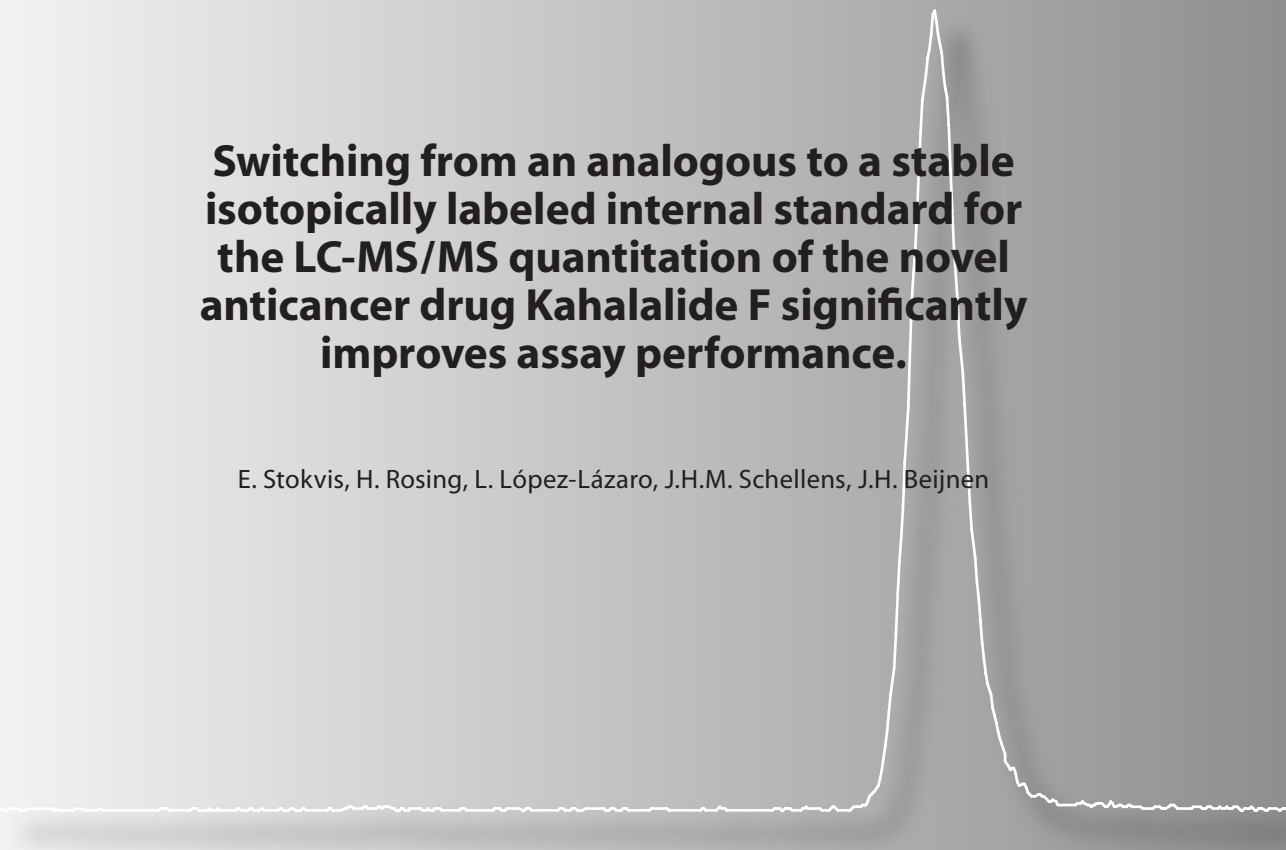

Submitted

## Abstract

The importance of a stable isotopically labeled (SIL) internal standard for the quantitative liquid chromatography tandem mass spectrometric (LC-MS/MS) assay for Kahalalide F in human plasma is highlighted. Similar results can be expected for other LC-MS/MS assays. Therefore, we emphasize the need for a SIL internal standard for accurate and precise LC-MS/MS assays of drugs in biological matrices.

## Introduction

An appropriate internal standard is essential in quantitative liquid chromatography mass spectrometric (LC-MS) assays. MS, known for its sensitivity and selectivity, is not quantitative by nature. The MS response depends on a compound's molecular structure and may vary due to several instrument related parameters, such as the condition, temperature, and pressure of the ion source, and experimental conditions, such as eluent and matrix components simultaneously present in the source [1]. Therefore, the most appropriate internal standards for MS assays are stable isotopically labeled (SIL) internal standards [2], which can be expected to exhibit the same ionization efficiencies as the analyte.
In an earlier publication we have described the development and validation of an LC-MS/MS assay for the quantitative analysis of the investigational anticancer agent Kahalalide F in human plasma and its subsequent implementation into a phase I clinical trial [3]. At that time only a structural analogue was available as internal standard. When a SIL internal standard became available for Kahalalide F, it was implemented into the assay. In this paper we describe the effects of that replacement. The presented results underline the importance of a SIL internal standard in the LC-MS/MS plasma assay of Kahalalide F.

## Experimental

Kahalalide F, SIL Kahalalide F ($^{2}H_{8}$-Kahalalide F), and the butyric acid derivative were provided by Pharma Mar S.A. (Colmenar Viejo, Spain). The experimental conditions of the assay have been described in detail before [3]. In summary, to 500 µL plasma sample 25 µL of internal standard solution was added (1000 ng/mL in methanol), after which the samples were loaded onto C18 solid phase extraction (SPE) cartridges. After washing, Kahalalide F and the internal standard were eluted from the cartridges using 0.1 N HCl in methanol. The solvent was evaporated under a flow of warm nitrogen and the dry extract was reconstituted with methanol – aqueous 10 mM ammonium hydroxide (50:50 v/v). After filtration of the samples 10 µL aliquots were injected onto a Zorbax Extend C18 analytical column (150 x 2.1 mm ID, 5 µm particle size; Agilent Technologies, Palo Alto, CA, USA) thermostatted at 30 °C. The LC system consisted of an HP1100 series binairy pump, degasser, autosampler and column oven (Agilent Technologies). An eluent of acetonitrile – aqueous 10 mM ammonium hydroxide (85:15, v/v) was delivered at a flow rate of 0.2 mL/

min. The eluate was split 1:4 before entering an API 2000 triple quadrupole MS equipped with an ESI source (Sciex, Thornhill, ON, Canada) controlled by Analyst™ software. A mass transition for Kahalalide F from m/z 740 (doubly charged molecular ion) to the $b_1$ ion at m/z 212 (Figure 1) was monitored in the positive multiple reaction monitoring (MRM) mode. For SIL Kahalalide F the same fragmentation reaction was monitored for quantitation (m/z 744 → 212). For the analogous internal standard a mass transition from m/z 719 (doubly charged molecular ion) to the $b_3$ ion at m/z 370 (Figure 1) was monitored. Validation experiments were executed using the analogous internal standard and subsequently the SIL internal standard according to FDA guidelines [4].

## Results

Kahalalide F is a tridecapeptide composed of a cyclic and a linear region with a methylhexanoic acid conjugated to the N-terminus (Figure 1). A butyric acid analogue of Kahalalide F was the only internal standard available during development and initial validation of the method and later replaced by a SIL internal standard.

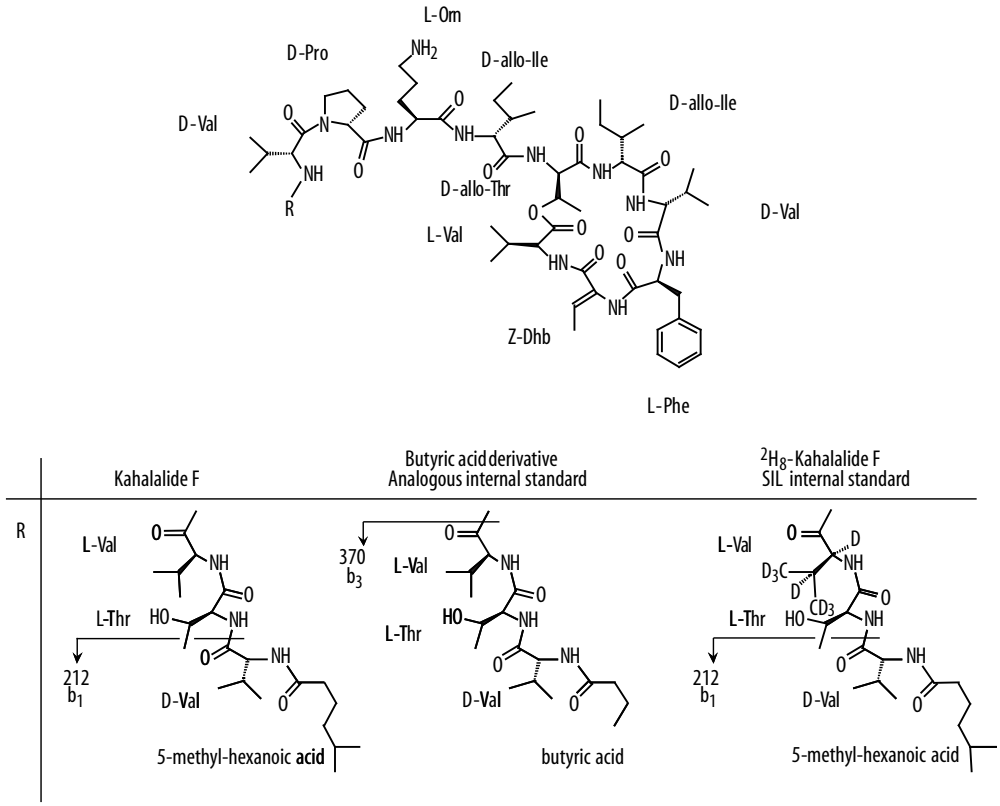


**Figure 1.** Structures of Kahalalide F, its butyric acid derivative, and SIL Kahalalide F.

Statistical evaluations were carried out in order to determine whether the implementation of the Kahalalide F SIL internal standard resulted in an improvement of the assay's performance. Calculated concentrations were documented for calibration standards and quality control samples determined using the two methods. Both for the analogous internal standard (n=284) and the SIL internal standard (n=340) calculated concentrations were corrected for nominal concentrations. A Levene's test for equality of variances was performed followed by an independent samples t-test to compare means. The mean bias was 96.8% for the analogous internal standard with a standard deviation of 8.6% and 100.3% for the SIL internal standard with a standard deviation of 7.6%. The Levene's test showed that the variance using the SIL internal standard was significantly lower ($p=0.02$) than with the use of the butyric acid analogue, meaning that the precision of the method has significantly improved by implementation of the SIL internal standard. In addition, the accuracy of the assay has improved significantly, since the mean bias using the analogous internal standard (96.8%) differs significantly from the true value of 100% ($p<0.0005$) and the bias using the SIL internal standard (100.3%) does not ($p=0.5$).
Another advantage of the implementation of the SIL internal standard was that it allowed a prolongation of the stability of the processed sample. Using the analogous internal standard stability after reconstitution in the autosampler was utmost 16 hours both at ambient temperatures and at 4 °C. This was due to a more rapid degradation of the butyric acid derivative in the final extract than of Kahalalide F. Using the SIL internal standard, analyte and internal standard are chemically identical, and stability in the final extract could be extended to at least 5 days at ambient temperatures and to at least 40 days at 4 °C. Deviations from the analyte concentrations at t=0 were within ±8.08% under these conditions.

## Discussion and Conclusion

During the development of several LC-MS/MS assays in our laboratory we have witnessed that an analogous internal standard often leads to insufficient accuracy and precision. On the other hand, the assay for Kahalalide F using the analogous internal standard was one of few where an analogue could be used as an internal standard in an LC-MS/MS assay. This may be due to the structural similarities between Kahalalide F and its butyric acid derivative. The two compounds differ only in the fatty acid side chain, a relatively small difference considering the size of the molecule, and, in addition, one not likely to have a large influence on the ionization efficiency. However, structural variations which result in differences in chromatographic properties may also influence ionization efficiency. When compounds do not co-elute and thus are not simultaneously ionized, the ionization conditions are not similar which may decrease the accuracy and precision of quantitation. As can be observed in Figure 2, Kahalalide F and the analogous internal standard did not co-elute, in contrary to, as expected, Kahalalide F and the SIL internal standard.
In our opinion similar results as described in this communication could be obtained for other LC-MS/MS assays using an analogous internal standard. Therefore, we want to

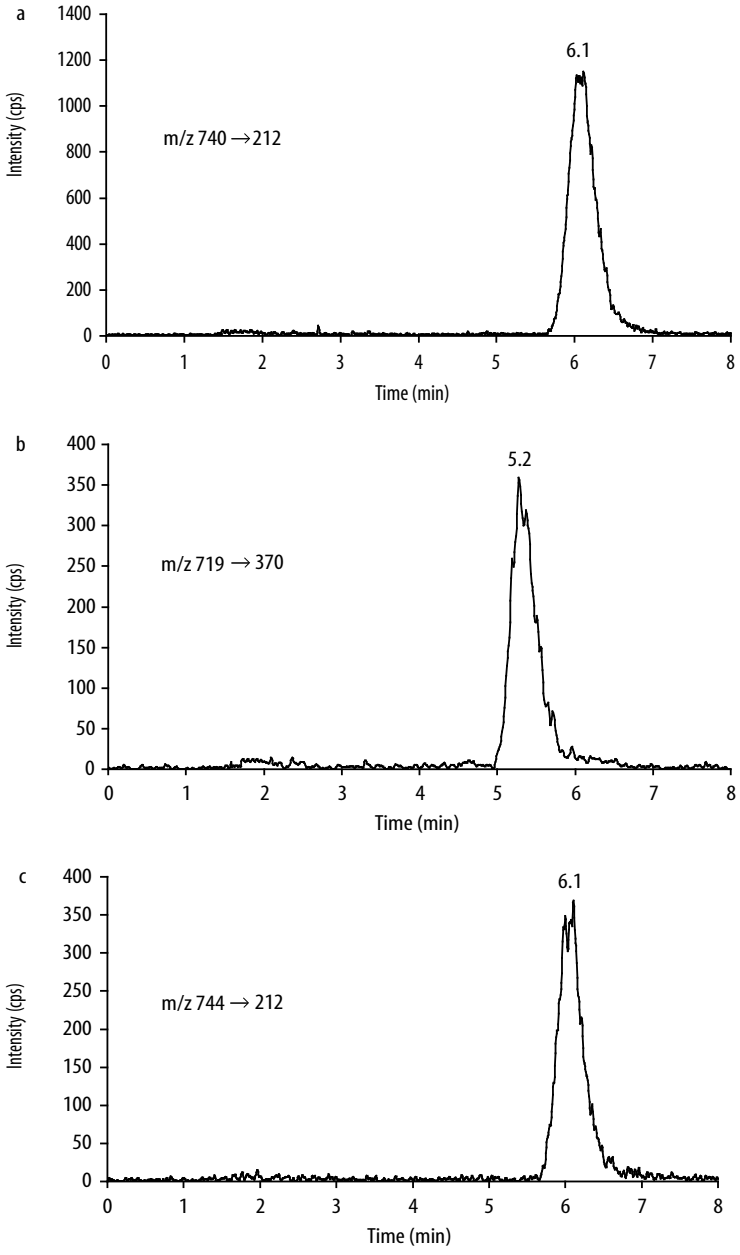


**Figure 2.** Typical MRM chromatograms for a calibration standard (50 ng/mL) for Kahalalide F (a), the analogous internal standard (b), and the SIL internal standard (c).

emphasize again the need of a SIL internal standard for accurate and precise LC-MS/MS assays of drugs in biological matrices, although we realize that this is not always available. In those cases we are forced to use a structural analogue as the second best choice. Profound validation will reveal the assay's performance.

## Acknowledgements

The authors would like to thank the Pharma Mar Synthetic Processes Department for providing the deuterated internal standard.

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# 2.3

## Quantitative analysis of ES-285, an investigational marine anticancer drug in human, mouse, rat, and dog plasma using coupled liquid chromatography and tandem mass spectrometry

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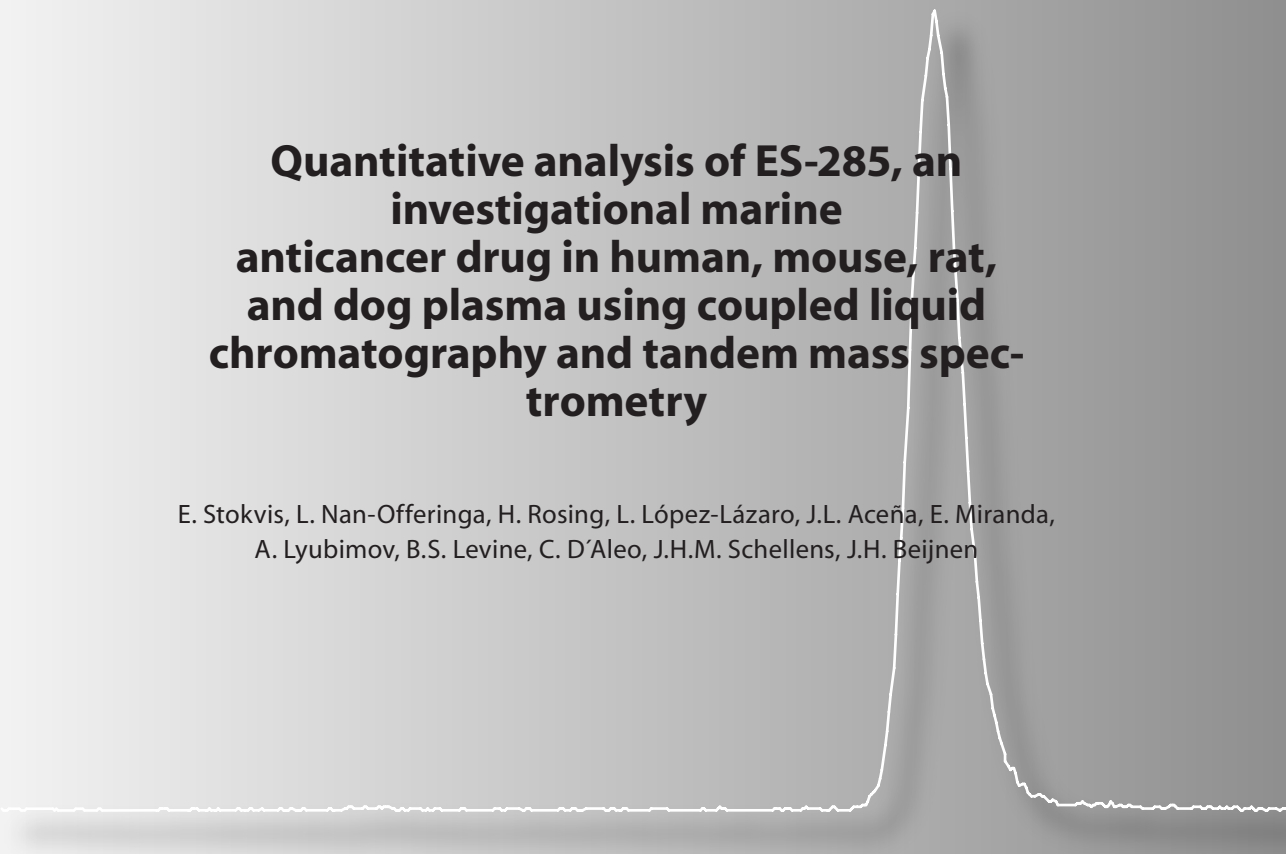

J Mass Spectrom 2003; 38: 548

## Abstract

A method was developed for the quantitative analysis of the novel anticancer agent ES-285 (spisulosine; free base) in human, mouse, rat, and dog plasma using high-performance liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS) in order to support pre-clinical and clinical studies with the drug. Sample preparation was carried out by protein precipitation with acetonitrile, containing isotopically labeled ($^2H_3$) ES-285 as internal standard. Ten microliter aliquots of the supernatant were directly injected onto an Inertsil ODS-3 column (50 x 2.0 mm ID, 5 μm). Elution was carried out using a mixture of methanol – 10 mM ammonium formate pH 4 in water (80:20, v/v) pumped at a flow rate of 0.2 mL/min with a run time of 8 min. Multiple reaction monitoring chromatograms obtained on an API365 triple quadrupole mass spectrometer were used for quantification. The lower limit of quantitation (LLOQ) was 10 ng/mL in human, mouse, rat, and dog plasma and the linear dynamic range extended to 500 ng/mL. A full validation of the method was performed in human plasma, and partial validations were performed in mouse, rat, and dog plasma. Accuracies as well as precisions were lower than 20% at the LLOQ concentration and lower than 15% for all other concentrations in all matrices. ES-285 was stable during all steps of the assay. Thus far this method has been used successfully to analyze over 500 samples in pre-clinical trials, and will be implemented in the planned clinical phase I studies.

## Introduction

ES-285 (spisulosine; anti-β-amino alcohol, (2S, 3R)-2-amino-3-hydroxy-octadecane, free base; Figure 1) is a novel anticancer agent of marine origin with a molecular weight of 285 Da. The compound was isolated from the marine clam *Spisula polynyma*, and has demonstrated *in vitro* antiproliferative activity against a variety of human tumor cell lines, such as colon, gastric, pancreas, pharynx, and renal tumors, but a particular selectivity for hepatomas and slowly growing tumors was noted [1]. During *in vivo* studies in mice significant tumor growth inhibition was observed by ES-285 of human renal tumors, melanoma, and prostate tumors [1]. ES-285 is believed to decrease the activity of Rho proteins [2]. Rho proteins regulate signal transduction from receptors in the membrane to a variety of cellular events such as cell adhesion and motility [3]. Adhesion of tumor cells

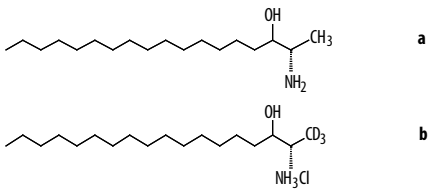


**Figure 1.** Structures of ES-285 (a) and the internal standard (b).

to host cell layers and subsequent transcellular migration are crucial processes in cancer invasion and metastasis [4,5]. Since Rho proteins play an important role in these processes, its inhibition may clearly have an antitumor effect.
An accurate and sensitive assay for the quantitative analysis of an investigational drug in biological samples is essential during studies in animals and humans in order to provide the data needed to determine future development of the drug [6-8]. Traditionally used high-performance liquid chromatography (HPLC) coupled to UV detection has been largely replaced by LC with tandem mass spectrometric detection (MS/MS). MS provides unrivalled sensitivity and selectivity, and is universal [6,9]. ES-285, for example, does not contain a chromophore thus eliminating the possibility of using UV or fluorescence detection. MS detection is the most obvious choice.
In this article, the development of the first method for the quantitative analysis of ES-285 in human plasma using HPLC electrospray (ESI)-MS/MS is described. In addition, a full validation in this matrix as well as partial validations in mouse, rat and dog plasma employing current FDA guidelines for bioanalytical method validation have been executed. The applicability of the method is demonstrated by its implementation in pre-clinical studies.

## Experimental

### Chemicals

ES-285 (free base; lot 1E064; Figure 1) and the $^{2}H_{3}$ ($D_{3}$)-internal standard (hydrochloride salt, lot 3E011A; Figure 1) were obtained from Pharma Mar S.A. (Madrid, Spain). Acetonitrile and methanol (both LC gradient grade) were purchased from Biosolve ltd. (Amsterdam, The Netherlands). Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany) and ammonium formate (analytical grade) was obtained from Sigma (St. Louis, MO, USA). Distilled water was used throughout the analyses. Drug free human plasma was obtained from the Central Laboratory for Blood Transfusion (Amsterdam, The Netherlands). Drug free mouse and rat plasma were kindly provided by Dr. O van Tellingen from the Department of Clinical Chemistry, The Netherlands Cancer Institute (Amsterdam, The Netherlands). Drug free dog plasma was obtained from pre-clinical trials.

### Instrumentation

The HPLC system consisted of a Perkin Elmer (Norwalk, CT, USA) 200 series pump and an ISS 200 autosampler. A mobile phase of methanol - 10 mM ammonium formate pH 4 in water (80:20, v/v) was pumped at a flow rate of 0.2 mL/min through an Inertsil ODS-3 column (50 x 2.0 mm I.D. 5 µm particle size; Chrompack, Middelburg, The Netherlands) protected with an in-line filter (Micro filter frit, 5 µm, Upchurch Scientific, Inc., Oak Harbor, WA, USA). The LC eluate was directed to the waste at the beginning of an analytical run, which was controlled by a WE-II actuator (Valco International, Schenkon, Switzerland). Between 3 and 7 min the LC eluate was introduced directly into an API365 triple quadrupole MS equipped with an ESI source and controlled by MassChrom 1.1 software (Sciex, Thornhill, ON, Canada). Sample injections of 10 µL were carried out and the total run time

was 8 min. The obtained multiple reaction monitoring (MRM) chromatograms were used for quantification using MacQuan 1.6 software (Sciex). ESI-MS/MS parameters used in this study are listed in Table 1.

**Preparation of calibration standards and quality control samples**

A stock solution of ES-285 was prepared in methanol at a concentration of 1 mg/mL. This solution was further diluted with methanol to obtain working solutions with concentrations ranging from 1-50 µg/mL. A fixed amount of working solution was diluted with control human or dog plasma in volumetric flasks in order to obtain calibration standards. Concentrations of the calibration standards ranged from 10-500 ng/mL. An independently prepared stock solution, with separate weighing of ES-285, was diluted with methanol in order to obtain working solutions with concentrations ranging from 1-100 µg/mL. These working solutions were spiked to human, mouse, rat, or dog plasma resulting in quality control samples at three concentration levels (25, 100, and 400 ng/mL). Furthermore, quality control samples at the lower limit of quantitation (LLOQ; 10 ng/mL) were prepared,

**Table 1.** Settings of the API365

| Parameter | | Setting |
|---|---|---|
| Duration | | 8 min. |
| Ionspray voltage (positive ion mode) | | +4000V |
| Orifice voltage | | 51 V |
| Ring voltage | | 200 V |
| Quad 0 | | -12 V |
| Inter Quad 0 | | -13 V |
| Stubbies | | -17 V |
| Rod Offset 1 | | -13 V |
| Inter Quad 2 | | -22 V |
| Rod Offset 2 | | -33 V |
| Inter Quad 3 | | -76 V |
| Rod Offset 3 | | -38 V |
| Q1 Resolution | | Unit |
| Q3 Resolution | | Low |
| Deflector | | -380 V |
| Channel Electron Multiplier | | 2700 V |
| Nebulizer gas (compressed air) | | 1.4 L/min |
| Curtain gas ($N_2$, 5.0) | | 1.1 L/min |
| Turbo gas (compressed air) | | 7 L/min |
| Temperature | | 300 °C |
| Collision Activated Dissociation Gas Thickness ($N_2$, 5.0) | | $1.46*10^{15}$ molecules/$cm^2$ |
| ES-285 | Q1 mass | 286 amu |
| | Q2 mass | 268 amu |
| | Dwell time | 200 ms |
| $D_3$-ES-285 | Q1 mass | 289 amu |
| | Q2 mass | 271 amu |
| | Dwell time | 200 ms |

and in human plasma an additional quality control sample with a concentration higher than the upper limit of quantification (ULOQ; 2500 ng/mL) was spiked. ES-285 solutions in plasma never contained more than 1% of methanol. A stock solution of the deuterated internal standard was prepared similar to that for ES-285. The stock solution was initially diluted with methanol and subsequently a working solution of 100 ng/mL internal standard in acetonitrile was prepared.

**Sample preparation**

Volumes of 100 µL plasma were transferred to 1.5 mL eppendorf tubes (Eppendorf Netheler Hinz GmbH, Hamburg, Germany) and 200 µL of internal standard working solution in acetonitrile (100 ng/mL) was added. The samples were shaken for 15 min at maximum speed (1250 rpm) and subsequently centrifuged for 15 min at 23,100 g. Immediately after centrifugation the supernatant was transferred into autosampler vials and 10 µL aliquots were injected onto the analytical column.

**Validation procedures**

A full validation according to the FDA guidelines was performed for the assay in human plasma [10]. For the assay in mouse, rat, and dog plasma partial validations were performed by means of accuracy and precision, specificity and selectivity, recovery, and stability in plasma under storage conditions according to FDA rules [10].

*Linearity*

Seven plasma calibration standards (10.1, 25.2, 50.4, 101, 252, 353, and 504) were prepared in singular and analyzed in duplicate in three separate analytical runs. Calibration curves were calculated by least-squares linear regression using a weighting factor of 1/x (the reciprocal of the concentration). Concentrations were back-calculated from the constructed calibration curve and deviations from the nominal concentrations should be within ±20% for the LLOQ and within ±15% for other concentrations [10,11].

*Accuracy and precision*

For the validation of the assay in human plasma, quality control samples at five concentration levels were prepared. Quality control samples with concentrations higher than the ULOQ were diluted ten times in human plasma prior to sample processing, in order to validate dilution of samples that were originally above the ULOQ. Five replicates of each sample were analyzed together with a calibration curve, independently prepared from the quality control samples, in three analytical runs. For the validation of the assay in mouse, rat, and dog plasma, quality control samples at four concentration levels were prepared. Five replicates of each sample were analyzed together with a calibration curve, independently prepared from the quality control samples in human plasma (for mouse and rat samples) or in dog plasma (for dog samples), in one analytical run. The accuracy was determined in percent difference between the mean concentration and the nominal concentration. The coefficient of variation was used to report the precisions. The intra- and inter-assay accuracies should be within ±20% for the LLOQ concentration and within

±15% for other concentrations. The precisions should be less than 20% and less than 15%, respectively [10].

*Specificity and selectivity*

From six individual batches of control drug-free human plasma samples containing neither analyte nor internal standard (double blank), samples containing only internal standard (blank), and LLOQ samples were prepared. Double blank, blank, and LLOQ samples were also prepared in human plasma spiked with typical levels of either caffeine, acetaminophen, or morphine. Furthermore, the ES-285 formulation solution, which contains ES-285.HCl and cyclodextrin, a formulation excipient known to improve solubility of lipophilic compounds [12], was spiked directly to drug-free human plasma and subsequently further diluted in human plasma to obtain a concentration within the dynamic range of the assay. These samples were prepared in order to determine whether endogenous compounds, co-medication, or the pharmaceutical vehicle cyclodextrin interferes at the mass transition chosen for ES-285 or the internal standard. Samples were processed according to the described procedures and analyzed. Deviations from the nominal concentrations should be within ±20% for the LLOQ samples [10] and within ±15% for other concentrations.

From three individual batches of control drug-free mouse and one batch of drug free rat plasma double blanks and blanks were prepared. Furthermore control rat and dog samples originating from pre-clinical studies were analyzed. Samples were processed and analyzed in order to determine potential interference from endogenous compounds in mouse, rat, or dog plasma. Peak areas of compounds co-eluting with ES-285 should not exceed 20% of the ES-285 peak area at the LLOQ.

*Recovery*

Recovery experiments were performed by comparing the analytical results of extracted samples at three concentration levels with matrix-free standard solutions.

*Stability*

The stability of ES-285 was evaluated in the stock and working solutions under both processing (ambient temperatures) and storage (-70 °C) conditions. In addition, the stability of the internal standard in the stock solution was determined under storage conditions (-70 °C). ES-285 and the internal standard were considered stable in the stock and working solutions when 90-110% of the fresh sample's ratio was found. The stability in human plasma after three freeze/thaw cycles was investigated by comparing quality control samples that have been frozen and thawed three times with freshly prepared quality control samples. Furthermore, the stability of ES-285 in human plasma under processing (ambient temperatures) and storage (-20 °C) conditions was evaluated. Stability of ES-285 under storage conditions (-20 °C) was also investigated in mouse, rat, and dog plasma. Finally, stability in the final extract for human plasma was determined in the autosampler. ES-285 was considered stable in the biological matrix or extracts thereof when 80-120% of the initial concentration was found.

**Implementation in pre-clinical trials**

The analytical method described in this article has been used to support pre-clinical trials of ES-285, one of which was a trial in rats. During this trial, undertaken by the University of Illinois, 6 animals received 15 mg/kg and 6 received 25 mg/kg of ES-285 intravenously by means of a bolus injection. Blood samples were collected at several time points and, after centrifuging, plasma was removed and stored at –20 °C until analysis.

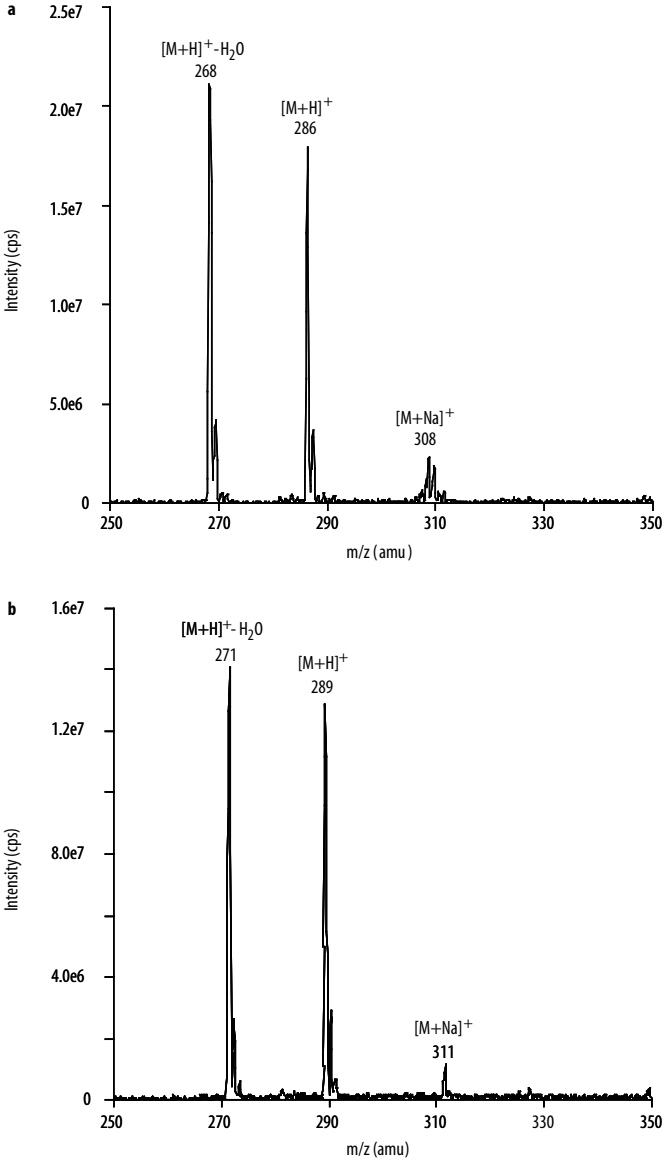


**Figure 2.** Positive Q1 spectrum of ES-285 (a) and D3-ES-285 (b) recorded on an API365 triple-quadrupole MS using continuous infusion.

## Results and Discussion

### Method development

A mass spectrum of ES-285 was recorded in the positive ion mode in the first quadrupole (Q1) of an API365 MS with an m/z range from 250 to 350 amu by continuous infusion of a solution containing approximately 10 µg/mL of ES-285 in methanol-water (80:20, v/v) (Figure 2a). The peak in the spectrum at m/z 286 corresponds to the $[M+H]^+$ ion of ES-285. The peak at m/z 268 corresponds to the loss of a water molecule from ES-285, which, judging from the relative intensities of the peaks, happens easily. The relatively small peak at m/z 308 corresponds to the sodium adduct of ES-285. ES-285 contains a primary amine moiety (Figure 1), which is a strong base ($pK_a$ approximately 10-11) and therefore protonated at neutral pH, thus explaining the strong signal for the $[M+H]^+$ ion. Furthermore, a

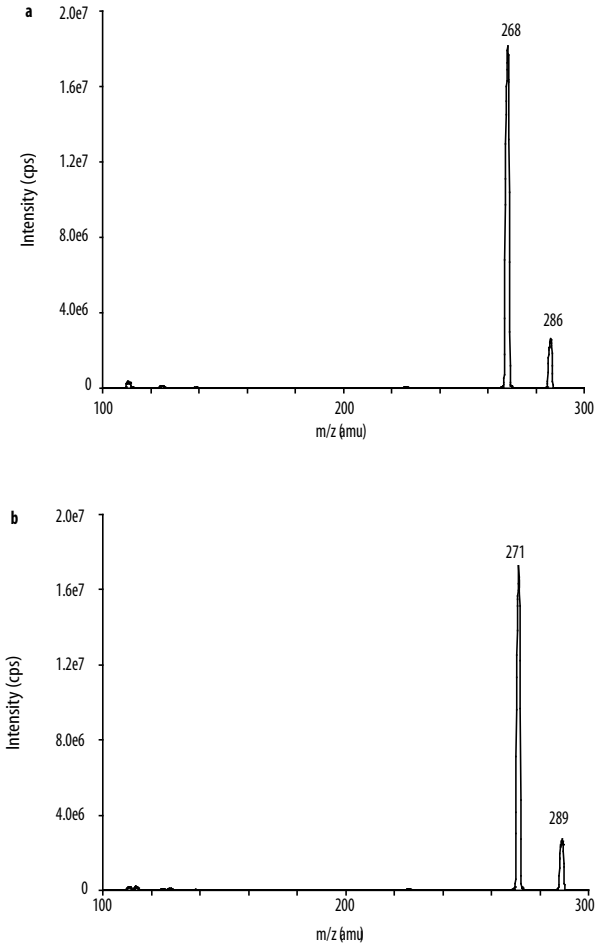


**Figure 3.** Positive tandem mass spectrum of ES-285 (a) and D3-ES-285 (b). The molecular ions (m/z 286 and m/z 289, respectively) were selected for MS/MS analysis.

mass spectrum of $D_3$-ES-285, used as internal standard, was recorded under the same conditions (Figure 2b). Results similar to those obtained for spisolusine were observed.
In order to determine the most suitable fragment ions to use for MRM analysis, MS/MS spectra were recorded for spisolusine and the internal standard selecting their molecular ions ($[M+H]^+$) for fragmentation (Figure 3a and b, respectively). However, for both compounds only the fragment ions corresponding to the loss of a water molecule were observed. Even with increasing collision gas values or collision energy values peaks corresponding to other fragments were not observed. Fragments resulting from the loss of a water molecule may not be suitable to use for MRM analyses, since the loss of water also occurs in the ion source, as observed in the Q1 spectra (Figure 2a and b), suggesting that the loss of water is not very specific.
The loss of water from a molecule is usually dependent of the pH of the solution. In order to determine the influence of the pH on the water elimination and whether this elimination can be influenced at a certain pH value, the intensities of the peaks corresponding to ES-285 (m/z 286) and the elimination of water from ES-285 (m/z 268), as well as the ratio of these intensities were determined as a function of pH in a range from 3 to 6. (Figure 4). Not only peak intensities for both ES-285 and dehydrated ES-285 varied as a result of a pH change, but also the ratio of the two intensities. However, by changing the pH from 3 to 6 the elimination of water from ES-285 was not inhibited. Thus, for stabilizing the elimination reaction the aqueous phase of the HPLC eluent was buffered at a pH value of 4 with an ammonium formate buffer since the most intensive signal for ES-285 was obtained at this pH value.
As a potential alternative for MRM and the subsequent use of the fragment at m/z 268,

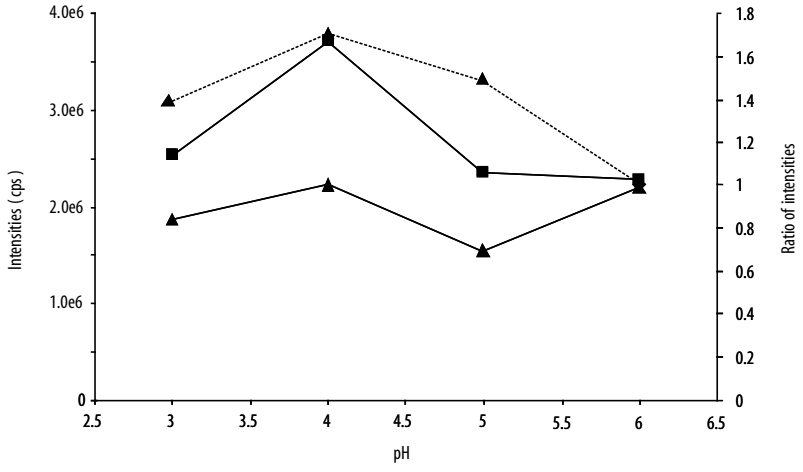


**Figure 4.** The intensities of the peaks corresponding to ES-285 (–■–), ES-285 after elimination of water (–▲–), and the ratio of these intensities (--▲--) as a function of the pH. Solutions of ES-285 in methanol – water (80:20, v/v) were prepared. The aqueous phase was buffered using either ammonium acetate (pH 5 and 6) or ammonium formate (pH 3 and 4) and the pH was adjusted using acetic acid and formic acid, respectively.

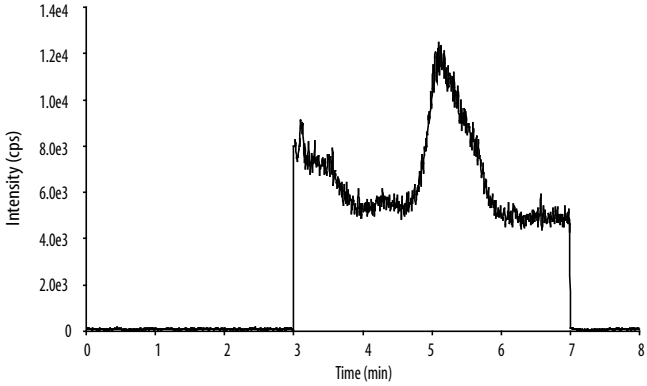


**Figure 5.** Selected ion monitoring (SIM) chromatogram at m/z 286 of a sample containing approximately 100 ng/mL of ES-285 in human plasma.

selective ion monitoring (SIM) of the molecular ions ($[M+H]^+$) of ES-285 at m/z 286 and the internal standard at m/z 289 was investigated. Compared to MRM, the SIM mode is less specific since it only selects the compound of choice by its molecular ion. ES-285 is a small molecule with a molecular mass in the lower mass regions which is an additional negative influence on the specificity in SIM mode since interferences are more likely to occur. A sample containing approximately 100 ng/mL ES-285 in human plasma was processed and in Figure 5 the SIM chromatogram of this sample is shown. Because of the broad peak, low signal to noise ratio, and high signal offset, all due to interferences, SIM was discarded and the use of MRM was further investigated.

Isotopically labeled ES-285, the internal standard, is chemically very similar to ES-285, and should therefore behave similar to ES-285 itself. As a result the fragment corresponding to the loss of water should, in theory, be useable. Multiple injections of a processed plasma sample were carried out using the transitions from the protonated molecular ion to the dehydrated fragment for both ES-285 and the internal standard in order to determine whether the measured MRM signals remain constant during multiple injections. After 60 injections the relative standard deviation of the ratio of the analyte and internal standard area was calculated. This value was 4.9%, which demonstrates high precision and the ability of the internal standard to correct for variations in the ratio of the ES-285 and dehydrated ES-285 areas. Therefore MRM was used throughout the analyses.

## Validation

*Linearity*

Calibration standards in control human plasma were analyzed in duplicate in three analytical runs in a range from 10 to 500 ng/mL. Representative MRM chromatograms of ES-285 at the LLOQ level and at a median level as well as a chromatogram of the internal standard for human plasma are shown in Figure 6. The calibration concentrations were back-calculated from the responses. The deviations from the nominal concentration were between –3.34 and 3.04% for all concentrations.

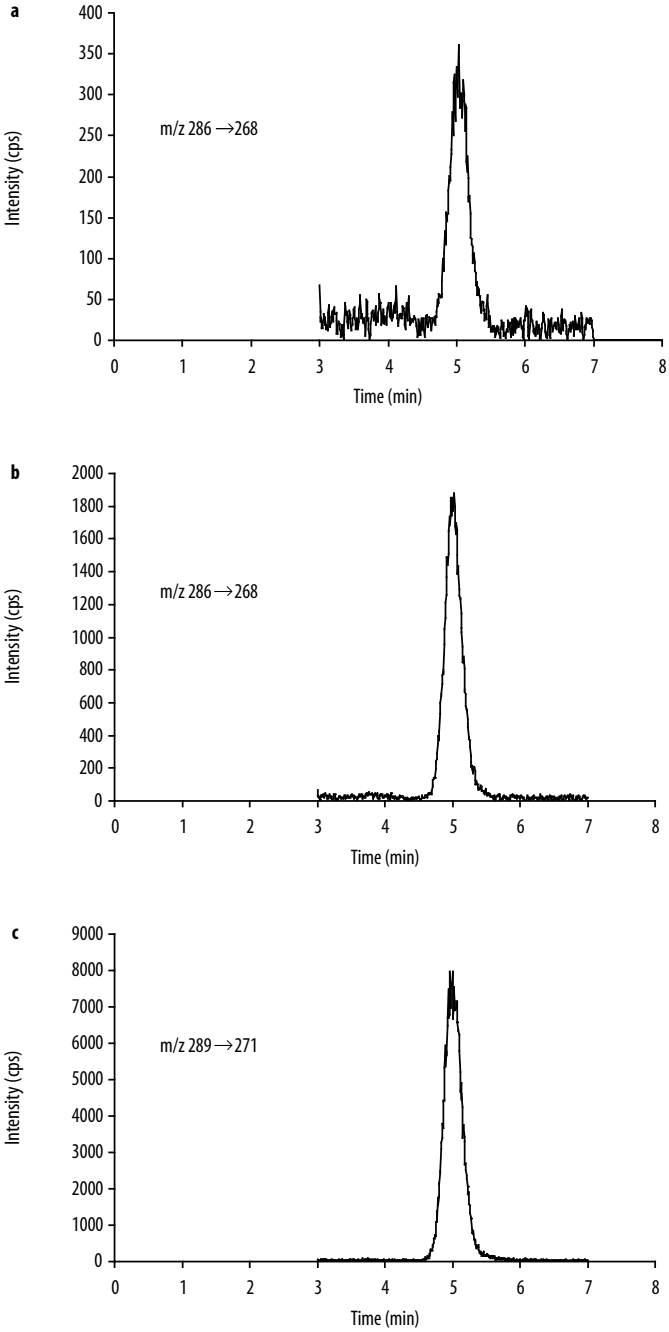


**Figure 6.** Representative MRM chromatograms of ES-285 and the internal standard. (a) Control blank human plasma spiked at the LLOQ level of 10 ng/mL for ES-285. (b) Control human blank plasma spiked at a concentration in the middle of the dynamic range (250 ng/mL). (c) The triple deuterated stable isotope of ES-285. Mass transitions are listed in the figure.

*Accuracy, precision, and recovery*

Accuracy data for ES-285 in human, mouse, rat and dog plasma are summarized in Table 2. For the assay in human plasma only inter-assay accuracies and precisions are shown. The mean recovery of ES-285 from human plasma was 109 ± 6.41%. From mouse and rat plasma recoveries were 94.0 ± 8.05% and 106 ± 13.7%, respectively. Since there are no significant differences between the recoveries for ES-285 from human, mouse, and rat plasma the quality control samples prepared in mouse and rat plasma were quantified using calibration standards prepared in human plasma. For the determination of quality control samples prepared in dog plasma calibration standards in dog plasma were used since the recovery from this matrix was lower than from human plasma (91.9 ± 0.388% and 109 ± 6.41%, respectively), although not significantly ($p = 0.05$). The inter-assay accuracies for human plasma were between –10.7 and 6.19% for all concentrations with inter-assay precisions lower than 9.18%. Intra-assay accuracies for human plasma were between 0.0928 and 17.0% for the LLOQ level and between –11.9 and 5.50% for other concentrations (data not shown). Accuracies for mouse, rat, and dog plasma were between –12.1 and 10.3% for all concentrations in the three matrices, with precisions lower than 11.5%.

*Specificity and selectivity*

MRM chromatograms of double blank and blank samples prepared in six individual batches of human plasma did not show peaks that co-eluted with either ES-285 or the internal standard nor interfered with the mass transition for either of the compounds.

**Table 2.** Assay performance data

| Species | Nominal concentration (ng/mL) | Measured concentration (ng/mL) | Accuracy (%) | Precision (%) | Number of replicates |
|---|---|---|---|---|---|
| Human* | 9.70 | 10.3 | 6.19 | 9.18 | 15 |
| | 24.3 | 21.7 | -10.7 | 3.69 | 15 |
| | 97.0 | 91.0 | -6.19 | 6.21 | 15 |
| | 388 | 393 | 1.29 | 7.24 | 15 |
| | 2426 | 2427 | 0.0412 | 6.37 | 15 |
| Mouse | 9.70 | 10.2 | 5.15 | 11.5 | 5 |
| | 24.3 | 22.4 | -7.82 | 4.56 | 5 |
| | 97.0 | 93.2 | -3.92 | 7.34 | 5 |
| | 388 | 405 | 4.38 | 6.75 | 5 |
| Rat | 9.70 | 10.3 | 6.50 | 6.18 | 5 |
| | 24.3 | 24.0 | -0.999 | 2.03 | 5 |
| | 97.0 | 106 | 9.28 | 2.85 | 5 |
| | 388 | 428 | 10.3 | 0.898 | 5 |
| Dog | 9.70 | 10.4 | 7.22 | 5.76 | 5 |
| | 24.3 | 22.0 | -9.47 | 5.99 | 5 |
| | 97.0 | 88.1 | -9.18 | 5.46 | 5 |
| | 388 | 341 | -12.1 | 1.89 | 5 |

* Inter-assay performance data are reported

Interference was also not observed for the tested co-medication. Deviations from the nominal concentrations at the LLOQ level were between –19.9 and 5.16%. Furthermore cyclodextrin did not interfere with the assay. Deviations from the nominal concentration were –8.32 and –13.1%. Peaks co-eluting with that of ES-285 with areas exceeding 20% of the ES-285 area at the LLOQ level were not observed in mouse, rat, or dog plasma.

*Stability*

ES-285 is stable in the stock and working solutions under both processing conditions (ambient temperatures) for at least 24 h and storage conditions (-70 °C) for at least 9 months. Stability of the deuterated internal standard in the stock solution was established for at least 7 months at –70 °C. Stability of ES-285 in the biomatrix was not critical under any of the tested conditions. No significant changes in concentration were observed after three freeze (-20 °C)/thaw cycles. Furthermore, ES-285 is stable under both processing conditions for 24 h and storage conditions (-20 °C) for 6 months in human plasma. In mouse and rat plasma stability was established for at least 5 months at –20 °C and in dog plasma for at least 6 months at –20 °C. Furthermore, no significant change in concentration of ES-285 was observed in the final extract after 7 days at ambient temperatures.

**Implementation in pre-clinical trials**

In Figure 7 the concentration *vs*. time plots of samples obtained from a rat receiving 15 mg/kg and of a rat receiving 25 mg/kg of ES-285 in the cyclodextrin formulation through an intravenous bolus injection are depicted. The y-axis is logarithmically scaled. Maximum concentrations of 3348 and 4620 ng/mL were obtained after dosing with 15 and 25 mg/kg, respectively. ES-285 concentrations were determined in plasma samples up to 6 h after administration for animals receiving 15 mg/kg and up to 10 h for animals receiving 25 mg/kg. Multiple elimination phases are visible.

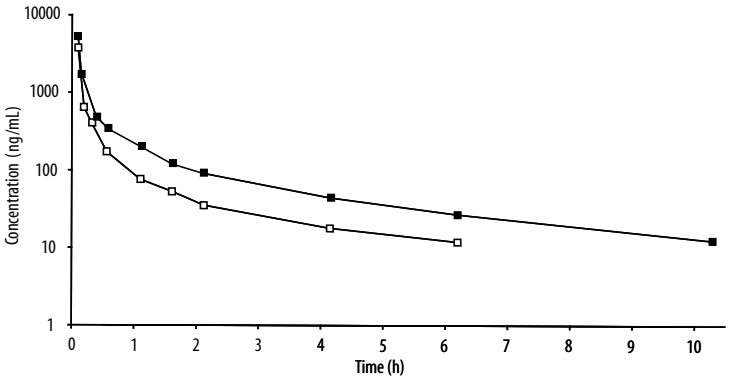


**Figure 7.** Concentration vs. time profiles for ES-285 in two rats treated with a bolus injection of 15 mg/kg (□) and 25 mg/kg (■) of ES-285, respectively.

## Conclusions

The presented reversed-phase LC-MS assay is simple, rapid, reliable and reproducible. The method utilizes a simple sample preparation with protein precipitation using acetonitrile and the supernatant was injected directly onto the analytical column. This method has shown its usefulness in pre-clinical trials and will be implemented in forthcoming clinical phase I investigations.

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# 2.4

## A more sensitive MS detector does not obviously lead to a more sensitive assay: experiences with ES-285

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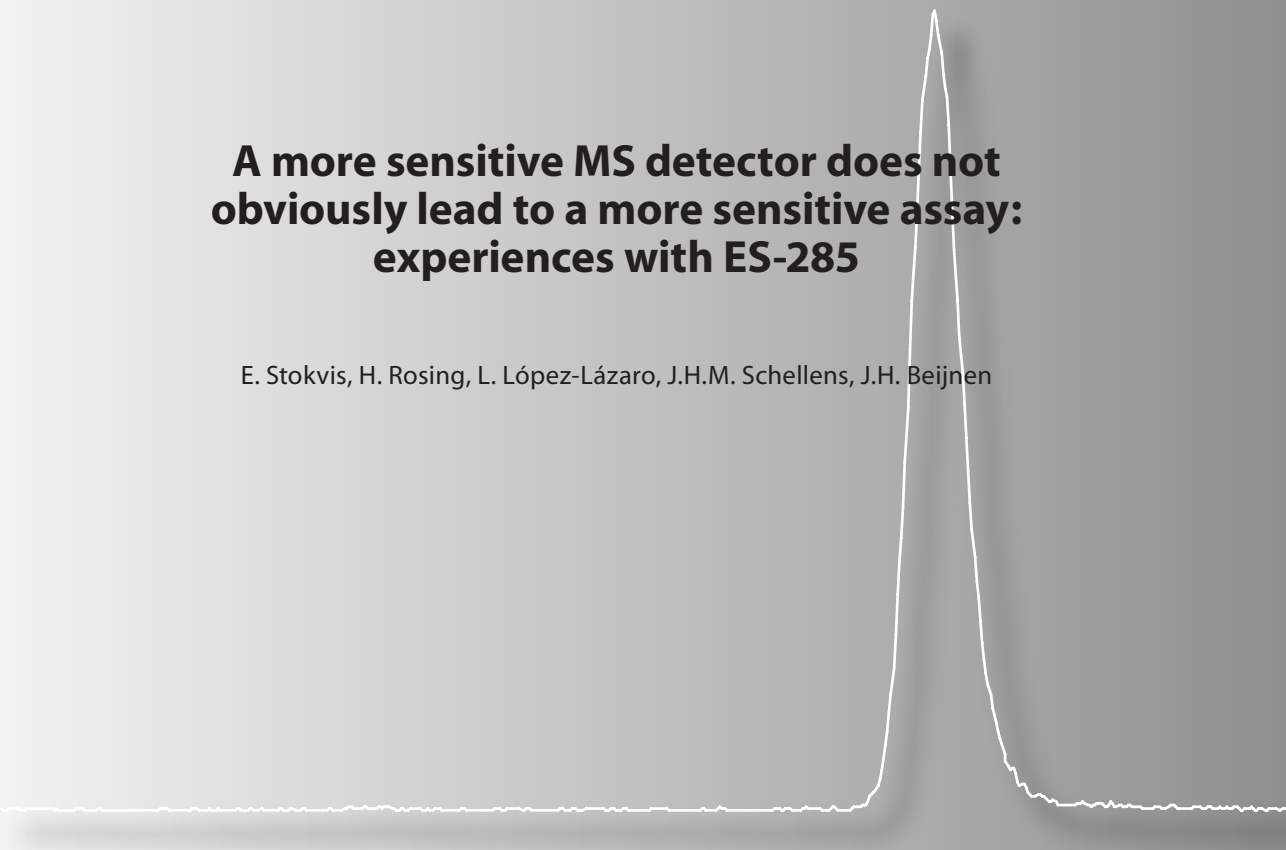

Submitted

## Abstract

In this paper the transfer of an existing method for the quantitative determination of the anticancer agent ES-285 in human plasma using liquid chromatography tandem mass spectrometry from an API 365 to an API 3000 mass spectrometer is described. The transfer appeared not straightforward. Problems arose resulting from carry over and interferences. In addition, due to the expansion of the calibration range, data ought to be weighted with a different factor to increase the accuracy of the lower concentrations. After finding appropriate solutions for these problems, the lower limit of quantitation could be lowered from 10 ng/mL to 1 ng/mL for ES-285 in human plasma. The usefulness and necessity of the modified assay was demonstrated by analysis of plasma samples from a patient receiving a low dosage of the drug.

## Introduction

ES-285 (spisulosine) is an investigational marine anticancer agent currently under investigation in several phase I trials. In an earlier publication we described the development and validation of a liquid chromatography tandem mass spectrometric (LC-MS/MS) assay for the quantitative analysis of ES-285 in human, mouse, rat, and dog plasma [1]. This assay has been used successfully for pharmacokinetic support of several pre-clinical trials with ES-285. From the data obtained in these studies it was calculated, however, that plasma concentrations of ES-285 in patients entered in phase I trials with the drug, are expected to be substantially lower than the lower limit of quantitation (LLOQ; 10 ng/mL) of that method. A reduction of the LLOQ of the method was thus needed. Our first approach was to transfer the original assay using an API 365 triple quadrupole MS to its more sensitive upgrade, the API 3000, while keeping the same sample pretreatment and LC conditions. However, we experienced that transferring an existing assay to a more sensitive MS is not as straightforward as it may appear to be. In this paper we discuss the problems that can occur as a result of such a transfer, problems that may lead to a smaller gain of sensitivity than was expected. Following a partial validation of the adapted method, it was applied to the pharmacokinetic support of a phase I trial with ES-285, demonstrating the importance of the LLOQ reduction.

## Experimental

The LC-MS/MS assay for the quantitative determination of ES-285 in human, mouse, rat, and dog plasma on an API 365 MS with an LLOQ of 10 ng/mL has been described before [1]. In short, 100 µL plasma samples were deproteinized using 200 µL acetonitrile containing the triply deuterated internal standard (100 ng/mL). After shaking and centrifugation of the samples, 10 µL aliquots of supernatant were directly injected onto an Inertsil ODS analytical column (50 x 2.0 mm I.D., 5 µm particle size; Chrompack, Middelburg, The Netherlands)

protected with an in-line filter (Micro filter frit, 5 µm, Upchurch Scientific, Inc., Oak Hoarber, WA, USA). The LC system consisted of a Perkin-Elmer (Norwalk, CT, USA) 200 series pump and autosampler. An eluent consisting of methanol – 10 mM ammonium formate pH 4 in water (80:20, v/v) was pumped at a flow rate of 0.2 mL/min. The LC eluate was introduced directly into an API 365 MS (Sciex, Thornhill, ON, Canada) equipped with an electrospray ion source between 3 and 7 min, which was controlled by a two position micro-electric actuator E (Valco Instruments, Schenkon, Switzerland). Total run time was 8 minutes and the obtained multiple reaction monitoring (MRM) chromatograms were used for quantitation.

After the transfer to an API 3000 MS the following changes were made. The internal standard concentration was lowered to 50 ng/mL and after the analysis of a series of calibration standards, quality control samples, or patient samples one injection of 100 µL of methanol was introduced. In addition, the API 3000 was equipped with an HP1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) consisting of a binairy pump, autosampler, and degasser.

## Results

### Transferring the method

The mass transitions used for MRM of ES-285 and internal standard were similar to those used in the original assay (m/z 286-268 and 289-271, respectively). Furthermore, the curtain gas (1.1 L/min), nebulizer gas (1.4 L/min), and turbo gas settings (7 L/min), as well as the source temperature (300 °C) remained the same. The compound related electrical settings were all automatically optimized as they differ from MS to MS. In addition, the value for the collision activated dissociation gas was optimized again because the LINAC™ collision cell in the API 3000 mass spectrometer differs from the API 365 collision cell. None of the chromatographic parameters were changed after transfer to the API 3000 MS.

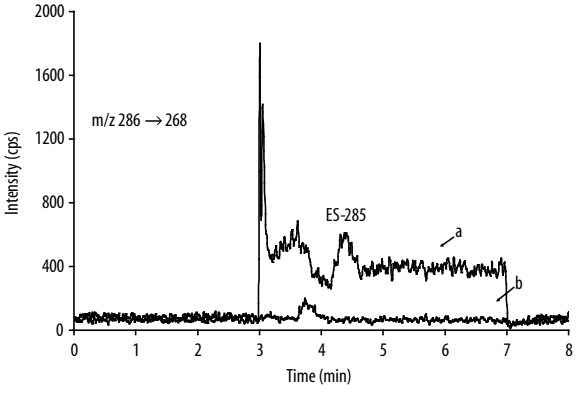


**Figure 1.** Carry-over effect of ES-285 in a double blank sample (a), and its reduction after the injection of 100 mL of methanol (b).

**Interferences**

An LLOQ of 1 ng/mL of ES-285 appeared feasible with a signal to noise ratio of ~12, even though an increase in noise was observed compared to the original assay. Interferences in the double blank and blank samples, however, were observed with areas higher than 20% of the LLOQ area. These interferences, not observed while using an API 365 MS, appeared to result from carry-over. Since ES-285 is easily dissolved in methanol, carry-over problems were solved by injecting 100 µL of methanol after every series of calibration standards, quality control samples, and pharmacokinetic samples as can be observed in Figure 1. Although interferences in double blank samples were now reduced to areas lower than 20% of the LLOQ area, interferences in the blank samples remained more than 20%. This could be attributed to the presence of ES-285 in the ES-285-$D_3$ internal standard solution. The amount of ES-285 in internal standard stock solutions and precipitation solutions was monitored. Solutions of ES-285-$D_3$ all contained approximately 0.2% of ES-285, meaning that at a concentration of 200 ng/mL of internal standard in plasma, approximately 0.4 ng/mL of ES-285 is added to the sample. This accounts for approximately 40% of the ES-285 LLOQ area. The percentage of ES-285 in internal standard solutions was similar in freshly prepared and stored solutions. This indicates that the presence of ES-285 in these ES-285-$D_3$ solutions is not due to stability issues causing H/D exchange in solution and thus generating ES-285 from ES-285-$D_3$, but more likely to impurity of the internal standard reference standard. The presence of ES-285 probably results from a contamination in the triply deuterated chemicals used for the synthesis of ES-285-$D_3$, because H/D exchange in not likely to be observed in a solid product. In order to reduce the amount of ES-285 in the blank sample, the concentration of the internal standard solution was lowered from 100 to 50 ng/mL and interferences in blank plasma samples with areas exceeding 20% of the LLOQ area were no longer observed.

**Dynamic range**

Calibration standards ranging from 1 to 500 ng/mL of ES-285 in human plasma were prepared, processed, and analyzed on the new system. An LLOQ of 1 ng/mL of ES-285 was feasible as shown in Figure 2. Furthermore, the upper limit of quantitation (ULOQ; 500 ng/mL) did not have to be reduced. Linear regression is based on the assumption that the variance of the response is independent of the analyte concentration. This, however, is not justified for many analytical methods, particularly when the calibration range expands more than a few orders of magnitude. Therefore, in order to increase the accuracy of the lower concentrations calibration data needs to be weighted. After expanding the calibration range for ES-285 in human plasma with one order of magnitude the most appropriate weighting factor for correct quantitation of the lower concentrations appeared to be the reciprocal of the squared concentration ($1/x^2$) contrary to the original assay which used a weighting factor of 1/x [1].

**Validation**

According to the FDA guidelines on bioanalytical method validation, a partial validation is sufficient following a small change in an existing assay [2]. In our opinion, after the transfer

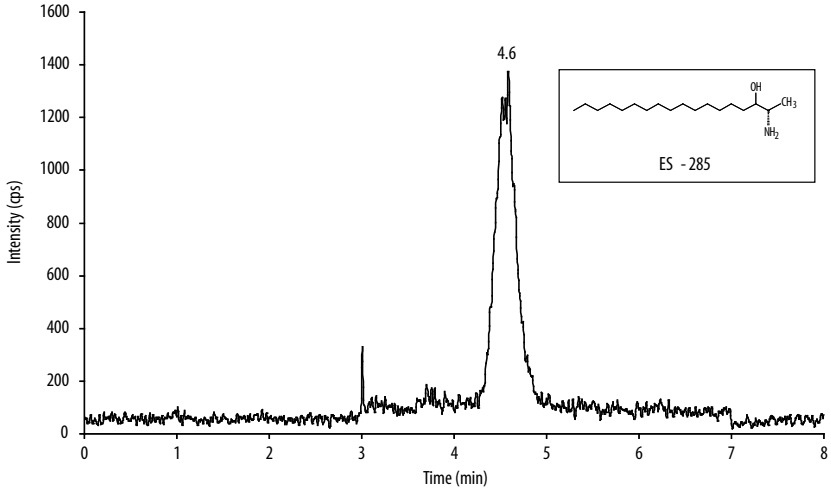


**Figure 2.** Representative chromatogram of ES-285 of an LLOQ sample from human plasma (1 ng/mL)

of this assay from an API 365 MS detector to an API 3000 the following parameters needed to be re-validated: linearity, accuracy and precision, specificity and selectivity, and long-term stability of ES-285 in plasma during storage at low concentrations.

Calibration standards in control human plasma were analyzed in duplicate in a range from 1 to 500 ng/mL in one analytical run. The calibration concentrations were back-calculated from the responses. The deviations from the nominal concentration were between –1.88 and 4.06% for all concentrations, with C.V. values less than 2.50%.

Quality control samples at four concentration levels were prepared (at the LLOQ level, at a level at approximately three times the LLOQ, at a level approximately mid-way the dynamic range, and at a level near the ULOQ). Five replicates of each sample were analyzed together with a calibration curve. Accuracy was determined in percent difference between the mean and nominal concentration. C.V. values were used to report the precision. Accuracy and precision results are tabulated in Table 1. Accuracy was –14.6% at the LLOQ level at 1 ng/mL and between –8.54 and –0.588% at the other three concentration levels. Precisions were less than 5.73%.

From six individual batches of control drug-free human plasma samples containing neither analyte nor internal standard (double blank), samples containing only internal

**Table 1.** Assay performance data

| Nominal concentration (ng/mL) | Measured concentration (ng/mL) | Accuracy (%) | Precision (%) | Number of replicates |
|---|---|---|---|---|
| 0.986 | 0.842 | -14.6 | 5.73 | 5 |
| 2.46 | 2.25 | -8.54 | 2.74 | 5 |
| 98.6 | 98.0 | -0.588 | 3.45 | 5 |
| 394 | 379 | -3.86 | 1.78 | 5 |

standard (blank), and LLOQ samples were prepared. Samples were processed according to the described procedures and analyzed. MRM chromatograms of double blank or blank samples prepared in six individual batches of human plasma did not show peaks that co-eluted with either ES-285 or the internal standard nor interfered with the mass transition for either of the compounds with areas exceeding 19.7 % of the area at LLOQ level. Interferences with the mass transition for ES-285-$D_3$ were not observed. Deviations from the nominal concentrations at LLOQ level were between –2.94 and –19.2%.

Long-term stability of ES-285 in plasma during storage at –20 °C was re-tested in the range 1 to 500 ng/mL since the earlier tests did not cover the range below 10 ng/mL. After 14 months of storage at –20 °C, ES-285 plasma quality control samples at the low (2.46 ng/mL) and high (394 ng/mL) level were analyzed and the calculated concentrations were compared to results from samples determined at t=0. Stability was established for at least 14 months.

**Clinical trial**

A concentration vs. time profile of a patient who received the starting dose of 2 mg/$m^2$ as a 3 h infusion (daily times five every three weeks) is presented in Figure 3. The data presented in the figure demonstrate the usefulness and necessity of the decrease of the LLOQ of the assay. Using the original method with an LLOQ of 10 ng/mL, ES-285 concentrations could not have been determined, while the more sensitive assay described in this paper allowed pharmacokinetic profiling up to 3.5 h after the start of the infusion.

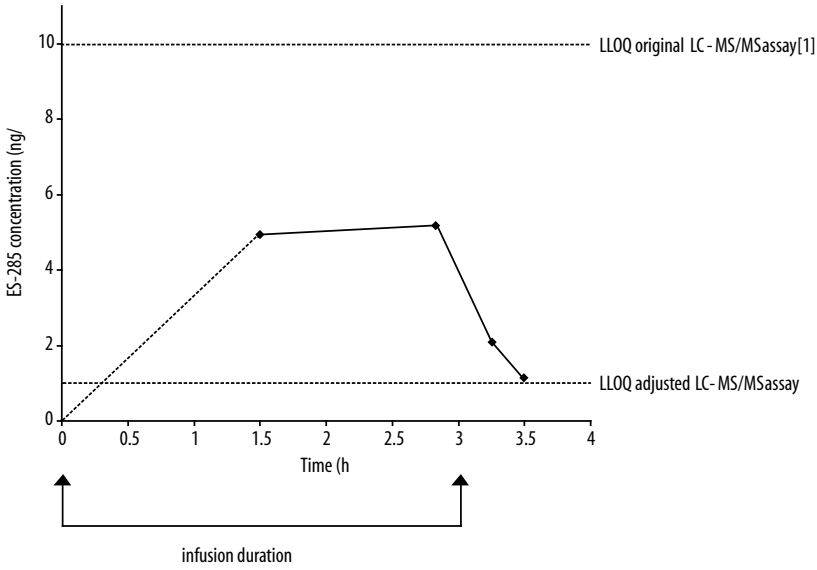


**Figure 3.** Concentration vs. time profile for ES-285 from a patient treated with 2 mg/m2 ES-285 intravenously administered in 3 h. The LLOQ level of the original assay (10 ng/mL) and the more sensitive assay described in this paper (1 ng/mL) are indicated in the figure.

## Conclusion

From the results described in this paper it is clear that transferring a method to a more sensitive mass spectrometer is not as straightforward as it may appear. Sensitivity is not gained instantly since noise levels, carry over and interferences are to be considered. These problems may not have occurred while using the original method, but can appear when attempting at a tenfold decrease of the LLOQ of an assay. Linearity, accuracy and precision, and specificity and selectivity experiments are important to validate the modified assay. These experiments indicate whether noise levels have increased proportionate to the analyte level, whether there are problems with carry over or interferences, such as we saw here with contamination of the internal standard reference standard. In conclusion, we have successfully lowered the LLOQ of an assay for the quantitative determination of ES-285 in human plasma from 10 ng/mL to 1 ng/mL. This tenfold gain in sensitivity was very appropriate for low dose pharmacokinetic profiling of the drug up to 3.5 h after the start of the infusion, which was not possible with the original method.

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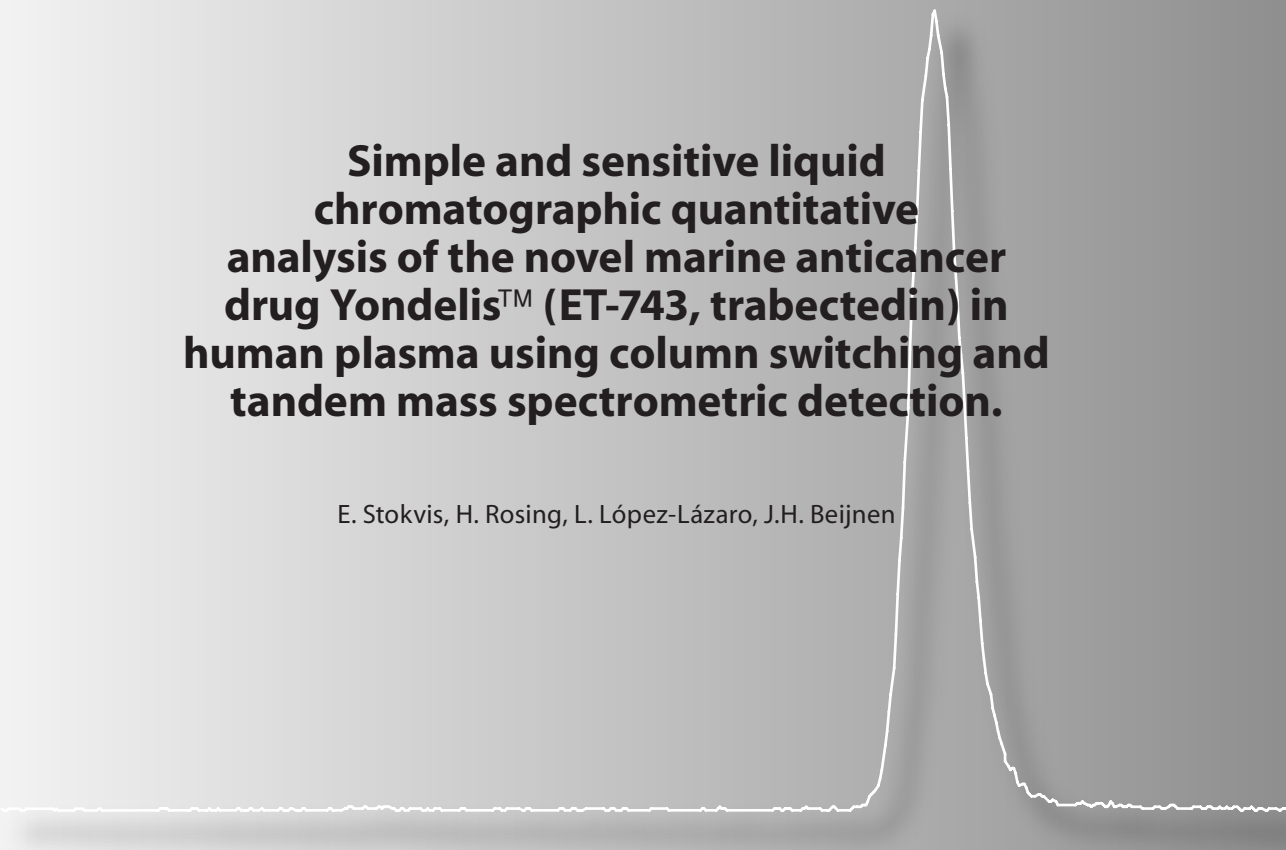
# 2.5

## Simple and sensitive liquid chromatographic quantitative analysis of the novel marine anticancer drug Yondelis™ (ET-743, trabectedin) in human plasma using column switching and tandem mass spectrometric detection.

E. Stokvis, H. Rosing, L. López-Lázaro, J.H. Beijnen



J Mass Spectrom, In press

## Abstract

The development of a simple and sensitive assay for the quantitative analysis of the marine anticancer agent Yondelis™ (ET-743, trabectedin) in human plasma using liquid chromatography (LC) with column switching and tandem mass spectrometric detection (MS/MS) is described. After protein precipitation with methanol, diluted extracts were injected onto a small LC column (10 x 3.0 mm I.D.) for on-line concentration and further clean-up of the sample. Next, the analyte and deuterated internal standard were back-flushed onto an analytical column for separation and subsequent detection in an API 2000 triple quadrupole mass spectrometer. The lower limit of quantitation (LLOQ) was 0.05 ng/mL using 100 µL of plasma with a linear dynamic range up to 2.5 ng/mL. Validation of the method was performed according to the most recent FDA guidelines for bioanalytical method validation. The time needed for off-line sample preparation has been reduced by a tenfold, when compared to an existing LC-MS/MS method for ET-743 in human plasma, employing a labor-intensive solid phase extraction (SPE) procedure for sample pretreatment. The presented column switching method was successfully applied in phase II clinical trials with Yondelis™ and pharmacokinetic monitoring.

## Introduction

Yondelis™ (ET-743, trabectedin; Figure 1) is a marine anticancer agent currently under investigation in several clinical phase II trials [1]. A sensitive assay for the quantitative analysis of ET-743 in human plasma using liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) with sample pre-treatment on non-endcapped cyano solid phase extraction (SPE) cartridges has been described by Rosing et al [2]. The lower limit of quantitation (LLOQ) was 0.01 ng/mL using 500 µL of plasma. The sensitivity was necessary in order to be able to determine ET-743 concentrations in plasma from patients enrolled in the phase I trials with Yondelis™ intravenously administered with starting doses in the low µg/m$^2$ range [3]. To achieve high sensitivity, a labor-intensive SPE sample pretreatment

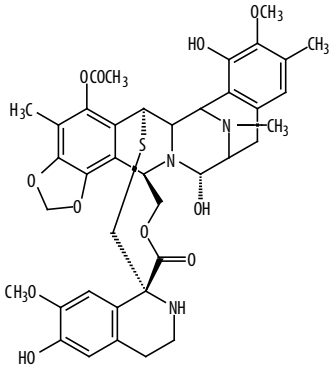



**Figure 1.** Structure of ET-743.

was needed as clean-up procedure and to concentrate the sample. At present, Yondelis™ is further developed in phase II trials using 3 hour or 24 h infusions with dose levels in the mg/m$^2$ range. These trials involve extensive sample collection for pharmacokinetic studies, generating a large number of samples that need to be analyzed. The time consuming sample pre-treatment is becoming a drawback of the existing method. In addition, phase II studies have commenced in children for which we prefer to utilize smaller sample volumes. Therefore, we have investigated means to reduce sample processing time and sample volume. In order to reduce sample preparation time the manual element had to be eliminated from the sample preparation process. There are several ways to accomplish this, such as automated SPE (examples in [4,5]), 96 well technologies (examples in [6,7]) or on-line sample clean-up (examples in [8-15]). We have investigated the potentials of on-line sample pre-treatment for the determination of ET-743.

## Experimental

### Chemicals

ET-743 (ET-743; lot WKSTD-21) and ET-743-D$_3$ (lot ET743 D3 P) reference standards were obtained from Pharma Mar S.A. (Colmenar Viejo, Madrid, Spain). Methanol (LC gradient grade) was purchased from Biosolve ltd. (Amsterdam, the Netherlands). Distilled water was used throughout the analyses. Drug free human plasma was obtained from the Central Laboratory for Blood Transfusion (Sanquin Amsterdam, The Netherlands).

### Instrumentation and analytical settings

A scheme presenting the analytical set-up is presented in Figure 2. The trapping column was a high efficiency Chromguard Reversed Phase (RP) 10 x 3.0 mm I.D. guard column with housing (Varian Inc, Palo Alto, CA, USA). Three sample aliquots of 100 µL were injected onto

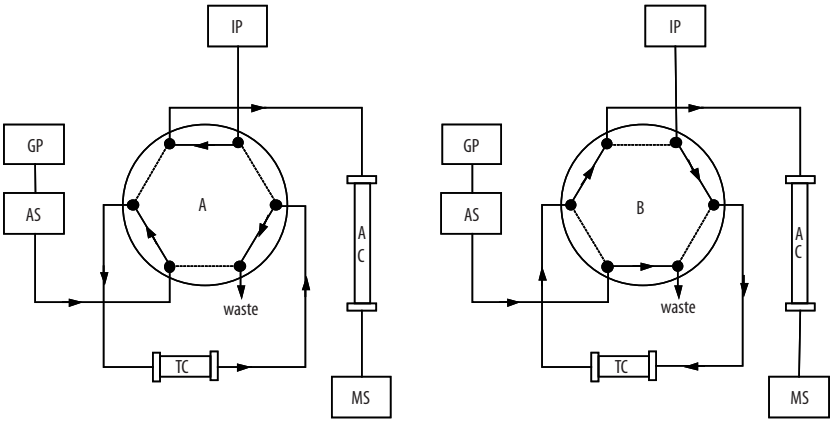



**Figure 2.** Analytical set-up for the assay. IP isocratic pump, GP gradient pump, AS autosampler, TC trapping column, AC analytical column, MS mass spectrometer.

the trapping column using an HP1100 autosampler (Agilent Technologies). The autosampler wash solvent was methanol. A two position ten-port valve was integrated into the MS equipment and six positions were used. The loading solvent consisted of methanol-water (10:90, v/v) pumped at a flow rate of 0.3 mL/min by an HP1100 binary pump equipped with a degasser (Agilent technologies, Palo Alto, CA, USA) and the eluate was directed to the waste. After loading the samples, the trapping column was washed by linearly increasing the methanol component of the loading eluent to 50%. After 4.5 min of loading the sample and subsequent washing of the trapping column, the valve was switched to position B and ET-743 and the internal standard were back-flushed from the trapping column onto the analytical column (Zorbax RX-C18, 150 x 2.1 mm I.D., 5 µm particle size, Agilent) and subsequently to the MS. An eluent consisting of methanol-water (85:15, v/v) was provided by an isocratic P1000 pump (Thermo Separations Products, San Jose, CA, USA) at a flow rate of 0.3 mL/min. An API 2000 triple quadrupole MS was used, equipped with an electrospray ion source (Sciex, Thornhill, ON, Canada). The MS was operated using Analyst™ software version 1.2 (Sciex). Positive ions were created at atmospheric pressure and the mass analyzer was operated in the multiple reaction monitoring (MRM) mode using unit resolution for the quadrupoles. Selected MRM ion chromatograms were used for quantitation using Analyst™ software. After 3.5 min the valve was switched back to position A for conditioning of the trapping column with the loading solvent for 3 min. Detailed LC and MS settings are presented in Table 1.

**Preparation of calibration standards and quality control samples**

A ET-743 stock solution was prepared in methanol at a concentration of 10 µg/mL. This solution was further diluted with methanol to obtain working solutions with concentrations between 5 and 250 ng/mL. Aliquots of working solutions were diluted with drug free human plasma in volumetric flasks in order to obtain seven non-zero calibration standards, ranging from 0.05 to 2.5 ng/mL in plasma. An independently prepared stock solution, with separate weighing of ET-743, was diluted with methanol in order to obtain working solutions with concentrations ranging from 5 to 500 ng/mL. These working solutions were spiked to drug free heparinized human plasma resulting in quality control samples at three concentration levels (0.125, 1, and 2 ng/mL). In addition, quality control samples at the LLOQ level (0.05 ng/mL) and quality control samples exceeding the upper limit of quantitation (ULOQ; 5 ng/mL) were prepared. ET-743 solutions in plasma never contained more than 1% of methanol, in order to maintain the integrity of the spiked plasma samples. An internal standard stock solution was prepared similar to that for ET-743. The stock solution was diluted with methanol to a final concentration of 1 ng/mL. All solutions were stored at –20 °C.

**Sample processing**

100 µL of plasma sample was diluted with 200 µL of internal standard solution in methanol (1 ng/mL ET-743-$D_3$). After shaking the samples vigorously for 15 min the mixture was centrifuged for 15 min at 23,100 g. Supernatant aliquots of 200 µL were transferred to conical shaped autosampler vials. 200 µL of water was added and the vials were vigor-

**Table 1.** LC and MS settings

| Device/Time | Setting | |
|---|---|---|
| **Injection** | | |
| -1 min | 100 µL | |
| -0.5 min | 100 µL | |
| 0 min | 100 µL | |
| **Gradient** | | |
| -1 to 0.5 min | Methanol-water 10:90 v/v | |
| 0.5 to 2.5 min | Methanol-water 10:90 v/v – 50:50 v/v | |
| 2.5 to 5 min | Methanol-water 50:50 v/v | |
| 5 to 11 min | Methanol-water 10:90 v/v | |
| **Switching valve** | | |
| -1 to 4.5 min | Position A | |
| 4.5 to 8 min | Position B | |
| 8 to 11 min | Position A | |
| **MS settings** | | |
| Ion spray voltage | 5500 V | |
| Source temperature | 350 °C | |
| Nebuliser gas (compressed air) | 30 psi | |
| Curtain gas ($N_2$) | 20 psi | |
| Turbo gas ($N_2$) | 80 psi | |
| Collision gas ($N_2$) | $1.25*10^{15}$ mol/cm$^2$ | |
| | ET-743 | ET-743-$D_3$ |
| Quadrupole 1 mass (amu) | 744 | 747 |
| Quadrupole 3 mass (amu) | 495 | 496* |
| Dwell time (s) | 150 | 150 |

*The three deuterium atoms of the internal standard are probably dispersed over different parts of the molecule.

ously shaken for 5 min. A volume of 300 µL (in three 100 µL injections) was injected onto the trapping column.

## Validation procedures

Validation of the method was performed according to the latest FDA guidelines [16].

*Linearity*

Seven non-zero plasma calibration standards were prepared and subsequently analysed in duplicate in three separate analytical runs. In order to establish the best fit and weighting for the calibration curves, back-calculated calibration concentrations were determined. The model showing the lowest % deviation of the determined concentrations from the nominal concentrations was considered the best fit. Deviations should be within ±15% except at LLOQ level where deviations within ±20 % are accepted. Precision, by means of the coefficient of variation (C.V.), is required to be less than 20% at LLOQ level, and less than 15% at all other concentration levels [16].

*Accuracy and precision*

For the validation of the assay, quality control samples were prepared with concentrations of 0.05, 0.125, 1, 2, and 5 ng/mL. Five replicates of each sample were analyzed together with a set of calibration standards, independently prepared from the control samples, in three analytical runs. The accuracy was determined as percent difference between the mean concentration and the nominal concentration. The coefficient of variation (C.V.) was used to report the precisions. Accuracies were required to be within ±15% and precisions were required to be less than 15%, except at the LLOQ level were accuracies within ±20% and precisions less than 20% are accepted. Samples higher than the ULOQ were diluted ten times in control drug-free human plasma before processing to a concentration within the calibration range [16].

*Specificity and selectivity*

From six individual batches of control drug-free human plasma, samples containing neither analyte nor internal standard (double blank), containing only internal standard (blank), or spiked at LLOQ level with ET-743 were prepared in order to determine whether endogenous compounds interfere at the mass transitions chosen for ET-743 and the internal standard. Samples were processed according to the described procedures and analyzed. Areas of peaks co-eluting with ET-743 should not exceed 20% of the area at LLOQ level, and areas of peaks co-eluting with the internal standard should not exceed 5% of the internal standard peak area. The samples spiked at LLOQ level should have an accuracy within 20% of the nominal value [16].

*Ion-suppression and recovery*

Control drug-free plasma was processed and 200 µL aliquots of supernatant were evaporated under a flow of warm nitrogen (40 °C). Dry extracts were dissolved with working solutions that represent 100% recovery containing ET-743 and internal standard in methanol-water (1:2, v/v). Ion-suppression was determined by comparing the analytical response of these samples to that of the working solutions. The loss of signal represents the ion suppression. Extraction recovery was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with working solutions as described above. These experiments were performed at three concentration levels, in triplicate. Overall recovery corresponds to the net response after subtraction of the ion-suppression and the signal loss due to the extraction. Ion-suppression and recovery experiments for the internal standard were performed in a similar manner [16].

*Stability*

The stability of ET-743 has already been extensively investigated prior to this study [2]. Only stability of ET-743- $D_3$ in the working solution used for precipitation, as well as stability of ET-743 in the processed extracts has been investigated. ET-743 is considered stable in the biological matrix or extracts thereof when 80-120% of the initial concentration is found. The internal standard is considered stable in the precipitation solution when 95-105% of

the original concentration is recovered. All stability experiments were performed in triplicate and in plasma at two concentration levels (0.125 and 2 ng/mL)

## Results and Discussion

The goal of this study was to develop an analytical method for the quantitative analysis of ET-743 in human plasma that was less labor-intensive and used smaller sample volumes than the existing method using SPE as sample pretreatment [2]. We investigated on-line sample processing as a means of reducing the workload for sample clean-up. The analytical procedure was optimized and the method validated.

### Optimization of the analytical method

*Sample processing*

The injection of crude unprocessed sample is possible but complicated for a matrix such as plasma. Injections of crude plasma require a lot from the performance of both the autosampler and trapping column. Conventional autosamplers are usually not capable of direct injection of plasma. The viscosity of plasma complicates the injection of accurate and precise volumes and can cause carry-over and clogging of the needle. Similarly, clogging of the trapping column can occur. To circumvent these problems, plasma can be filtered, centrifuged and/or diluted with aqueous or organic solvent before injection. Plasma samples containing ET-743 were diluted two times in water, 5% formic acid in water, or 7% perchloric acid, or diluted three times with methanol or acetonitrile/methanol (1:1, v/v). Samples were subsequently centrifuged for 15 min at 23,100 g. Clogging of the autosampler needle or trapping column did not occur, also after multiple injections, for any of the dilutions. However, after dilution of plasma with aqueous solutions peak intensities for ET-743 rapidly decreased with every injection, probably caused by modification of the column material by plasma proteins. Similar results were obtained using a trapping column specially intended for on-line sample preparation and direct injection of biological samples. After protein precipitation with methanol (1:2, v/v), on the other hand, acceptable peak shapes and recoveries were obtained even after multiple injections.

*Injection volume*

The injection volume should be maximized in order to obtain optimal sensitivity for the assay. However, the injection volume was limited to 100 µL due to the size of the autosampler injection needle and loop and not as a result of analytical issues. Therefore, we have constructed a means to inject several times 100 µL aliquots onto the trapping column without modifications to the autosampler. Two acquisition methods were employed. The first is an injection method, in which 100 µL of sample is injected and ET-743 is loaded onto the trapping column in 30 s. The valve and MS stand idle. The second is an analysis method, in which the final 100 µL aliquot is injected and loaded onto the trapping column, followed by washing of the trapping column and the subsequent back-flush. Injection of a total of 300 µL of sample (3 x 100 µL) was chosen since it produced acceptable sensi-

tivity within an acceptable time-span. The injection method was employed twice, before starting the analysis method. The resulting LLOQ was 0.05 ng/mL using 100 µL of plasma sample.

*Sample loading and washing*

Trapping columns are short, mostly reversed phase columns [13-15]. The analytes need to be retained, and a high number of theoretical plates is redundant. Trapping columns should be able to deal with crude sample without clogging up. Special trapping columns for on-line sample processing purposes do exist. However, in our experience, a simple wide-bore Chromguard guard column, normally used for protection of an analytical LC column from matrix constituents is just as suitable and much more cost-effective. Breakthrough experiments using working solutions showed that ET-743 is retained on the trapping column using an eluent containing 50% methanol in water v/v or less. Starting with a loading solvent of methanol – water (50:50, v/v), ion suppression was substantial, approximately 50%, probably caused by failing to adequately remove polar matrix components from the trapping column. Decreasing the methanol percentage from 50 to 10% for loading ET-743, however, resulted in lower peak intensities. A lower percentage of methanol, although more suited for removal of polar components may not be appropriate to adequately remove apolar matrix compounds from the trapping column. In order to be able to wash off both polar and apolar compounds a washing procedure for the trapping column was introduced. After loading the third 100 µL injection of ET-743 on the trapping column for 30 s using an eluent containing 10% methanol in water v/v, the percentage of methanol was increased to 50% in 2 min, and the trapping column was subsequently washed for another 2 min with 50% methanol. This procedure reduced the amount of ion suppression significantly to 6.54 ± 3.25% and 6.65 ± 9.24% for ET-743 and the internal standard, respectively.

*Sample back-flushing*

For an adequate elution of ET-743 from the trapping column during back-flush an eluent containing at least 85% of methanol was needed. Lower percentages of methanol resulted in broad peaks for ET-743. This loading and back-flushing system was coupled to an LC-MS/MS system using a minibore reversed phase analytical LC column. Excellent peak shapes for ET-743 were obtained with this system, while the eluents contain only methanol and water and the trapping column is a simple guard column.

**Validation of the analytical method**

*Linearity*

Calibration standards prepared in control human plasma were analysed in duplicate in three analytical runs with a dynamic range from 0.05 to 2.5 ng/mL plasma. A linear fit using a weighting factor of the inverse squared concentration appeared to be most appropriate to describe the data. Correlation coefficients were 0.9939 or higher. The calibration standards were back-calculated from the responses. The deviations from the nominal concentrations were between –4.02 and 6.78% for all concentrations in human plasma.

**Table 2.** Assay Performance Data

| Nominal concentration (ng/mL) | Measured concentration (ng/mL) | Inter-assay accuracy (%) | Inter-assay precision (%) | Number of replicates |
|---|---|---|---|---|
| 0.0499 | 0.0511 | 2.40 | 12.4 | 15 |
| 0.128 | 0.123 | -3.91 | 8.45 | 15 |
| 1.02 | 1.01 | -0.980 | 5.77 | 15 |
| 2.04 | 1.96 | -3.92 | 5.05 | 15 |
| 5.10 | 5.36 | 5.10 | 7.50 | 15 |

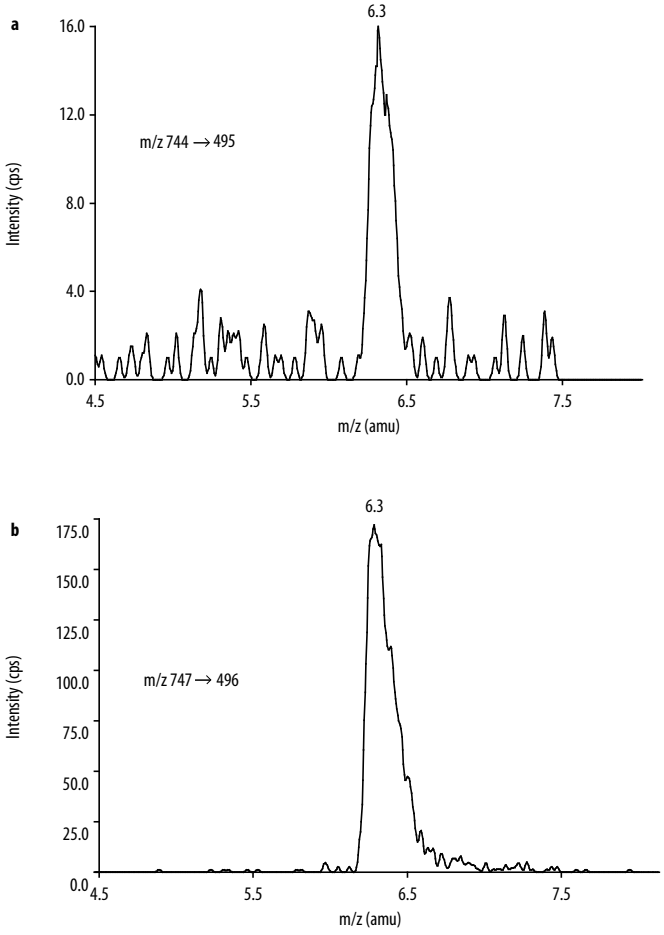



**Figure 3.** Representative chromatograms for ET-743 (a) and the internal standard (b) from an LLOQ sample containing 0.05 ng/mL in plasma.

C.V. values were less than 8.35% for all concentration levels.

*Accuracy and precision*
In Table 2 the inter-assay accuracy and precision data are presented. The presented data are within FDA requirements. Intra-assay accuracy (data not shown) was within ± 13.0% at LLOQ level and within ± 10.8% at other concentration levels, with C.V. values less than 13.1% and 9.77%, respectively.

*Specificity and selectivity*
MRM chromatograms of double blank, blank, and LLOQ samples prepared in six individual batches of human plasma did not show peaks that co-eluted with either ET-743 or the internal standard nor interfered with the mass transition for either of the compounds. Deviations from the nominal concentrations at LLOQ level were between –12.0 and 8.22%. Representative chromatograms from an LLOQ sample are presented in Figure 3.

*Ion-suppression and recovery*
The mean ion-suppression of ET-743 was 6.54 ± 3.25%. Ion-suppression of the internal standard was 6.65 ± 9.24% Extraction recoveries for ET-743 and the internal standard were 128 ± 5.54% and 149 ± 10.8%, respectively. The recovery experiments were probably hampered by adsorption of ET-743 to the container wall in protein-free aqueous 100% recovery working solutions. This results in an overestimation of the ET-743 recovery. Although an absolute recovery could not be determined, the C.V. values for the extraction recovery (5.54% and 10.8% for ET-743 and the internal standard, respectively) demonstrate the reproducibility of the method.

*Stability*
Stability of ET-743-$D_3$ has been established in the working solution used for precipitation (1 ng/mL ET-743-$D_3$ in methanol) after 48 h at ambient temperatures and at least 1 month at –20 °C. ET-743 is stable in the processed extract after protein precipitation for 13 days at ambient temperatures and at 4 °C.

*Cross validation*
A batch of 23 samples from a clinical Phase I study with Yondelis was analysed using both the SPE method [2] and the newly developed column switching method. The obtained results were compared using unweighted Deming regression analyses using the computer program Method Validator (version 1.1.10.0; by P. Marquis, http://perso.easynet.fr/~philimar). The results of the cross-validation are presented in Figure 4. The ET-743 concentrations obtained using the column switching method (y-axis) are plotted against the concentrations obtained using the SPE method (x-axis). The intercept of the calculated line did not differ significantly from 0, and the slope did not differ significantly from 1, both within the 95% level of significance. Thus, the calculated line does not differ significantly from the line of identity and therefore it can be concluded that the two methods yield similar results.

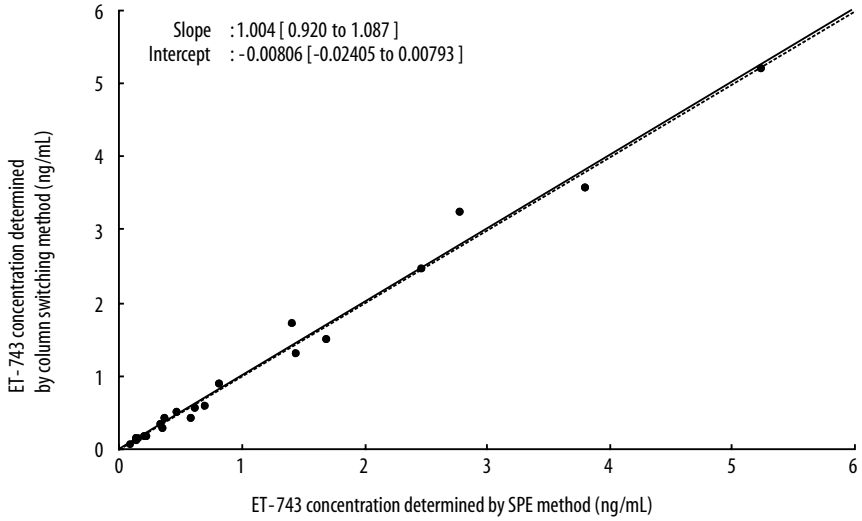



**Figure 4.** Cross validation results of ET-743 concentrations in human plasma samples obtained with the column switching method (y-axis) vs. the concentrations obtained using the SPE method (x-axis) [2]. The continuous line represents the calculated line and the dotted line the line of identity.

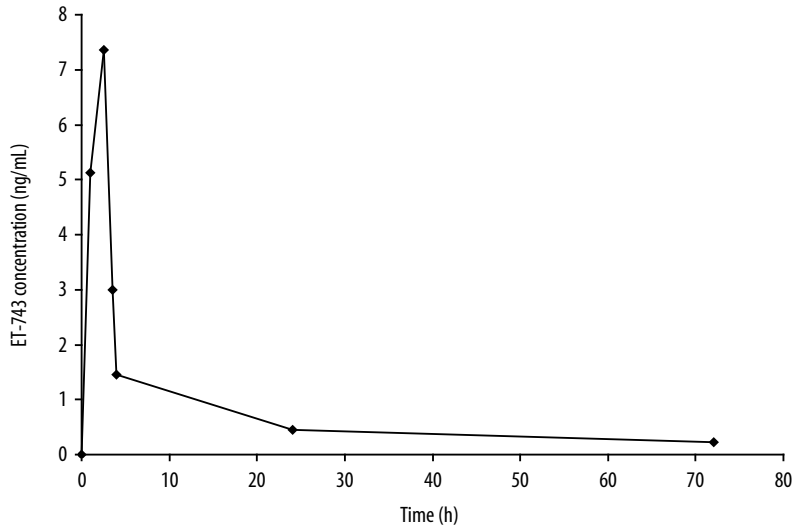



**Figure 5.** Concentration *vs.* time curve from a patient receiving 1.2 mg/m2 Yondelis™ intravenously in 3 h.

**Phase II study**

The assay described in this paper was used for the analysis of ET-743 in phase II clinical studies. In Figure 5 a concentration vs. time plot is presented for ET-743 from a patient to whom 1.2 mg/m$^2$ ET-743 was administered intravenously in 3 h. Measured concentrations were still above the LLOQ level at 72 h after administration. The results show the applicability of the assay for phase II pharmacokinetic studies with ET-743.

## Conclusion

An assay for the quantitative analysis of ET-743 in human plasma is described. Off-line protein precipitation was performed and column switching was used for sample concentration and additional clean-up. The time reduction permitted by this procedure compared to the original SPE LC-MS/MS is substantial. The time needed for off-line sample preparation has been reduced tenfold. Additionally, only 100 µL of plasma sample is used for the new assay compared to 500 µL for the SPE LC-MS/MS assay. The new assay is sensitive with an LLOQ of 0.05 ng/mL, simple, rapid, and cost-effective. A full validation according to FDA guidelines was performed. The assay was successfully used to support phase II clinical studies with the drug.

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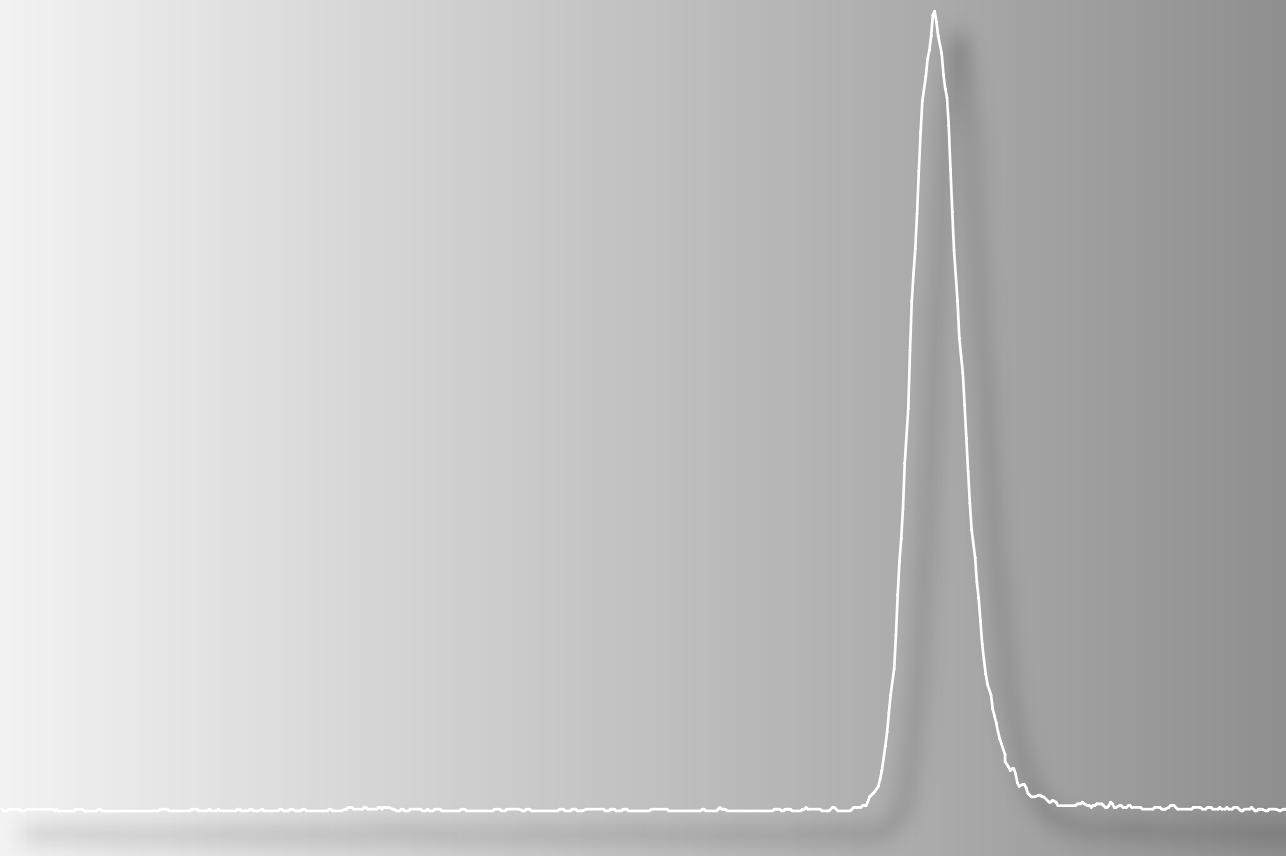
# Chapter 3



## Matrix Metalloproteinase Inhibitors

# 3.1

## Quantitative analysis of the novel anticancer drug ABT-518, a matrix metalloproteinase inhibitor, plus the screening of six metabolites in human plasma using high-performance liquid chromatography coupled with electrospray tandem mass spectrometry

E Stokvis, H Rosing, M Crul, MJ Rieser, AJR Heck, JHM Schellens, JH Beijnen

J Mass Spectrom. In press

## Abstract

A liquid chromatography tandem mass spectrometric (LC-MS/MS) assay for the quantitative analysis of the novel anticancer drug ABT-518 and the screening of six potential metabolites in human plasma has been developed and validated to support a phase I study with the drug. ABT-518 is an inhibitor of matrix metalloproteinases, which are associated with tumor growth and development of metastasis. Plasma samples were prepared for analysis using a simple solid phase extraction method on phenyl cartridges. LC separation was performed on a Zorbax extend C18 column (150 x 2.1 mm I.D., 5 µm particle size) using a mobile phase of methanol – aqueous 10 mM ammonium hydroxide (80:20, v/v) pumped at a flow rate of 0.2 mL/min. An API 2000 triple quadrupole mass spectrometer was used for specific and sensitive detection. The best chromatographic speed (total run time was 8 min) and peak shapes were obtained by employing an alkaline mobile phase (pH in aqueous phase approximately 10). Furthermore, an alkaline eluent was favored in order to obtain a better overall sensitivity for the protonated analytes. The dynamic range was from 10 to 1,000 ng/mL from 500 µL of plasma for ABT-518 and the metabolites were detected in the same order of magnitude. Inter-assay accuracies for ABT-518 on 5 different concentration levels were between –9.24 and 6.93% and inter-assay precisions were always lower than 10.7%. Analyte stability was not critical during either storage or processing. This method was successfully applied in a phase I clinical study of ABT-518. The active drug, ABT-518, and all of the metabolites included in the assay could be identified in plasma from dosed patients. We believe that the method described in this report using an alkaline mobile phase in combination with a basic stable analytical column may also be generally useful for the bioanalysis of other basic drugs.

## Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-binding enzymes whose principle function is to digest extracellular matrix proteins associated with growth and development of tissue [1,2]. MMPs are highly regulated under normal conditions. Increased MMP activity, however, is associated with growth, invasion, and metastasis of solid tumors as it allows tumor cells to migrate through the extracellular matrix [1,3,4]. Experimental evidence suggests that gelatinase A and B, two examples of MMPs are particularly important in tumor progression [1]. Other MMPs may be critical in the remodeling of normal tissue [5]. Synthetic MMP inhibitors (MMPIs) have been developed and potencies have been reported in the low nanomolar range. However during clinical trials toxicities were reported [6], which may result from inhibition of all MMPs [5]. Therefore highly selective gelatinase inhibitors were investigated for their antitumor activity. ABT-518 ([S-(R*,R*)]-N-[2,2-dimethyl-1,3-dioxol-4-yl)-2-[[4-[4-(trifluoromethoxy)-phenoxy] phenyl] sulfonyl] ethyl]-N-hydroxyformamide; Figure 1) demonstrated sub-nanomolar activity versus gelatinase B and antitumor activity in several tumor models, but only marginal activity towards other MMPs [5,7]. Based on pre-clinical data it was decided to incorporate

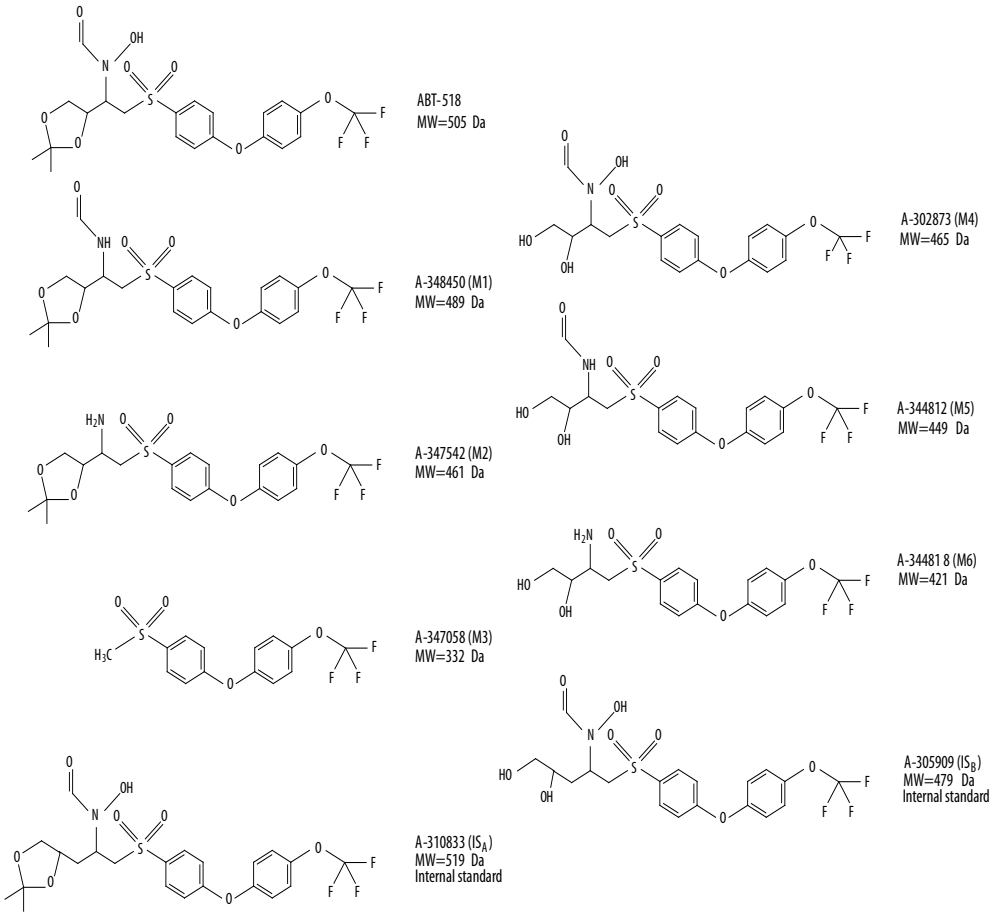



**Figure 1.** Chemical structures of ABT-518, metabolites and internal standards.

six potential metabolites of ABT-518 in the assay (Figure 1).

High-performance liquid chromatography (HPLC) coupled with electrospray mass spectrometry (ESI-MS) is generally considered the preferred technique for the determination of drugs and their metabolites in biological samples [8-11]. In particular, tandem triple quadrupole mass spectrometry (TQ-MS/MS) used in the multiple reaction monitoring (MRM) mode provides unrivaled sensitivity and selectivity. For the bioanalysis of nine structurally related compounds such as ABT-518, metabolites and internal standards, MS detection is highly favorable as its selectivity alleviates the need for a complete chromatographic separation of the compounds.

This is the first report to describe the development, validation, and implementation in a clinical phase I trial of a liquid chromatographic (LC)-ESI-TQ-MS/MS method for the quantitative determination of ABT-518 and the screening of six potential metabolites in human plasma.

## Experimental

### Chemicals

ABT-518 (A-291518, lot 66-489-ZW-00) reference standard as well as potential metabolites M1 to M6 (A-348450, A-347542, A-347058, A-302873, A-344812, and A-344818) and internal standards $IS_A$ and $IS_B$ (A-310833 and A-305909) (Figure 1) were obtained from Abbott (Abbott Park, IL, USA). Methanol (LC gradient grade) was purchased from Biosolve ltd. (Amsterdam, The Netherlands). Ammonia solution 25%, di-sodium hydrogen phosphate (dihydrate), potassium di-hydrogen phosphate (all analytical grade), and water for chromatography were obtained from Merck (Darmstadt, Germany). Water for chromatography was used throughout the analyses. Drug free human plasma was obtained from the Central Laboratory for Blood Transfusion (Sanquin Amsterdam, The Netherlands).

### Instrumentation

Chromatographic separations of the analytes were carried out using a HP1100 liquid chromatograph (Agilent technologies, Palo Alto, CA, USA) consisting of a binary pump, auto sampler, degasser, and column oven. A mobile phase of methanol - aqueous 10 mM ammonium hydroxide (80:20, v/v) was pumped at a flow rate of 0.2 mL/min through a Zorbax Extend C18 column (150 x 2.1 mm ID, particle size 5 µm; Agilent technologies) protected with an in-line filter (Micro filter frit, 5 µm, Upchurch Scientific Inc., Oak Harbor, WA, USA) and thermostatted at 30 °C. The eluent was split 1:4 before entering an API 2000 triple quadrupole MS equipped with an electrospray ion source (Sciex, Thornhill, ON, Canada). Sample injections of 50 µL were carried out and the resulting MRM chromatograms were used for quantification using Analyst™ software version 1.1 (Sciex). The MS was operated in the positive ion mode and at unit resolution. The ion spray voltage was +5500 V and the source temperature was set at 300 °C. The nebulizer gas (compressed air), turbo gas ($N_2$), curtain gas ($N_2$), and collision activated dissociation gas ($N_2$) were operated at 35, 65, 25, and 2 psi, respectively. Total run time was 8 minutes. In order to avoid carry-over, the autosampler needle was rinsed after a series of calibration standards, quality control samples, and pharmacokinetic samples with 10 times 100 µL of methanol.

### Preparation of calibration standards and quality control samples

Stock solutions of ABT-518, metabolites and internal standards were prepared separately in methanol at a concentration of 1 mg/mL. Aliquots of the analyte stock solutions were diluted further with methanol to obtain one working solution with a concentration of 100 µg/mL of each analyte. This working solution was subsequently spiked to control human plasma in order to obtain plasma working solutions with concentrations of 1 and 0.1 µg/mL. From these last two plasma working solutions calibration standards were prepared in control human plasma in concentration ranges from 10 – 1,000 ng/mL. The calibration standards were prepared freshly for every analytical run. A second working solution containing 100 µg/mL of each of the analytes in methanol was prepared from independently prepared stock solutions with separate weighing of the analytes. This working solution was diluted further in control human plasma in order to obtain plasma working solutions with

concentrations of 2.5 and 1 µg/mL. These plasma working solutions were used to prepare quality control samples in control human plasma at three concentration levels (30, 600, and 900 ng/mL) and additional levels at the lower limit of quantitation (LLOQ; 10 ng/mL) and at a concentration exceeding the upper limit of quantitation (ULOQ; 3,500 ng/mL). Plasma samples never contained more than 1% (v/v) of methanol, in order to maintain the integrity of the spiked plasma samples. Aliquots of the internal standard stock solutions were diluted further with methanol to obtain a working solution containing 100 µg/mL of each internal standard. This working solution was diluted further with methanol – water (25:75, v/v) in order to obtain a solution with a final concentration of 4 µg/mL of the internal standards. All solutions were stored at nominally –20 °C.

**Sample processing**

Before extraction the Bond Elut Phenyl Solid Phase Extraction (SPE) cartridges (100 mg, Varian, Harbour City, CA, USA) were activated using 1.0 mL of methanol and 2.0 mL of water. To a volume of 500 µL of plasma, 50 µL of internal standard solution (4 µg/mL) was added and 500 µL of a 10 mM phosphate buffer (pH 6). This mixture was loaded onto the cartridges with a dispense flow rate of approximately 1 mL/min. After washing with 1.0 mL of methanol – water (5:95, v/v), elution was carried out using 0.75 mL of methanol. Subsequently, the eluate was diluted with 0.75 mL of water and after mixing and filtration using Micro-spin filters (0.45 µm, Alltech Ass. Inc., Deerfield, IL, USA) a volume of 50 µL was injected onto the analytical column.

**Validation procedures**

*Linearity*

Seven plasma calibration standards were prepared and subsequently analyzed in duplicate in three separate analytical runs. Calibration curves were calculated using linear or quadratic regression without weighting and using 1/(concentration) and 1/(concentration)$^2$ as weighting factors. In order to establish the best fit and weighting, back-calculated calibration concentrations were determined. The model with the lowest total bias and the most constant bias across the concentration range was considered the best fit. Deviations of the calculated concentrations from the nominal concentrations should be within ±15% (±20% for the LLOQ) for at least two-thirds of the non-zero calibration standards [12].

*Accuracy and precision*

For the validation of the assay, quality control samples were prepared with concentrations of 10, 30, 600, 900, and 3,500 ng/mL. The latter was diluted ten times in control human plasma prior to sample processing in order to demonstrate the ability to dilute samples originally above the ULOQ. Five replicates of each sample were analyzed together with a set of calibration standards, independently prepared from the control samples, in three analytical runs. The accuracy was determined as percent difference between the mean concentration and the nominal concentration. The coefficient of variation (C.V.) was used to report the precision. The intra- and inter-assay accuracies were required to be within

±20% at the LLOQ level and within ±15% for other concentrations. The precisions were required to be less than 20% at the LLOQ and less than 15% at other concentrations [12].

*Specificity and selectivity*
Six individual batches of control drug-free human plasma samples containing neither analyte nor internal standard were processed in order to determine whether endogenous compounds interfere at the mass transitions chosen for ABT-518, metabolites or the internal standards. In addition, plasma samples were prepared containing a single analyte at the ULOQ level in order to determine whether the analytes interfere with one another in mass transition and retention time. Samples were processed according to the described procedures and analyzed. Areas of peaks co-eluting with the analytes should not exceed 20% of the area at LLOQ level, and areas of peaks co-eluting with the internal standards should not exceed 5% of the internal standard peak area.

*Ion suppression and recovery*
Control drug-free plasma was processed by SPE and the eluate was evaporated to dryness ($N_2$, 40 °C). Dry extracts were dissolved using analyte standard solutions in methanol-water (50:50, v/v) that represent 100% recovery. Ion suppression was determined by comparing the analytical response of these samples to that of the standard solutions. SPE recovery was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with standard solutions as described above. These experiments were performed at three concentration levels, in triplicate. Overall recovery corresponds to the net response after subtraction of the ion suppression and the signal loss due to the extraction.

*Stability*
The stability of ABT-518 and metabolites was investigated in the stock and working solutions, in plasma during storage, during processing, after three freeze (-20 °C) - thaw cycles, and in the final extract. Analytes are considered stable in the biological matrix when 80-120% of the initial concentration is found. Stability in stock and working solutions was also investigated for the internal standards. Analytes and internal standards are considered stable in the stock and working solutions when 95-105% of the original concentration is recovered.

**Clinical study**
During the phase I clinical study patients received ABT-518 orally once daily on day 1 and day 4 to 29. The starting dose was 25 mg. Several blood samples were collected on study days 1 and 22 and on the other days only pre-dose samples were collected. Depending on their physical condition and toxicity profile, patients were allowed to continue therapy with ABT-518 once a day at the same dose level. Pre-dose samples were then withdrawn weekly. Blood samples were collected in heparinized tubes, immediately cooled, and centrifuged within one hour (4 °C, 10 min at 1,200 g). The plasma layer was removed and stored in polypropylene tubes at –20 °C until analysis.

## Results and Discussion

### Metabolism of ABT-518

In Figure 1 the chemical structures of ABT-518, potential metabolites, and internal standards are shown. Based on pre-clinical data it was decided to incorporate these six metabolites in the assay. These potential metabolic conversions of ABT-518 may occur via several pathways. Metabolite M1 is formed after reduction of the N-hydroxy moiety of the retrohydroxamate group in the ABT-518 molecule and metabolite M2 may be formed from M1 after subsequent elimination of the N-linked aldehyde moiety by hydrolysis of the amide, thus modifying the retrohydroxamate to a primary amine. Metabolite M4 is formed from ABT-518 by modification of the cyclic ketal to form a diol moiety. Metabolite M5 may be formed by reduction of the N-hydroxy group from M4 or by dealkylation of the cyclic ketal moiety from M1. In a similar way M6 may be formed out of M5 or M2. It is not clear whether only one of these reactions occurs or both. Metabolite M3 is formed by a rather unusual cleavage of a carbon-carbon bond in the backbone structure of the molecule, resulting in a methyl sulfone endgroup and may be formed from the parent drug and each of the metabolites. $IS_A$ and $IS_B$, the internal standards, are structural analogues of ABT-518 and metabolite M4, respectively, and have an additional internal methylene group compared to the parent drug and metabolite M4.

### Mass Spectrometry

A mass spectrum of ABT-518, metabolites, and internal standards in a mixture of methanol – water (80:20, v/v) was recorded in the first quadrupole (Q1) in the positive ion mode with a mass range from 320 to 570 amu (Figure 2). Analyte concentrations were in the same order of magnitude, approximately 1 µg/mL. However, the analyte responses differ significantly from one compound to the other. This is probably due to differences in ionization efficiency, which is dependent on the compound's molecular structure. For example, metabolites M2 and M6 both contain a basic primary amine moiety and are readily protonated. The most intense peaks in the Q1 mass spectrum correspond to the $[M+H]^+$ ions of these two metabolites at m/z 462 and 422 respectively. The sodium adducts of the two metabolites are also formed and visible in the mass spectrum at m/z 484 and 444, respectively. Sodium adducts are observed often in electrospray mass spectra and sometimes more abundant than the $[M+H]^+$ ions, especially when solutions of neutral pH are used and/or the molecule lacks basic sites [13]. Furthermore the ion at m/z 404 most-likely results from the loss of water (-18 amu) from the M6 molecular ion. Since a water molecule can be expected to eliminate from the diol moiety, it explains why it does not occur for M2. Peaks corresponding to the loss of water were not observed for the other diol moiety containing compounds, M4, M5, and $IS_B$, but this probably results from the lower overall responses of these compounds. M6 differs from M2 in the cyclic ketal moiety which is modified (Figure 1) and the relative responses in the Q1 mass spectrum suggest that this modification results in lower ionization efficiency. Probably, the diol has a larger electron withdrawing effect on the amine than the cyclic ketal, reducing the basic character of the amine, and therefore lowering the ionization efficiency of M6 in comparison to

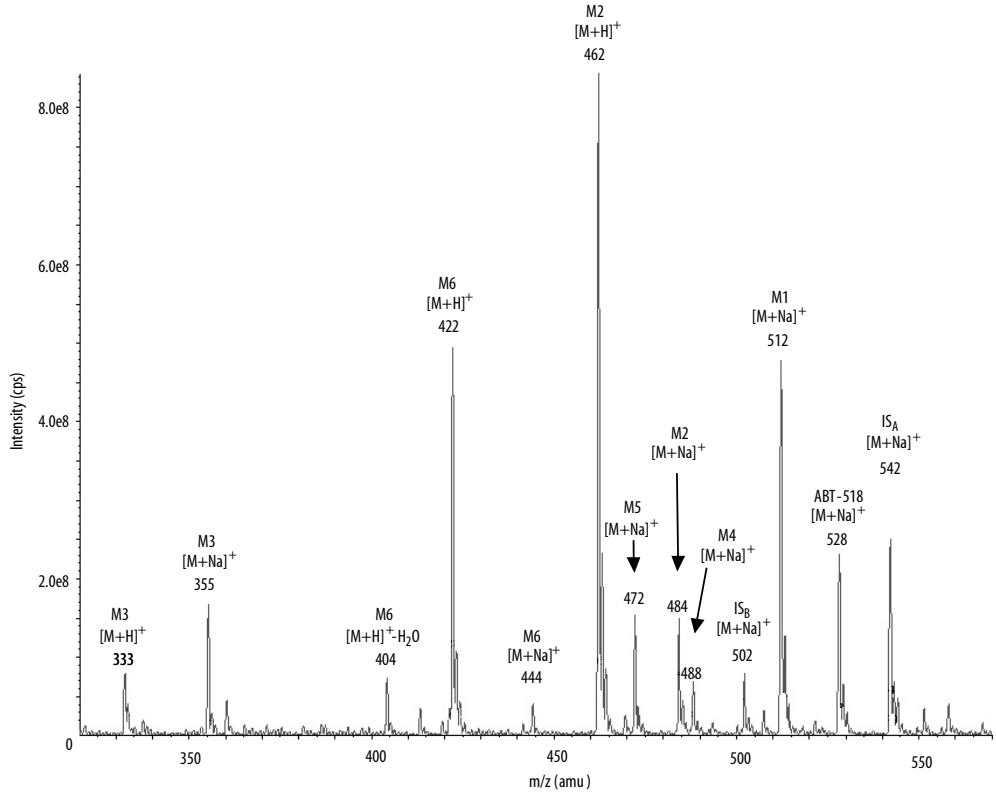



**Figure 2.** Positive ESI-Quadrupole mass spectrum of a solution containing approximately 1 µg/mL of ABT-518, metabolites, and internal standards in methanol-water (80:20, v/v).

M2. The peak at m/z 333 corresponds to the $[M+H]^+$ ion of metabolite M3. The sodium adduct of this metabolite is present in the spectrum at m/z 355. The remaining peaks in the spectrum correspond to the sodium adducts of ABT-518, the internal standards and the three remaining metabolites (M1, M4, and M5). All these compounds contain an amide group. Unlike amines, amides are relatively non-basic [14]. Therefore $[M+H]^+$ ions of these compounds are not detected under the neutral conditions (methanol – water, 80:20 v/v) used to record this spectrum. Sodium adducts, however, are present. The ion at m/z 528 corresponds to the sodium adduct of ABT-518, the ion at m/z 542 to that of $IS_A$. These two compounds are almost identical apart from the internal standard's additional methylene group, and therefore show similar ion efficiencies. This was also observed for the metabolite M4 and $IS_B$. In addition, ABT-518 and $IS_A$ give more intense signals in the mass spectrum than M4 and $IS_B$, suggesting again the lower ionization efficiency of the diol moiety compared to the cyclic ketal.

In brief, an increase in signal is observed after N-reduction of the retrohydroxamate moiety of ABT-518 (formation of M1) and an additional increase when the amide is subsequently hydrolyzed to form M2. The same observations are made for M4, M5, and M6, however, with overall lower peak intensities than their cyclic ketal containing analogues.

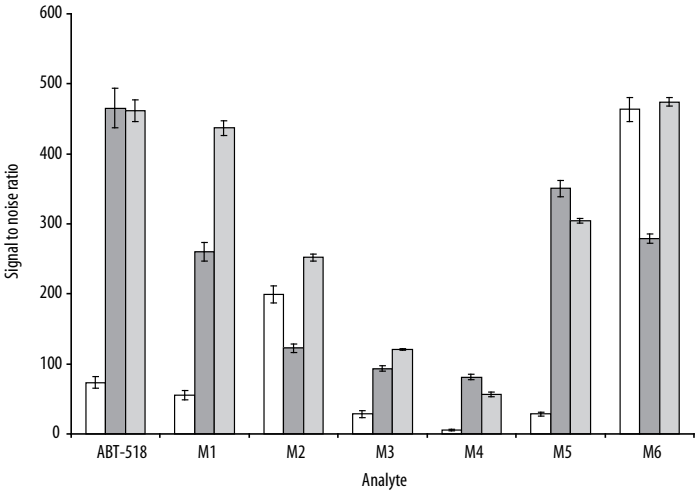



**Figure 3.** Comparison of signal to noise ratios for ABT-518 and metabolites resulting from flow injection analyses using neutral, acidic, or alkaline eluents. (Neutral: methanol-water (80:20, v/v); Acidic: methanol-10 mM ammonium acetate/acetic acid in water pH 5 (80:20, v/v); Alkaline: methanol-10 mM ammonium hydroxide in water (80:20, v/v)).

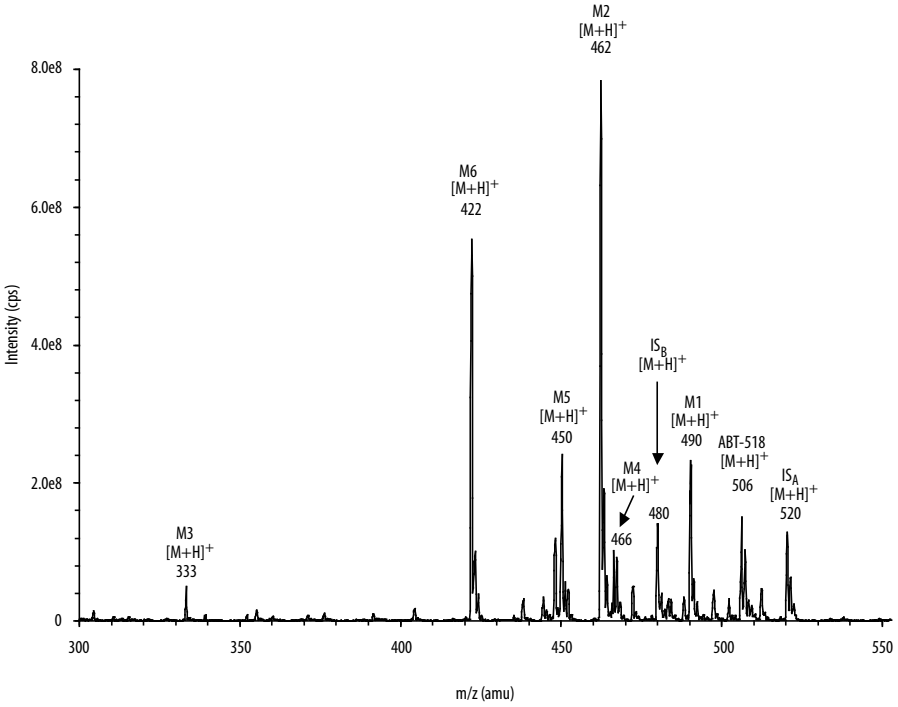



**Figure 4.** Positive ESI-Quadrupole mass spectrum of a solution containing approximately 1 µg/mL of ABT-518, metabolites, and internal standards in methanol-10 mM ammonium hydroxide in water (80:20, v/v).

**Method development**

In the bioanalysis of multiple analytes mass spectrometry has become irreplaceable, especially when analogous compounds, such as ABT-518, its metabolites and internal standards, are concerned. A chromatographic baseline separation of these compounds, if feasible, would result in prohibitively long run times. Due to the selectivity of the TQ-MS operating in the MRM mode complete chromatographic separation of the analytes is not necessary.

Normally, the $[M+H]^+$ molecular ions are used in MRM analysis, since $[M+H]^+$ ions are usually more readily fragmented with only low collision gas values than sodium adducts, which are usually more abundant in electrospray but are hardly fragmented and then only at very high collision gas values and high collision energies [15]. The composition of the aqueous phase of the LC eluent influences the formation of $[M+H]^+$ ions or sodium adducts. We have compared signal to noise ratios of $[M+H]^+$ ions of ABT-518 and metabolites when analyzed with flow injection analysis (FIA; without analytical column) using neutral, acidic, or basic eluents. These eluents consisted of 80% of methanol and either 20% of water (pH~7), 10 mM ammonium acetate/acetic acid in water (pH~5), or 10 mM ammonium hydroxide in water (pH~10), respectively. The results are presented in Figure 3. When using a neutral eluent relatively low signal to noise ratios were obtained, except for the primary amine containing metabolites M2 and M6. Apart from compounds with a relatively strong basic character, such as amines, protonation from neutral solutions is difficult because of the low abundance of protons in these solutions. Comparison of the signal to noise ratios obtained using either an acidic or alkaline eluent reveals small differences for ABT-518, M3, M4, and M5. However, for M1, M2, and M6 an alkaline eluent is clearly favored in terms of sensitivity, while an apparent preference for an acidic eluent is not observed. In conclusion, a alkaline eluent is preferred in order to obtain the highest overall sensitivity for the analytes. In Figure 4, a Q1 mass spectrum of the analytes and internal standards in the alkaline mixture (approximate concentration of each compound 1 µg/mL in methanol – 10 mM ammonium hydroxide (80:20, v/v)) is presented. The figure demonstrates that predominantly protonated molecular ions are formed under these conditions. However, the mutual peak ratios as first observed in Figure 2 have not changed. The protonated molecular ions of the analytes were selected in the first quadrupole and induced to fragment in the collision cell. Fragment ions were subsequently analyzed in the third quadrupole. The resulting product ion MS/MS spectra and proposed fragmentation schemes are presented in Figure 5. The base peak in the MS/MS spectrum of ABT-518 probably corresponds to the fragmentation of the cyclic ketal moiety resulting in the loss of a fragment of 58 amu. The elimination of the N-linked aldehyde moiety is also observed. Furthermore, the peak at m/z 317 corresponds to the cleavage of the bond between the backbone methylene moiety and the carbon containing the retrohydroxamate group. The product ion spectrum of M1 shows similar characteristics, however, the elimination of the N-linked aldehyde is not observed, which may be due to changes in the charge distribution of the retrohydroxamate moiety following the elimination of the N-linked hydroxyl group. The product ion mass spectrum of M2 shows an additional fragment corresponding the cleavage of the bond between the carbon containing the retrohydroxamate moiety and

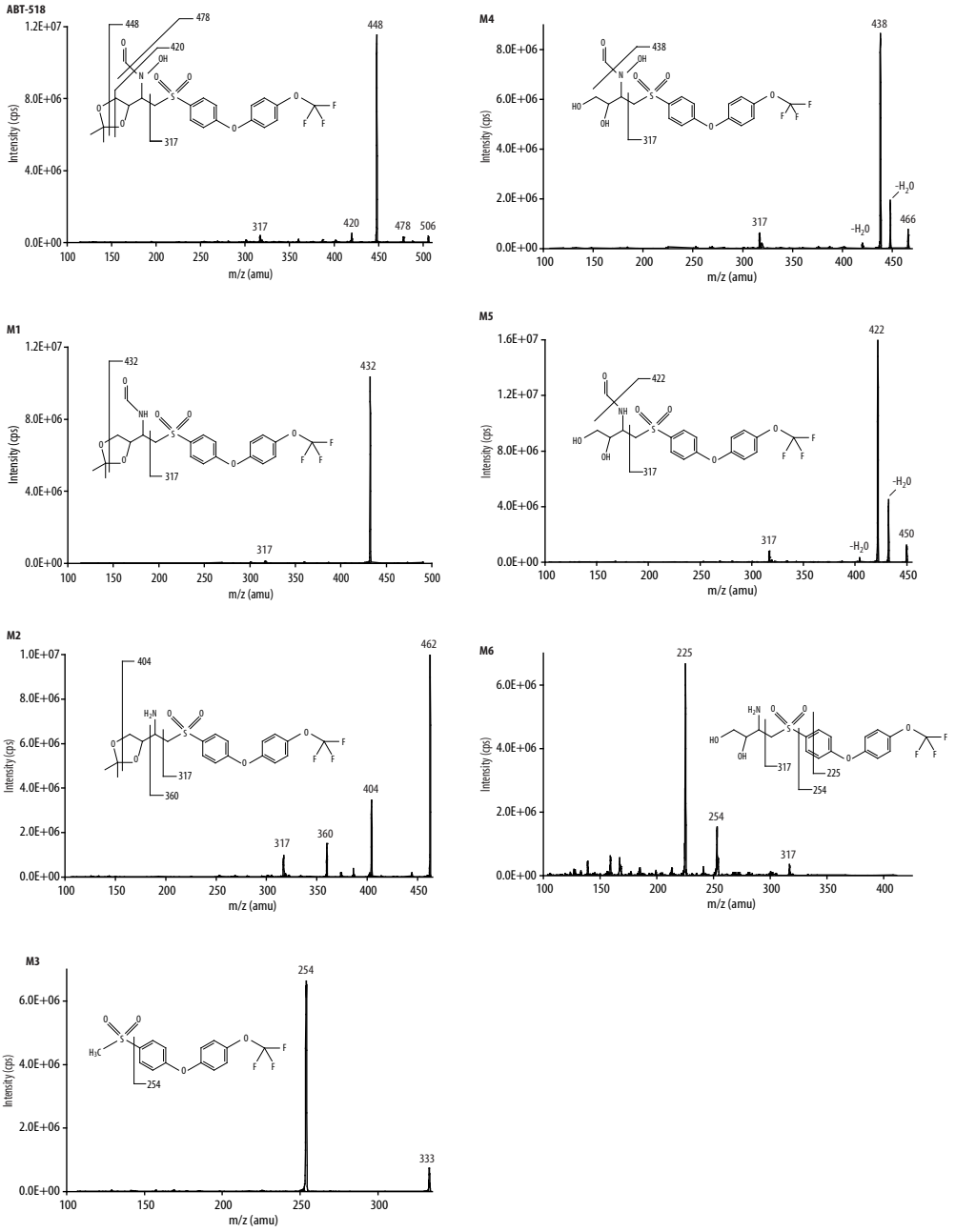



**Figure 5.** Product ion MS/MS spectra of ABT-518 and its metabolites. Potential fragmentation schemes are presented for each analyte.

the cyclic ketal moiety. Contrary to the MS/MS spectra of ABT-518 and M1, the base peak in this spectrum is the protonated molecular ion. Metabolite M3 differs greatly from the other compounds as it lacks the retrohydroxamate and cyclic ketal moiety. The base peak fragment ion corresponds to a cleavage of the bond between the phenyl and sulfone moiety. The product ion spectra of diol metabolites M4 and M5 both show as base peak the elimination of the N-linked aldehyde moiety. Probably, the diol groups influence this fragmentation. In addition, the elimination of water is observed. Metabolite M6, however, does not contain the aldehyde moiety, and shows a fragmentation pattern which differs from all other analytes. The base peak fragment ion probably corresponds to the cleavage of the sulfone-linked phenyl moiety. The mass transitions from the protonated molecular ions to the base peak fragment ions were used for MRM in LC-MS/MS.

One would expect it to be more difficult to positively ionize compounds from an alkaline eluent than from a neutral and definitely from an acidic eluent, since the proton abundance is even lower than in a neutral solution, but the opposite seems to be true. Positive ionization in the electrospray ion source in the presence of ammonium hydroxide most likely results from ion-molecule-reactions between the analyte molecule and ammonium ions or collision induced dissociation of the ammonium adducts of the analyte under influence of the electrospray voltage [16]. The neutral, acidic, and alkaline eluents were also compared for their respective effect on the chromatography. The first two eluents were pumped over a regular minibore reversed phase $C_{18}$ column, while for the alkaline eluent a special column with a basic-stable stationary phase was used. Both under neutral and acidic conditions the chromatography of the compounds containing a hydroxy formamide moiety (ABT-518, M4, and both internal standards) resulted in extensively tailing peaks. Alkaline conditions produced excellent peak shapes for all compounds. Thus, an eluent consisting of methanol-10 mM ammonium hydroxide in water (80:20, v/v) appeared to be most appropriate for the LC-MS/MS analysis of ABT-518 and metabolites from both mass spectrometric and chromatographic point of view. Furthermore, the LC runtime was only 8 minutes using this mobile phase. Representative LC-MS/MS chromatograms from a study sample and a double blank sample are presented in Figure 6 and 7, respectively.

During development carry-over was substantial, for all compounds in similar quantities. Apart from classic carry-over, an increase in the noise level was observed with every injection. In order to circumvent carry-over the upper limit of quantitation was lowered as well as the internal standard concentrations. Although these measures reduced carry-over significantly, the extensive carry-over could only be eliminated when the autosampler needle was rinsed after every series of calibration standards, quality control samples, or study samples with large quantities of methanol.

**Sample pretreatment**

Since protein precipitation did not produce acceptable peak shapes or recoveries for the analytes and internal standards, a simple and straightforward SPE method was used for sample pretreatment. Phenyl SPE cartridges provided the most appropriate means of sample preparation for all compounds. The overall recovery (SPE plus ion-suppression) for ABT-518 was 50.8 ± 4.09.%. For the metabolites the overall recoveries ranged from 43.9 to

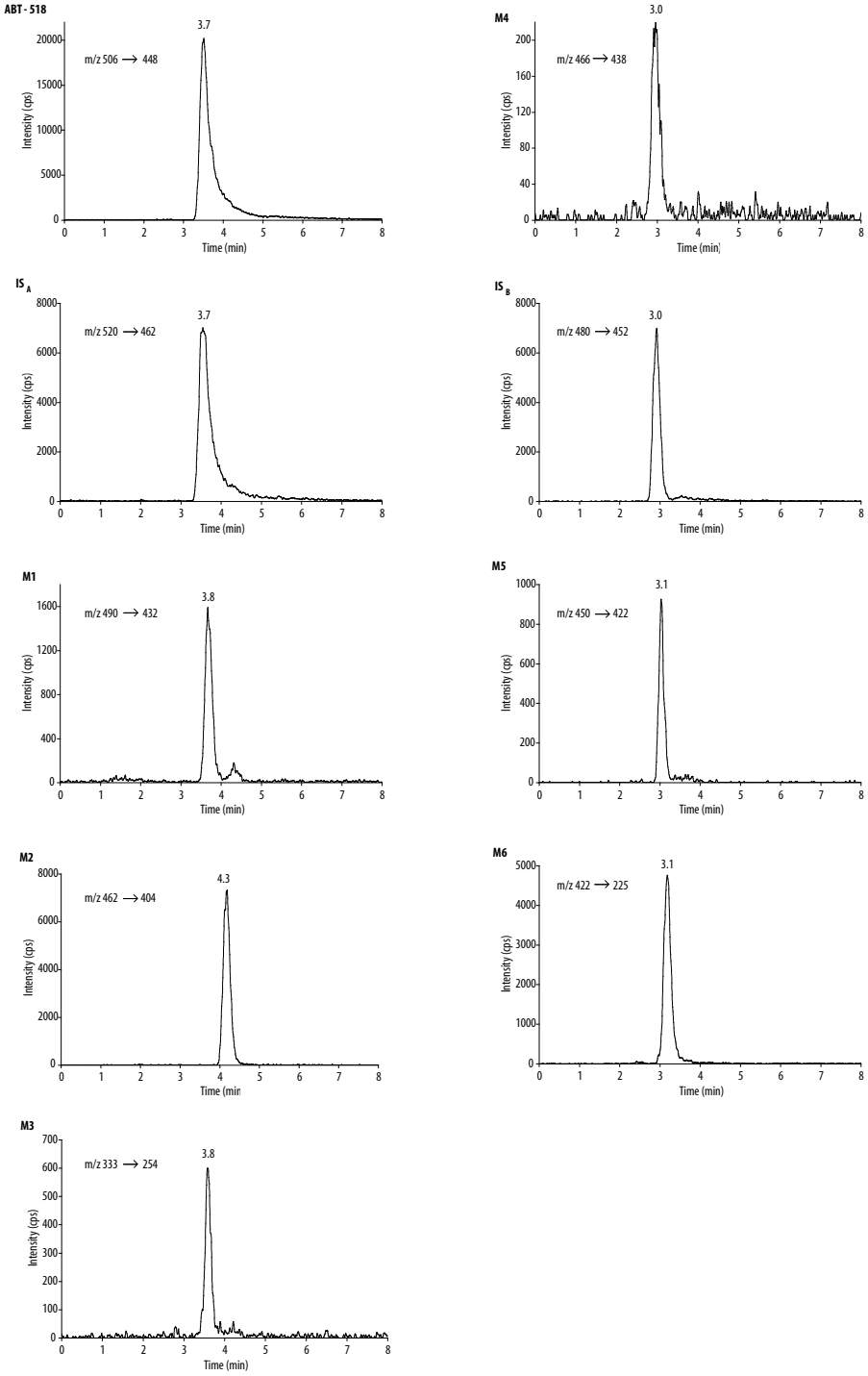



**Figure 6.** Representative HPLC-ESI-TQ-MS/MS MRM chromatograms of the analytes and internal standard from a representative study sample.

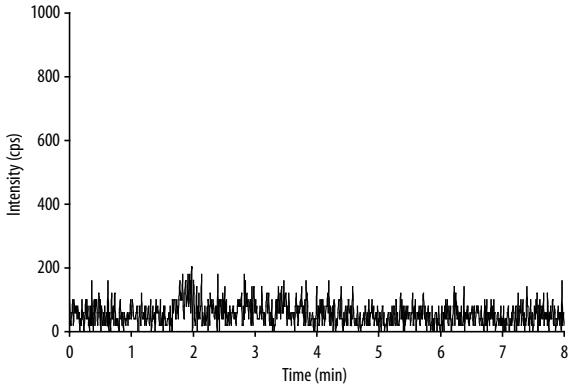



**Figure 7.** Representative HPLC-ESI-TQ-MS/MS MRM chromatogram of a double blank sample.

87.9% with C.V. values ranging from 0.778 to 18.5%. After elution of the sample from the phenyl SPE cartridges with methanol, the eluate was diluted 1:1, v/v with water, and 50 µL of this mixture was injected onto the analytical system. This dilution step was chosen in stead of evaporation of the eluate and reconstitution in a smaller amount of solvent since it is less time consuming and cleaner extracts were produced.

**Results of the Validation for ABT-518**

*Linearity*

Calibration standards were analyzed in duplicate in three analytical runs to determine the ABT-518 concentrations in a range of 10 to 1,000 ng/mL. Analyses of ABT-518 were performed using $IS_A$ as internal standard. Linear fits with a weighting factor of 1/(concentration) appeared to be most appropriate to describe the signal versus concentration curves. For every calibration curve the calibration concentrations were back-calculated from the response (Table 1). The deviation from the nominal concentration for all ABT-518 concentrations was between –5.14 and 3.81%, with C.V. values lower than 4.61%.

*Accuracy and precision*

The inter-assay performance data of ABT-518 are summarized in Table 2. Inter-assay accuracies are between –9.24 and 6.93%. The intra-assay accuracies (data not shown) for all tested ABT-518 concentrations were between –12.1 and 13.7%. Precisions were less than 10.7% for all tested concentrations.

*Specificity and selectivity*

Chromatograms of six batches of control drug-free plasma contained no co-eluting peaks >20% of the ABT-518 area at the LLOQ level, and no co-eluting peaks >5% of the area of $IS_A$. Furthermore, no co-eluting peaks were observed originating from any of the metabolites at ULOQ level in the ABT-518 window that exceeded 20% of the ABT-518 area at LLOQ level.

**Table 1.** Calibration concentrations of ABT-518 back-calculated from the nominal concentrations.

| Run | ABT-518 concentration (ng/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| Number | 10.5 | 26.3 | 52.5 | 105 | 525 | 788 | 1050 |
| 1 | 10.5 | 26.9 | 52.0 | 107 | 519 | 801 | 1090 |
| | 10.6 | 27.2 | 49.0 | 107 | 504 | 779 | 1030 |
| 2 | 11.1 | 25.9 | 50.5 | 108 | 540 | 776 | 1090 |
| | 11.5 | 25.2 | 49.1 | 104 | 516 | 740 | 1070 |
| 3 | 10.3 | 28.1 | 50.5 | 116 | 512 | 772 | 1130 |
| | 11.4 | 25.9 | 47.9 | 102 | 481 | 751 | 1080 |
| Mean | 10.9 | 26.5 | 49.8 | 107 | 512 | 770 | 1082 |
| S.D. | 0.502 | 1.06 | 1.45 | 4.80 | 19.4 | 21.6 | 32.5 |
| C.V. (%) | 4.61 | 3.99 | 2.92 | 4.47 | 3.78 | 2.81 | 3.01 |
| Dev. (%) | 3.81 | 0.760 | -5.14 | 1.90 | -2.48 | -2.28 | 3.05 |

S.D. Standard deviation; C.V. Coefficient of variation; Dev. Deviation

**Table 2.** Inter-assay performance data for ABT-518

| Nominal concentration (ng/mL) | Measured concentration (ng/mL) | Inter-assay accuracy (%) | Inter-assay precision (%) | Number of replicates |
|---|---|---|---|---|
| 10.1 | 10.8 | 6.93 | 10.7 | 15 |
| 30.3 | 27.5 | -9.24 | 5.09 | 15 |
| 606 | 591 | -2.48 | 3.03 | 15 |
| 909 | 852 | -6.27 | 5.09 | 15 |
| 3540 | 3529 | -0.311 | 9.52 | 15 |

*Stability*

In Table 3 the results of stability experiments for ABT-518 are summarized. ABT-518 is stable in the stock solution for at least 24 h at ambient temperatures and 6 months at –20 °C, in the working solutions for at least 24 h at ambient temperatures and 3 months at –20 °C, since all deviations from time zero were less than 5%. Stability in plasma has been established after three freeze (-20 °C)/ thaw cycles, during at least 24 h at ambient temperatures, and 4 months at –20 °C. In addition, ABT-518 is stable in the final extract for at least 4 days at ambient temperatures and for at least 6 days at 2-8 °C. The internal standard ($IS_A$) is stable in the working solutions for at least 4 months at –20 °C (data not shown).

**Results of the Validation for the Metabolites**

*Linearity*

Metabolites M1, M2, and M3 were analyzed without using an internal standard, since results deteriorated with the use of either one of the internal standards compared to quantitation without an internal standard. Calibration curves were calculated by linear regression using a weighting factor of 1/(concentration). For the three other metabolites (M4, M5, and M6) quadratic fits using a weighting factor of 1/(concentration) appeared

to be the most appropriate means to describe the signal versus concentration curves. Analyses of these compounds were performed using $IS_B$ as internal standard. The calibration concentrations for the metabolites were back-calculated from the responses (data not shown) and they were between –6.57 and 7.67% for all concentrations.

*Accuracy and Precision*

The intra- and inter-assay accuracies for the metabolites were within 30% at all concentration levels for all metabolites, with the exception of M3. For M3 an intra-assay precision at LLOQ level (10 ng/mL) of 38.9% was found and an intra-assay accuracy of –37.8% at the low concentration level (30 ng/mL). With a signal to noise ratio of 5 the LLOQ for M4 was established at 25 ng/mL.

*Specificity and Selectivity*

Specificity and selectivity of the assay for the metabolites were satisfactory, since peaks originating from endogenous compounds or any of the analytes either did not co-elute with the analyte of interest or had areas lower than 20% of the LLOQ area. Furthermore the area of peaks co-eluting with $IS_B$ never exceeded 5% of the $IS_B$ area.

**Table 3.** Stability data of ABT-518

| Matrix | Conditions* | Initial conc (ng/mL) | Found conc (ng/mL) | Dev. (%) | C.V. (%) | Number of replicates |
|---|---|---|---|---|---|---|
| Methanol (stock solution) | Ambient, 24 h | $1.05*10^6$ | $1.05*10^6$ | 0.00 | 0.629 | 3 |
| | -20 °C, 6 months | $1.05*10^6$ | $1.04*10^6$ | -0.952 | 1.25 | 3 |
| Methanol (working solution) | Ambient, 24 h | $1.05*10^5$ | $1.01*10^5$ | -3.81 | 0.635 | 3 |
| | -20 °C, 3 months | $1.05*10^5$ | $1.06*10^5$ | 0.952 | 1.67 | 3 |
| Plasma | 3 freeze(-20 °C)/thaw cycles | 30.3 | 28.0 | -7.59 | 3.85 | 3 |
| | | 909 | 904 | -0.550 | 2.64 | 3 |
| | Ambient, 24 h | 30.3 | 34.8 | 14.9 | 4.61 | 3 |
| | | 909 | 826 | -9.13 | 0.559 | 3 |
| | -20 °C, 4 months | 30.3 | 29.6 | -2.42 | 4.13 | 3 |
| | | 909 | 756 | -16.8 | 1.78 | 3 |
| Methanol-water (50:50, v/v) | Autosampler, ambient, 4 days | 30.3 | 30.7 | 1.32 | 5.39 | 3 |
| | | 909 | 819 | -9.90 | 0.711 | 3 |
| | Autosampler, 2-8 °C, 6 days | 30.3 | 34.3 | 13.2 | 14.2 | 3 |
| | | 909 | 964 | 6.05 | 1.80 | 3 |

conc. concentration; Dev. Deviation; C.V. Coefficient of Variation; * Temperatures are nominal

*Stability*
All metabolites were stable in the stock and working solutions for at least 24 h at ambient temperatures. Furthermore metabolites and $IS_B$ were stable for at least 2 months in the stock solution at –20 °C, since deviations in concentration from time zero never exceeded 5%. All metabolites were stable for at least 2 months in human plasma at –20 °C and in the final extract for at least 4 days at ambient temperatures as deviation from time zero was less than 20%. Furthermore, metabolites are stable after three freeze (–20 °C)/thaw cycles. Deviations of –20.7 and –22.0 % from cycle zero for the low concentration were observed for metabolites M3 and M5, respectively. These results were random and do not result from instability problems in view of the analytical accuracy. Deviations measured for other concentrations or metabolites were less than 20%. The stability in human plasma after 24 h at ambient temperatures was established for all metabolites at both concentration levels. A deviation from time zero of 25.2 and 29.8% was observed for the low concentration level of M1 and M2, respectively.

**Internal standards**
In quantitative mass spectrometry, internal standards are needed for accurate determination of concentrations [17]. Since ionization efficiency in ESI is highly dependent on the structure of a compound, internal standards are preferably stable isotopically labeled analogues of the analyte or a compound with a structure closely resembling the parent drug. For the quantitative analysis of ABT-518 a structural analogue, $IS_A$, was available. A stable isotopically labeled internal standard was not available. In the Q1 mass spectra of ABT-518, metabolites and internal standards (Figure 2 and 4) it is observed that ABT-518 and $IS_A$ show similar responses in the mass spectrometer indicating similar ionization efficiencies. This suggests that $IS_A$ can be an appropriate internal standard for ABT-518 from a mass spectrometric point of view. From both figures, however, it is also clear that $IS_A$ has a different ionization efficiency than metabolites M1, M3, and especially M2, potentially complicating the quantitative analysis of these metabolites. Similarly, metabolite M4 and internal standard $IS_B$ show similar responses, but M5 and M6 show very different responses. The absence of appropriate internal standards for the majority of the metabolites may explain why accuracy and precision data for the metabolites were not within FDA requirements and, in addition, the need for quadratic fits of some calibration curves. However, since stable isotopically labeled internal standards or better resembling analogues are not available, the described method was used for the quantitative determination of ABT-518 and screening of the metabolites. An analytical run for the metabolites was accepted when calculated concentrations for calibration standards and quality control samples did not deviate more than ±30% from the nominal concentrations.

**Clinical study**
After the validation, the method was used to support a clinical phase I trial of ABT-518. At the starting dose of 25 mg four patients were included and $C_{max}$ values of 273, 869, 462, and 1090 ng/mL were obtained for ABT-518. Metabolites were detected, except for M1 and M3. Dose limiting toxicities did not occur at the first dose level and the dose was escalated

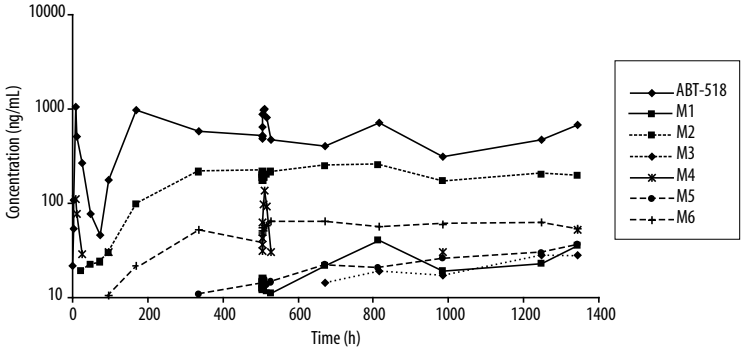



**Figure 8.** Concentration *vs.* time profiles of ABT-518 and metabolites over days 1 to 64 in a patient treated with a of 50 mg on day 1 and days 4 to 64.

100% to 50 mg. At this dose level all metabolites included in the assay could be detected. The first patient entered at 50 mg was allowed to receive ABT-518 after day 29. In Figure 8 concentration vs. time plots over all days of treatment are presented for ABT-518 and metabolites from this patient treated with 50 mg of ABT-518. A maximum concentration of ABT-518 of 1040 ng/mL was measured eight hours after administration of the drug. Approximately 72 hours after administration ABT-518 levels reach their minimum, however concentrations were still above the LLOQ. The concentration vs. time curve for M4 shows a maximum concentration of 136 ± 40.8 ng/mL. The concentration vs. time curve of ABT-518 and M4 show similarities in shape, indicating that M4 is formed immediately after administration of ABT-518. The other metabolites show different concentration vs. time profiles than ABT-518 and M4. The first of these metabolites to be detected was M2, 24 hours after administration of the drug. For this metabolite the highest plasma levels were obtained around 250 ± 75 ng/mL. Metabolite M6 is first detected after 96 hours, M5 after approximately 350 hours, M1 after 500 hours, and M3 after 700 hours. These metabolites are probably all formed simultaneously, but some in concentrations which are too low to detect at first. After approximately 300 hours, a pharmacokinetic steady-state is reached.

## Conclusion

The results described in this paper demonstrate the usefulness of the assay in the bioanalytical and pharmacokinetic support of the phase I trial with ABT-518. We believe that the method we have described here using an alkaline mobile phase in combination with a basic stable analytical column may also be applied for the bioanalysis of other basic drugs.

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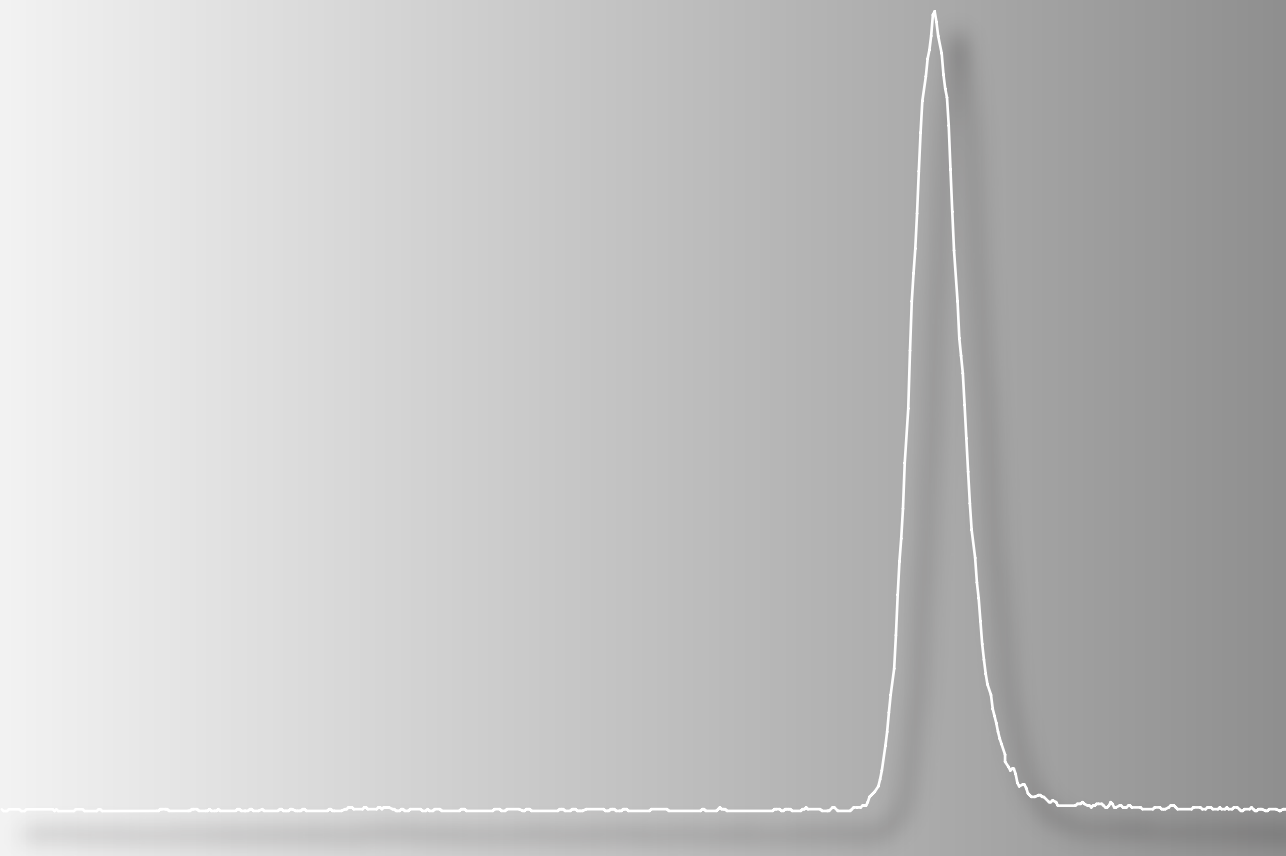
# Chapter 4

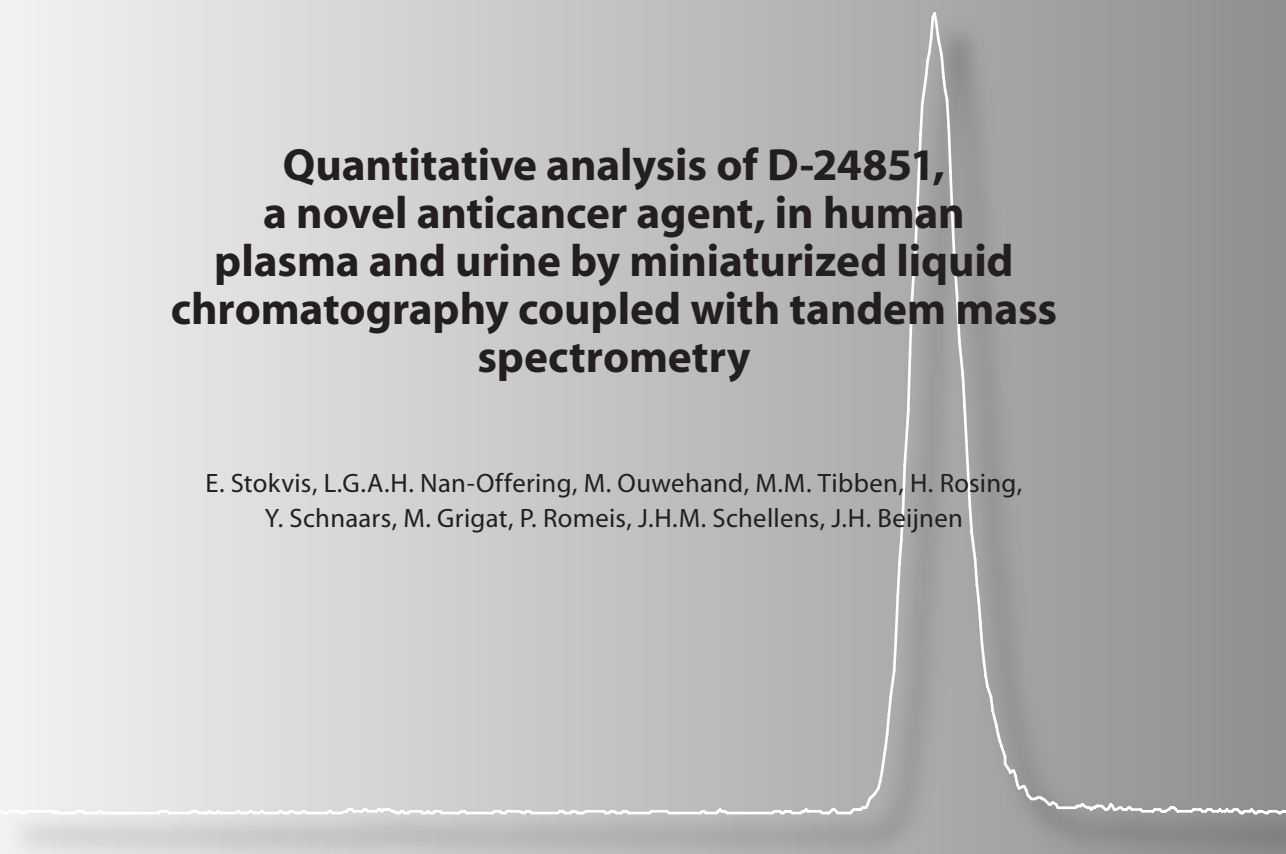


## Tubulin Binding Anticancer Drugs

# 4.1

## Quantitative analysis of D-24851, a novel anticancer agent, in human plasma and urine by miniaturized liquid chromatography coupled with tandem mass spectrometry

E. Stokvis, L.G.A.H. Nan-Offering, M. Ouwehand, M.M. Tibben, H. Rosing, Y. Schnaars, M. Grigat, P. Romeis, J.H.M. Schellens, J.H. Beijnen



Submitted

## Abstract

The development of a liquid chromatography tandem mass spectrometric assay for the quantitative analysis of the novel tubulin inhibitor D-24851 in human plasma and urine is described. D-24851 and the deuterated internal standard were extracted from 250 µL of plasma or urine using hexane-ether (1:1, v/v). Subsequently, 10 µL aliquots of reconstituted extracts were injected onto an Inertsil ODS analytical column (50 x 2.0 mm ID, 5 µm particle size) with a flow rate of 0.2 mL/min. An API 365 triple quadrupole mass spectrometer was used in the multiple reaction monitoring mode for sensitive detection. For human plasma a dynamic range of 1 – 1,000 ng/mL was validated, and for human urine a range of 0.25 - 50 ng/mL. Validation was performed according to the most recent FDA guidelines and all results were within requirements. The assay has been successfully applied to support a phase I clinical trial with orally administered D-24851.

## Introduction

Tubulin has been an important target in cancer chemotherapy for decades, since inhibition of polymerization and de-polymerization of tubulin interferes with the segregation of chromosomes and therefore cell division [1,2]. Tubulin inhibitors, such as the taxanes and *Vinca* alkaloids, are effective in the treatment of different malignancies, although development of drug resistance often limits their potential. In addition, these drugs are associated with neurotoxicity since microtubules are also involved in the axonal transport in neurons. D-24851, N-(pyridin-4-yl)-[1-(4-chlorbenzyl)-indol-3-yl]-glyoxylamide (Figure 1), is a compound of synthetic nature and has shown potent cytotoxic activity towards several tumor cell lines as well as their resistant phenotypes both *in vitro* and *in vivo*. Curative doses of D-24851 in rats were well tolerated without systemic toxicities, including a lack of neurotoxicity in vivo [3]. In order to pharmacokinetically support a phase I trial with D-24851, a liquid chromatography tandem mass spectrometric (LC-MS/MS) assay for the quantitative determination of D-24851 in human plasma and urine was developed. In this paper the development of the first bioanalytical LC-MS/MS method for the quantita-

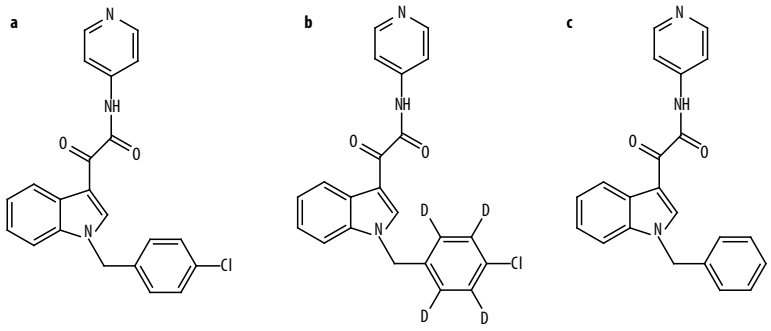



**Figure 1.** Structures of D-24851 (a) and internal standards D-24851-D4 (b) and D-24843 (c).

tive analysis of D-24851, the validation according to the most recent FDA guidelines on bioanalytical method validation, and the implementation into clinical pharmacokinetic studies are described.

## Experimental

### Chemicals

D-24851 and internal standards D-24851-$D_4$ and D-24843 were supplied by Baxter Oncology (Frankfurt am Main, Germany). Methanol and hexane (both HPLC supra-gradient) were purchased from Biosolve Ltd. (Amsterdam, The Netherlands). Di-methyl sulfoxide (DMSO), ammonium acetate, formic acid (all pro analysis) and ether (uvasol) originated from Merck (Darmstadt, Germany). Distilled water was used throughout the analyses. Drug free human plasma was obtained from the Central Laboratory for Blood Transfusion (Sanquin Amsterdam, The Netherlands).

### Instrumentation

The HPLC system consisted of a PE200 series pump and an ISS200 autosampler (Perkin Elmer, Norwalk, CT, USA). A mobile phase of methanol - 5 mM ammonium acetate, 0.004% formic acid in water (80:20, v/v) was pumped at a flow rate of 0.2 mL/min through an Inertsil ODS-3 column (50 x 2.0 mm I.D. 5 µm particle size; Chrompack, Middelburg, The Netherlands) protected with an in-line filter (Micro filter frit, 5 µm, Upchurch Scientific, Inc., Oak Harbor, WA, USA). The LC eluate was introduced directly into an API 365 triple quadrupole MS equipped with an electrospray ion source and controlled by Analyst™ 1.2 software (Sciex, Thornhill, ON, Canada). Sample injections of 10 µL were carried out and the total run time was 5 min. The quadrupoles were operated in the positive ion mode with unit resolution and the obtained multiple reaction monitoring (MRM) chromatograms were used for quantification. Mass transitions of m/z 390 $\rightarrow$ 268, m/z 394 $\rightarrow$ 272, and m/z 356 $\rightarrow$ 234 were optimized for D-24851, D-24851-$D_4$, and D-24843, respectively with dwell times of 150 ms. Nebulizer and turbo gas (both compressed air) were operated at 2.5 and 7 L/min, respectively, while the curtain gas and collision gas (both $N_2$) were operated at 1.4 L/min and $2.1*10^{15}$ molecules/$cm^2$, respectively. The ionspray voltage was kept at 5500 V, with a source temperature of 350 °C.

### Preparation of calibration standards and quality control samples

Two independent stock solutions of D-24851 were prepared in DMSO at a concentration of 1 mg/mL. One solution was further diluted with methanol to obtain working solutions with concentrations ranging from 0.05 to 100 µg/mL. Working solutions were diluted with control human plasma or urine in volumetric flasks in order to obtain calibration standards, ranging from 1 to 1,000 ng/mL in plasma and from 0.25 to 50 ng/mL in urine. The second stock solution was diluted with methanol in order to obtain working solutions with concentrations ranging from 0.3-100 µg/mL. These working solutions were spiked to human plasma and urine in order to obtain quality control samples at three concentration

levels (3, 100, and 800 ng/mL in plasma and 0.6, 25, and 40 ng/mL in urine). Furthermore, quality control samples at the lower limit of quantitation (LLOQ; 1 ng/mL and 0.25 ng/mL in plasma and urine, respectively) and at a concentration exceeding the upper limit of quantification (ULOQ; 2,500 ng/mL and 125 ng/mL in plasma and urine, respectively) were prepared. Plasma or urine solutions of D-24851 never contained more than 1% of methanol to maintain the integrity of the biomatrix. A stock solution of the deuterated internal standard was prepared similar to that for D-24851. The stock solution was diluted with methanol to a final concentration of 1,000 ng/mL for the assay in plasma or 100 ng/mL for the assay in urine.

**Sample processing**

To 250 µL volumes of plasma or urine 25 µL of internal standard solution was added followed by 1,250 µL of hexane-ether (1:1, v/v). After mixing for 5 min, the samples were centrifuged for 5 min at 23,100 g. The aqueous layer was frozen in an ethanol – dry ice mixture and the organic layer was decanted into a clean tube. The organic solvent was evaporated under a gentle stream of nitrogen gas at 20 °C. The residue was reconstituted with 100 µL of eluent by vortex mixing for 30 s and vigorous shaking for 10 min. After centrifuging for 5 min at 23,100 g the supernatant was transferred to a glass autosampler vial and 10 µL was injected onto the analytical column.

**Validation procedures**

A full validation according to the FDA guidelines was performed for the assay in human plasma and urine [4].

*Linearity*

Eight non-zero plasma and urine calibration standards were processed and analyzed in duplicate in three separate analytical runs. Calibration curves were calculated by least-squares linear regression using a weighting factor of $1/x^2$ (the reciprocal of the squared concentration). Concentrations were evaluated on basis of the corresponding calibration curve and deviations from the nominal concentrations should be within ±20% for the LLOQ and within ±15% for other concentrations. Precisions were reported by means of the coefficient of variation (C.V.) and should not exceed ±20% at LLOQ level or ±15% for other concentration levels [4].

*Accuracy and precision*

Quality control samples at five concentration levels were prepared in both matrices. Aliquots of quality control samples in plasma with concentrations exceeding the ULOQ were diluted five times in human plasma prior to sample processing. In urine, a five times larger volume of internal standard solution was added followed by reconstitution of the dry extract in 500 µL of eluent instead of a volume of 100 µL. These experiments were performed in order to validate dilution of samples that were originally above the ULOQ. Five replicates of each sample were analyzed together with a calibration curve, independently prepared from the quality control samples, in three analytical runs. The accuracy

was determined in percent difference between the mean concentration and the nominal concentration. The coefficient of variation was used to report the precisions. The intra- and inter-assay accuracies should be within ±20% for the LLOQ concentration and within ±15% for other concentrations. The precisions should be not exceed ±20% and ±15%, respectively [4].

*Specificity and selectivity*
From six individual batches of control drug-free human plasma and urine samples containing neither analyte nor internal standard (double blank), samples containing only internal standard (blank), and LLOQ samples were prepared. These samples were prepared in order to determine whether endogenous compounds interfere at the mass transition chosen for D-24851 or the internal standard. Samples were processed according to the described procedures and analyzed. Deviations from the nominal concentrations should be within ±20% for the LLOQ samples. Peak areas of compounds co-eluting with D-24851 should not exceed 20% of the D-24851 peak area at the LLOQ or 5% of the internal standard peak area [4].

*Ion suppression and recovery*
Control drug-free plasma and urine was processed and dry extracts were dissolved with working solutions containing D-24851 and internal standard in eluent that represent 100% recovery. Ion-suppression was determined by comparing the analytical response of these samples to that of the working solutions. The loss of signal represents the ion suppression. Liquid-liquid extraction recovery was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with working solutions as described above. These experiments were performed at three concentration levels, in triplicate. Overall recovery corresponds to the net response after subtraction of the ion-suppression and the signal loss due to the extraction. Ion suppression and recovery experiments for the internal standard were performed similarly.

*Stability*
The stabilities of D-24851 and D-24851-$D_4$ were evaluated in the stock and working solutions under both processing (ambient temperatures) and storage (-20 °C) conditions. D-24851 and the internal standard are considered stable in the stock and working solutions when 95-105% of the original concentration is recovered. The stability in human plasma and urine after three freeze (-20 °C) /thaw cycles was investigated by comparing quality control samples that have been frozen and thawed three times with freshly prepared quality control samples. Furthermore, the stability of D-24851 in human plasma and urine under processing (ambient temperatures) and storage (-20 °C) conditions was evaluated. Finally, stability in the dry extract at 4 °C and in the reconstituted extract in the autosampler (ambient temperatures) was determined for both matrices. D-24851 is considered stable in the biological matrix or extracts thereof when 80-120% of the initial concentration is recovered.

### Application of the LC-MS/MS assay in phase I clinical trial

The analytical method described in this paper is used to support a phase I trial with D-24851. In this trial, executed at the Netherlands Cancer Institute, D-24851 is administered orally. The starting dose was 20 mg. Blood samples were collected at several time points and, after centrifuging, plasma was removed and stored at –20 °C until analysis. Immediately after collection and homogenization of urine samples, 250 µL aliquots were transferred to 2 mL tubes and stored at –20 °C until analysis.

## Results

### Mass spectrometry

For the quantitative analysis of D-24851 two potential internal standards were available. At first only D-24843 (Figure 1c), a structural analogue of D-24851, lacking the chlorine atom, was available, later replaced by stable isotopically labeled (SIL) D-24851 (Figure 1b), in which the four hydrogen atoms on the chlorobenzyl moiety are exchanged by deuterium atoms. A Q1 spectrum was recorded from a solution containing ~1 µg/mL of the three compounds in water - methanol (80:20, v/v) containing 0.5% formic acid. The compounds are easily protonated due to the two basic amine moieties. In the Q1 mass spectrum in Figure 2 the protonated molecular ion ($[M+H]^+$) of D-24851 is observed at m/z 390, with the $^{37}Cl$ isotope of D-24851 two mass units higher. The $[M+H]^+$ ion of the deuterated internal standard and its $^{37}Cl$ isotope are found at m/z 394 and 396, respectively, while the molecular ion of the analogous internal standard is observed at m/z 356. The less intense peaks in the spectrum correspond to fragment ions, which are formed in the ion source as a result of the high ionspray voltage (5500 V). Product ion spectra of the three compounds were recorded by allowing the protonated molecular ions to fragment

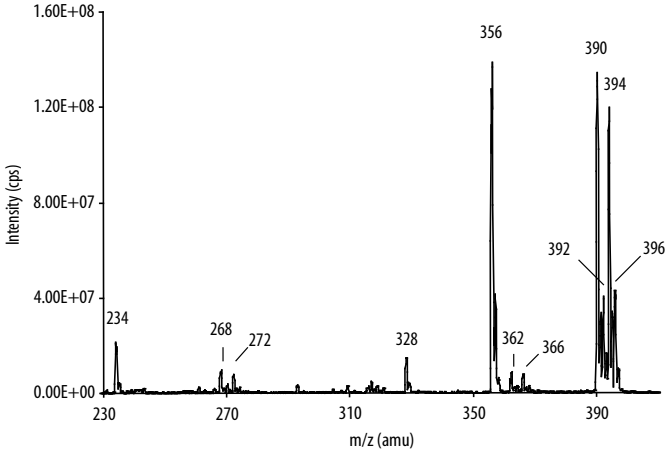



**Figure 2.** Q1 mass spectrum of a mixture of ~1 µg/mL of D-24851, D-24851-$D_4$ and D-24843 in methanol – water (80:20, v/v) and 0.5% formic acid, continuously infused at a flow rate of 3 mL/h.

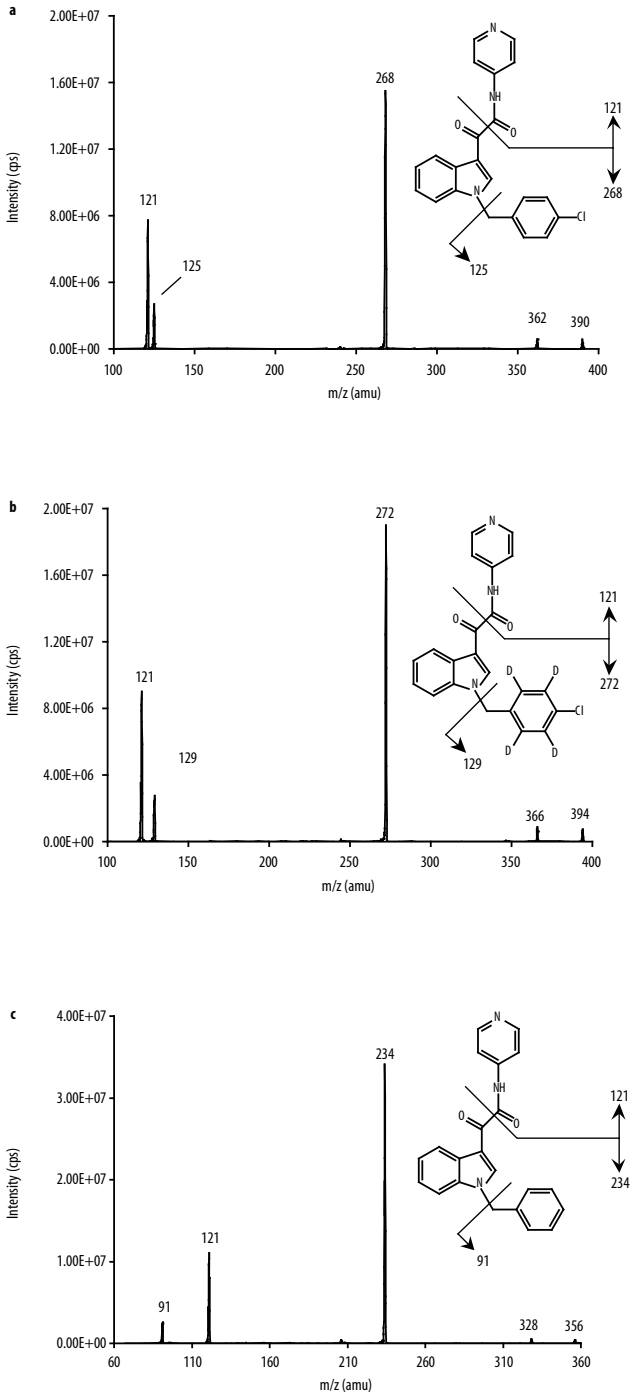



**Figure 3.** Product ion mass spectra of D-24851 (a) and internal standards D-24851-D4 (b) and D-24843 (c). The protonated molecular ions were used for MS/MS.

in the collision cell and analysis of the resulting fragments in Q3. The resulting product ion spectra are presented in Figure 3.

In the product ion mass spectrum of D-24851 (Figure 3a) the main product ion observed at m/z 268 results from $\alpha$-cleavage of the di-keton bond, as indicated in the figure, resulting in protonated 1-(4-chlorobenzyl)-indol-3-carbaldehyde. The fragment ion at m/z 121 results from the same cleavage, leaving the charge on the pyridine-4-amide. The fragment ion at m/z 125 results from cleavage of the nitrogen-carbon bond between the indole and 1-methyl-4-clorobenzene moiety resulting in positively charged 1-methyl-4-clorobenzene. This proposed fragmentation scheme is supported by the product ion mass spectrum of D-24851-$D_4$ (Figure 3b) in which the four hydrogen atoms of the 4-chlorobenzene moiety are replaced by four deuterium atoms. As expected the fragments containing the 4-chlorobenzyl moiety (m/z 268 and 125) are observed four mass units higher in the D-24851-$D_4$ product ion spectrum, while the protonated pyridine-4-amide product ion found at m/z 121 for D-24851 is also observed at m/z 121 for deuterated D-24851. In addition, the product ion spectrum of D-24843 (Figure 3c), the structural analogue of D-24851 with no chloride atom also supports the proposed fragmentation scheme. The benzyl containing fragments are observed 34 mass units lower in the D-24843 product ion mass spectrum at m/z 234 and 91, respectively.

The product ion at m/z 362 in the MS/MS spectrum of D-24851 (m/z 366 for deuterated D-24851 and m/z 328 for D-24843) represents a neutral loss of 28 amu from the molecular ion, which corresponds to either carbon monoxide or ethylene. In order to obtain more information on this fragment ion, $MS^3$ was performed. This was possible since this fragment ion is also formed in the ion source and is present in the Q1 spectrum (Figure 2). Thus, a product ion spectrum of the fragment ion at m/z 362 could be obtained (Figure 4). From Figure 4 it is clear that the unknown fragment is formed after cleavage or rearrangement

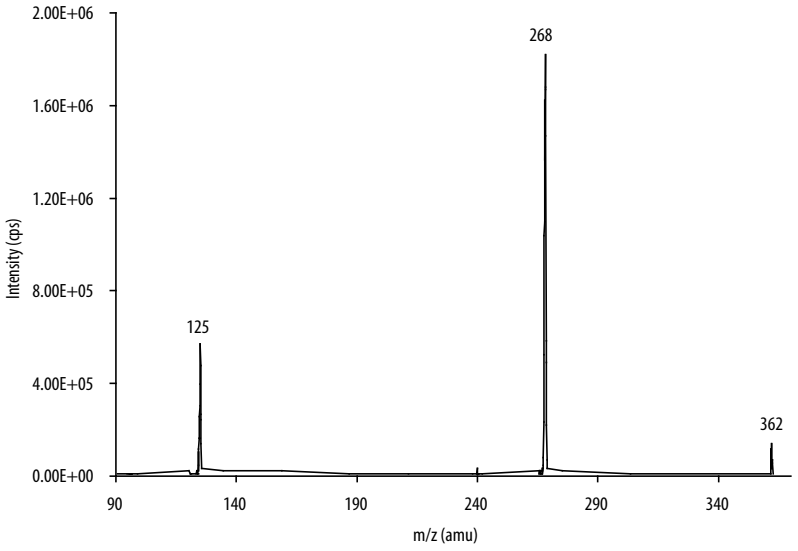



**Figure 4.** Product ion mass spectrum of the D-24851 fragment ion at m/z 362.

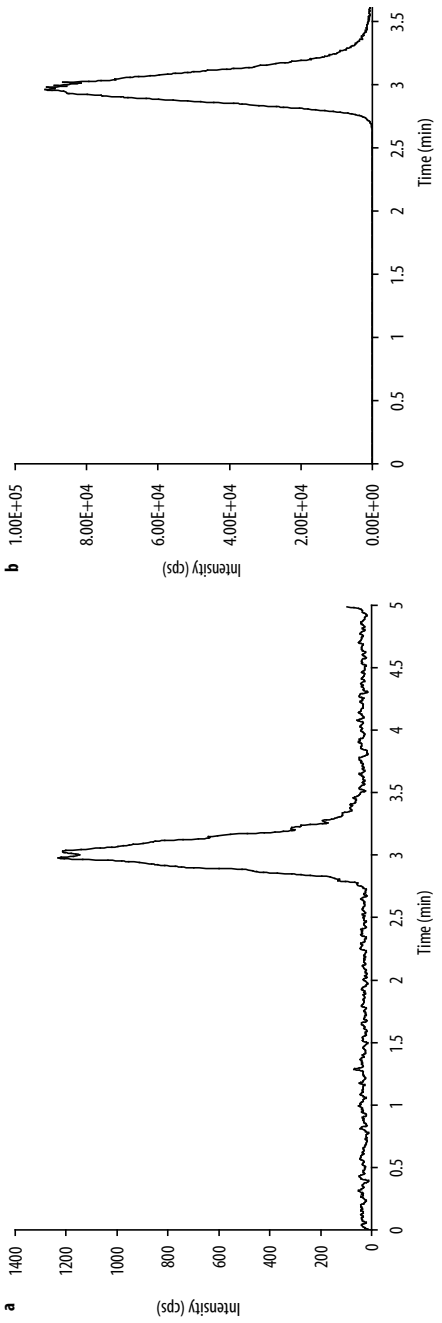



**Figure 5.** Typical MRM chromatograms for the LLOQ level from plasma for D-24851 (a; 1 ng/mL) and the internal standard (b; 100 ng/mL) and from urine for D-24851 (c; 0.25 ng/mL) and the internal standard (d; 10 ng/mL).

occurring in the pyridine or amide moiety since the rest of the molecule is observed intact at m/z 268. It is unclear how the fragment ion at m/z 362 is formed, but in our opinion a rearrangement resulting in the elimination of carbon monoxide is more likely than the elimination of ethylene from the pyridine moiety, since the glyoxyl amid is more reactive than the pyridine.
The product ion at m/z 268 was used for quantitation of D-24851, the ions at m/z 272 and 234 for D-24851-$D_4$ and D-24843, respectively, in combination with their parent masses as precursor ions.

**Chromatography**
The most appropriate combination of eluent and analytical column for quantitation of D-24851 was methanol – aqueous 5 mM ammonium acetate, 0.004% formic acid (80:20, v/v) pumped at a flow rate of 0.2 mL/min through an Inertsil ODS-3 analytical column. Ammonium acetate and formic acid were added to the eluent for better chromatography and to promote formation of protonated ions. Peak shapes were excellent under these conditions and the LC run time was only 5 minutes (Figure 5).

**Sample pretreatment**
Different methods of sample pretreatment were investigated. These experiments were performed at a concentration of 100 ng/mL D-24851 in human plasma. Recoveries were determined by comparing responses from plasma samples processed according to the mentioned procedures to results from D-24851 standard solutions in methanol representing 100% recovery. The recovery values that are reported represent the sum of the signal loss due to ion suppression and the extraction from the plasma matrix. The results are presented in Table 1. Protein precipitation of plasma samples containing D-24851 using acetonitrile or methanol was tested since it is fast and straightforward. Protein precipitation, however, resulted in very low recoveries and, in addition, broad peaks (0.8 min compared to 0.5 min for D-24851 standard solutions) were observed after protein precipitation using acetonitrile. Protein precipitation was discarded as a means of sample pretreatment due to the low recoveries. Subsequently solid phase extraction (SPE) on $C_2$ cartridges was tested. The SPE eluate was injected directly onto the analytical column. The resulting chromatograms showed a very high noise offset for D-24851 of approximately

**Table 1.** Sample pretreatment experiments

| Sample pretreatment | Conditions | Recovery | Remarks | No. of replicates |
|---|---|---|---|---|
| PP | Acetonitrile | 8.48 ± 0.971 | Broad peaks | 4 |
| PP | Methanol | 14.6 ± 3.23% | Narrow peaks | 4 |
| SPE | $C_2$ cartridges | 42.8 ± 11.8% | High noise offset | 3 |
| LLE | Hexane | 28.3 ± 1.83% | Clean extracts | 3 |
| LLE | Hexane-ether (1:1, v/v) | 82.6 ± 2.97% | Clean extracts | 3 |
| LLE | Hexane-isopropanol-ether (8:5:2, v/v) | 103 ± 22.6% | Turbid extracts | 3 |

PP Protein Precipitation; SPE Solid Phase Extraction; LLE Liquid-Liquid Extraction

1,000 cps and recoveries were low. Therefore, SPE was discarded for sample pretreatment as well. Finally, liquid-liquid extraction (LLE) was investigated as a means of sample pretreatment using hexane, hexane-ether (1:1, v/v), and hexane-isopropanol-ether (8:5:2, v/v/v) as extraction solvents. The latter produced the highest recoveries, however variability was high and turbid extracts were obtained, which may clog the LC column and in addition may cause ion suppression. Because acceptable and reproducible recoveries and clean extracts were obtained, sample pretreatment was performed using LLE with hexane-ether (1:1, v/v).

**D-24843 internal standard**

During the first stages of the development of the assay only D-24843, a structural analogue of D-24851 lacking the chlorine atom (Figure 1), was available as internal standard. Calibration standards in human plasma were prepared and after addition of D-24843 working solution the samples were processed according to the described procedures. Samples were analyzed in duplicate in one analytical run. Deviations from the nominal concentrations and relative standard deviation were calculated. In Table 2 the results of these experiments are presented. Deviations exceeding ± 20% were obtained on five different occasions. Relative standard deviations were extensive and in only one case lower than 15%. These results show that D-24843 is unsuitable as internal standard for the quantitative analysis of D-24851 with LC-MS/MS. The data were re-calculated without using the D-24843 internal standard (external standard calibration). Deviations of back-calculated concentrations from the nominal concentrations exceeded ±20% in two cases. Deviations were –24.6 and 26.2% at 10.1 ng/mL and 252 ng/mL, respectively. Relative standard deviation was 20.5% at 252 ng/mL but ≤15% for all other concentrations. These data show that external standard quantitation yields even better results than using D-24843 as internal standard.

There are examples of accurate and reproducible LC-MS/MS assays using an analogous internal standard for quantitation, e.g. [5]. Whether an analogous internal standard is appropriate depends on several factors including difference in ionization efficiency between the analogous internal standard and the analyte. Ionization efficiency as well

**Table 2.** Internal standard data

| Conc. (ng/mL) | D-24843 | | | External standard | | | D-24851-$D_4$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | Accuracy (%) | | Precision (%) | Accuracy (%) | | Precision (%) | Accuracy (%) | | Precision (%) |
| | 1 | 2 | | 1 | 2 | | 1 | 2 | |
| 1.00 | 12.9 | -22.9 | 25.3 | 11.9 | 4.95 | 4.90 | -2.92 | -0.604 | 1.64 |
| 5.00 | 13.9 | -22.9 | 26.0 | -24.6 | -3.37 | 15.0 | 4.23 | 10.5 | 4.42 |
| 25.0 | 23.8 | -13.5 | 26.4 | 2.38 | -4.37 | 4.77 | 6.05 | 5.65 | 0.285 |
| 101 | 14.9 | -7.72 | 16.0 | 5.94 | -1.88 | 5.53 | 5.74 | 9.77 | 2.85 |
| 252 | 27.8 | -14.3 | 29.7 | 26.2 | -2.78 | 20.5 | 0.403 | 0.403 | 0.00 |
| 1010 | -13.2 | -30.5 | 12.3 | -12.9 | -6.93 | 4.20 | -11.5 | -8.06 | 2.42 |

Conc. Concentration ; Dev. Deviation; C.V. Coefficient of Variation

as the amount of suppression caused by co-eluting matrix components depends on a compound´s structure. Furthermore, co-elution of analyte and internal standard is important to ensure the same amount of ion suppression for both of them. The lack of the chloride atom may cause substantial differences in ionization efficiency between D-24843 and D-24851. In addition, D-24843 elutes almost a minute before D-24851 from the analytical column and therefore does not experience the same amount of suppression. When a stable isotopically labeled (SIL) internal standard for D-24851 became available it was implemented into the assay. As demonstrated by the data in Table 2, the SIL internal standard of D-24851 improves the LC-MS/MS analysis of D-24851 dramatically.

**Development of the urine assay**

In human plasma the assay for the quantitative analysis of D-24851 was developed and validated in a range of 1 - 1,000 ng/mL with a signal to noise ratio of ~30 at the LLOQ level (Figure 5). The same method and procedures were applied to urine samples. However, due to the low solubility of lipophilic D-24851 in an aqueous and protein free matrix such as urine, linearity of the assay could only be assured up to a maximum of 50 ng/mL. As a result of the lipophilicity of D-24851 only low concentrations of the unchanged drug are expected in patient's urine, thus an ULOQ of 50 ng/mL in urine was considered to be sufficient. A test run of urine samples obtained from patients enrolled at the first dose level (20 mg) of the phase I study with oral D-24851 indicated that an LLOQ of 1 ng/mL is insufficient for the assay in urine and a lower LLOQ is desirable. Since the signal to noise ratio at the 1 ng/mL level is ~30, it was possible to lower the LLOQ to 0.25 ng/mL without changing the assay (Figure 5).

In addition, adsorption of the drug to container walls was observed, especially after cooling or freezing the samples. Nevertheless, the extraction solution (hexane-ether 1:1, v/v) redissolves all drug present in the sample. Therefore, the appropriate aliquots of urine sample for processing (250 µL) have to be prepared immediately after preparation of calibration standards and quality control samples or collection of the patient samples. As a result of this, dilution of samples with concentrations exceeding the ULOQ of 50 ng/mL is performed by adding a five times larger volume of internal standard solution and reconstitution of the dry extract in 500 µL of eluent instead of 100 µL. A linear dynamic range of 0.25-50 ng/mL was feasible for D-24851 in urine, and a full validation according to the FDA guidelines on bioanalytical method validation was performed [4].

**Validation**

*Linearity*

Calibration standards in control human plasma were analyzed in a dynamic range of 1 - 1,000 ng/mL and in control human urine of 0.25 - 50 ng/mL in duplicate in three analytical runs. The calibration concentrations were back-calculated from the responses. The deviations from the nominal concentration were between –9.05 and 8.56% for all concentrations in plasma and between –6.97 and 5.67% in urine, with precisions less than 8.00 and 9.78%, respectively.

**Table 3.** Assay performance data for D-24851 in plasma and urine

| Species | Nominal concentration (ng/mL) | Measured concentration (ng/mL) | Inter-assay accuracy (%) | Inter-assay precision (%) | Number of replicates |
|---|---|---|---|---|---|
| Plasma | 1.00 | 1.04 | 4.00 | 7.34 | 15 |
| | 3.00 | 3.26 | 8.67 | 2.72 | 15 |
| | 100 | 108 | 8.00 | 4.34 | 15 |
| | 800 | 743 | -7.12 | 1.27 | 15 |
| | 2500 | 2480 | -0.700 | 2.98 | 15 |
| Urine | 0.252 | 0.245 | -2.78 | 13.2 | 15 |
| | 0.600 | 0.546 | -9.00 | 6.27 | 15 |
| | 25.2 | 24.6 | -2.38 | 3.06 | 15 |
| | 40.0 | 42.9 | 7.25 | 5.34 | 15 |
| | 128 | 124 | -3.12 | 8.43 | 15 |

*Accuracy and precision*

Inter-assay accuracy and precision data for D-24851 in human plasma and urine are summarized in Table 3. All data met the requirements [4]. Intra-assay accuracies (data not shown) for human plasma were between –7.16 and 8.62 % for all concentrations with intra-assay precisions lower than 7.34%. Intra-assay accuracies for human urine (data not shown) were between –14.1 and 14.1% for all concentrations with precisions lower than 10.5%.

*Ion suppression and Recovery*

In plasma samples the mean ion-suppression for D-24851 was 16.1 ± 7.66%. The ion-suppression of the internal standard was 14.8 ± 8.63%. In urine they were 6.89 ± 5.49% and 5.80 ± 4.99%, respectively. Extraction recovery for D-24851 was 95.7 ± 9.66% from plasma and 85.9 ± 8.35% from urine. For the internal standard extraction recoveries were 86.5 ± 8.49% and 91.1 ± 7.47%, respectively.

*Specificity and selectivity*

MRM chromatograms of double blank, blank, and LLOQ samples prepared in six individual batches of human plasma or urine did not show peaks that co-eluted with either D-24851 or the internal standard nor interfered with the mass transition for either of the compounds. Deviations from the nominal concentrations at the LLOQ level were between –11.7 and –1.61% for human plasma and between–19.8 and –5.95 % for human urine.

*Stability*

D-24851 is stable in the stock solution under both processing conditions for at least 24 h and storage conditions for at least 11 months. Stability of the deuterated internal standard in the stock solution at –20 °C was established for at least 9 months. Stability of D-24851 in plasma or urine was not critical under any of the tested conditions. No significant changes in concentration were observed after three freeze/thaw cycles or after 24 h at ambient temperatures. Stability in human plasma during storage has been established for at least

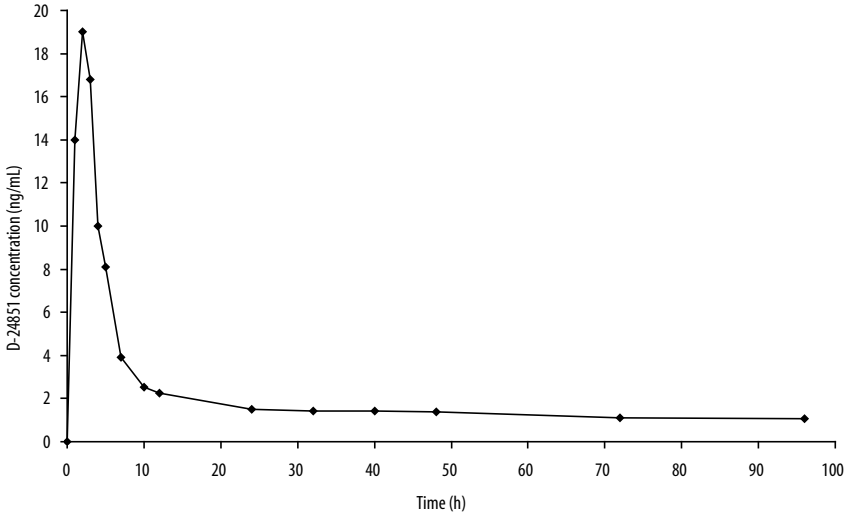



**Figure 6.** Plasma concentration *vs.* time curve for D-24851 obtained from a patient receiving the starting dose of 20 mg D-24851 orally in a phase I study with the drug.

9 months and further testing is still ongoing. Long-term stability of D-24851 in human urine at –20 °C is ongoing. D-24851 is stable in the dry extract from plasma for at least 3 days at 2-8 °C and in the final extract for at least 3 days at ambient temperatures. In human urine stability in the dry extract was guaranteed for at least 7 days at 2-8 °C and in the final extract for at least 7 days at ambient temperatures.

**Application of the LC-MS/MS assay in a clinical phase I trial**

From a patient receiving a single dose of 20 mg of D-24851 orally, plasma samples were collected prior to administration and up to 96 hours after drug intake. Urine samples were collected during the first day of the first course. In Figure 6 a concentration vs. time profile for D-24851 in plasma is presented. Up to 96 hours after administration the concentrations for D-24851 were still higher than the LLOQ of 1 ng/mL. At this starting dose level, the D-24851 concentrations in urine were lower than the LLOQ level at 0.25 ng/mL.

## Conclusion

An LC-MS/MS assay for the quantitative determination of D-24851 in human plasma and urine was developed and subsequently validated according to FDA guidelines. The assay has been successfully applied in a clinical phase I trial with oral D-24851.

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# 4.2

## An easy and sensitive assay for the quantitative analysis of paclitaxel in human and mouse plasma and brain tumor tissue using coupled liquid chromatography and tandem mass spectrometry

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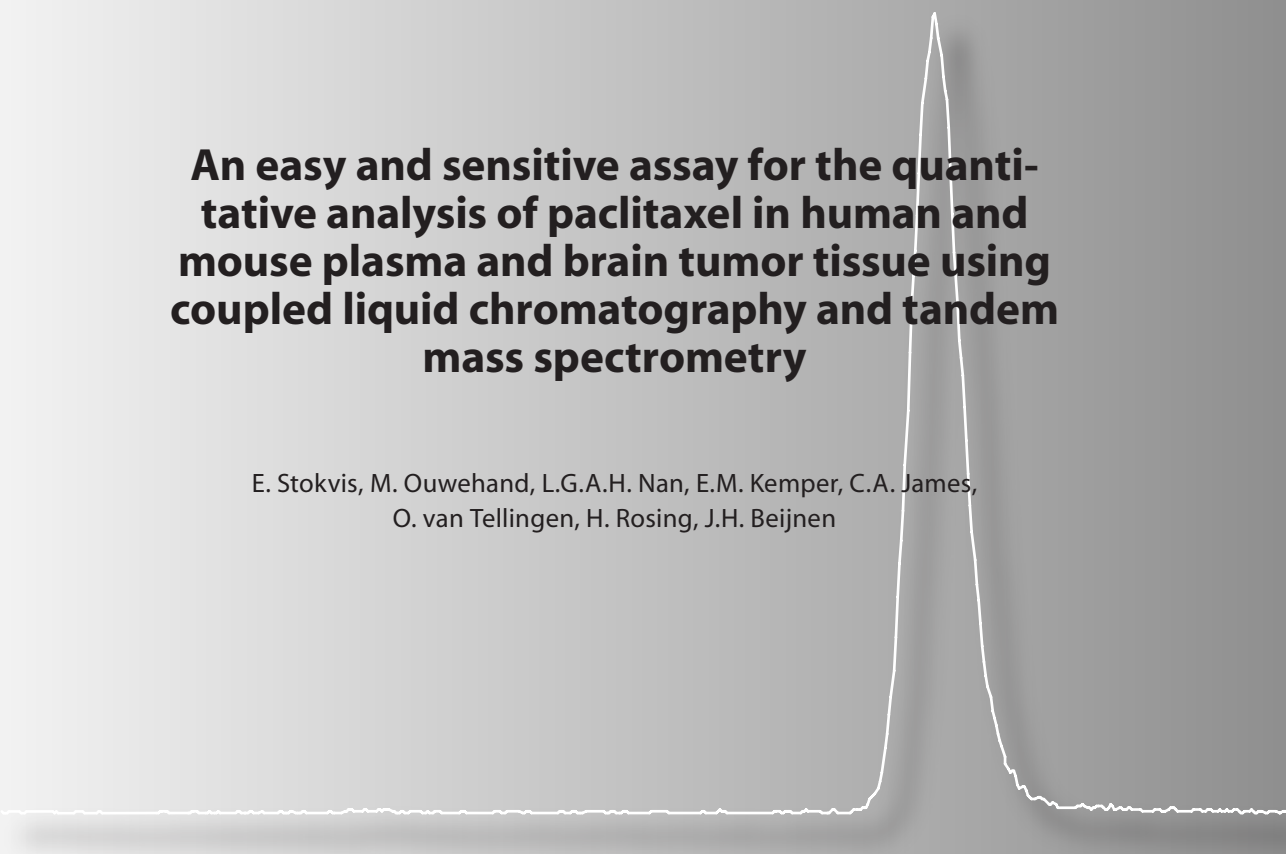

Submitted

## Abstract

The development and validation of an assay for the determination of paclitaxel in human plasma, human brain tumor tissue, mouse plasma, and mouse brain tumor tissue is described. Paclitaxel was extracted from the matrices using liquid-liquid extraction with *tert*-butylmethylether, followed by chromatographic analysis using an alkaline eluent. Positive ionization electrospray tandem mass spectrometry was performed for selective and sensitive detection. The method was validated according to the FDA guidelines on bioanalytical method validation. Validation results indicate that calibration standards in human plasma can be used to quantify paclitaxel in all tested matrices. In human samples, the validated range for paclitaxel was from 0.25 to 1,000 ng/mL using 200 µL plasma aliquots and from 5 to 5,000 ng/g using 50 µL tumor homogenate aliquots (0.2 g tissue/mL control human plasma). In mice, the ranges were from 1 to 1,000 ng/mL and from 5 to 5,000 ng/g using 50 µL plasma aliquots and 50 µL tumor homogenate aliquots (0.2 g tissue/mL control human plasma), respectively. The method can be applied to studies generating only small sample volumes (e.g. mouse plasma, tumor tissue), but also to studies in human plasma requiring a low lower limit of quantitation. The assay was applied successfully to several studies with both human and mouse samples.

## Introduction

Paclitaxel (Taxol®; Figure 1) is an antitumor agent derived from the bark of the Pacific yew tree *Taxus brevifola*. The drug inhibits mitosis through stabilization of the polymerization and de-polymerization of microtubules. Paclitaxel is mainly used in therapy for breast, lung, and ovarian cancer but is also under investigation for other therapeutic indications. Monitoring of paclitaxel concentrations in human plasma is important since studies have indicated that the toxicity and efficacy of paclitaxel are correlated to drug exposure [1,2].

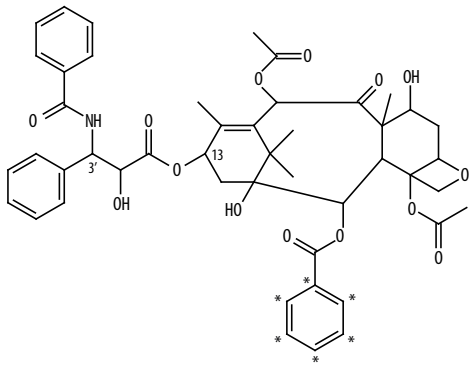


**Figure 1.** Structure of paclitaxel. The asterisks represent the $^{13}C$ atoms in the $^{13}C_6$-paclitaxel internal standard.

With the introduction of routine liquid chromatography-mass spectrometry (LC-MS) hyphenation, MS detection has become the method of choice for the determination of paclitaxel, due to its sensitivity and selectivity. Several assays have been described using LC-MS for the quantitative bioanalysis of paclitaxel [3-9]. Most assays employ solid phase extraction (SPE) as a means of sample pretreatment [3-6]. Since SPE is generally a time consuming procedure, other means of sample pretreatment were investigated. Liquid-liquid extraction (LLE) procedures with diethylether or *tert*-butylmethylether have been described for paclitaxel [7-9]. Alexander et al. describe a simple LLE procedure with *tert*-butylmethylether for the extraction of paclitaxel and two metabolites from 400 μL human and dog plasma volumes. Lower limit of quantitation (LLOQ) values of 0.1 ng/mL were obtained, which are the lowest reported for paclitaxel so far [7]. Guo et al. describe an LC-MS assay for the quantitation of paclitaxel in mouse plasma and brain tissue using SPE for sample processing. LLOQ values of 36 ng/mL and 486 ng/g were obtained, respectively [3]. Since the latter LLOQ values were not sufficiently low for our studies, we have attempted to obtain lower values.
Our goal was to develop and to validate a sensitive assay for the quantitation of paclitaxel in human and mouse plasma and brain tumor tissue to support clinical and pre-clinical trials. Only minute amounts of mouse tumor tissue and plasma as well as human tumor tissue are available which challenges us to achieve a low LLOQ for paclitaxel in these matrices. For human plasma larger volumes are available, and hence it was possible to achieve a lower LLOQ. Due to the scarcity of control drug free mouse plasma, mouse tumor tissue, and human tumor tissue we have investigated the potential of using calibration standards prepared in human plasma for the quantitation of paclitaxel in these matrices.

## Experimental

### Materials

Paclitaxel (lot TECH6 00600-A; Figure 1) was obtained from Hauser Inc. (El Segundo, CA, USA) and the $^{13}C_6$-paclitaxel internal standard (lot 15075/03; Figure 1) was kindly supplied by Pharmacia Corporation (Nerviano, Italy). Methanol (LC gradient grade) was purchased from Biosolve ltd. (Amsterdam, The Netherlands). Ammonia solution 25% and *tert*-butylmethylether (both analytical grade) were obtained from Merck (Darmstadt, Germany). Ethanol (technical grade) was purchased from Klinipath (Duiven, The Netherlands). Distilled water was used throughout the analyses. Drug free human plasma was obtained from the Central Laboratory for Blood Transfusion (Sanquin Amsterdam, The Netherlands). Drug free mouse plasma, mouse tumor tissue and human tumor tissue originated from the Department of Clinical Chemistry, The Netherlands Cancer Institute (Amsterdam, The Netherlands).

### Instrumentation

Chromatographic separations were carried out using an HP1100 liquid chromatograph

(Agilent technologies, Palo Alto, CA, USA) consisting of a binary pump, autosampler, degasser, and column oven. A mobile phase of methanol - aqueous 10 mM ammonia (70: 30, v/v) was pumped at a flow rate of 0.2 mL/min through a Zorbax Extend C18 column (150 x 2.1 mm ID, particle size 5 μm; Agilent technologies) protected with an in-line filter (Micro filter frit, 5 μm, Upchurch Scientific Inc., Oak Harbor, WA, USA). Sample injections of 25 μL were carried out and the autosampler was thermostatted at 10 °C. The LC eluate was let directly into an API 3000 triple quadrupole MS equipped with an electrospray (ESI) ion source (Sciex, Thornhill, ON, Canada). The quadrupoles were operated with unit resolution in the positive ion mode. The resulting multiple reaction monitoring (MRM) chromatograms were used for quantification using Analyst™ software version 1.2 (Sciex). Run time was 9 min. Mass transitions of m/z 854 → 509 and m/z 860 → 515 were optimized for paclitaxel and $^{13}C_6$-paclitaxel, respectively, with dwell times of 150 ms. Nebulizer and turbo gas (both compressed air) were operated at 1.6 and 7 L/min, respectively, while the curtain gas and collision gas (both $N_2$) were operated at 1.2 L/min and 1.8*$10^{15}$ molecules/$cm^2$, respectively. The ionspray voltage was kept at 5500 V, with a source temperature of 300 °C.

**Preparation of stock and working solutions**

Two stock solutions of paclitaxel with separate weighing were prepared in methanol at a concentration of 1 mg/mL. These solutions were further diluted with methanol to obtain working solutions. One set of working solutions was used to prepare calibration standards, the other to prepare quality control samples. A stock solution of $^{13}C_6$-paclitaxel was prepared in methanol at a concentration of 1 mg/mL and was subsequently diluted with methanol to a final concentration of 1 μg/mL. All solutions were stored at –20 °C.

**Preparation of calibration standards and quality control samples for paclitaxel in human plasma**

Calibration standards were prepared in a range from 0.25 to 1,000 ng/mL by diluting a fixed amount of working solution in control human plasma in volumetric flasks. Quality control samples were prepared in a similar way from the other set of working solutions in human plasma at concentrations of 0.75, 500, and 800 ng/mL. Furthermore, additional quality control samples at the LLOQ level (0.25 ng/mL) and with a concentration higher than the upper limit of quantitation (ULOQ; 2,500 ng/mL) were prepared. Sample aliquots of 200 μL were transferred to 2.0 mL polypropylene tubes (Eppendorf Netheler Hinz GmbH, Hamburg, Germany) and stored at –20 °C.

**Preparation of quality control samples for paclitaxel in human tumor tissue, mouse plasma and mouse tumor tissue**

Quality control samples were prepared in human tumor tissue, mouse plasma, and mouse tumor tissue. For calibration the standards in human plasma were used. In mouse plasma quality control samples were prepared at levels of 3, 500, and 800 ng/mL. Furthermore, quality control samples at the LLOQ level (1 ng/mL) were prepared. Blank human and mouse tumor tissue homogenates were prepared by homogenizing 0.2 g of tumor tissue with 1 mL of human plasma. Quality control samples were prepared at levels of 15, 2,500,

and 4,000 ng/g in human and mouse tumor tissue. Furthermore, quality control samples at the LLOQ level (5 ng/g) were prepared in human tumor homogenate. All quality control samples were diluted four times prior to processing by mixing sample aliquots of 50 µL with 150 µL of control human plasma in 2.0 mL tubes (Eppendorf). Samples were stored at –20 °C.

**Sample processing**

To 200 µL sample volumes 25 µL of internal standard working solution in methanol (1,000 ng/mL) and subsequently 1,000 µL of *tert*-butylmethylether was added. The samples were shaken automatically for 10 min at 1,250 rpm (Labinco, Breda, The Netherlands) and subsequently centrifuged for 5 min at 23,100 g. The aqueous layer was frozen in an ethanol – dry ice mixture and the organic layer was decanted into a clean tube. The organic solvent was evaporated under a gentle stream of nitrogen gas at 40 °C. The residue was reconstituted with 100 µL of methanol – water (70:30, v/v) by vortex mixing for 30 s. After centrifuging for 5 min at 23,100 g the supernatant was transferred to a glass autosampler vial with insert and 25 µL was injected onto the analytical column.

**Validation procedures**

For the paclitaxel assay in human plasma a full validation program according to the FDA guidelines was executed [10]. For the paclitaxel quantitation in human tumor homogenate, mouse plasma and mouse tumor homo-genate, partial validations were performed. This includes accuracy and precision, specificity and selectivity, and stability in the dry and final extract, and long-term stability during storage at –20 °C, following FDA requirements [10]. In mouse tumor homogenate only accuracy and precision were assessed due to the limited amount of sample available for validation.

*Linearity*

Nine non-zero calibration standards (0.25, 0.5, 1, 5, 10, 100, 250, 500, and 1,000 ng/mL) were prepared in human plasma and analyzed in duplicate in three separate analytical runs. Calibration curves were calculated by least-squares linear regression using a weighting factor of $1/x^2$ (the reciprocal of the squared concentration). Concentrations were back-calculated from the constructed calibration curve and deviations from the nominal concentrations should be within ±20% for the LLOQ and within ±15% for other concentrations with coefficient of variation (C.V.) values less than 20 and 15%, respectively [10].

*Accuracy and precision*

Five replicates of each human plasma sample were analyzed together with a calibration curve, independently prepared from the quality control samples, in three analytical runs. Quality control samples with concentrations higher than the ULOQ were diluted four times in human plasma prior to sample processing, in order to validate dilution of samples that were originally above the ULOQ. For partial validations five replicates of each sample were analyzed together with a calibration curve, independently prepared from the quality control samples in human plasma in one analytical run. The accuracy was determined

in percent difference between the mean concentration and the nominal concentration. The coefficient of variation (C.V.) was used to report the precisions. The intra- and inter-assay accuracies should be within ±20% for the LLOQ concentration and within ±15% for other concentrations. The precisions should be less than 20% and less than 15%, respectively [10].

*Specificity and selectivity*

From six individual batches of control drug-free human plasma samples containing neither analyte nor internal standard (double blank), samples containing only internal standard (blank), and LLOQ samples were prepared. In addition, from six individual batches of control drug-free mouse plasma, one batch of control drug-free mouse tumor homogenate, and one batch of control drug-free human tumor homogenate double blanks were prepared. The samples were prepared to determine whether endogenous compounds interfere at the mass transitions chosen for paclitaxel or the internal standard. Samples were processed according to the described procedures and analyzed. Peak areas of compounds co-eluting with the analyte or internal standard should not exceed 20% of the analyte peak area at the LLOQ or 5% of the internal standard area. Deviations from the nominal concentrations should be within ±20% for the LLOQ samples [10].

*Ion suppression and recovery*

Control drug-free human plasma was processed and dry extracts were dissolved with working solutions that represent 100% recovery containing the analyte and internal standard in methanol – water (70:30, v/v). Ion-suppression was determined by comparing the analytical response of these samples to that of the working solutions. The loss of signal represents the ion suppression. Liquid-liquid extraction recovery was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with working solutions as described above. These experiments were performed at three concentration levels, in triplicate. Overall recovery corresponds to the net response after subtraction of the ion-suppression and the signal loss due to the extraction. Ion suppression and recovery experiments for the internal standard were performed in a similar way.

*Stability*

The stability of paclitaxel was evaluated in human plasma after three freeze/thaw cycles by comparing quality control samples that have been frozen (-20 °C) and thawed three times with freshly prepared quality control samples. Furthermore, stability in human plasma under processing conditions (ambient temperatures) was evaluated. Long-term stability studies of paclitaxel under storage conditions (-20 °C) in human tumor homogenate and mouse plasma are ongoing. Furthermore, stability in the dry extract at 2-8 °C and in the final extract at ambient temperatures was determined for all matrices except mouse tumor homogenate. Finally, re-injection reproducibility was tested after 24 h in the autosampler. The analytes are considered stable in the biological matrix or extracts thereof when 80-120% of the initial concentration was found.

**Implementation of the paclitaxel assay**

The analytical method described in this article has been used to support a study in mice and humans investigating the exposure of brain tumor tissue to paclitaxel compared to the systematic exposure to paclitaxel. Mice received 10 mg/kg of paclitaxel intravenously by means of a bolus injection while humans received 70 mg/m$^2$ intravenously in 3 hours. Blood samples were collected at several time points and, after centrifuging, plasma was

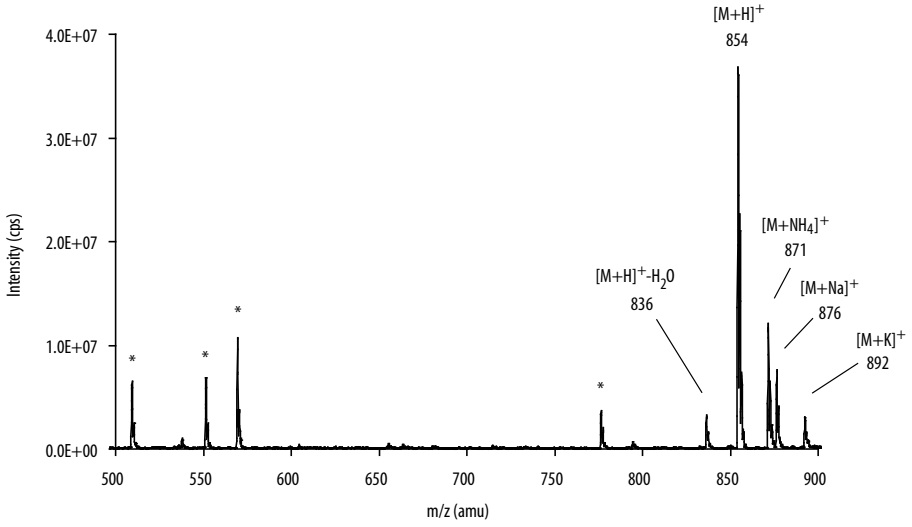


**Figure 2.** Q1 mass spectrum of paclitaxel. Peaks labeled with an asterisk correspond to fragment ions.

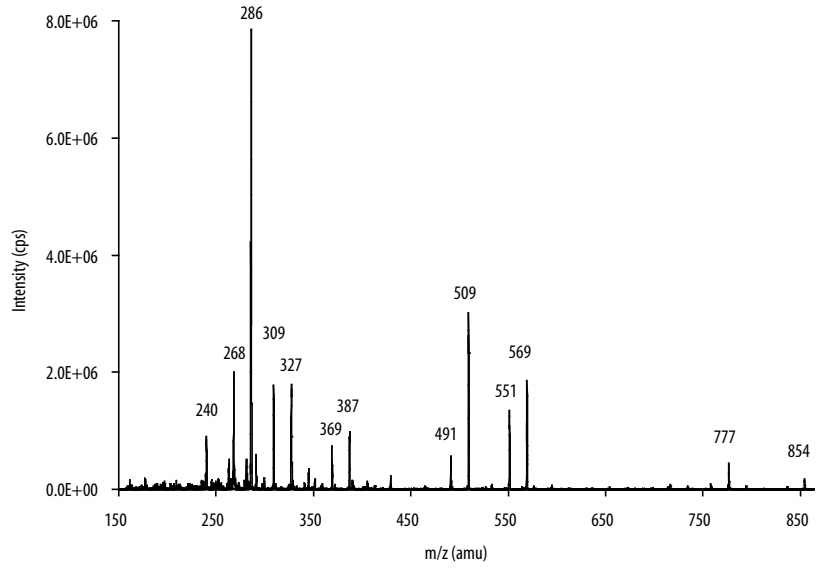


**Figure 3.** Product ion MS/MS spectrum of paclitaxel from the protonated molecular ion at m/z 854.

removed and stored at –20 °C until analysis. Approximately 24 hours after the end of the paclitaxel infusion tumor tissue was surgically removed. Tumor tissue was homogenized in human plasma in a concentration of 0.2 g/mL.

## Results

### Mass spectrometry

In Figure 2 a Q1 mass spectrum of paclitaxel is presented. Apart from the protonated species, also ammonium, sodium, and potassium adducts are visible. In addition, a peak corresponding to the elimination of water from the molecular ion is observed. The peaks in the spectrum labeled with an asterisk, represent fragment ions formed in the ion source, probably as a result of the high ionspray voltage. The protonated molecular ion of paclitaxel was induced to fragment in the collision cell and the resulting product ion spectrum is presented in Figure 3. A proposed fragmentation pattern for paclitaxel is presented in Figure 4. The main fragment ion corresponds to the cleavage of the side chain at the C-13 position, predominantly leaving the charge on the side chain (m/z 286). Other fragment ions for paclitaxel result mainly from the elimination of water, acetic acid or benzoic acid

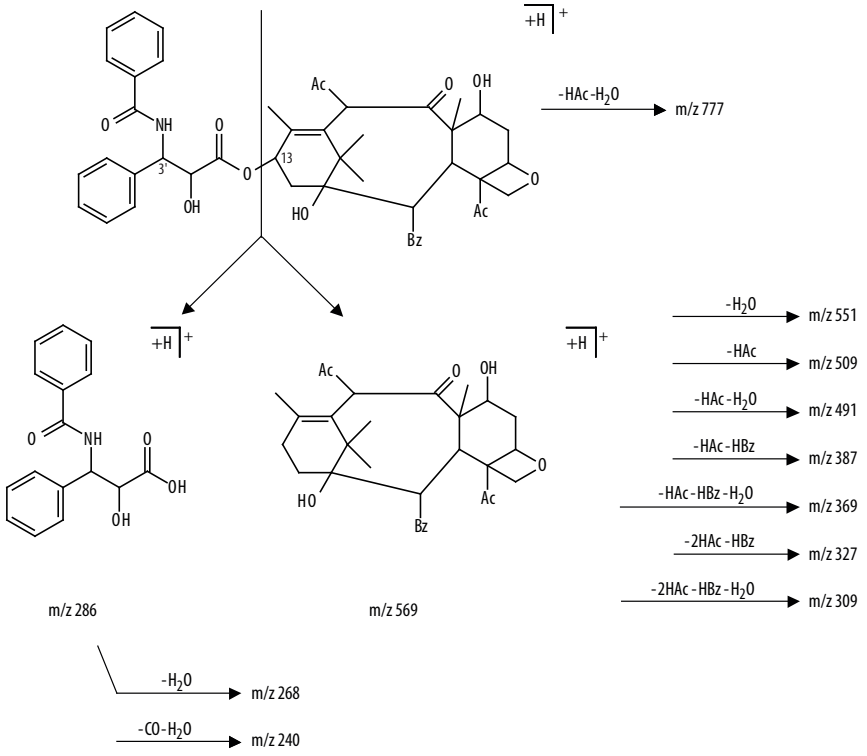


**Figure 4.** Proposed fragmentation scheme for paclitaxel. Ac Acetate; Bz Benzoate.

from the product ion at m/z 569. Interestingly, neither benzaldehyde nor benzene are eliminated from the C-3' benzamide moiety in the C-13 side chain, although $\alpha$-cleavage of the carbonyl is a common fragmentation reaction for amides [11]. This may result from a $\pi$-system stabilizing this part of the molecule. After optimization of the MS parameters the fragment ion at m/z 509 was most abundant and used for quantitative MRM of paclitaxel.

**Chromatography**

Several assays have been described for the determination of paclitaxel in biological fluids using LC-MS techniques [3-9]. In all assays the analytes are chromatographically separated from matrix components using acidic mobile phases containing mostly acetic or formic

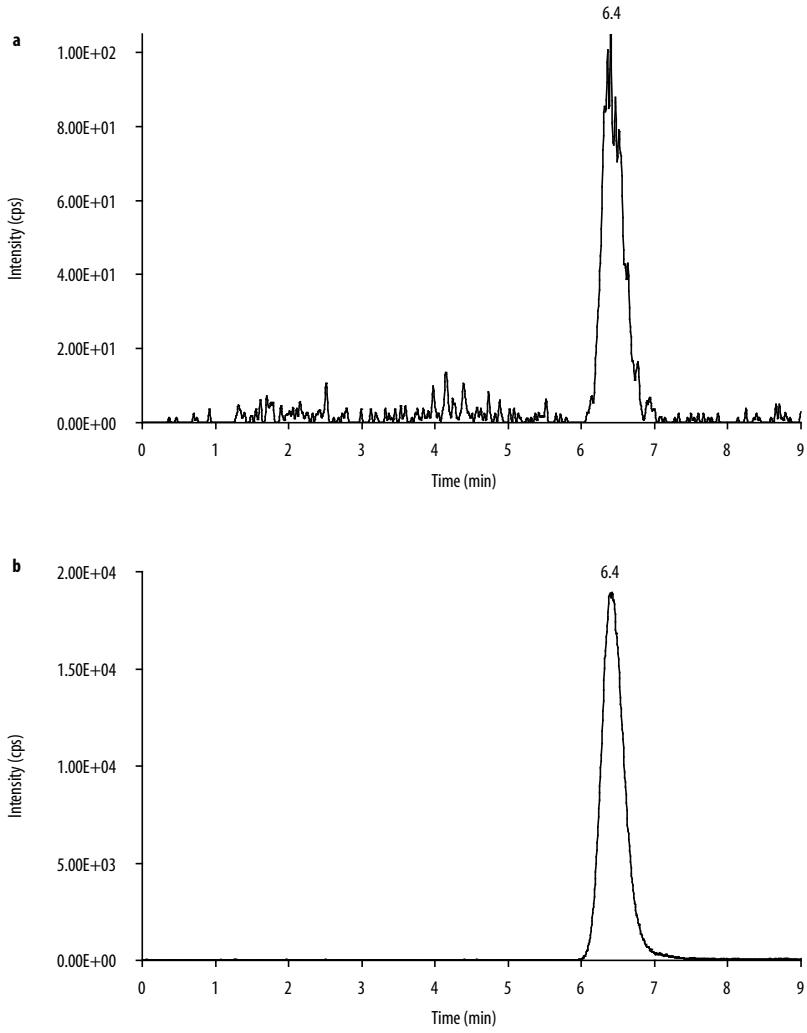


**Figure 5.** Representative chromatograms for paclitaxel (a) and $^{13}C_6$-paclitaxel (b) at the LLOQ level (0.25 ng/mL) in human plasma.

acid in a mixture of water and either methanol or acetonitrile. In our earlier work we have described that the use of mobile phases containing ammonium hydroxide in combination with positive ionization may be very well suited for the bioanalysis of basic drugs [12]. Several mobile phase additives were tested in flow injection analysis experiments to determine their influence on the MS response of paclitaxel. It appeared that the addition of ammonium acetate, ammonium formate, or ammonium hydroxide to the aqueous phase of the eluent resulted in higher signal to noise ratios for the analyte than the addition of acetic acid, formic acid, or no addition to the eluent. During LC-MS/MS experiments the best results for paclitaxel in terms of signal to noise ratio and peak width were then obtained using an alkaline mobile phase containing 10 mM ammonium hydroxide. Positive ionisation in the electrospray ion source in the presence of ammonium hydroxide most likely results from ion-molecule reactions between the analyte molecule and ammonium ions or collision-induced dissociation of ammonium adducts of the analyte under influence of the electrospray voltage [13]. Representative chromatograms of paclitaxel and the internal standard at the LLOQ level of 0.25 ng/mL from human plasma are shown in Figure 5.

**Sample pretreatment**

LLE was investigated for the processing of samples, since it is a simpler and often faster procedure than the more commonly used SPE for the extraction of paclitaxel from biological matrices. LLE with *tert*-butylmethylether was suitable to obtain good recoveries for paclitaxel (72.2 ± 4.69%). The procedure described here was adapted from Alexander et al. [7], however, the time-consuming procedure of freezing the aqueous layer in a –60 °C freezer was replaced by instant freezing in an ethanol-dry ice bath.

**Validation procedures**

*Linearity*

The calibration concentrations were back-calculated from the responses. For all concentrations of paclitaxel, the deviations from the nominal concentration were between –2.28 and 3.84% with C.V. values less than 8.42%.

*Accuracy and precision*

Assay performance data for paclitaxel is summarized in Table I. For the assay of paclitaxel in human plasma only inter-assay accuracies and precisions are shown. Intra-assay accuracy and precision from human plasma for paclitaxel were within ±12.0% and less than 12.1%, respectively, for all concentrations (data not shown). All data met the requirements [9]. The data demonstrate that calibration standards prepared in human plasma can be used for the quantitation of paclitaxel in human tumor tissue, mouse plasma, and mouse tumor tissue.

*Specificity and selectivity*

MRM chromatograms of double blank and blank samples prepared in six individual batches of human plasma did not show peaks that co-eluted with paclitaxel with areas

**Table 1.** Assay performance data for paclitaxel

| Matrix | Nominal concentration (ng/mL)[1] | Calculated concentration (ng/mL) | Accuracy (%) | Precision (%) | Number of replicates |
|---|---|---|---|---|---|
| Human plasma[2] | 0.249 | 0.231 | -7.36 | 10.9 | 15 |
| | 0.747 | 0.684 | -8.38 | 6.25 | 15 |
| | 498 | 454 | -8.78 | 2.90 | 15 |
| | 796 | 731 | -8.20 | 3.95 | 15 |
| | 2490 | 2338 | -6.10 | 3.58 | 15 |
| Human tumor[3] | 4.98 | 5.90 | 18.5 | 14.5 | 5 |
| | 14.9 | 14.7 | -1.34 | 2.73 | 5 |
| | 2,490 | 2,270 | -8.84 | 1.46 | 5 |
| | 3,980 | 3,515 | -11.7 | 2.27 | 5 |
| Mouse plasma[3] | 0.996 | 1.13 | 13.9 | 8.77 | 5 |
| | 2.99 | 3.17 | 5.89 | 11.3 | 5 |
| | 498 | 495 | -0.602 | 2.00 | 5 |
| | 796 | 778 | -2.31 | 0.965 | 5 |
| Mouse tumor[3] | 14.9 | 13.5 | -9.40 | 4.12 | 5 |
| | 2,490 | 2,385 | -4.22 | 5.21 | 5 |
| | 3,980 | 3,590 | -9.80 | 6.27 | 5 |

[1]Concentrations in tumor tissue are reported in ng/g; [2]Inter-assay precisions are reported; [3]Calibration standards in human plasma were used.

exceeding 20% of the area at LLOQ level nor showed peaks that co-eluted with the internal standard with areas that exceeded 5% of the internal standard area. Deviations from the nominal concentrations at the LLOQ level in human plasma were between –18.9 and –13.1% for paclitaxel. MRM chromatograms of double blank samples prepared in six individual batches of mouse plasma did not show peaks that co-eluted with either paclitaxel or the internal standard nor interfered with the mass transition for either of the compounds. For human and mouse tumor tissue homogenate the batches that were used for the preparation of quality control samples were tested for interferences. No peaks co-eluting with paclitaxel or interfering with the mass transitions of paclitaxel or the internal standard were observed.

*Ion suppression and recovery*

In human plasma samples the mean ion-suppression for paclitaxel and its internal standard were 16.1 ± 5.01% and 22.2 ± 7.42%, respectively. Extraction recovery from human plasma was 72.2 ± 4.69% for paclitaxel and 42.5 ± 9.86% for the internal standard.

*Stability*

Paclitaxel is stable in human plasma for at least three freeze (-20 °C)/thaw cycles. Paclitaxel is stable for at least 7 days in the dry extract at 2-8 °C and in the final extract at ambient temperatures. Furthermore, paclitaxel was stable for at least 7 days in the dry extract at 2-8 °C and in the final extract at ambient temperatures from human tumor homogenate and mouse plasma. Finally, re-injection reproducibility was established after 24 hours.

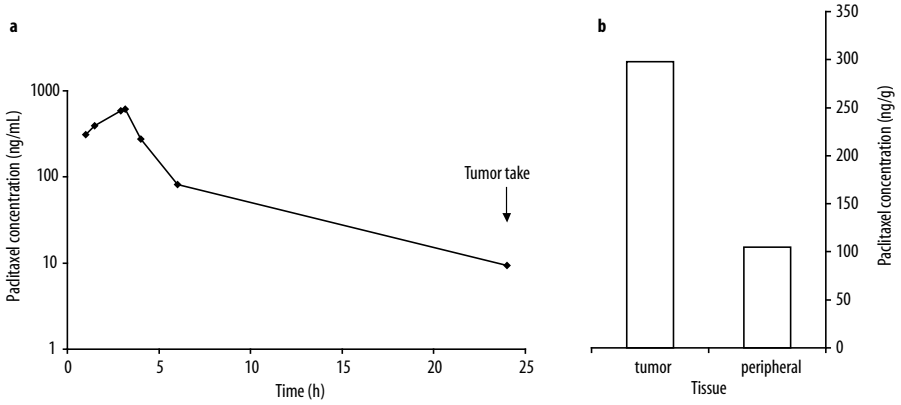


**Figure 6.** Concentration versus time curve of a patient receiving 70 mg/m$^2$ paclitaxel intravenously in 3 hours (a). Approximately 24 h after start of infusion, tumor tissue was surgically removed. The paclitaxel concentrations obtained in brain tumor tissue and peripheral normal brain tissue are presented in the bar diagram (b).

### Implementation of the paclitaxel assay

The correlation between plasma concentrations and tumor exposure was investigated after intravenous administration of paclitaxel in humans and in mice. The results from the first patient enrolled in this study are presented in Figure 6. The tissue samples were divided in multiple portions and histological analysis indicated the presence of tumor tissue and normal brain tissue with infiltrating tumor. Paclitaxel concentrations were higher in tumor tissue than in the infiltrated normal tissue. Furthermore, concentrations in tumor were higher than in plasma at the time of tumor take (24 h after start of the infusion). In mice, the same phenomenon was observed.

## Conclusions

The development and validation of an assay for the determination of paclitaxel in human plasma, human tumor tissue, mouse plasma, and mouse tumor tissue is described. The validated range for paclitaxel was from 0.25 to 1,000 ng/mL using 200 μL human plasma sample aliquots and from 1 to 1,000 ng/mL using 50 μL mouse plasma. In human and mouse tumor tissue the validated range was from 5 to 5,000 ng/g using 50 μL tissue homogenates (0.2 g tissue/mL human plasma). The obtained LLOQs for human plasma were comparable to the LLOQ obtained in an earlier study [7], but the presented assay is almost hundred-fold more sensitive than described earlier for mouse brain tissue and thirty-six times more sensitive than described for mouse plasma [3].

Validation results demonstrate that paclitaxel concentrations can be accurately and precisely quantified in human plasma, human brain tumor tissue, mouse plasma, and mouse brain tumor tissue when using calibration standards in human plasma. The assay was applied successfully to studies in both humans and mice.

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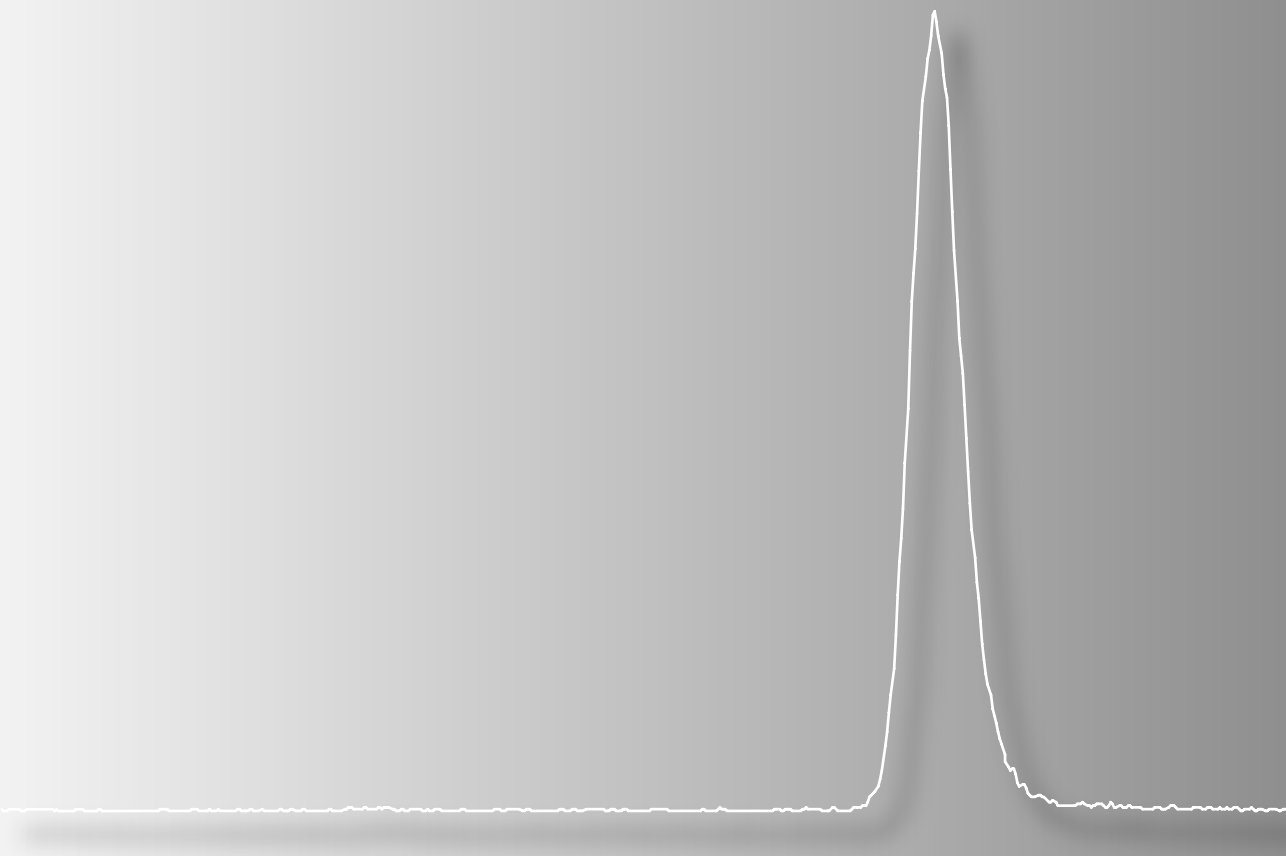
# Chapter 5

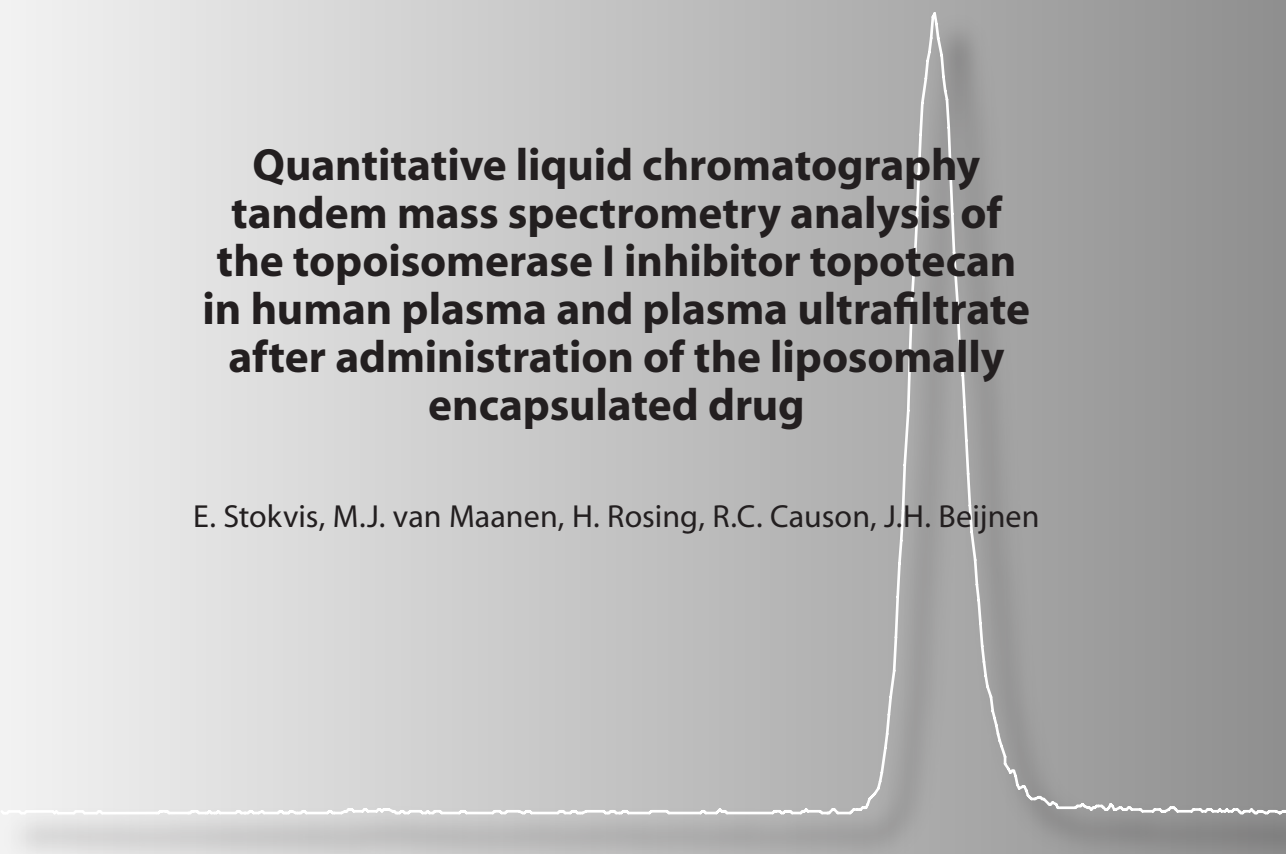


## Topoisomerase I inhibitors

# 5.1

## Quantitative liquid chromatography tandem mass spectrometry analysis of the topoisomerase I inhibitor topotecan in human plasma and plasma ultrafiltrate after administration of the liposomally encapsulated drug

E. Stokvis, M.J. van Maanen, H. Rosing, R.C. Causon, J.H. Beijnen



Submitted

## Abstract

This is the first report to describe a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the quantitative bioanalysis of the topoisomerase I inhibitor topotecan. Topotecan was determined as the total of its lactone and hydroxy carboxylate forms in human plasma and plasma ultrafiltrate after administration of the liposomally encapsulated drug. Sample aliquots were deproteinized with methanol after addition of the $[^2H_6]$-topotecan internal standard. Liquid chromatography was performed using an eluent containing methanol-water and trifluoroacetic acid (TFA). In order to achieve the target lower limit of quantitation (LLOQ) of 0.1 ng/mL in plasma ultrafiltrate, a TFA fix was required to increase the MS signal after suppression caused by TFA. Without the TFA fix the target LLOQ of 1 ng/mL in plasma was obtained. Both assays were validated according to the FDA guidelines on bioanalytical method validation. The validated ranges were 1-500 ng/mL and 0.1-50 ng/mL in plasma and plasma ultrafiltrate, respectively, from 500 µL sample volumes. Validation results demonstrate that the assay is accurate, precise and selective. This method is applied to pharmacokinetic studies of topotecan in human plasma and plasma ultrafiltrate after administration of liposomal topotecan.

## Introduction

Topotecan is a semi-synthetic analogue of the plant alkaloid camptothecin. It inhibits the cellular enzyme topoisomerase I, which is involved in the replication and regulation of single stranded DNA during the cell cycle. Inhibition of topoisomerase I damages DNA and causes cell death. Topotecan is applied to the treatment of metastatic ovarian carcinoma and is under investigation for other indications. The drug undergoes reversible hydrolysis of its lactone moiety to a hydroxy carboxylate form, which is believed to be devoid of topo-isomerase I inhibitory activity (Figure 1). At physiological pH an equilibrium between the two forms exists. Studies have indicated that total (lactone and hydroxy carboxylate) topotecan levels are related with pharmacodynamic outcomes [1].

For the bioanalysis of topotecan traditionally sensitive liquid chromatography (LC)-fluorescence techniques were used [2-8]. In order to improve the selectivity of the assay, we have developed an LC-tandem mass spectrometry (MS/MS) method for the quantitative analysis of total topotecan levels in human plasma and plasma ultrafiltrate. This assay will be used to support a study investigating the pharmacokinetics of topotecan after administration of the liposomally encapsulated drug. Topotecan is determined as the free, non-liposomal encapsulated fraction in plasma ultrafiltrate and the total of its liposomal and free fraction in human plasma.

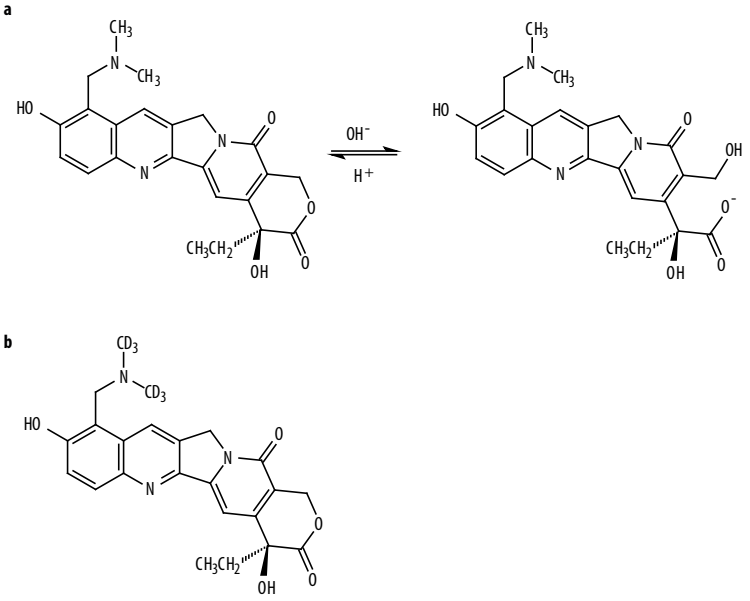



**Figure 1.** Structure of topotecan and schematic representation of the reversible hydrolysis of the lactone ring into the open-ringed hydroxy carboxylate form (a) and molecular structure of $D_6$-topotecan (b).

## Experimental

### Materials

Topotecan (S-9-dimethylaminomethyl-10-hydroxy-camptothecin; lot WRS2; hydrochloride salt; Figure 1a) and the $^2H_6$ ($D_6$)-topotecan internal standard (lot R937/1/1; hydrochloride salt; Figure 1b) were obtained from GlaxoSmithKline (Ware, Hertfordshire, UK). A solution of liposomal topotecan reference (4.04 mg/mL topotecan, lot U02082) originated from Inex Pharmaceuticals (Vancouver, BC, Canada). Methanol (LC gradient grade) was purchased from Biosolve ltd. (Amsterdam, The Netherlands). Hydrochloric acid 37% (analytical grade), trifluoroacetic acid (TFA; uvasol), 2-propanol (for LC), and propionic acid (for synthesis) were from Merck (Darmstadt, Germany). Distilled water was used throughout the analyses. Drug free human plasma was obtained from Sanquin (Amsterdam, The Netherlands).

### Instrumentation

Chromatographic separations were carried out using an HP1100 liquid chromatograph (Agilent technologies, Palo Alto, CA, USA) consisting of a binary pump, autosampler, degasser, and column oven. Sample volumes of 50 µL were injected and the autosampler was thermostatted at 4 °C. For the determination of topotecan in plasma a mobile phase of 0.05% TFA in methanol - water (40:60, v/v) was pumped at a flow rate of 1 mL/min through a Luna C8(2) column (250 x 4.6 mm ID, particle size 5 µm; Phenomenex, Torrance, CA, USA), thermostatted at 35 °C. The eluate was split ¼ before entering an API 3000 triple quadrupole MS equipped with an electrospray (ESI) ion source (Sciex, Thornhill, ON, Canada). The quadrupoles operated with unit resolution in the positive ion mode. The obtained multiple

reaction monitoring (MRM) chromatograms were used for quantitation using Analyst software version 1.2 (Sciex). Run time was 8 min. Mass transitions of m/z 422 → 377 and m/z 428 → 377 were optimized for topotecan and $D_6$-topotecan, respectively, with dwell times of 150 ms. Nebulizer and turbo gas (both compressed air) were set at 1.6 and 7 L/min, respectively, while the curtain gas and collision gas (both $N_2$) were operated at 1.2 L/min and $2.1x10^{15}$ molecules/$cm^2$, respectively. The ionspray voltage was kept at 5500 V, with a source temperature of 400 °C. In order to avoid carry-over, the autosampler needle was rinsed ten times after a series of calibration standards, quality control samples, and pharmacokinetic samples (12-14 sample injections) with 100 µL of methanol, followed by an injection of 0.2% TFA in methanol - water (40:60, v/v).

For the quantitative analysis of topotecan in plasma ultrafiltrate a mobile phase composed of 0.2% TFA in methanol - water (40:60, v/v) was pumped at a flow rate of 0.5 mL/min through a Luna C8(2) column (250 x 3.0 mm ID, particle size 5 µm; Phenomenex), thermostatted at 35 °C. The eluate was split ¼ and subsequently mixed (2:1, v/v) with propionic acid-2-propanol (3:1, v/v) before entering the API 3000 triple quadrupole MS. The curtain gas was set at 1.8 L/min and the source temperature was kept at 300 °C. Other settings were as described above.

**Preparation of calibration standards and quality control samples in plasma**

Two sets of working solutions were prepared in human plasma from a stock solution containing 100 µg/mL liposomal topotecan in human plasma. Calibration standards were prepared in human plasma in a range of 1-500 ng/mL from one set of working solutions. Quality control samples were prepared from the other set of working solutions in human plasma at concentrations of 3, 100, and 400 ng/mL. Additional quality control samples at the lower limit of quantitation (LLOQ; 1 ng/mL) and at the upper limit of quantitation (ULOQ; 500 ng/mL) were prepared. Sample aliquots of 500 µL were transferred to 2.0 mL polypropylene tubes (Eppendorf Netheler Hinz GmbH, Hamburg, Germany) and processed according to the described procedures.

**Preparation of calibration standards and quality control samples in plasma ultrafiltrate**

Two topotecan stock solutions were prepared with separate weighing in acidified methanol (1 mM HCl in methanol) with concentrations of 1 mg/mL. These solutions were further diluted with acidified methanol to obtain working solutions. Control human plasma ultrafiltrate was obtained by centrifuging 10 mL human plasma for 30 minutes at 3,000 g in a YM centriplus centrifugal filter device (30 kDa; Millipore, Billerica, MA, USA). Calibration standards were prepared in plasma ultrafiltrate in a range of 0.1-50 ng/mL from one set of working solutions. Quality control samples were prepared from the other set of working solutions in plasma ultrafiltrate at concentrations of 0.3, 20, and 40 ng/mL. Additional quality control samples at the lower limit of quantitation (LLOQ; 0.1 ng/mL) and at the upper limit of quantitation (ULOQ; 50 ng/mL) were prepared. Sample aliquots of 500 µL were transferred to 2.0 mL polypropylene tubes (Eppendorf) and processed according to the described procedures.

**Preparation of internal standard solutions**
A stock solution of $D_6$-topotecan was prepared in a similar way as the stock solution of topotecan. This solution was further diluted with acidified methanol to obtain a working solution with a final concentration of 200 ng/mL.

**Sample processing**
To 500 µL of plasma or plasma ultrafiltrate 50 µL of internal standard working solution in acidified methanol (200 ng/mL) was added. The samples were vortex mixed for 10 s and subsequently 1,000 µL of methanol was added. After vortex mixing for 10 s, samples were centrifuged for 15 min at 23,100 g. The supernatant was transferred to a clean tube and subsequently evaporated to dryness at nominally 60 °C under nitrogen. The residue was reconstituted with 150 µL of 2% TFA in methanol – water (40:60, v/v) by vortex mixing for 60 s and subsequent sonication for 15 min. The samples were centrifuged for 10 min at 23,100 g and the supernatant was transferred to a glass autosampler vial. A volume of 50 µL was injected onto the analytical column.

**Validation procedures**
All validation experiments were performed according to the FDA guidelines on bioanalytical method validation [9].

*Linearity*
Eight non-zero calibration standards were prepared and subsequently analyzed in duplicate in three separate analytical runs. In order to establish the best fit and weighting for the calibration curves, back-calculated calibration concentrations were determined. The model showing the lowest % total bias and the most constant bias across the range was considered the best fit. Deviations of the calculated concentrations from the nominal concentrations should be within ±15% for at least 85% of the non-zero calibration standards, except for the LLOQ concentration, for which deviations within ±20% are accepted [9].

*Accuracy and precision*
Quality control samples were prepared with topotecan concentrations of 1, 3, 100, 400, and 500 ng/mL in plasma spiked with liposomal topotecan or 0.1, 0.3, 20, 40, and 50 ng/mL in plasma ultrafiltrate. Six replicates of each sample were analyzed together with a set of calibration standards, independently prepared from the control samples, in three analytical runs. The accuracy was determined as percent difference between the mean concentration and the nominal concentration. The coefficient of variation (C.V.) was used to report the precisions. Accuracies were required to be within ±15% and precisions were required to be less than 15% for all concentrations, except for the LLOQ level at which accuracies within ±20% and precisions less than 20% are accepted [9].

*Specificity and selectivity*
From six individual batches of control drug-free human plasma and plasma ultrafiltrate samples containing neither analyte nor internal standard (double blank), containing only

internal standard (blank), and spiked with liposomal topotecan or topotecan at the LLOQ level were prepared in order to determine whether endogenous compounds interfere at the mass transitions chosen for topotecan or the internal standard. Samples were processed according to the described procedures and analyzed. Areas of peaks co-eluting with topotecan should not exceed 20% of the area at the LLOQ level, and areas of peaks co-eluting with the internal standard should not exceed 5% of the internal standard peak area. The samples spiked at LLOQ level should have an accuracy within ±20% of the nominal value [9].

*Ion suppression and recovery*

For the determination of ion suppression control drug-free plasma and plasma ultrafiltrate were processed and dry extracts were dissolved with solutions that represent 100% recovery containing liposomal topotecan and internal standard or topotecan and internal standard in acidified methanol, respectively. Ion suppression was determined by comparing the analytical response of these samples to that of the solutions that represent 100% recovery. The loss of signal represents the ion suppression. Extraction recovery was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with solutions of topotecan or liposomal topotecan and internal standard in acidified methanol as described above. These experiments were performed at three concentration levels, in triplicate. Overall recovery corresponds to the net response after subtraction of the ion suppression and the signal loss due to the extraction. Ion suppression and recovery experiments for the internal standard were performed similarly.

*Stability*

The stability of topotecan in plasma and plasma ultrafiltrate samples was investigated during processing at ambient temperatures, during storage at nominally –20 °C, and after three freeze-thaw cycles from nominally –20 °C. Stability of the dry extract and re-injection reproducibility were tested. When 85-115% of the initial concentration of the analyte is found in the biomatrix or extracts thereof, the sample is considered stable. Stability of the internal standard in acidified methanol was determined during processing at ambient temperatures and will be established when 95-105% of the original concentration is recovered. All stability experiments were performed at least in duplicate and in plasma or plasma ultrafiltrate at a minimum of two concentration levels.

## Results and Discussion

### Method development

Q1 mass spectra of topotecan and $D_6$-topotecan were recorded in both positive and negative ion mode from solutions containing methanol and water, or methanol, water, and either ammonium acetate, ammonium hydroxide, or formic acid. For both topotecan and the internal standard the highest MS responses were obtained in the positive ion mode from a solution containing formic acid. A mass spectrum of the two compounds is presented in Figure 2. At neutral or alkaline pH, part of topotecan is present in the open hydroxy carboxylate form. In the negative ion mode, peaks corresponding to the open ring form of topotecan (m/z 438) and the internal standard (m/z 444) were not observed. In positive ion mode, however, peaks corresponding to the open-ring form were observed at m/z 440 and m/z 446, but only from an alkaline solution (Figure 3), and not, as might be expected, also from solutions of neutral pH. Product ion spectra of topotecan and $D_6$-topotecan recorded from the protonated molecular ions at m/z 422 and 428, respectively, are presented in Figure 4. In both spectra, the largest peak is observed at m/z 377, which corresponds to the elimination of dimethylamine from topotecan or di[$D_6$]methylamine from $D_6$-topotecan. For both compounds, the mass transition from the protonated molecular ion to the fragment ion at m/z 377 was monitored in the MRM mode.

For the development of the chromatographic system on the Luna C8(2) methanol-water and acetonitrile-water mixtures with addition of formic acid were tested. Methanol was more suitable as an organic modifier than acetonitrile as it yielded symmetrical peaks. Formic acid was not suitable as an additive since topotecan eluted without retention (methanol-water-formic acid, 20:80:0.05, v/v/v). Probably formic acid is not an appropriate ion-pairing agent. The addition of 0.05% TFA, on the other hand, resulted in excellent peak shapes and

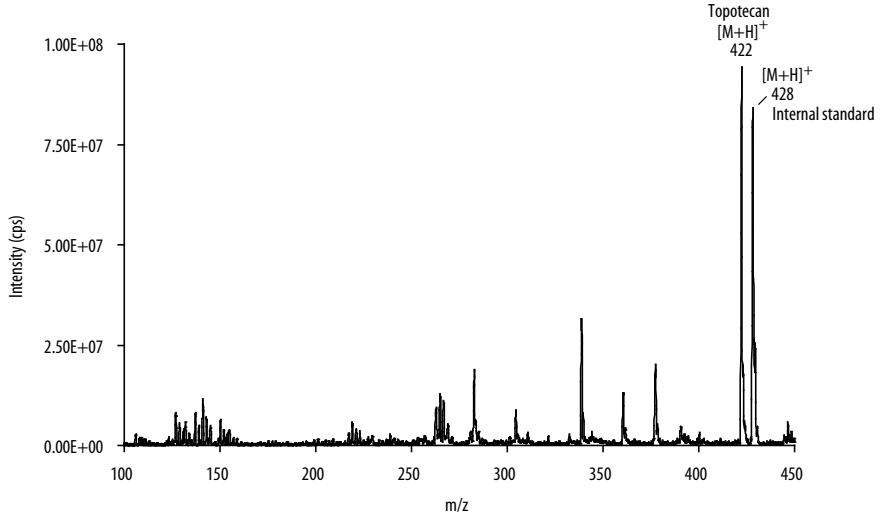



**Figure 2.** Q1 mass spectrum of topotecan and D6-topotecan from a solution containing water-methanol-formic acid.

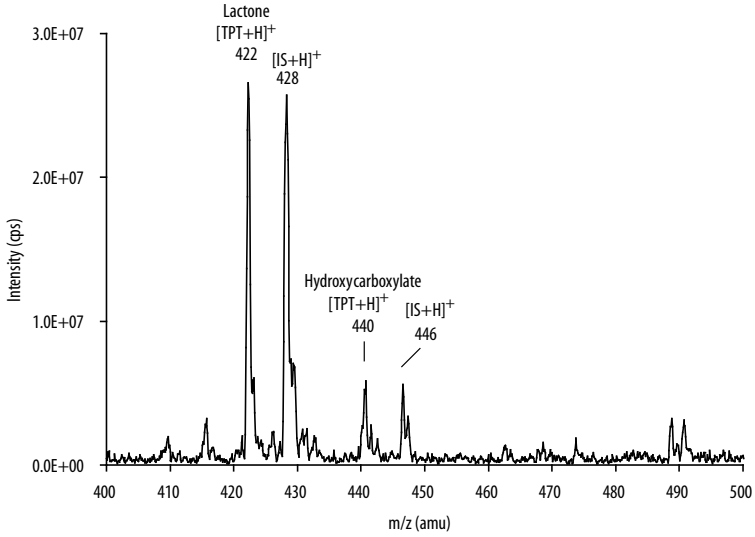



**Figure 3.** Q1 mass spectrum of topotecan (TPT) and the internal standard (IS) from a solution containing water-methanol-ammonium hydroxide.

retention with a k' of 1.7. TFA is a commonly used mobile phase additive for pH adjustment or ion pairing. Unfortunately, TFA and other volatile strong acids are also known to cause signal suppression for basic compounds when analyzed by ESI-MS, typically resulting in a ten-fold reduction of sensitivity. This effect has been attributed to the high conductivity and high surface tension of solutions containing TFA, resulting in an inefficient spray process. In addition, the observed signal reduction appears to be due to the strong bond between the TFA anion and analyte cation, which inhibits the release of the protonated analyte from the droplets [10,11]. With an eluent consisting of methanol-water and 0.05% TFA the target LLOQ of 1 ng/mL topotecan in plasma was obtained. In plasma ultrafiltrate, however, the target LLOQ of 0.1 ng/mL could not be reached. Therefore, measures to increase MS response have been investigated. First, the chromatographic system used was miniaturized by using an LC column with a smaller internal diameter (3.0 mm in stead of 4.6 mm) and reduction of the LC flow rate (1 to 0.5 ml/min). Secondly, reduction of the TFA content in the eluent was investigated (Figure 5). A ten-fold reduction of the TFA concentration yields an increase in signal to noise ratio of approximately four. Unfortunately, already at 0.025% TFA, the topotecan peak divides into two peaks and at concentrations <0.025% TFA, topotecan is not retained on the LC column. Thus, other measures were needed to obtain the target LLOQ of 0.1 ng/mL for this assay. Post-column addition of a mixture of propionic acid and 2-propanol (3:1, v/v) as described by Kuhlmann [10] and Apffel [11] was investigated. This so called TFA fix is used to enhance the MS signal after suppression caused by TFA. 2-Propanol decreases the conductivity and surface tension of the droplets. The addition of propionic acid removes the TFA anion from the analyte. Since TFA is more volatile than propionic acid, it will evaporate out of the droplets faster, resulting finally in the replacement of the TFA anions by propionate

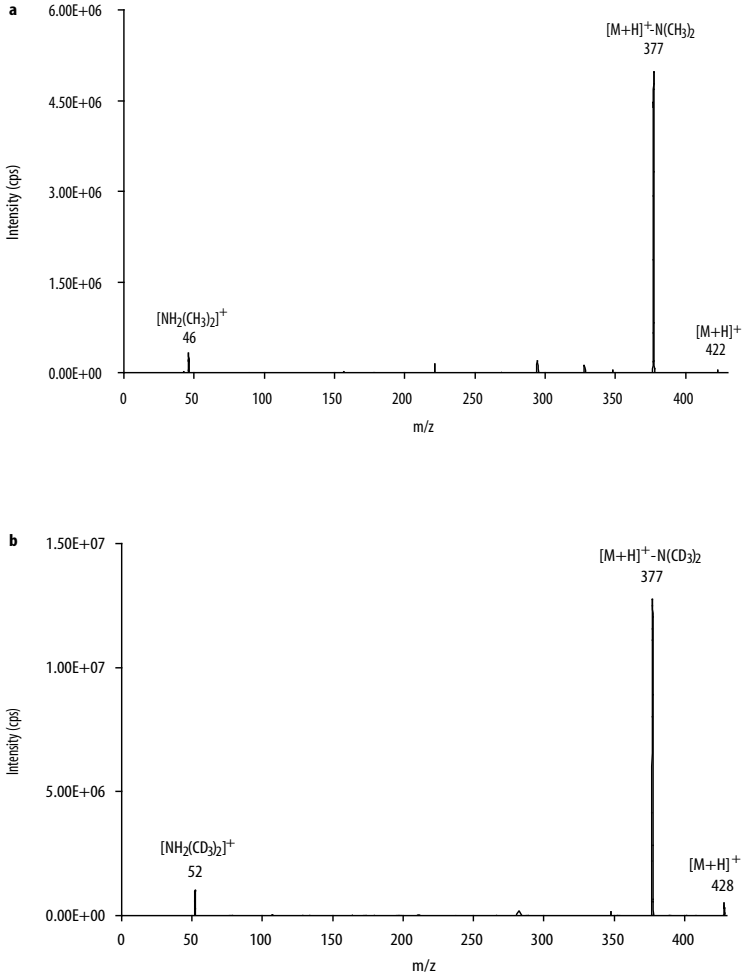



**Figure 4.** Product ion MS/MS spectra of topotecan from m/z 422 (a) and of $D_6$-topotecan from m/z 428 (b).

anions that bind with the analyte. The bond between the propionate anion and the analyte is easily broken, thereby releasing the protonated analyte ion. The influence of the TFA fix in combination with several TFA concentrations has been investigated (Figure 5). The results demonstrate that the TFA fix increases the topotecan signal to noise ratio three- to tenfold. In addition, the signal gain does not appear to be related to the TFA content in the eluent between 0.05 and 0.2%. Since peak shapes were optimal with a concentration of 0.2% TFA, this percentage was used. The LC eluate was split ¼ and subsequently mixed 2:1, v/v with propionic acid - 2-propanol (3:1, v/v). Representative chromatograms of topotecan and the internal standard from plasma at the LLOQ value of 1 ng/mL and from plasma ultrafiltrate at the LLOQ value of 0.1 ng/mL are presented in Figure 6.

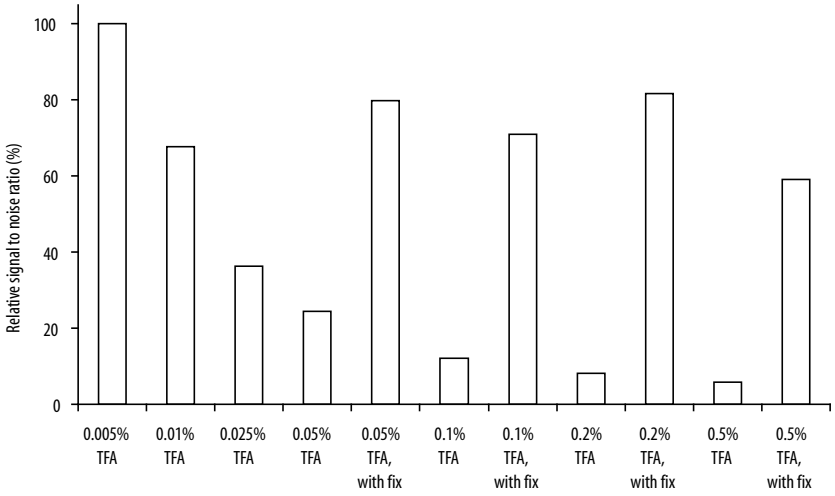



**Figure 5.** Influence of TFA concentration in the eluent and the use of the TFA fix on the topotecan signal to noise ratio. Fix: post-column addition of propionic acid and 2-propanol (3:1, v/v).

## Validation

*Linearity*

Calibration standards prepared with liposomal topotecan in plasma and with topotecan in plasma ultrafiltrate were analyzed in duplicate in three analytical runs with a dynamic range from 1 to 500 ng/mL (plasma) and 0.1 to 50 ng/mL (plasma ultrafiltrate), respectively. Linear fits using weighting factors of the inverse squared concentration ($1/x^2$) appeared to be the most appropriate to fit the data. Correlation coefficients of 0.9974 or higher were obtained for both assays. The calibration standards were back-calculated from the responses. The deviations from the nominal concentrations were between –4.69 and 1.95% for all concentrations in human plasma and between –2.00 and 2.25% for all concentrations in ultrafiltrate. C.V. values were less than 10.6% for all concentrations for both assays. All results met the requirements.

*Accuracy and precision*

In Table 1 the intra- and inter-assay accuracy and precision data for the topotecan quantitation in human plasma are presented. Intra-assay accuracy was within ±13.0% and inter-assay accuracy was within ±8.42%, with precisions less than 5.92% and 10.3%, respectively. In Table 2 the intra- and inter-assay performance data for the topotecan quantitation in plasma ultrafiltrate are tabulated. Intra-assay accuracy was within ±9.45% and inter-assay accuracy was within ±3.65%, with precisions less than 11.7% and 9.99%, respectively. All results met the requirements.

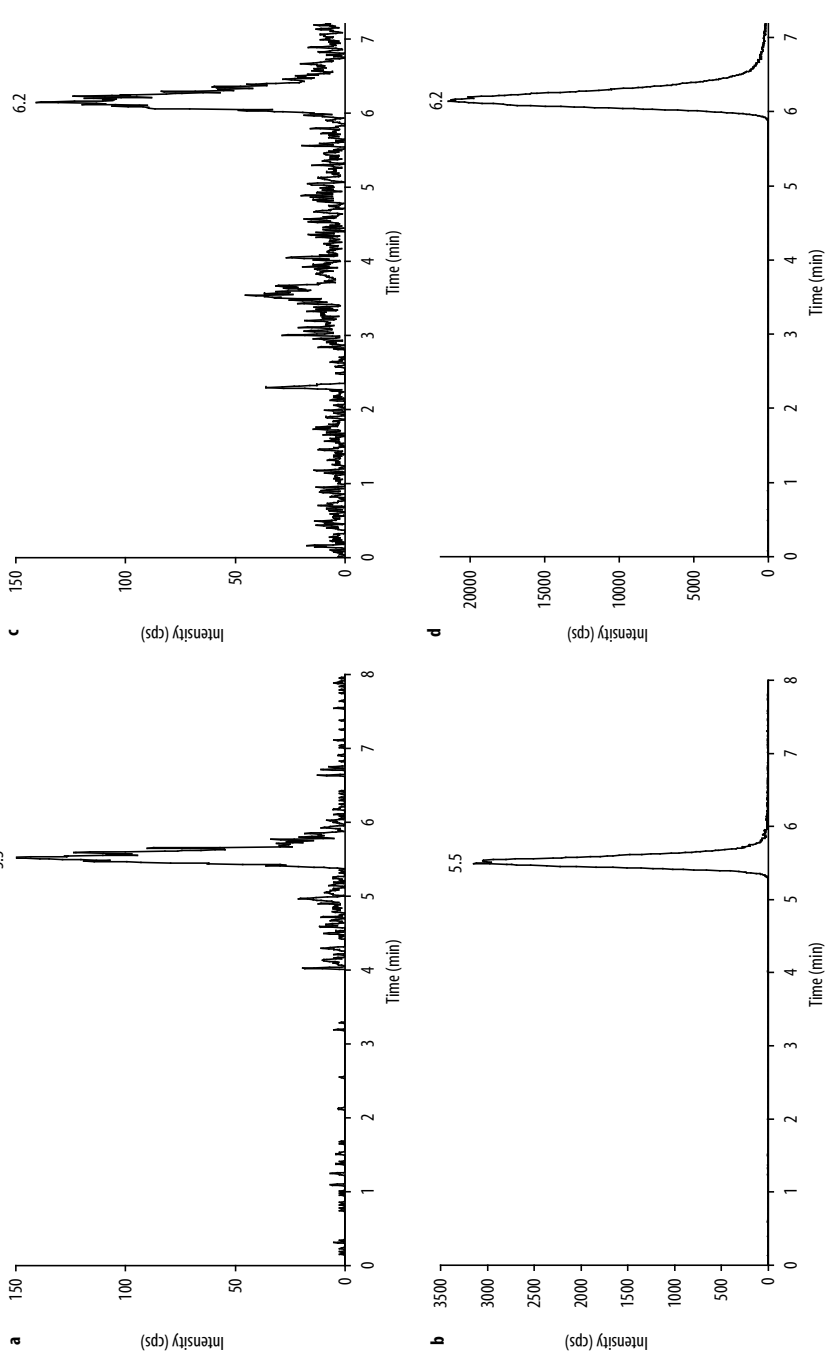



**Figure 6.** Representative chromatograms of topotecan (a) and the internal standard (b) in human plasma spiked with liposomal topotecan at the LLOQ level of 1 ng/mL, and of topotecan (c) and the internal standard (d) in plasma ultrafiltrate at the LLOQ level of 0.1 ng/mL.

**Table 1.** Assay performance data for analysis of topotecan in human plasma spiked with liposomal topotecan

| Run | Nominal concentration (ng/mL) | Mean calculated concentration (ng/mL) | Accuracy (% Bias) | Precision (% CV) | Number of replicates |
|---|---|---|---|---|---|
| 1 | 1.01 | 1.10 | 8.91 | 3.51 | 6 |
| 2 | 1.01 | 0.879 | -13.0 | 3.36 | 6 |
| 3 | 1.01 | 0.986 | -2.38 | 5.92 | 6 |
| Total | 1.01 | 0.989 | -2.08 | 10.3 | 18 |
| 1 | 3.03 | 3.20 | 5.61 | 1.44 | 6 |
| 2 | 3.03 | 2.71 | -10.6 | 3.57 | 6 |
| 3 | 3.03 | 2.72 | -10.2 | 1.60 | 6 |
| Total | 3.03 | 2.88 | -4.95 | 8.36 | 18 |
| 1 | 101 | 96.7 | -4.26 | 2.58 | 6 |
| 2 | 101 | 89.5 | -11.4 | 3.23 | 6 |
| 3 | 101 | 91.2 | -9.70 | 5.09 | 6 |
| Total | 101 | 92.5 | -8.42 | 4.91 | 18 |
| 1 | 404 | 404 | -0.00 | 1.88 | 6 |
| 2 | 404 | 371 | -8.17 | 0.835 | 6 |
| 3 | 404 | 388 | -3.96 | 2.08 | 6 |
| Total | 404 | 387 | -4.21 | 3.91 | 18 |
| 1 | 505 | 472 | -6.53 | 2.34 | 6 |
| 2 | 505 | 469 | -7.13 | 2.92 | 6 |
| 3 | 505 | 521 | 3.17 | 1.50 | 6 |
| Total | 505 | 487 | -3.56 | 5.45 | 18 |

**Table 2.** Assay performance data for topotecan analysis in plasma ultrafiltrate

| Run | Nominal concentration (ng/mL) | Mean calculated concentration (ng/mL) | Accuracy (% Bias) | Precision (% CV) | Number of replicates |
|---|---|---|---|---|---|
| 1 | 0.105 | 0.0976 | -7.05 | 5.32 | 6 |
| 2 | 0.105 | 0.108 | 2.86 | 11.7 | 6 |
| 3 | 0.105 | 0.110 | 4.76 | 8.05 | 6 |
| Total | 0.105 | 0.105 | 0.00 | 9.99 | 18 |
| 1 | 0.301 | 0.280 | -6.98 | 4.33 | 6 |
| 2 | 0.301 | 0.286 | -4.98 | 4.92 | 6 |
| 3 | 0.301 | 0.305 | 1.33 | 4.74 | 6 |
| Total | 0.301 | 0.290 | -3.65 | 5.84 | 18 |
| 1 | 20.1 | 18.2 | -9.45 | 0.731 | 6 |
| 2 | 20.1 | 20.8 | 3.48 | 1.17 | 6 |
| 3 | 20.1 | 20.0 | -0.498 | 2.20 | 6 |
| Total | 20.1 | 19.7 | -1.99 | 5.84 | 18 |
| 1 | 40.1 | 36.8 | -8.23 | 1.07 | 6 |
| 2 | 40.1 | 42.7 | 6.48 | 2.06 | 6 |
| 3 | 40.1 | 42.4 | 5.74 | 3.80 | 6 |
| Total | 40.1 | 40.6 | 1.25 | 7.24 | 18 |
| 1 | 50.2 | 46.6 | -7.17 | 1.05 | 6 |
| 2 | 50.2 | 54.0 | 7.57 | 1.57 | 6 |
| 3 | 50.2 | 51.6 | 2.79 | 2.70 | 6 |
| Total | 50.2 | 50.8 | 1.20 | 6.49 | 18 |

*Specificity and selectivity*
MRM chromatograms of double blank, blank, and LLOQ samples prepared in six individual batches of both plasma and plasma ultrafiltrate did not show peaks that co-eluted with either topotecan or the internal standard nor interfered with the mass transition for either of the compounds. Deviations from the nominal concentrations at LLOQ level were between –13.1 and –0.990% in plasma and between –11.9 and 1.32% in plasma ultrafiltrate.

*Ion suppression and recovery*
The mean ion suppression of topotecan was 15.8 ± 41.8% in human plasma and 39.0 ± 26.2% in plasma ultrafiltrate. Ion suppression values of the internal standard were 16.3 and 39.5%, respectively. Extraction recovery of topotecan was 41.8 ± 19.4% from human plasma and 64.2 ± 12.4% from ultrafiltrate. Extraction recovery of the internal standard was 52.4% from plasma and 73.8% from ultrafiltrate at a concentration of 20 ng/mL for both assays.

*Stability*
The internal standard is stable for at least 24 h at ambient temperatures in acidified methanol. Stability of topotecan in plasma and in plasma ultrafiltrate has been established for at least 24 h at ambient temperatures and after three freeze and thaw cycles from nominally –20 °C to ambient temperatures. Investigations into long-term stability of topotecan in plasma or plasma ultrafiltrate during storage at nominally –20 °C are ongoing. Furthermore, topotecan was stable in the dry extract from ultrafiltrate for at least 13 days at nominally 2-8 °C and in the dry extract from plasma for at least 5 days at nominally 2-8 °C. Finally, re-injection reproducibility was established for topotecan in plasma and plasma ultrafiltrate after 24 h at nominally 4 °C .

## Conclusion

The development and validation of assays for the quantitative analysis of topotecan in human plasma after the administration of liposomal topotecan and of topotecan in plasma ultrafiltrate is described. The validated ranges were 1-500 ng/mL and 0.1-50 ng/mL in plasma and plasma ultrafiltrate, respectively. Validation experiments were performed that amply met with FDA requirements. Results demonstrate that the assays are accurate, precise and selective.
This is the first article to describe an LC-MS/MS assay for the quantitative bioanalysis of topotecan. Tandem MS detection is less sensitive than fluorescence detection with LLOQ values as low as 0.05 ng/mL from 100 µL plasma samples [3]. However, with MS detection better selectivity is obtained. In addition, this is also the first report describing the use of an internal standard for the quantitation of topotecan, which has evidently improved the accuracy of the analysis. This LC-MS/MS method for topotecan can thus be considered a valuable extension of available assays. The assay will be applied in a clinical phase I study with liposomal topotecan.

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# 5.2

## Quantitative analysis of the P-glycoprotein inhibitor Elacridar (GF120918) in human and dog plasma using liquid chromatography with tandem mass spectrometric detection.

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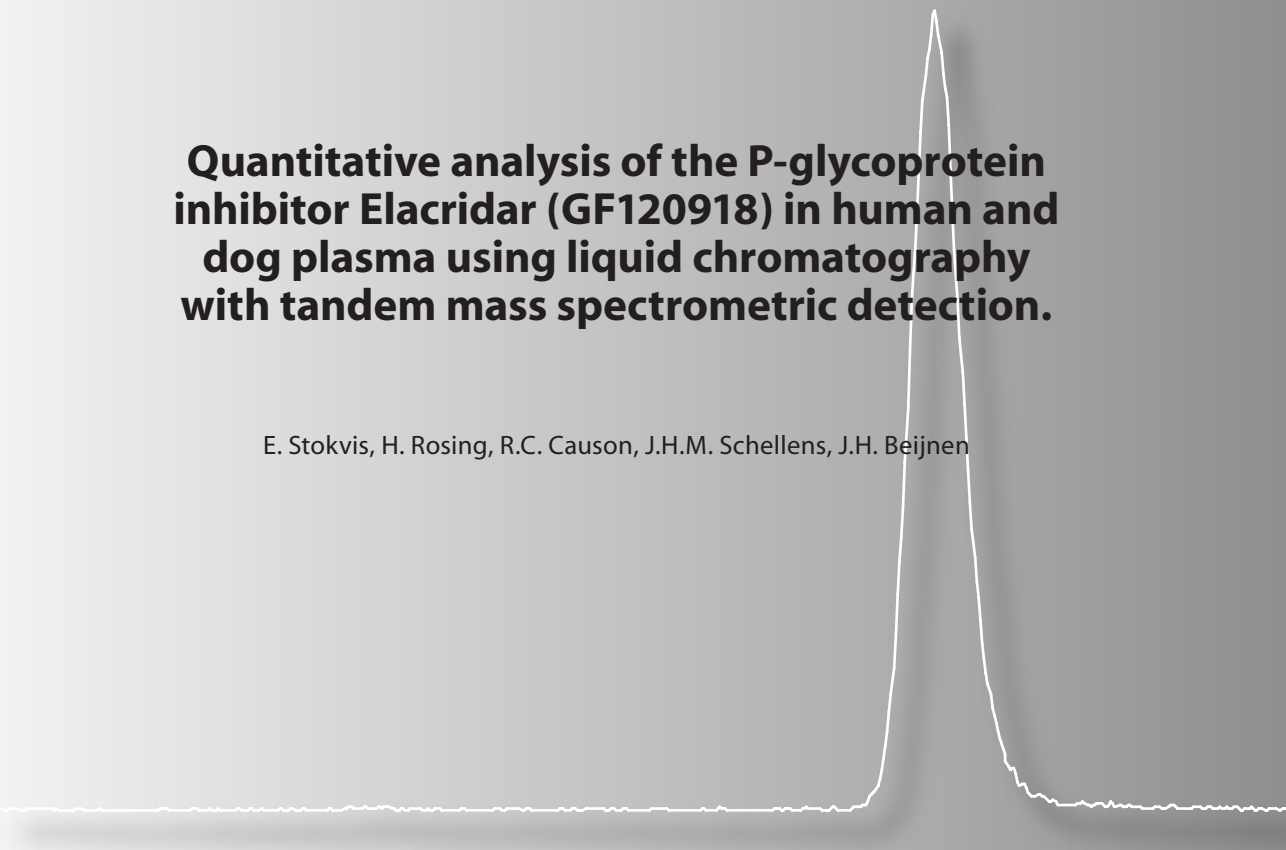

Submitted

## Abstract

A liquid chromatography tandem mass spectrometric (LC-MS/MS) method for the determination of the P-glycoprotein and Breast Cancer Resistance Protein inhibitor Elacridar in human and dog plasma is described. The internal standard was stable isotopically labeled Elacridar. Sample pretreatment involved liquid-liquid extraction with *tert*-butyl methyl ether. Analysis of Elacridar and internal standard was performed by reversed phase LC on a basic stable minibore analytical column with an eluent consisting of acetonitrile and aqueous ammonium hydroxide. An API 2000 triple quadrupole mass spectrometer with an electrospray ion source was used in the positive ion multiple reaction monitoring mode. Run time per sample was only six minutes. The method is sensitive and specific, with a dynamic range from 1 to 500 ng/mL from 100 µL human or dog plasma. Accuracy of the method was within 15% bias and precision was lower than 15% for all tested concentration levels and in both matrices. The method is simple and the liquid-liquid extraction produces clean samples. This method was successfully applied to support the pharmacokinetics of a clinical trial in which orally applied Elacridar was used as a bioavailability enhancer.

## Introduction

Elacridar (GF120918) is a potent and selective inhibitor of the drug pumps P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP; A3CG2). Expression of P-gp or BCRP in cancer cell lines is associated with multi-drug resistance (MDR) and is held responsible for chemotherapeutic treatment failure in cancer patients [1,2]. Furthermore, the native presence of these proteins in the apical membrane of the epithelial cells in the intestine limits the entry of substrates e.g. several anticancer drugs, into the bloodstream by actively pumping the drug back into the intestinal lumen [2-4].

Elacridar was discovered in the search for potent and selective P-gp inhibitors. Many MDR reversing agents appeared to be weak competitive P-gp inhibitors or potent inhibitors of Cytochrome P4503A4 (CYP3A4), causing undesirable pharmacokinetic interactions [5]. Elacridar, on the other hand, is about 100-fold more potent than the widely used P-gp inhibitor cyclosporin A and has very low affinity for CYP3A4 [6,7]. Recently, it was demonstrated that Elacridar is also a potent inhibitor of BCRP [8]. BCRP is primarily associated with *in vitro* resistance against the anticancer drug topotecan (a topo-isomerase I inhibitor) as well as its low oral bioavailbility in patients [8-10]. Therefore, in clinical studies with oral dosing of topotecan Elacridar could be an excellent choice to obtain maximum bioavailability of topotecan [11].

We have developed a liquid chromatography tandem mass spectrometric (LC-MS/MS) method for the quantitative analysis of Elacridar in human and dog plasma in order to investigate the pharmacokinetics of Elacridar during pre-clinical and clinical bioavailability combination studies with topotecan. The method has been validated according to the most recent FDA guidelines [12] and has successfully been applied in a pharmacokinetic

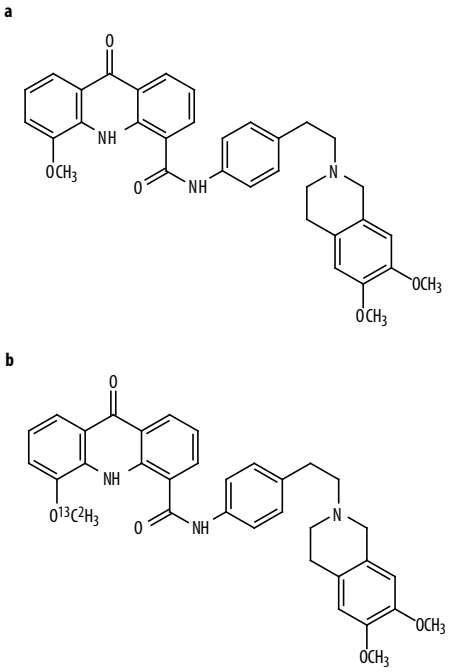


**Figure 1.** Structures of Elacridar and [$^{2}H_{3}{}^{13}C$]-Elacridar.

study. Reported bioanalytical assays for Elacridar [6,13,14] based on LC with fluorescence detection were time-consuming and lacked sensitivity for low dosing trials with Elacridar.

## Experimental

### Chemicals

Elacridar (GF120918; GG918; N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolyl)-ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; hydrochloride salt; lot MDRI1004; Figure 1a) reference standard as well as the internal standard reference standard ([$^{2}H_{3}{}^{13}C$]-Elacridar; hydrochloride salt; lot R7548/167/1 l; Figure 1b) were supplied by GlaxoSmithKline (Ware, Hertfordshire, UK). Methanol and acetonitrile (LC gradient grade) were purchased from Biosolve ltd. (Amsterdam, the Netherlands). Ammonia solution 25%, *N*,*N*-dimethylacetamide (DMA), and *tert*-butyl-methylether (all analytical grade) were obtained from Merck (Darmstadt, Germany). Ethanol (technical grade) was purchased from Klinipath (Duiven, The Netherlands). Distilled water was used throughout the analyses. Drug free human plasma was obtained from the Central Laboratory for Blood Transfusion (Sanquin Amsterdam, The Netherlands). Drug free dog plasma was obtained from GlaxoSmithKline.

**Instrumentation**

Chromatographic separations of Elacridar and internal standard were carried out using a HP1100 liquid chromatograph (Agilent technologies, Palo Alto, CA, USA) consisting of a binary pump, auto sampler, degasser, and column oven. A mobile phase of acetonitrile - aqueous 10 mM ammonia (70:30, v/v) was pumped at a flow rate of 0.2 mL/min through a Zorbax Extend C18 column (150 x 2.1 mm ID, particle size 5 μm; Agilent technologies) protected with an in-line filter (Micro filter frit, 5 μm, Upchurch Scientific Inc., Oak Harbor, WA, USA). Sample injections of 10 μL were carried out and the eluate was let directly into an API 2000 triple quadrupole MS equipped with an electrospray (ESI) ion source (Sciex, Thornhill, ON, Canada). The quadrupoles were operated with unit resolution in the positive ion mode. The resulting multiple reaction monitoring (MRM) chromatograms were used for quantification using Analyst™ software version 1.2 (Sciex). Run time was 6 min. ESI-MS/MS parameters and specific Elacridar and internal standard parameters used in this study are listed in Table 1. Since the auto sampler did not have a possibility to rinse the outside of the autosampler needle, it was rinsed after a series of calibration standards, quality control samples, and twelve to fourteen pharmacokinetic samples with five times 100 μL of acetonitrile in order to prevent carry over.

**Preparation of calibration standards and quality control samples**

An Elacridar stock solution was prepared in DMA at a concentration of 0.1 mg/mL. This solution was further diluted with methanol to obtain working solutions with concentrations of 0.1, 1, and 10 μg/mL. Aliquots of working solutions were diluted in drug free human or dog plasma in volumetric flasks in order to obtain eight calibration standards, ranging from 1-500 ng/mL in both matrices. An independently prepared stock solution, with separate weighing of Elacridar, was diluted with methanol as described above. The

**Table 1.** ESI-MS/MS settings

| Parameter | | Setting |
|---|---|---|
| Ionspray voltage (positive ion mode) | | 5500 V |
| Curtain gas ($N_2$) | | 25 psi |
| Temperature | | 350 °C |
| Nebulizer gas (compressed air) | | 40 psi |
| Turbo gas ($N_2$) | | 70 psi |
| Collission Activated Dissociation gas ($N_2$) | | $2.66*10^{15}$ molecules/$cm^2$ |
| Collission Cell Entrance Potential | | 22 V |
| Collission Cell Exit Potential | | 4 V |
| | **Elacridar** | **Internal standard** |
| Q1 mass (u) | 564 | 568 |
| Q3 mass (u) | 252 | 256 |
| Dwell time | 150 ms | 150 ms |
| Declustering Potential | 131 V | 111 V |
| Focussing Potential | 280 V | 350 V |
| Entrance Potential | 11.5 V | 12 V |
| Collission Energy | 51 V | 49 V |

working solutions were spiked to drug free human and dog plasma resulting in quality control samples at three concentration levels (3, 250, and 400 ng/mL). In addition, quality control samples at the lower limit of quantitation (LLOQ; 1 ng/mL) and the upper limit of quantitation (ULOQ; 500 ng/mL) were prepared. Elacridar solutions in plasma never contained more than 5% of methanol, in order to maintain the integrity of the spiked plasma samples.
An internal standard stock solution was prepared similar to that of Elacridar. The stock solution was diluted with methanol to obtain a working solution with a final concentration of 200 ng/mL. Human plasma samples were stored at nominally both –70 °C and –20 °C, while dog plasma samples were stored at nominally –20 °C. All other solutions were stored at nominally –20 °C.

**Sample processing**
A volume of 25 μL of 200 ng/mL internal standard solution in methanol was added to 100 μL of plasma. Elacridar and internal standard were then extracted from plasma using 1.5 mL of *tert*-butyl-methylether. After vortex mixing for 30 s followed by shaking vigorously for 15 min, the mixture was centrifuged for 5 min at 20,000 g. The aqueous layer was frozen in an ethanol-dry ice mixture and the organic layer was decanted into a clean tube. The organic solvent was evaporated under a gentle stream of nitrogen gas at nominally 40 °C. The residue was reconstituted with 100 μL of acetonitrile-water (70:30, v/v) by vortex mixing for 30 s and vigorous shaking for 10 min. After centrifuging for 5 min at 20,000 g the supernatant was transferred to a glass autosampler vial and 10 μL was injected onto the analytical column.

**Validation procedures**
Validation was performed according to FDA guidelines in both matrices [12]. However, for linearity as well as accuracy and precision it was possible to set more stringent requirements.

*Linearity*
Eight non-zero plasma calibration standards were prepared and subsequently analyzed in duplicate in three separate analytical runs. In order to establish the best fit and weighting for the calibration curves, back-calculated calibration concentrations were determined. The model showing the lowest % total bias and the most constant bias across the range was considered the best fit. Deviations of the calculated concentrations from the nominal concentrations should be within ±15% for at least 85% of the non-zero calibration standards, including the LLOQ concentration, for which deviations within ±20% are accepted according to the FDA guidelines [12].

*Accuracy and precision*
For the validation of the assay, quality control samples were prepared with concentrations of 1, 3, 250, 400, and 500 ng/mL. Six replicates of each sample were analyzed together with a set of calibration standards, independently prepared from the control samples, in three

analytical runs. The accuracy was determined as percent difference between the mean concentration and the nominal concentration. The coefficient of variation (C.V.) was used to report the precisions. Accuracies were required to be within ±15% and precisions were required to be less than 15% for all concentrations, while according to the FDA guidelines accuracies within ±20% and precisions less than 20% are accepted at the LLOQ level [12].

*Specificity and selectivity*

From six individual batches of control drug-free human plasma and dog plasma, samples containing neither analyte nor internal standard (double blank), containing only internal standard (blank), and spiked at the LLOQ level with Elacridar (LLOQ sample) were prepared in order to determine whether endogenous compounds interfere at the mass transitions chosen for Elacridar or the internal standard. Samples were processed according to the described procedures and analyzed. Areas of peaks co-eluting with Elacridar should not exceed 20% of the area at the LLOQ level, and areas of peaks co-eluting with the internal standard should not exceed 5% of the internal standard peak area. The samples spiked at LLOQ level should have an accuracy within ±20% of the nominal value.

*Ion suppression and recovery*

Control drug-free plasma was processed and dry extracts were reconstituted with solutions that represent 100% recovery containing Elacridar and internal standard in acetonitrile-water (70:30, v/v). Ion suppression was determined by comparing the analytical response of these samples to that of the solutions that represent 100% recovery. The loss of signal represents the ion suppression. Liquid-liquid extraction recovery was determined by comparing the analytical response of processed quality control samples with the analytical response of blank samples reconstituted with solutions of Elacridar and internal standard in acetonitrile-water (70:30, v/v) as described above. These experiments were performed at three concentration levels, in triplicate. Overall recovery corresponds to the net response after subtraction of the ion suppression and the signal loss due to the extraction. Ion suppression and recovery experiments for the internal standard were performed similarly.

*Stability*

The stability of Elacridar was investigated in the stock solution during storage and processing. In human plasma the stability of Elacridar was investigated during storage and after three freeze-thaw cycles at both nominally –70 °C and nominally –20 °C, while for dog plasma these stability experiments were performed at nominally –20 °C. Furthermore stability of the analyte was tested for both matrices during processing and in the final extract. Elacridar is considered stable in the biological matrix when 85-115% of the initial concentration is found. Stability in the stock solution during storage was also investigated for the internal standard. Elacridar and the internal standard are considered stable in stock solutions when 95-105% of the original concentration is recovered. All stability experiments were performed in triplicate and in plasma at two concentration levels (3 and 400 ng/mL)

**Clinical study**

A phase I clinical study was started with topotecan in combination with Elacridar in order to determine the minimal dose of Elacridar required to achieve maximal oral bioavailability of topotecan. In addition, in previous studies using Elacridar as an enhancer of oral bioavailability, it was always dosed one hour prior to the other agent, while simultaneous administration is desired for practical reasons and patient comfort. Therefore, the appropriate dosing schedule of Elacridar and topotecan was also investigated. Elacridar doses of 100, 300, 500, 700, and 1000 mg were tested. Several blood samples were collected on study days 1 and 8 for Elacridar. Blood samples were collected in heparinized tubes, immediately cooled, and centrifuged within one hour (nominally 4 °C, 10 min at 1,200 g). The

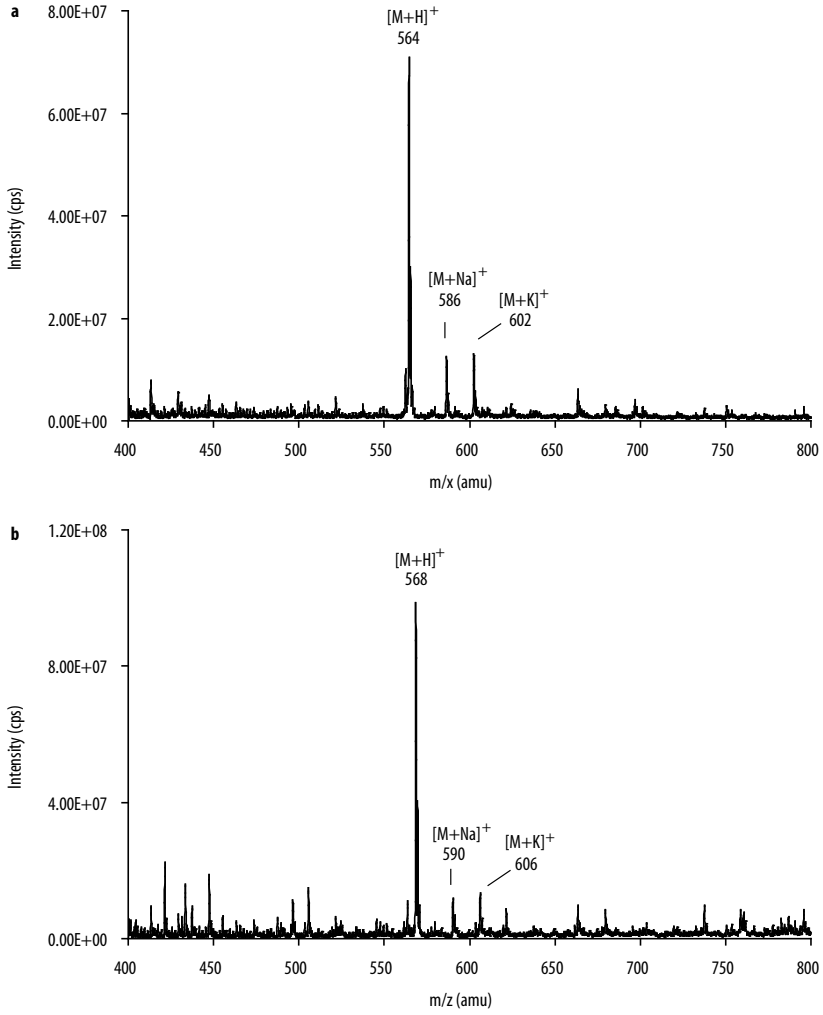


**Figure 2.** Positive ion Q1 spectrum of Elacridar (a) and the internal standard (b), recorded with an API 2000 MS from 400 to 800 mass units. A solution of ~1 µg/mL of Elacridar and internal standard in a solution of methanol-10 mM ammonium hydroxide in water (80:20, v/v) was continuously infused (5 µl/min).

plasma layer was removed and stored in polypropylene tubes at nominally –70 °C until analysis.

## Results and Discussion

### Mass Spectrometry

Elacridar (GF120918; Figure 1a) is an acridone carboxamide derivative and contains a tertiary and a secondary amine moiety, suggesting uncomplicated positive ionisation in the electrospray source. The internal standard for this method was deuterium and $^{13}C$ labeled Elacridar ([$^{2}H_{3}{}^{13}C$]-Elacridar; Figure 1b). A solution of approximately 1 µg/mL of Elacridar or internal standard in a mixture of methanol-10 mM ammonium acetate (80: 20, v/v) were continuously infused (5 µL/min) into the ESI source and mass spectra were recorded in the first quadrupole (Q1) of the MS (Figure 2). The $[M+H]^+$ ions of Elacridar and internal standard at m/z 564 and m/z 568, respectively, were most abundantly present in the spectrum and only small peaks corresponding to sodium and potassium adducts were observed. MS/MS analysis of the compounds was performed by selecting the m/z values for the molecular ions and inducing them to fragment in the collision cell using nitrogen gas, after which the fragments were detected in the third quadrupole of the MS. Figure 3 shows the product ion spectra of Elacridar and internal standard. In the product ion mass spectrum of Elacridar the most intense peak at m/z 252, corresponds to fragmentation of the amide bond leaving the charge on the acridone aldehyde. This fragmentation reaction also occurs for the internal standard, with the fragment ion peak found four mass units higher than in the spectrum in Figure 3a, since the acridone moiety bears the stable isotopes. The potential pathway for generating the fragments found in the product ion spectrum for Elacridar is presented in Figure 3a. The assignment of fragments was facilitated by the product ion spectrum of the stable isotopically labeled internal standard. If a peak corresponds to a fragment containing the acridone group, then the corresponding fragment in the internal standard's product ion spectrum should be found four mass units higher. The peaks in the product ion spectrum of the internal standard (Figure 3b) that are observed four mass units higher than those in the product ion spectrum of Elacridar are underlined.

Internal cleavage of the acridone moiety did not occur probably because of a stable pi-system. However, the hydrated isoquinoline structure is susceptible to fragmentation. Furthermore, the peak at m/z 120 in both product ion spectra probably corresponds to *p*-ethylaniline resulting from cleavage of both the amide bond and the bond between the isoquinoline nitrogen and the ethyl moiety as indicated in Figure 3. The fragments at m/z 546 and 382 result from the elimination of a water molecule from the parent compound and the fragment ion at m/z 400, respectively. The fragment ion at m/z 270 most-likely corresponds to the addition of water to the acridone aldehyde at m/z 252. For quantitative detection of Elacridar and the internal standard the most abundant mass transitions from the molecular ions to the acridone aldehyde fragments at m/z 252 and 256, respectively, were chosen.

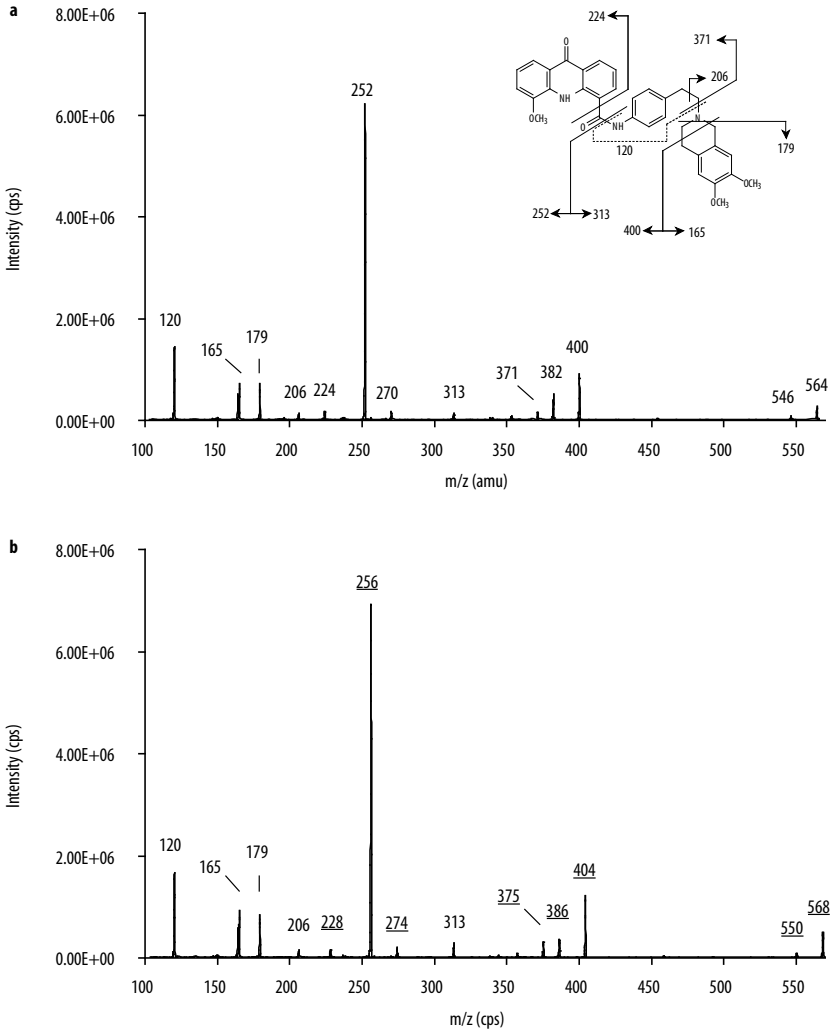


**Figure 3.** Product ion spectrum of Elacridar (a) and the internal standard (b) recorded in Q3 of an API 2000 MS after selection in Q1 of the $[M+H]^+$ ions at m/z 564 and 568, respectively and induction of fragmentation in the collision cell with nitrogen gas. The potential pathway for generation of fragments consistent with the peaks observed in the spectrum is presented. The peaks in the product ion spectrum of the internal standard (b) that are observed four mass units higher than those in the product ion spectrum of Elacridar (a) are underlined.

### Liquid Chromatography and Sample Preparation

MS detection under neutral, acidic, and alkaline conditions was investigated in order to determine which eluent composition resulted in the most sensitive detection for Elacridar. The neutral eluent consisted of methanol and water, while ammonium acetate and acetic acid were added for acidic conditions and ammonium hydroxide was added for alkaline conditions. The signal to noise ratio obtained for Elacridar with positive ionization from an

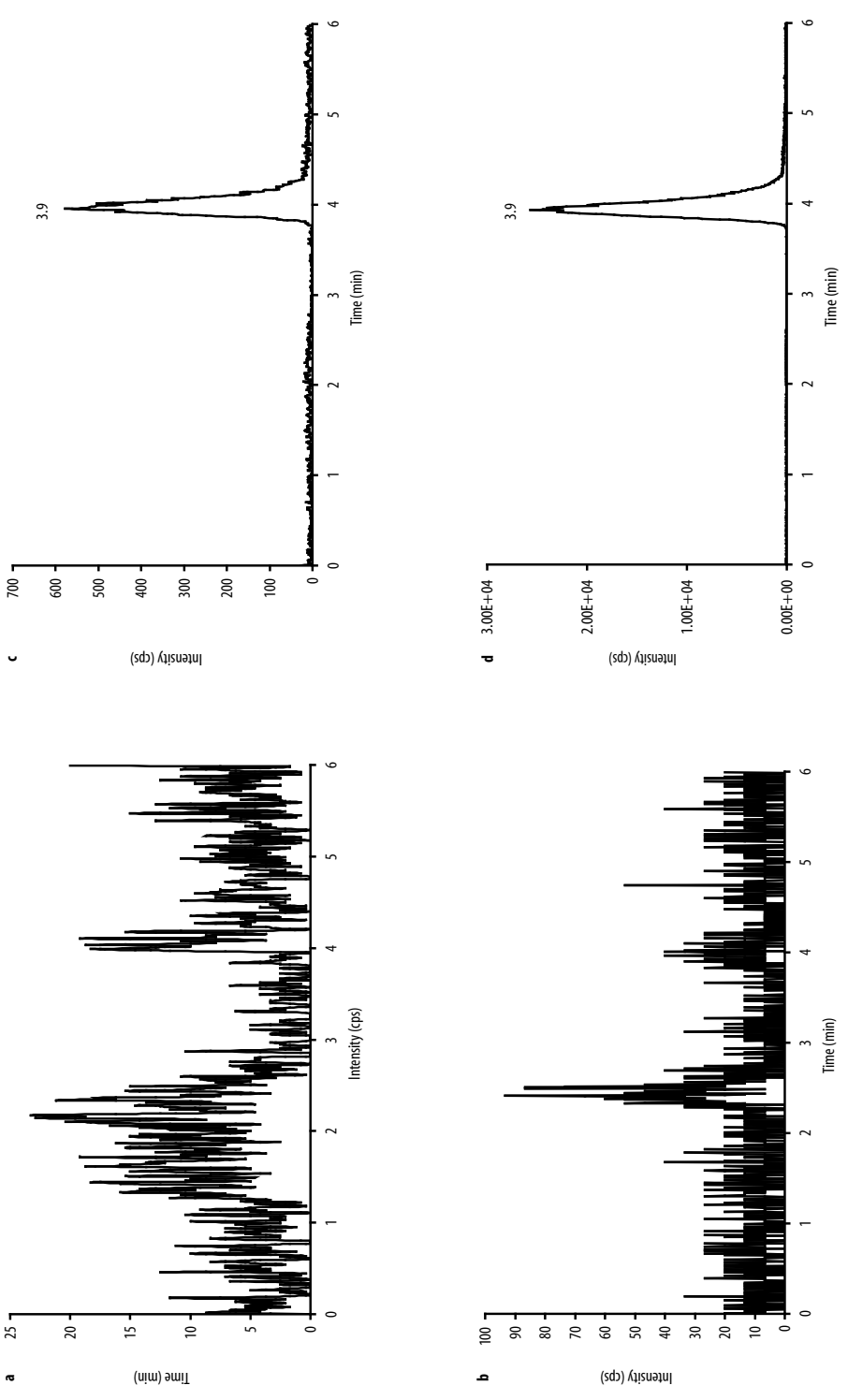


**Figure 4.** Representative chromatograms of (a) a double blank sample (Elacridar MRM transition), (b) a double blank sample (internal standard MRM transition), (c) Elacridar at LLQ level (1 ng/mL) and (c) the internal standard (50 ng/mL) from human plasma.

alkaline eluent was ~1.5 times higher than from an acidic eluent and ~40 times higher than from a neutral eluent. Positive ionization in the electrospray ion source in the presence of ammonium hydroxide most likely results from ion-molecule reactions between the analyte molecule and ammonium ions or collision-induced dissociation of ammonium adducts of the analyte under influence of the electrospray voltage [15]. An alkaline eluent was applied to the LC-MS/MS analysis of Elacridar in human and dog plasma and resulted in excellent peak shapes, short retention times and sensitive detection. Acetonitrile appeared to be more appropriate as an organic modifier for LC than methanol since it produced narrower and more symmetric peaks. Using an eluent consisting of acetonitrile-10 mM ammonium hydroxide in water (70:30, v/v) pumped at a flow rate of 0.2 mL/min through a Zorbax Extend C18 column (150 x 2.1 mm I.D. 5 µm particle size) suited for alkaline mobile phases, the retention time was only 3.9 min (k'~0.7), resulting in a total analytical run time of 6 minutes. Representative chromatograms of a double blank sample, of Elacridar at LLOQ level (1 ng/mL), and the internal standard (50 ng/mL) are presented in Figure 4.

Volumes of 1.5 mL of tert-butyl methyl ether resulted in clean extracts and acceptable recoveries for Elacridar when extracted from 100 µL of plasma, allowing the extraction to be performed in small 2 mL tubes.

**Table 2.** Assay performance data for Elacridar in human plasma

| Run | Nominal concentration (ng/mL) | Mean calculated concentration (ng/mL) | Accuracy (% Bias) | Precision (% CV) | Number of replicates |
|---|---|---|---|---|---|
| 1 | 1.11 | 1.10 | -0.901 | 8.33 | 6 |
| 2 | 1.11 | 1.11 | -0.150 | 5.75 | 6 |
| 3 | 1.11 | 1.03 | -6.77 | 5.80 | 6 |
| Total | 1.11 | 1.08 | -2.61 | 7.09 | 18 |
| 1 | 3.09 | 3.40 | 9.92 | 2.84 | 6 |
| 2 | 3.09 | 3.23 | 4.37 | 2.00 | 6 |
| 3 | 3.09 | 3.24 | 4.80 | 2.10 | 6 |
| Total | 3.09 | 3.29 | 6.36 | 3.30 | 18 |
| 1 | 251 | 246 | -2.19 | 1.84 | 6 |
| 2 | 251 | 254 | 1.06 | 1.53 | 6 |
| 3 | 251 | 247 | -1.59 | 0.810 | 6 |
| Total | 251 | 249 | -0.907 | 2.01 | 18 |
| 1 | 406 | 389 | -4.19 | 1.55 | 6 |
| 2 | 406 | 400 | -1.40 | 2.05 | 6 |
| 3 | 406 | 396 | -2.42 | 1.53 | 6 |
| Total | 406 | 395 | -2.67 | 2.03 | 18 |
| 1 | 503 | 486 | -3.45 | 1.59 | 6 |
| 2 | 503 | 480 | -4.54 | 1.07 | 6 |
| 3 | 503 | 478 | -5.00 | 2.65 | 6 |
| Total | 503 | 481 | -4.33 | 1.90 | 18 |

## Validation of the method for human and dog plasma

*Linearity*

Calibration standards prepared in control human plasma and control dog plasma were analyzed in duplicate in three analytical runs with a dynamic range from 1 to 500 ng/mL plasma. A linear fit using a weighting factor of the inverse squared concentration ($1/x^2$) appeared to be most appropriate to fit the data. Correlation coefficients of 0.9966 or higher were obtained for both matrices. The calibration standards were back-calculated from the responses. The deviations from the nominal concentrations were between –6.13 and 9.68% for all concentrations in human plasma and between –2.99 and 2.38% for all concentrations in dog plasma. C.V. values were less than 6.71% for all concentrations in both matrices.

*Accuracy and precision*

In Table 2 the intra- and inter-assay accuracy and precision data for the method in human plasma are presented. Intra-assay accuracy was within ±9.92% and inter-assay precision was within ±6.36%, with precision less than 8.33% and 7.09%, respectively. In Table 3 the intra- and inter-assay performance data for the method in dog plasma are tabulated. Intra-

**Table 3.** Assay performance data for Elacridar in dog plasma

| Run | Nominal concentration (ng/mL) | Mean calculated concentration (ng/mL) | Accuracy (% Bias) | Precision (% CV) | Number of replicates |
|---|---|---|---|---|---|
| 1 | 1.10 | 1.11 | 1.06 | 1.55 | 6 |
| 2 | 1.10 | 1.13 | 2.88 | 4.85 | 6 |
| 3 | 1.10 | 1.17 | 6.67 | 4.20 | 6 |
| Total | 1.10 | 1.14 | 3.54 | 4.29 | 18 |
| 1 | 3.09 | 3.17 | 2.54 | 2.34 | 6 |
| 2 | 3.09 | 2.94 | -4.72 | 11.2 | 6[a] |
| 3 | 3.09 | 3.24 | 4.96 | 1.73 | 6 |
| Total | 3.09 | 3.13 | 1.26 | 6.84 | 18 |
| 1 | 258 | 258 | -0.194 | 1.03 | 6 |
| 2 | 258 | 251 | -2.84 | 1.33 | 6 |
| 3 | 258 | 262 | 1.42 | 6.63 | 6 |
| Total | 258 | 257 | -0.538 | 4.19 | 18 |
| 1 | 425 | 404 | -5.06 | 2.79 | 6 |
| 2 | 425 | 412 | -3.18 | 2.30 | 6 |
| 3 | 425 | 410 | -3.45 | 3.58 | 6 |
| Total | 425 | 408 | -3.90 | 2.90 | 18 |
| 1 | 515 | 508 | -1.42 | 8.39 | 6 |
| 2 | 515 | 495 | -3.95 | 5.36 | 6 |
| 3 | 515 | 518 | 0.518 | 2.86 | 6 |
| Total | 515 | 507 | -1.62 | 5.92 | 18 |

[a]One result was rejected (outlier).

**Table 4.** Stability data of Elacridar and internal standard

| Matrix | Conditions[a] | Nominal conc. (ng/mL) | Dev. (%) | C.V. (%) | Number of replicates |
|---|---|---|---|---|---|
| **Elacridar** | | | | | |
| DMA (stock solution) | -20 °C, 9 months | $96.5*10^4$ | -2.13 | 0.476 | 3 |
| Human plasma | 3 freeze(-20 °C)/thaw cycles | 3.09 | 3.99 | 0.462 | 3 |
| | | 406 | 3.91 | 2.70 | 3 |
| | 3 freeze(-70 °C)/thaw cycles | 3.09 | -0.420 | 2.69 | 3 |
| | | 406 | 6.04 | 0.665 | 3 |
| | Ambient, 24 h | 3.09 | 0.412 | 2.76 | 3 |
| | | 406 | 1.10 | 2.00 | 3 |
| | -20 °C, 11 months | 3.09 | -9.25 | 1.99 | 3 |
| | | 406 | -4.97 | 2.56 | 3 |
| | -70 °C, 11 months | 3.09 | -8.67 | 3.84 | 3 |
| | | 406 | -2.40 | 2.21 | 3 |
| | Final extract, ambient, 3 days | 3.09 | -4.12 | 1.71 | 3 |
| | | 406 | -5.43 | 1.83 | 3 |
| Dog plasma | 3 freeze(-20 °C)/thaw cycles | 3.09 | -2.76 | 1.88 | 3 |
| | | 425 | 0.305 | 0.761 | 3 |
| | Ambient 24 h | 3.09 | 0.854 | 0.952 | 3 |
| | | 425 | 2.75 | 14.7 | 3 |
| | -20 ° C, 12 months | 3.09 | -12.0 | 0.364 | 3 |
| | | 425 | -9.57 | 0.637 | 3 |
| | Final extract,ambient, 4 days | 3.09 | 3.20 | 3.53 | 3 |
| | | 425 | 1.08 | 5.43 | 3 |
| **Internal standard** | | | | | |
| DMA (Stock solution) | -20 °C, 7 months | $92.1*10^4$ | -2.98 | 1.10 | 3 |

Conc. Concentration; Dev. Deviation; C.V. Coefficient of Variation; DMA N,N-dimethylacetamide; [a] Temperatures are nominal.

assay accuracy was within ±6.67% and inter-assay accuracy was within ±3.90%, with precisions less than 11.2% and 6.84%, respectively.

*Ion suppression and recovery*

The mean ion suppression of Elacridar was 11.7 ± 0.960% in human plasma and –9.80 ± 7.14% in dog plasma. Ion suppression of the internal standard was –9.80 and –8.48%, respectively. A negative ion "suppression" or ion enhancement, indicates that the presence of matrix components creates a more ideal environment for the compounds to ionize than when they are not present. Liquid-liquid extraction recovery of Elacridar was 67.9 ± 5.41% from human plasma and 77.7 ± 5.45% from dog plasma. Extraction recovery of the internal standard was 73.0% in human plasma and 78.1% in dog plasma at a concentration of 50 ng/mL in both matrices.

*Specificity and selectivity*

MRM chromatograms of double blank, blank, and LLOQ samples prepared in six individual batches of both human and dog plasma did not show peaks that co-eluted with either Elacridar or the internal standard nor interfered with the mass transition for either of the compounds. Deviations from the nominal concentrations at LLOQ level were between –17.4 and –7.24% in human plasma and between –3.81 and 6.67% in dog plasma.

*Stability*

An overview of stability experiments and results is presented in Table 4. Elacridar is stable in the stock solutions in DMA for at least 9 months at nominally –20 °C and the internal standard for at least 7 months. Stability of Elacridar in human plasma has been established after 24 h at ambient temperatures, 11 months at both nominally –20 °C and nominally –70 °C, and after three freeze and thaw cycles from both nominally –20 °C and nominally –70 °C to ambient temperatures. Furthermore, Elacridar was stable in the final extract from human plasma for at least three days at ambient temperatures. In dog plasma stability of Elacridar has been established after 24 h at ambient temperatures and after three freeze and thaw cycles from nominally –20 ° C to ambient temperatures. Long-term stability of Elacridar in dog plasma has been established for at least 12 months at nominally –20 °C. Stability of Elacridar in the final extract from dog plasma has been assessed for at least four days at ambient temperatures.

**Clinical trial**

In Figure 5 the concentration *vs*. time profiles obtained in four patients treated with 100 mg of Elacridar during a dose escalation and dosing schedule study with the drug are

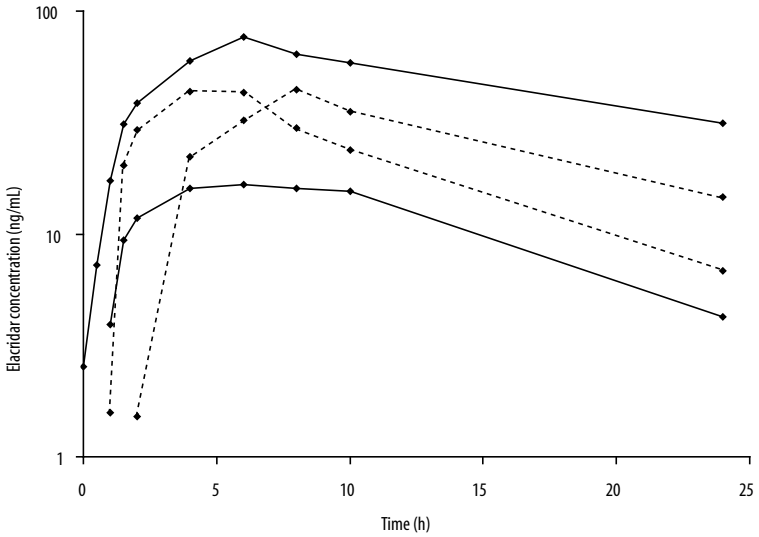


**Figure 5.** Concentration vs time profiles of four patients that received 100 mg of Elacridar. The dotted line represents the simultaneous dosing of Elacridar and topotecan, the continuous line dosing of Elacridar one hour prior to topotecan.

shown. The dotted lines in the figure indicate that the patients received Elacridar and topotecan simultaneously, while the continuous line represents dosing of Elacridar one hour prior to topotecan. Significant differences between the two dosing regimens were neither observed for Elacridar nor for topotecan (data not shown). Concentrations were still above the LLOQ at 24 hours after dosing of Elacridar. These results demonstrate the applicability of the method to support clinical trials with Elacridar and topotecan.

## Conclusion

In this paper the development, validation and implementation into day-to-day routine use of an LC-MS/MS method for the quantitative analysis of Elacridar is described. Elacridar and the internal standard were extracted from human or dog plasma using liquid-liquid extraction followed by chromatography under alkaline conditions. In both matrices, a linear dynamic range from 1 to 500 ng/mL was obtained. The method is easy to perform, accurate, precise, selective, and it has shown its applicability in the support of a clinical trial with Elacridar. Thus far, approximately 500 study samples have been assayed.

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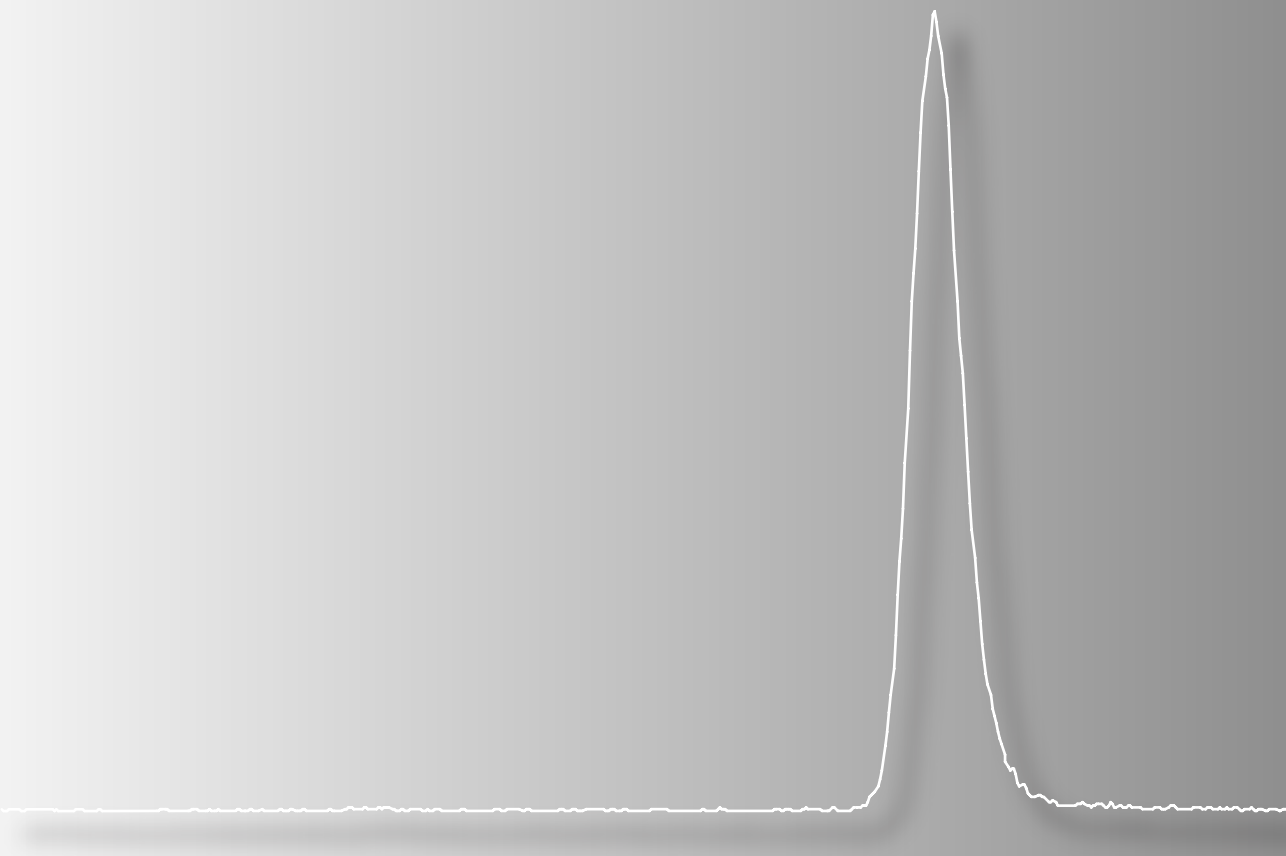
# Chapter 6

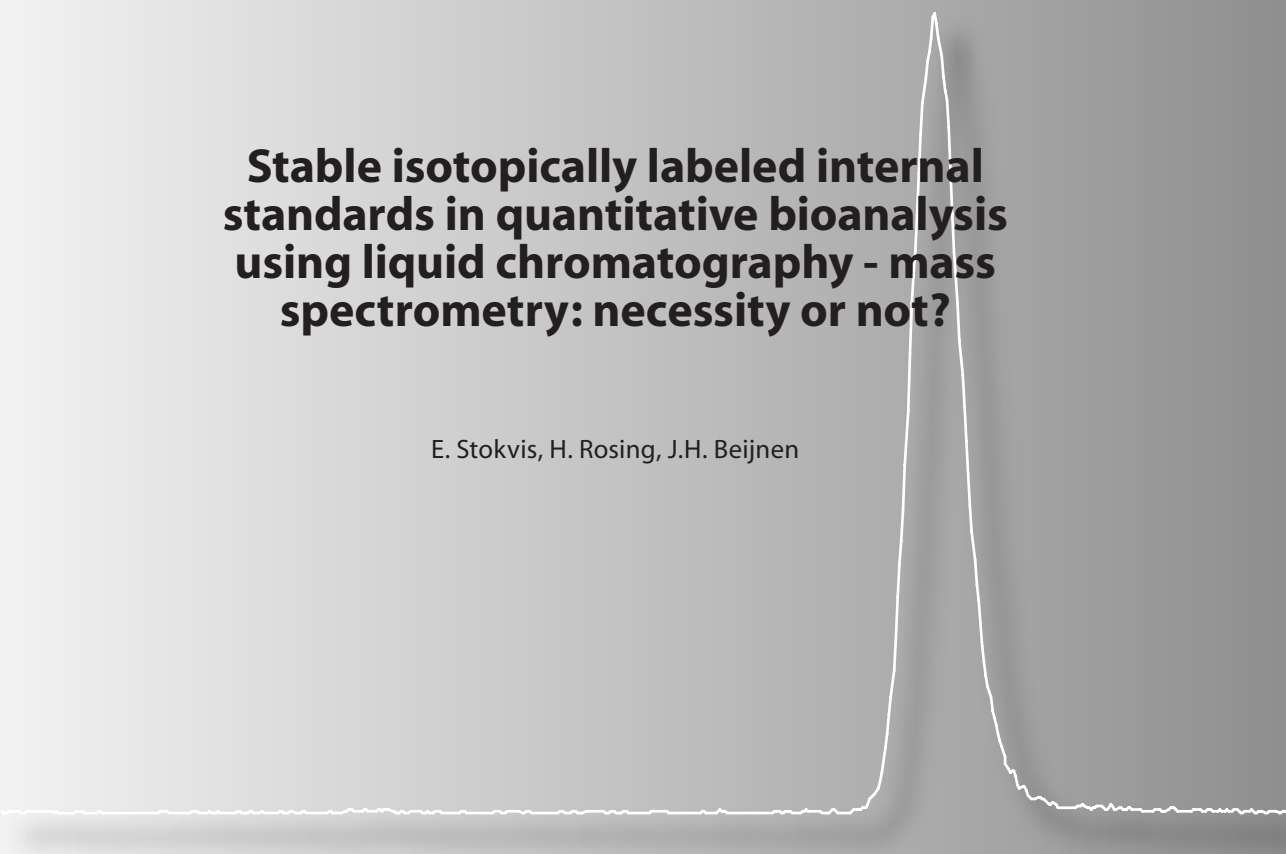


## Internal Standard Studies

# 6.1

# Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography - mass spectrometry: necessity or not?

E. Stokvis, H. Rosing, J.H. Beijnen



Submitted

## Abstract

It appears a general belief that stable isotopically labeled (SIL) internal standards yield better assay performance results for quantitative bioanalytical liquid chromatography mass spectrometry (LC-MS) assays than any other internal standard. In this article we describe our experiences with structural analogues and SIL internal standards and their merits and demerits. SIL internal standards are the first choice, but deuterium labeled compounds may demonstrate unexpected behavior, such as different retention times or recoveries than the analyte. In addition, a SIL internal standard with identical chemical properties as the analyte may cover up assay problems with stability, recovery, and ion suppression. Since SIL internal standards are not always available or very expensive, structural analogues can be used, however with consideration of several issues, which usually display during method validation.

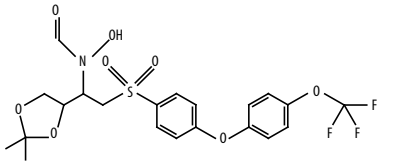
## Introduction

The implementation of internal standards in quantitative bioanalysis is an accepted and commonly used procedure. An internal standard (correct name ´processed internal standard`) is meant to correct for variability in dilutions, evaporation, degradation, recovery, adsorption, derivatization, and instrumental parameters such as injection volume, and even more so for gas chromatography (GC) than liquid chromatography (LC) based assays. With the introduction of LC - mass spectrometry (MS) for quantitative bioanalysis, the purpose of an internal standard has become mainly to correct for errors of detection [1].

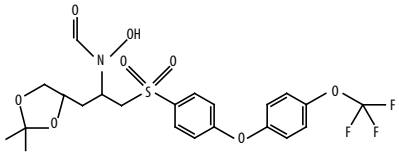
MS detection represents unrivaled sensitivity and selectivity and was therefore a clear candidate for LC based quantitative assays, although MS is not quantitative by nature. When a compound is introduced into the ion source only a portion of the total number of molecules is ionized. This portion (or ionization efficiency) depends largely on the molecular structure of the compound, but, in addition, may vary during day-to-day operation as a result of several parameters that are difficult or nearly impossible to control, such as temperature and pressure of the ion source, and the performance of the detector. Therefore, internal standards are essential in quantitative assays employing MS detection, since instrumental changes are made largely irrelevant because they affect only absolute responses, not ratios [2].

Quantitative detection using MS is further complicated by the effect of matrix components, for instance plasma or urine constituents. When the analyte is introduced into the ion source it will compete for ionization with other compounds introduced into the source simultaneously. Matrix components are infamous for decreasing the analyte signal, so called ion suppression, especially in electrospray ionization (ESI) based MS detection. The degree of ion suppression caused by matrix components may vary largely between matrices. Unfortunately, the degree of ion suppression caused by matrix components also depends on the analyte's structure. This means that if an analyte and internal standard

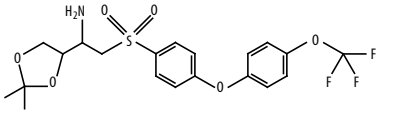
are not sufficiently similar in structure, the ratio of analyte and internal standard detector response may vary as a result of different degrees of ion suppression, thus compromising the quantitation. Therefore, internal standards in quantitative bioanalytical LC-MS assays are either structural analogues or stable isotopically labeled (SIL) analogues of the analyte. Structural analogues may differ in functional groups or backbone structure from the analyte, but rules for their structures have not been defined. SIL internal standards are compounds in which several atoms are replaced by their stable isotopes, such as $^{2}H$ (D, deuterium), $^{13}C$, $^{15}N$, or $^{17}O$. Labeling with three to eight $^{2}H$ or $^{13}C$ atoms or a combination of both is most common. Since a compound and its SIL analogue will theoretically co-elute, it is important that the mass difference between the compounds is at least 3 amu, in order to be able to separate them in the mass analyzer and to prevent "cross-talk". When the difference is less than 3 amu the isotope peaks of the analyte may interfere with the signal of the internal standard. Furthermore, the SIL internal standard should be pure enough to prevent any contributions to the analyte response. SIL internal standards are the preferred internal standards for MS detection since they are chemically identical to the analyte, but unfortunately they are not always available. Interestingly, although it is common knowledge that a SIL internal standard provides better assay performance results than any other internal standard, not many studies have been performed to demonstrate this [3]. On the other hand, several assays have been reported describing the use of an analogous internal standard with excellent results and there are reports describing the disadvantages of SIL internal standards [1,4,5]. In this article we have compared the use of structural analogues and SIL internal standards for several (investigational)



ABT-518



Internal standard



Metabolite

**Figure 1.** Structures of matrix metalloproteinase inhibitor ABT-518, its analogous internal standard, and a metabolite found in human plasma.

anticancer agents. Merits and demerits of the internal standards in our experiences with these assays are discussed.

## Case studies

### Assay performance

During the validation and routine use of a bioanalytical assay accuracy and precision are of major importance [6]. Therefore, the comparison of analogous and SIL internal standards was performed initially by means of those two parameters.

For the determination of the matrix metalloproteinase inhibitor ABT-518 an analogous internal standard with an additional internal methylene moiety in the backbone structure was available (Figure 1). This internal standard showed to be appropriate for a correct quantification of the drug in human plasma. A validation was executed according to the FDA guidelines on bioanalytical method validation [6] and results were always within requirements [7]. The assay, however, also included metabolites. One metabolite (Figure 1) is formed after reduction of the N-hydroxy moiety followed by hydrolysis of the amide. For the quantitative determination of this metabolite no dedicated internal standard was available and thus it was attempted to use the same as for the parent drug. This internal standard, however, was not suitable for the determination of the metabolite, which was reflected in the unacceptable validation results (accuracy >15%, precision >15%). In fact, the best results were obtained without the use of any internal standard. From equimolar amounts of ABT-518, its metabolite and internal standard a Q1 spectrum was recorded (Figure 2). Under the same MS conditions response for the metabolite was approximately

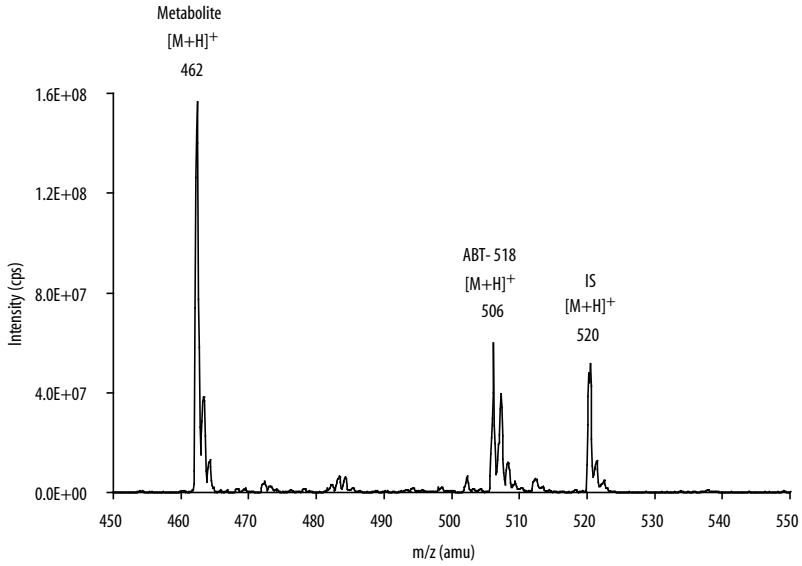



**Figure 2.** Q1 spectrum of ABT-518, the analogous internal standard (IS) and a human metabolite recorded from equimolar amounts.

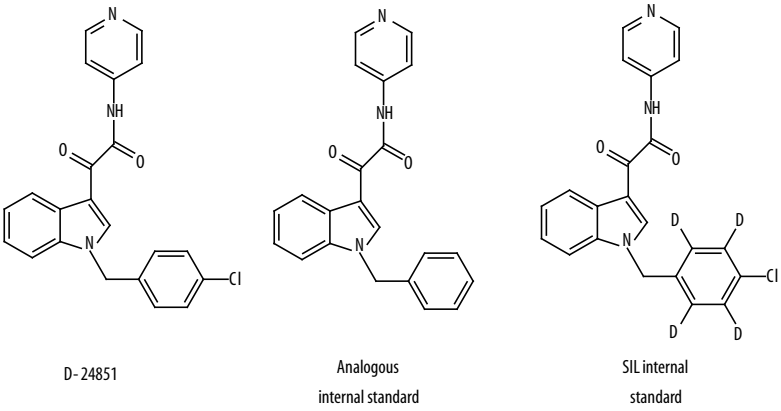



**Figure 3.** Structures of tubulin inhibitor D-24851, the analogous internal standard, and the SIL internal standard.

four times higher than for the internal standard, which can be attributed to the higher proton affinity of the amine moiety in the metabolite than of the amide group present in the internal standard molecule. The parent drug and internal standard, however, show similar ionization efficiencies [7].

During development of an assay for the tubulin inhibitor D-24851 (Figure 3) only an analogous internal standard was available lacking the chlorine atom at the benzyl moiety. This internal standard appeared inappropriate for use in the assay as is demonstrated in Table 1 (accuracy >15%, precision >15%). Quantitation without the use of an internal standard yielded even better results. On the other hand, for an LC-UV assay for the determination of D-24851, this internal standard was suitable (unpublished data). This suggests that the problems with accuracy and precision are caused by ion suppression rather than differences in extraction recovery. Apparently, the presence of the chlorine atom has a profound influence on the analyte's charge distribution which results in unacceptable

**Table 1.** Accuracy and precision data from calibration standards of D-24851 analyzed using different internal standards

| | **Analogous internal standard** | | | **No internal standard** | | | **SIL internal standard** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Conc. (ng/mL)** | **Accuracy (%)** | | **Precision (%)** | **Accuracy (%)** | | **Precision (%)** | **Accuracy (%)** | | **Precision (%)** |
| | 1 | 2 | | 1 | 2 | | 1 | 2 | |
| 1.00 | 12.9 | -22.9 | 25.3 | 11.9 | 4.95 | 4.90 | -2.92 | -0.604 | 1.64 |
| 5.00 | 13.9 | -22.9 | 26.0 | -24.6 | -3.37 | 15.0 | 4.23 | 10.5 | 4.42 |
| 25.0 | 23.8 | -13.5 | 26.4 | 2.38 | -4.37 | 4.77 | 6.05 | 5.65 | 0.285 |
| 101 | 14.9 | -7.72 | 16.0 | 5.94 | -1.88 | 5.53 | 5.74 | 9.77 | 2.85 |
| 252 | 27.8 | -14.3 | 29.7 | 26.2 | -2.78 | 20.5 | 0.403 | 0.403 | 0.00 |
| 1010 | -13.2 | -30.5 | 12.3 | -12.9 | -6.93 | 4.20 | -11.5 | -8.06 | 2.42 |
| Sum. | 106 | 112 | 136 | 83.8 | 24.3 | 54.9 | 30.8 | 35.0 | 11.6 |

Conc. Concentration; Sum. Absolute summation of the relative accuracy and precision values

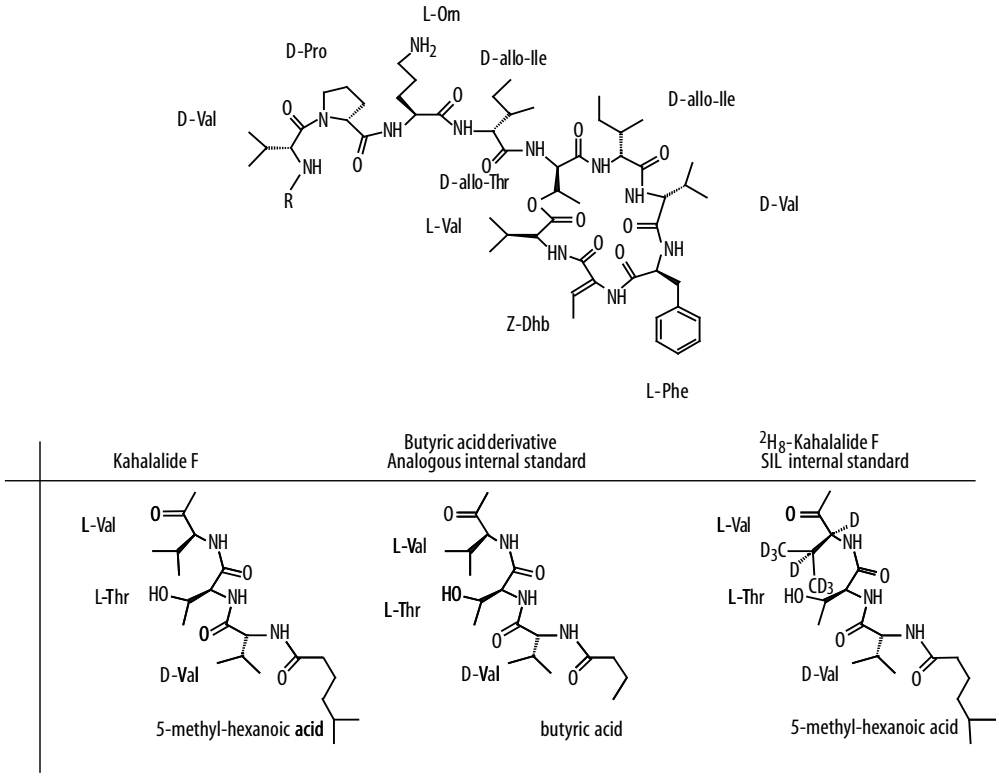



**Figure 4.** Structures of depsipeptide kahalalide F, the analogous internal standard, and the SIL internal standard.

differences in ionization efficiency between analyte and internal standard. The quadruply deuterated SIL internal standard yielded excellent results (Table 1).

For the LC-MS/MS assay of the depsipeptide marine anticancer agent kahalalide F, which contains a 5-methyl-hexanoic acid conjugated to the N-terminal, a butyric acid analogue was available as internal standard (Figure 4) [8]. The assay was validated according to the FDA guidelines and all data were within requirements. When a SIL $D_8$-internal standard became available, it was implemented into the assay. Re-validation results fulfilled all FDA requirements. The data was also statistically evaluated to determine whether the implementation of the SIL internal standard had influenced the assay performance. Calculated concentrations were documented for calibration standards and quality control samples as determined using the two methods. A Levene's test for equality of variances was performed followed by an independent samples t-test to compare the means. The mean bias was 96.8% for the use of the analogous internal standard with a standard deviation of 8.6% (n=284) and 100.3% for the SIL internal standard with a standard deviation of 7.6% (n=340). The Levene's test showed that the variance using the SIL internal standard was significantly lower (p=0.02) than with the use of the butyric acid analogue, indicating that the precision of the method has significantly improved by implementation of the SIL

internal standard. In addition, the accuracy of the assay has improved significantly, since the bias using the SIL internal standard did not deviate significantly from the true value of 100% (p=0.5) while the bias using the analogous internal standard did (p<0.0005).
The results of these studies suggest that when analyte and analogous internal standard differ in functional groups, the internal standard is less likely to be appropriate than when the difference is in the carbon backbone of the molecule. Atoms such as oxygen, nitrogen, sulfur, halogens, etc, are more likely to alter a compound's charge distribution, and as a result ionization efficiency, than carbon-hydrogen moieties. Statistics showed that the performance of the kahalalide F assay improved significantly after substitution of a well functioning analogous internal standard with a SIL internal standard. These experimental data underline the theoretical superiority of SIL internal standards over analogous internal standards.

**Stability**

Although a positive effect of the implementation of a SIL internal standard is probably most appreciated in terms of accuracy and precision, there may be other reasons for the preference of SIL internal standards. When the butyric acid analogue of kahalalide F was used as internal standard, stability of the drug in the processed extract was limited to utmost 16 h at both ambient and refrigerated temperatures because the analogous internal standard demonstrated a higher rate of degradation in the processed extract than kahalalide F, resulting in an overestimation of the analyte concentration [8]. Following the

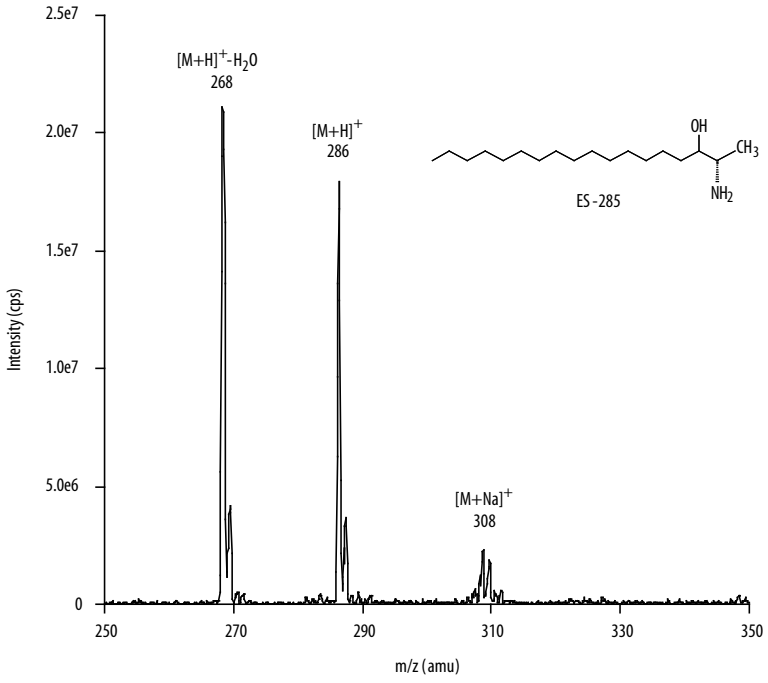



**Figure 5.** Q1 spectrum of ES-285

implementation of the more stable SIL internal standard, the time of stability in the final extract could be prolonged to at least 5 days at ambient temperatures and at least 40 days at refrigerated temperatures.

**Unusual mass transitions**

Anticancer agent ES-285 (2-amino-3-hydroxy octadecane) is a molecule with a lipid-like structure. For quantitation of ES-285 the transition from the molecular ion to a fragment ion corresponding to the elimination of water from ES-285 was monitored in MRM mode. The only peak in the product ion mass spectrum corresponded to this fragment ion. This transition was not considered to be robust for a correct quantification, especially since this ion is also present in the Q1 spectrum of ES-285 and thus formed also in the source (Figure 5). In fact, the dehydrated ES-285 peak is the base peak in the Q1 spectrum, which indicates that elimination of a water molecule from ES-285 happens readily. As a result of this the peak area ratio of ES-285 and dehydrated ES-285 present in Q1 may vary too much as a result of small changes in instrument parameters which hampers a correct quantitation of ES-285. Selected ion monitoring (SIM) was tested as an alternative for MRM monitoring of the described transition, but was discarded due to sensitivity problems. Fortunately, a triply deuterated SIL internal standard became available, which allowed the mass transition from the molecular ion to the fragment ion corresponding to the elimination of water to be used. Plasma was spiked with ES-285 and after processing, a sample was analyzed 60 times, and variation in the ratio (by means of the relative standard deviation) was only 4.9%. It was believed that this robustness could not have been obtained using an analogous internal standard [9].

**Potential metabolites**

An important factor to consider in the selection of an analogous internal standard is whether this compound may be formed *in vivo* by metabolic reactions or degradation of the drug. For instance, for the determination of the marine anticancer agent ET-743 (Trabectedin, Yondelis™) a structural analogue, ET-729, was available, corresponding to *N*-desmethyl-ET-743. ET-743, however, may undergo N-dealkylation to form ET-729. Therefore, many clinical plasma samples were analyzed without addition of an internal standard for the presence of ET-729. ET-729 was not detected and could therefore be utilized as internal standard in the bioanalytical assay of ET-743 [10].

**Deuterium labeled internal standards**

It is generally assumed that an analyte and SIL internal standard have equal physico-chemical properties. Deuterated compounds, however, may show unexpected results. Both Wieling and Kato et al. report on different retention times for the analyte and deuterated internal standard from reversed phase LC (Figure 6) [1,4]. In both cases the deuterated internal standard eluted first. Wieling's explanation for this phenomenon is that deuteriums have a stronger binding with carbon atoms than hydrogens, thereby introducing small differences in physico-chemical properties [1]. Kato et al. describe that they only observed differences in retention times when a neutral eluent was applied (ammonium

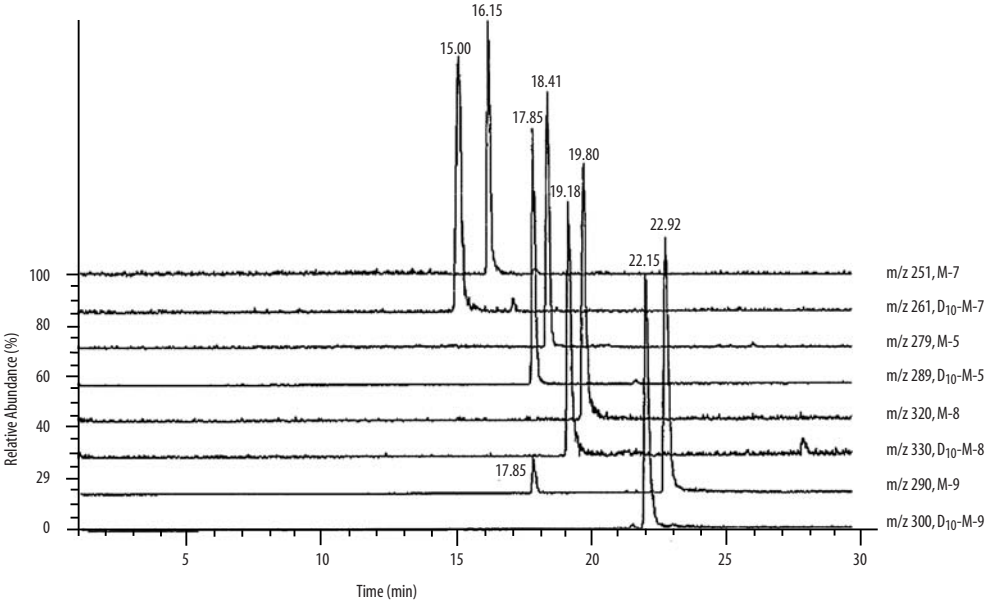



**Figure 6.** Reversed phase LC separation of pibutidine metabolites and their deuterium-labeled analogues [4].

**Table 2.** Hydrogen/deuterium exchange of the $^{13}CD_3$ and $^{13}C_7$-labeled rofecoxib [5]

| **Experiment** | **Content (%) of unlabeled and labeled rofecoxib** | | | | |
|---|---|---|---|---|---|
| | **Unlabeled** | **$^{13}CH_3$** | **$^{13}CDH_2$** | **$^{13}CD_2H$** | **$^{13}CD_3$** |
| A. $^{13}CD_3$ rofecoxib | | | | | |
| 1. Standard in cyclooctane | | | | | >99.5 |
| 2. Standard in acetonitrile | 0.24 | 17.33 | 7.15 | 9.66 | 65.62 |
| 3. Spiked in human plasma, stored: | | | | | |
| a. 0 h | 0.50 | 15.39 | 4.39 | 4.19 | 75.53 |
| b. 3 h | 0.27 | 15.28 | 4.22 | 4.43 | 75.80 |
| c. 6 h | 0.28 | 19.76 | 4.11 | 4.91 | 70.94 |
| | **Unlabeled** | **$^{13}C_5$** | **$^{13}C_6$** | **$^{13}C_7$** | |
| B. $^{13}C_7$ rofecoxib | | | | | |
| 1. Standard in cyclooctane | 0.0 | 0.2 | 5.6 | 94.2 | |
| 2. Standard in acetonitrile | 0.0 | 0.24 | 5.43 | 93.95 | |
| 3. Spiked in human plasma, stored: | | | | | |
| a. 0 h | 0.0 | 0.24 | 5.40 | 94.36 | |
| b. 3 h | 0.0 | 0.11 | 5.56 | 94.32 | |
| c. 6 h | 0.0 | 0.24 | 5.46 | 94.33 | |

acetate (20 mM, pH 7.0)–methanol-acetonitrile). When an acidic solvent system was used, co-elution of analyte and deuterated internal standard was established [4].

Another phenomenon observed by Wieling for deuterated internal standards was a different extraction recovery in the case of haloperidol and deuterated haloperidol, the latter's recovery being 35% lower [1]. This difference may also result from differences in physico-chemical properties but, in addition, may be due to exchange of part of the deuterium atoms by hydrogen atoms. Normally, deuterium atoms that are covalently linked to carbon atoms are not easily exchanged, however Chavez-Eng et al. describe deuterium exchange for the deuterated internal standard of rofecoxib (Figure 7a and b; Table 2) [5]. For comparison the sample was dissolved in cyclooctane and analyzed using gas chromatography. The results demonstrated the purity of the standard and thus that the presence of partially or unlabeled internal standard observed in other solutions is not due to a contamination of the reference standard. The SIL internal standard ($^{13}CD_3$-rofecoxib) demonstrated a loss of deuterium in acetonitrile solutions, which is according to the authors probably due to the traces of water usually present in acetonitrile [5]. Exchange into the $^{13}CH_3$ species was mainly observed. In plasma, exchange was observed to a lesser extend, but was still substantial. After six hours of incubation of $^{13}CD_3$-rofecoxib in human plasma at room temperature, the amount of $^{13}CH_3$-rofecoxib has increased 28%. These results clearly indicated that $^{13}CD_3$-rofecoxib was not suitable as an internal standard in the bioanalytical assay. Another SIL internal standard for rofecoxib was available, $^{13}C_7$-rofecoxib (Figure 7c). Similar experiments were performed as described for the deuterated internal standard and the $^{13}C_7$-labeled internal standard did not show

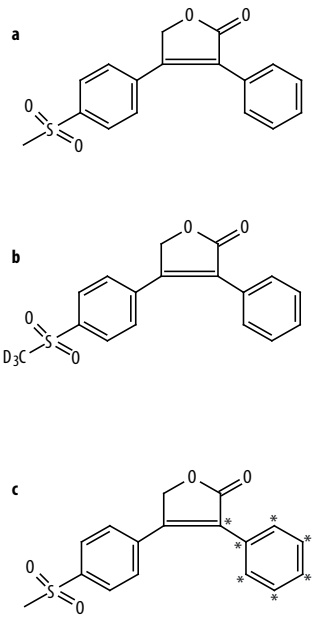



**Figure 7.** Structures of rofecoxib (a) and the $^{13}CD_3$ (b) and $^{13}C_7$ (c) labeled internal standards. The asterisks correspond to $^{13}C$ atoms.

degradation into the unlabeled or partially labeled species. $^{13}C$ labeling is clearly preferred over D incorporation.
The results of this study suggest that SIL internal standards can not be seen as one class of compounds. Deuterium labeled internal standards may demonstrate unwanted behavior, that can compromise a correct quantitation of the analyte.

**Matrix effects**
It is considered very important that an analyte and its analogous internal standard co-elute in order to ensure the same amount of matrix effect for the two compounds. Otherwise, different degrees of ion suppression may be imposed on the two compounds. Interestingly, Sancho et al. demonstrated in a mechanistic study on xenobiotics that co-elution of analyte and analogous internal standard in the presence of high matrix levels is less appropriate for a correct quantitation than non co-elution when matrix levels are low [11]. This unexpected effect may result from high matrix levels that enlarge any differences in ionization efficiency between the two analogous compounds, while this is minimized with low matrix levels. The data described by Fu et al. in their manuscript investigating the influence of matrix effects on the determination of the HIV protease inhibitor indinavir support this theory. They state that when structural analogues are utilized as internal standard, they may only partially compensate for variable ionization effects caused by matrix components when minimum sample preparation or little chromatographic separation of the compounds from matrix components is applied [3]. These findings may suggest that an analogous internal standard is less suitable for high throughput analyses.
On the other hand, Jemal et al. demonstrated that low background matrix levels are essential as well for a correct quantitation of melvalonic acid using a SIL internal standard in urine [12]. In one batch of urine the matrix effect was 26% higher for the deuterated internal standard than for the analyte, which could only be corrected by developing a method that displayed low matrix levels. The observed problems with the consistency of the analyte/internal standard response ratio may result from different chemical properties for deuterated and their unlabeled species, as was described above.

**Miscellaneous**
For quantitative LC-MS/MS a triple quadrupole mass analyzer in the MRM mode is commonly used. This mode ensures unrivaled sensitivity and selectivity. However, it is important to realize that much more is happening than can be seen in the ion chromatogram. Internal standards in general, but especially SIL internal standards can effectively cover-up analytical problems with for instance instability or ion suppression and recovery. For a bioanalytical assay it is essential to determine ion suppression as a part of the validation. Preferably, ion suppression, but also extraction recovery, are determined by comparison of absolute peak areas, since ratios can theoretically be 100% even though the absolute ion suppression may be >90%, which may cause sensitivity problems. Thus, apart from optimal assay performance, sample clean-up is also very important, e.g. for assay sensitivity.
Stability of the analyte and internal standard in the processed extract can give similar

problems. When employing an appropriate internal standard the ratio of analyte and internal standard responses may demonstrate stability, however, the absolute areas may have decreased so dramatically that the LLOQ level falls below the detection limit of the assay.

## Conclusion

In this article several merits and demerits of analogous and SIL internal standards in several bioanalytical quantitative LC-MS assay have been discussed. SIL internal standards are preferred for a correct quantity determination using LC-MS and LC-MS/MS assays. $^{13}C$, $^{15}N$ or $^{17}O$ labeled compounds may be more appropriate that deuterium labeled compounds. However, SIL internal standards are not always available or very expensive, especially when seeking exclusively non-deuterium labeled SIL internal standards. Then, structural analogues can be used, with consideration of the structural similarities between the internal standard and the analyte.

Finally, it is important to realize, especially for less experienced operators that when using SIL internal standards problems with for instance stability, extensive ion suppression or low extraction recoveries may not be observed in the MRM mode, but may cause sensitivity problems. It is advised to determine recovery and ion suppression during the validation of the method.

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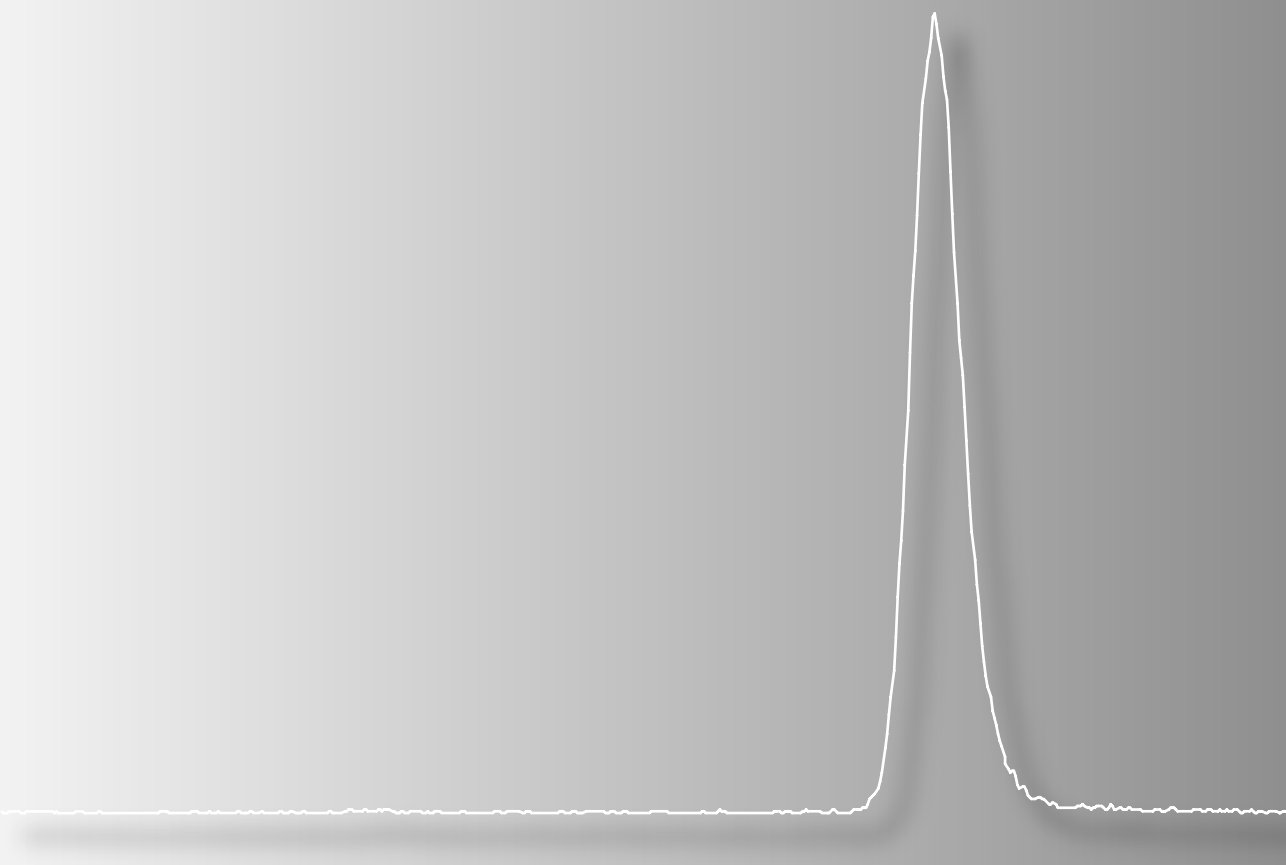


# Summary

## Summary

In this thesis, the development and validation of liquid chromatography tandem mass spectrometric (LC-MS/MS) methods for the quantitative bioanalysis of anti-cancer drugs are described. The monitoring of these drugs in biological fluids and tissues is important during both pre-clinical and clinical development and often in routine clinical use. Traditionally, liquid chromatography (LC) in combination with ultraviolet (UV), fluorescence, or electrochemical detection is employed for this purpose. The successful hyphenation of LC and mass spectrometry (MS), however, has dramatically changed this. MS detection provides better sensitivity and selectivity than UV detection and, in addition, is applicable to a significantly larger group of compounds than fluorescence or electrochemical detection.
LC-MS has now become the method of first choice for the quantitative bioanalysis of many anticancer agents as is demonstrated by the large number of articles that have appeared on the subject so far. In **chapter 1** an overview of these publications is presented. In almost every case, MS detection provides better sensitivity than other detection techniques. Furthermore, due to the selectivity of MS detection less chromatographic separation is required and thus shorter run times can be achieved. As a result high-throughput strategies are explored by an increasing number of researchers.

In **chapter 2** methods for the analysis of marine derived anticancer agents are described. Kahalalide F (KF) is a cyclic depsipeptide, which has shown anticancer activity both *in vitro* and *in vivo* especially against human prostate cancer cell lines. Since very low doses ($\mu g/m^2$ range) were administered to patients enrolled in a phase I trial, a sensitive method was developed and validated for the quantitative analysis of KF in human plasma using LC-MS/MS as described in **chapter 2.1**. LC performed with a mobile phase containing trifluoroacetic acid (TFA), an additive commonly used for separating peptides, resulted in a substantial suppression of the signal for KF on electrospray ionization (ESI)-MS/MS. An alternative approach employing an alkaline mobile phase containing ammonium hydroxide provided an excellent response for KF when detected in the positive ion mode. Plasma samples were prepared for LC-MS/MS by solid phase extraction (SPE) on C18 cartridges. A butyric acid analogue of KF was used as the internal standard. The lower limit of quantitation (LLOQ) using a 500 $\mu$L sample volume was 1 ng/mL and the linear dynamic range extended to 1,000 ng/mL.
Internal standards are very important in quantitative MS assays, since MS is not quantitative by nature. Stable isotopically labeled (SIL) internal standards are especially suited, since they are in theory chemically identical to the analyte. In **chapter 2.2** the replacement of the butyric acid analogue internal standard for the KF assay with a SIL internal standard ($^2H_8$-KF) is described. The results demonstrate that, although validation results of the original assay were within requirements, the assay performance had significantly improved after the replacement. In addition, stability in the reconstituted extract, originally established at utmost 16 hours, increased to at least 5 days at ambient temperatures and at least 40 days at nominally 2-8 °C, due to similar stability profiles of

the analyte and SIL internal standard.
In **chapter 2.3** the development and validation of a method for the quantitative analysis of the novel marine anticancer agent ES-285 in human, mouse, rat, and dog plasma using LC- MS/MS is presented. Sample preparation was carried out by a simple protein precipitation procedure with acetonitrile, containing isotopically labeled $^{2}H_{3}$-ES-285 as internal standard. Aliquots of the supernatant were directly injected onto the analytical system. An unusual mass transition was monitored in the multiple reaction monitoring mode from the molecular ion to a fragment ion corresponding to the elimination of water. Normally, this transition is not considered to be very robust. However it was demonstrated by excellent validation results that, using the SIL internal standard, the transition was suitable. The LLOQ was 10 ng/mL in all matrices (100 µL) and this method has been used successfully to analyze over 500 samples in pre-clinical trials. For the application of the assay in clinical studies, however, the LLOQ was expected to be insufficient as ES-285 is administered in very low doses. In **chapter 2.4** the transfer of the existing method for the quantitative determination of ES-285 in human plasma using LC-MS/MS from an API 365 to a more sensitive API 3000 mass spectrometer is described. The transfer appeared not straightforward. Problems arose resulting from carry over and interferences. In addition, due to the expansion of the calibration range, data needed to be weighted with a different factor to increase the accuracy of the lower concentrations. After finding appropriate solutions for these issues, the LLOQ could be lowered from 10 to 1 ng/mL. The usefulness and necessity of the modified assay was demonstrated by analysis of plasma samples from a patient receiving a low dosage of the drug.
The development and validation of a simple and sensitive assay for the quantitative analysis of the marine anticancer agent Yondelis™ (ET-743, trabectedin) in human plasma using LC with column switching and tandem MS/MS are presented in **chapter 2.5**. After protein precipitation with methanol, diluted extracts were injected onto a small LC guard for on-line concentration and further clean-up of the sample. Next, the analyte and deuterated internal standard were back-flushed onto an analytical column for separation and subsequent detection in an API 2000 triple quadrupole mass spectrometer. The LLOQ was 0.05 ng/mL using 100 µL of plasma with a linear dynamic range up to 2.5 ng/mL. The time needed for off-line sample preparation has been reduced by a tenfold, when compared to an existing LC-MS/MS method for ET-743 in human plasma, employing a labor-intensive SPE procedure for sample pretreatment. The presented column switching method could be successfully applied in phase II clinical trials with Yondelis™ and pharmacokinetic monitoring.

ABT-518 is an inhibitor of matrix metalloproteinases, which are associated with tumor growth and development of metastasis. An LC-MS/MS assay for the quantitative analysis of ABT-518 and the screening of six potential metabolites in human plasma has been developed and validated to support a phase I study with the drug as presented in **chapter 3.1**. Plasma samples were prepared for analysis using a simple SPE method on phenyl cartridges. The best chromatographic speed (total run time was 8 min) and peak shapes were obtained by employing an alkaline mobile phase as described earlier for

the bioanalysis of KF. Furthermore, an alkaline eluent was favored in order to obtain a better overall sensitivity for the protonated analytes. Two analogous internal standards were available for this assay, one of which appeared to be appropriate for a correct quantitation of the parent drug. For quantitation of the metabolites, however, these internal standards were not suitable. The differences in molecular structure between the metabolites and internal standards caused large differences in ionization efficiency. Therefore, only screening of the metabolites with accuracies of ±30% and precisions less than 30% was performed. The dynamic range was from 10 to 1,000 ng/mL from 500 µL of plasma for ABT-518 and the metabolites were detected at the same levels. ABT-518 and all of the metabolites included in the assay were identified in plasma from patients treated with ABT-518.

Tubulin inhibitors are an important part of current chemotherapeutic treatment. The development of an LC-MS/MS assay for the quantitative analysis of the novel tubulin inhibitor D-24851 in human plasma and urine is described in **chapter 4.1**. D-24851 and the deuterated internal standard ($^{2}H_{4}$-D-24851) were extracted from 250 µL of plasma or urine using liquid-liquid extraction (LLE) with hexane-diethylether (1:1, v/v). This SIL internal standard was needed for a correct quantitation of the drug, since an analogous internal standard, which had been successfully applied in an LC-UV assay, resulted in unacceptable assay performance results. For human plasma a dynamic range from 1 to 1,000 ng/mL was validated, and for human urine a range from 0.25 to 50 ng/mL. The assay has been successfully validated and subsequently applied to support a phase I clinical trial with orally administered D-24851.
Tubulin inhibitor paclitaxel has been used in cancer treatment for many years. An assay for the determination of paclitaxel in human plasma, human brain tumor tissue, mouse plasma, and mouse brain tumor tissue has been developed and validated to support pre-clinical and clinical studies with the drug (chapter 4.2). Paclitaxel was extracted from the matrices using LLE with tert-butylmethylether, followed by chromatographic analysis using an alkaline eluent in combination with positive ESI tandem mass spectrometry. Validation results indicate that calibration standards in human plasma can be used to quantify paclitaxel in all tested matrices. In human samples, the validated range for paclitaxel was from 0.25 to 1,000 ng/mL using 200 µL plasma aliquots and from 5 to 5,000 ng/g using 50 µL tumor homogenate aliquots (0.2 g tissue/mL control human plasma). In mice, the ranges were from 1 to 1,000 ng/mL and from 5 to 5,000 ng/g using 50 µL plasma aliquots and 50 µL tumor homogenate aliquots (0.2 g tissue/mL control human plasma), respectively. The method was applied successfully to studies generating only small sample volumes (e.g. mouse plasma, tumor tissue), but also to studies in human plasma requiring a low LLOQ.

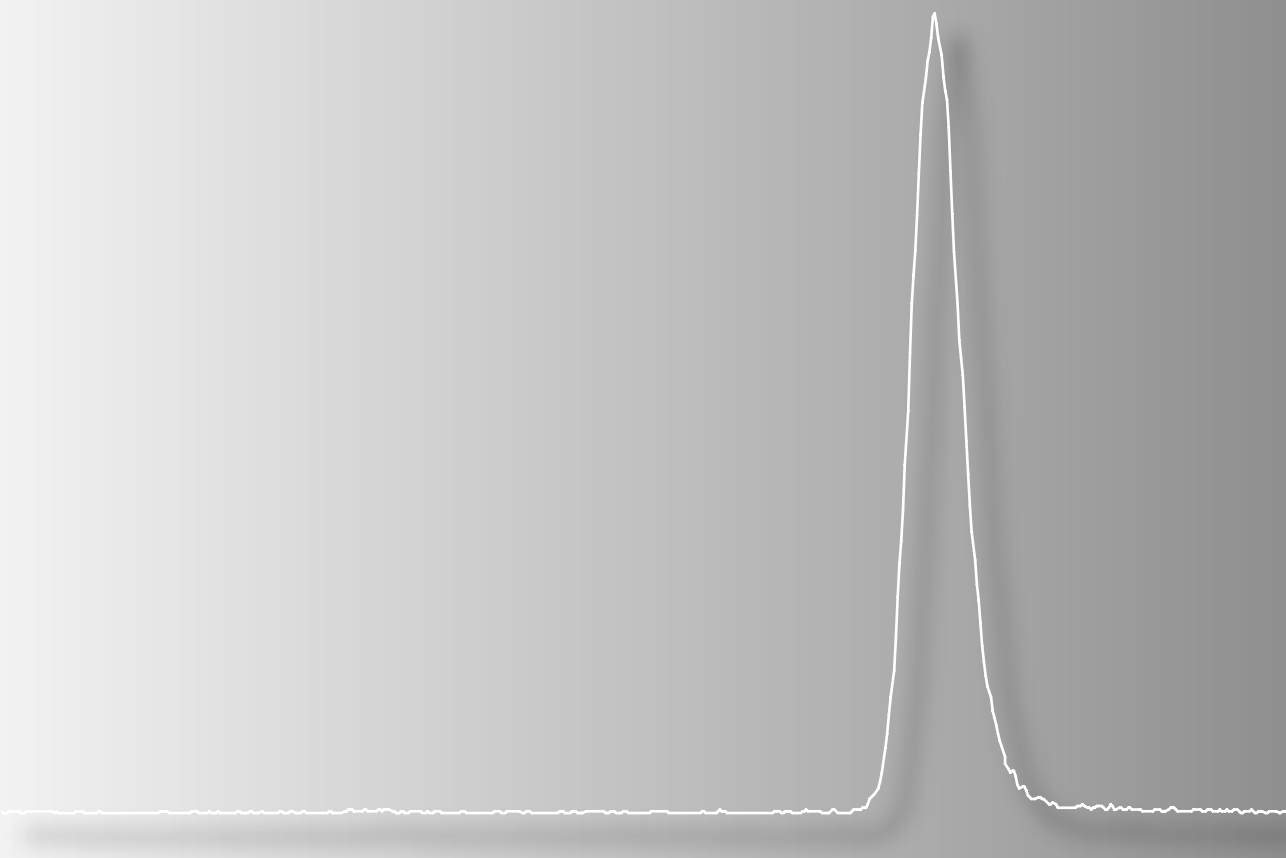
For the bioanalysis of topoisomerase I inhibitor and camptothecin derivative, topotecan, traditionally LC-fluorescence is used. In **chapter 5.1** the first LC-MS/MS assay for the quantitative bioanalysis of topotecan is presented. Topotecan was determined as the total of its lactone and hydroxy carboxylate forms in human plasma and plasma ultra-

filtrate after administration of the liposomally encapsulated drug. Sample aliquots were deproteinized with methanol after addition of the $^{2}H_{6}$-topotecan internal standard. LC was performed using an eluent containing methanol-water and TFA. TFA was necessary to obtain acceptable chromatography. Due to the pH dependent equilibrium between the closed and open ring forms of topotecan, LC under basic conditions was not possible. In order to achieve the target LLOQ of 0.1 ng/mL in plasma ultrafiltrate, a TFA fix (post-column addition of a mixture of 2-propanol and propionic acid) was required to increase the MS signal after suppression caused by TFA. The target LLOQ of 1 ng/mL in plasma could be obtained without the TFA fix. The validated ranges were 1-500 ng/mL and 0.1-50 ng/mL in plasma and plasma ultrafiltrate, respectively, using 500 µL sample volumes. This method will be applied to upcoming pharmacokinetic studies of topotecan in human plasma and plasma ultrafiltrate after administration of liposomal topotecan.
Currently, oral administration of topotecan is tested in clinical trials to increase patient comfort and decrease hospital admittance time. Due to the low oral bioavailability of topotecan, which is contributed to the expression of drug transporters P-glycoprotein and Breast Cancer Resistance Protein in the gastro-intestinal tract, the drug is administered in combination with an inhibitor of these proteins, elacridar. Pharmacokinetic profiling of this inhibitor is also important during phase I trials. Therefore, an LC-MS/MS method for the determination of elacridar in human plasma has been designed (**chapter 5.2**). The internal standard was stable isotopically labeled elacridar. Sample pretreatment involved LLE with *tert*-butylmethylether. Also for this assay the alkaline eluent in combination with positive ionization appeared to be most suited for sensitive analysis, good chromatographic performance, and short run times. A dynamic range from 1 to 500 ng/mL from 100 µL human or dog plasma was achieved.

From the analytical methods presented in chapters 2 to 5, it can be concluded that LC-MS/MS is very suitable for sensitive and selective quantitation of anticancer agents in biological samples. Furthermore, LC with an alkaline mobile phase in combination with MS detection in the positive ion mode appeared very useful for the analysis of four agents described in this thesis (KF, ABT-518, paclitaxel, and elacridar). This system may be applicable to other basic drugs.

In the final chapter of this thesis (**chapter 6.1**) the use of internal standards is discussed. SIL internal standards are the first choice, but deuterium labeled compounds may demonstrate unexpected behavior, such as different retention times or recoveries than the analyte. In addition, a SIL internal standard with identical chemical properties as the analyte may cover up assay problems with stability, recovery, and ion suppression.

In this thesis the development and validation of LC-MS/MS methods for several anticancer agents are described. These assays were developed in order to support clinical pharmacological studies. LC-MS/MS appeared to be very suitable for this purpose. Hopefully, this thesis has made a relevant contribution to cancer pharmacotherapy.



# Samenvatting

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In dit proefschrift worden de ontwikkeling en validatie van LC-MS/MS methoden voor de kwantitatieve bioanalyse van cytostatica beschreven. Het bepalen van deze klasse van geneesmiddelen in biologische vloeistoffen en weefsels is een belangrijk onderdeel van het preklinische en klinische ontwikkelingstraject van een nieuw cytostaticum. In de afgelopen jaren is voornamelijk vloeistofchromatografie (LC) in combinatie met ultraviolet (UV), fluorescentie of electrochemische detectie voor dit doel gebruikt. De succesvolle koppeling van LC en massaspectrometrie (MS) heeft dit echter drastisch veranderd. De gevoeligheid en selectiviteit bij MS detectie is beter dan UV detectie. Bovendien is de techniek toepasbaar op een bredere groep verbindingen dan fluorescentie of electrochemische detectie.
LC-MS is nu de methode geworden die bij voorkeur gebruikt wordt voor de kwantitatieve bioanalyse van veel cytostatica zoals blijkt uit het grote aantal artikelen dat over dit onderwerp is verschenen. In **hoofdstuk 1** wordt een overzicht gegeven van deze publicaties. In bijna alle gevallen laat MS een verbeterde gevoeligheid zien ten opzichte van andere detectietechnieken. Bovendien is door de selectiviteit van MS detectie geen volledige chromatografische scheiding nodig, waardoor methoden met kortere analysetijden, d.w.z. high throughput (letterlijk: snelle doorvoer) methoden konden worden ontwikkeld.

In **hoofdstuk 2** worden methoden beschreven voor de analyse van nieuwe antikanker geneesmiddelen afkomstig uit zeeorganismen. Kahalalide F (KF) is een cyclisch depsipeptide waarvoor *in vitro* en *in vivo* antitumor activiteit is aangetoond, voornamelijk gericht tegen humane prostaatkankercellijnen. Omdat KF in zeer lage doseringen (µg/$m^2$ niveau) wordt toegediend aan patiënten die deelnemen aan een fase I studie, was een gevoelige methode vereist. De ontwikkeling van een gevoelige LC-MS/MS methode voor KF staat beschreven in **hoofdstuk 2.1**. De mobiele fase bevatte oorspronkelijk trifluorazijnzuur (TFA), een eluens toevoeging die vaak toegepast wordt voor de chromatografische scheiding van peptiden. Het signaal van KF in electrospray (ESI)-MS werd echter sterk onderdrukt in aanwezigheid van TFA. Met een basisch, ammonium hydroxide bevattend eluens, werd daarentegen een uitstekend MS signaal voor KF gevonden waarbij de stof positief werd geioniseerd. De monstervoorbewerking van plasmamonsters werd uitgevoerd m.b.v. vastefase-extractie (SPE) op C18 kolommen. De interne standaard was een butaanzuuranaloog van KF. De laagste bepalingsgrens (LLOQ) is 1 ng/mL wanneer 500 µL monster wordt opgewerkt, met een lineair bereik tot 1.000 ng/mL. Interne standaarden zijn erg belangrijk voor kwantitatieve MS bepalingen, met name omdat MS van nature niet kwantitatief is. Interne standaarden gemerkt met stabiele isotopen ("stable isotopically labeled" SIL interne standaarden) zijn hiervoor in het bijzonder geschikt, omdat zij, theoretisch, chemisch identiek zijn aan de te bepalen verbinding. In **hoofdstuk 2.2** wordt de vervanging van de analoge interne standaard voor de bepaling van KF (het butaanzuurderivaat) door een SIL interne standaard ($^2H_8$-KF) beschreven. De resultaten tonen aan dat hiermee de juistheid en precisie van de

methodiek significant verbeteren. Bovendien kon de stabiliteit in het gereconstitueerde extract, oorspronkelijk vastgesteld op maximaal 16 uur, verlengd worden tot minstens 5 dagen bij kamertemperatuur en minstens 40 dagen bij 2-8 °C, doordat KF en de SIL interne standaard, in tegenstelling tot de analoog, vergelijkbare stabiliteitskarakteristieken vertonen.
In **hoofdstuk 2.3** worden de ontwikkeling en validatie van een methode voor de kwantitatieve analyse van het nieuwe cytostaticum ES-285 in humaan, muizen-, ratten- en hondenplasma m.b.v LC-MS/MS beschreven. Monstervoorbewerking bestond uit een eenvoudige eiwitprecipitatie met acetonitril dat de SIL interne standaard ($^{2}H_{3}$-ES-285) bevatte. Tien microliter van het supernatant werd direct geïnjecteerd in het analytisch systeem. Een ongebruikelijke en niet als robuust beschouwde massatransitie van het moleculair ion naar een fragment dat overeenkomt met de afsplitsing van water werd gemeten. Echter, met het gebruik van een SIL interne standaard bleek deze transitie zeer geschikt zoals blijkt uit de uitstekende validatieresultaten. De LLOQ was 10 ng/mL in alle matrices en deze methode is daarna toegepast voor de analyse van meer dan 500 monsters in preklinische onderzoeken. Van het gebruik van deze methode in klinische studies werd echter verwacht dat de LLOQ onvoldoende laag zou zijn, omdat ES-285 in zeer lage doseringen wordt toegediend. In **hoofdstuk 2.4** wordt de overstap van de bestaande methode voor de kwantitatieve bepaling van ES-285 in humaan plasma m.b.v. LC-MS/MS op een API 365 massaspectrometer naar een gevoeliger API 3000 systeem beschreven. Deze overstap bleek niet zonder complicaties te verlopen. Er ontstonden problemen door "carry-over" en interferenties. Door de uitbreiding van de calibratielijn naar 1 ng/mL was tevens een andere weegfactor vereist om de juistheid van de lagere concentraties te verbeteren. Nadat er geschikte oplossingen waren gevonden voor deze problemen, kon de LLOQ verlaagd worden van 10 naar 1 ng/mL. Bij de analyse van plasmamonsters van patiënten die ES-285 kregen toegediend bleek de noodzaak van metingen in het 1 tot 10 ng/mL bereik.
De ontwikkeling en validatie van een gevoelige methode voor de kwantitatieve analyse van Yondelis™ (ET-743, trabectedin) in humaan plasma m.b.v. LC met kolomschakeling en tandem MS worden beschreven in **hoofdstuk 2.5**. Na eiwitprecipitatie m.b.v. methanol werden verdunde extracten op een korte LC kolom (10 x 3.0 mm I.D.) geïnjecteerd, "on-line" geconcentreerd en daarna volgde er verdere zuivering van het monster. Vervolgens werden ET-743 en de gedeutereerde interne standaard met een omgekeerde stroom van de voorkolom geelueerd. Via een analytische kolom volgde detectie in een API 2000 massa spectrometer. De LLOQ was 0.05 ng/mL vanuit 100 µL plasma met een lineair bereik tot 2.5 ng/mL. De tijd die nodig was voor "off-line" monstervoorbewerking is tien maal verkort vergeleken met de bestaande LC-MS/MS methode waarbij gebruik wordt gemaakt van een arbeidsintensieve SPE monstervoorbewerking. De ontwikkelde methode is toegepast in verschillende fase II studies met farmacokinetische bepaling van Yondelis™.

ABT-518 is een remmer van matrix metalloproteinase enzymen die een belangrijke rol spelen bij tumorgroei en de ontwikkeling van metastasen. Een LC-MS/MS methode voor de kwantitatieve analyse van ABT-518 en de semi-kwantitatieve bepaling van zes

potentiële metabolieten in humaan plasma is ontwikkeld en gevalideerd, ter ondersteuning van een fase I studie (**hoofdstuk 3.1**). Monstervoorbewerking werd uitgevoerd m.b.v. een SPE methode op fenyl kolommen. De kortste chromatografische analysetijd (8 min) en optimale piekvormen werden verkregen door gebruik te maken van een alkalisch eluens zoals ook toegepast voor de bioanalyse van KF. Verder bleek een alkalische mobiele fase ook de hoogste gevoeligheid te geven voor de geprotoneerde verbindingen. Er waren twee analoge interne standaarden beschikbaar voor deze bepaling, waarvan één geschikt bleek te zijn om een correcte kwantificering van ABT-518 te bewerkstellingen. De interne standaarden waren echter niet bruikbaar voor kwantificering van de metabolieten volgens de FDA (Food and Drug Administration) normen. De verschillen in structuur tussen deze interne standaarden en de metabolieten veroorzaakten grote verschillen in ionisatie-efficiëntie. Het bleek wel mogelijk de metabolieten semi-kwantitatief te bepalen met eisen voor juistheid en precisie lager dan 30%. Het gevalideerde bereik voor ABT-518 (en de metabolieten) was 10 tot 1.000 ng/mL vanuit 500 µL plasma. ABT-518 en de zes metabolieten konden allen worden geanalyseerd in plasma van patiënten die met ABT-518 werden behandeld.

Tubuline remmers vormen een belangrijke groep oncolytica. De ontwikkeling van een LC-MS/MS methode voor de kwantitatieve analyse van de nieuwe tubuline remmer D-24851 in humaan plasma en urine wordt beschreven in **hoofdstuk 4.1**. D-24851 en de gedeutereerde interne standaard ($^{2}H_{4}$-D-24851) werden geëxtraheerd vanuit 250 µL plasma of urine d.m.v. vloeistof-vloeistof extractie (LLE) met hexaan-diethylether (1:1, v/v). In humaan plasma was het gevalideerde concentratiegebeid van 1 tot 1.000 ng/mL, in urine van 0.25 tot 50 ng/mL. De methode is uitgebreid volgens de huidige FDA richtlijnen gevalideerd en vervolgens toegepast voor de ondersteuning van een klinische studie met oraal gedoseerd D-24851.
De tubuline remmer paclitaxel wordt al vele jaren toegepast bij de behandeling van verschillende vormen van kanker. Een methode voor de bepaling van paclitaxel in humaan plasma, humaan hersentumorweefsel, muizenplasma, en muizen hersentumorweefsel werd ontwikkeld en gevalideerd voor de ondersteuning van preklinische en klinische studies (**hoofdstuk 4.2**). Paclitaxel werd geëxtraheerd vanuit de verschillende matrices d.m.v. LLE met *tert*-butylmethylether, gevolgd door chromatografische analyse met een alkalisch eluens in combinatie met positieve ESI tandem MS. Resultaten van de validatie tonen dat de calibratiestandaarden bereid in humaan plasma ook gebruikt kunnen worden om paclitaxel te kwantificeren in de andere geteste matrices. In de humane monsters ligt het gevalideerde bereik tussen 0.25 en 1.000 ng/mL vanuit 200 µL plasma en tussen 5 en 5.000 ng/g vanuit 50 µL tumorhomogenaat (0.2 g weefsel/mL blanco humaan plasma). In muizenmonsters is het bereik tussen 1 en 1.000 ng/mL vanuit 50 µL plasma en tussen 5 en 5.000 ng/g vanuit 50 µl tumorhomogenaat (0.2 g weefsel/mL blanco humaan plasma). De methode bleek met name geschikt voor studies waarbij slechts kleine hoeveelheden monster beschikbaar waren (bijv. muizenplasma en tumorweefsel).

Voor de bioanalyse van de topoisomerase I remmer en camptothecine derivaat, topotecan,

wordt traditioneel LC-fluorescentie gebruikt. In **hoofdstuk 5.1** wordt de eerste LC-MS/MS methode voor de kwantitatieve bioanalyse van topotecan beschreven. Topotecan werd bepaald als het totaal van zijn lacton en hydroxy-carboxylaat vormen in humaan plasma en plasma ultrafiltraat na toediening van liposomaal topotecan. De monsters werden onteiwit m.b.v. methanol na toevoeging van de $^{2}H_{6}$-topotecan interne standaard. De mobiele fase bevat methanol, water en TFA. TFA was noodzakelijk om acceptabele chromatografie te verkrijgen. Vanwege het pH afhankelijke evenwicht tussen de open en gesloten ringvorm van topotecan, was LC met een basisch eluens niet mogelijk. Om de beoogde LLOQ van 0.1 ng/mL te bereiken in plasma ultrafiltraat was een zogenaamde TFA fix (het na de kolom bijmengen van een mengsel van 2-propanol en propionzuur) noodzakelijk om het MS signaal te verbeteren na suppressie door TFA. De beoogde LLOQ van 1 ng/mL in plasma kon bereikt worden zonder de TFA fix. Het gevalideerde bereik voor topotecan was van 1 tot 500 ng/mL in plasma en van 0.1 tot 50 ng/mL in plasma ultrafiltraat vanuit 500 µL monster. Deze methode zal worden toegepast bij toekomstige studies waarbij patiënten liposomaal geformuleerd topotecan krijgen toegediend.
Op dit moment wordt de orale toediening van topotecan in ons instituut getest. De orale biologische beschikbaarheid van topotecan is echter laag en variabel, hetgeen wordt toegeschreven aan de expressie van de geneesmiddelentransporterende eiwitten P-glycoproteïne en het "Breast Cancer Resistance Protein" in het maagdarmkanaal. De functies van deze eiwitten kunnen worden geblokkeerd m.b.v. het middel elacridar. Het bepalen van de farmacokinetiek van elacridar is daarbij ook van belang. Daarom werd een LC-MS/MS methode voor de bepaling van elacridar in plasma ontwikkeld (**hoofdstuk 5.2**). SIL elacridar werd gebruikt als interne standaard. De monstervoorbewerking bestond uit LLE met *tert*-butylmethylether. Ook voor deze methode was een alkalisch eluens in combinatie met positieve ionisatie het meest geschikt voor een gevoelige analyse, goede chromatografie en korte analysetijden. Het gevalideerde bereik was van 1 tot 500 ng/mL waarbij 100 µL humaan plasma (of hondenplasma) in bewerking werd genomen.

Uit de resultaten beschreven in de hoofdstukken 2 tot en met 5 kan worden geconcludeerd dat LC-MS/MS zeer geschikt is voor een gevoelige en selectieve kwantificering van oncolytica in biologische matrices. Bovendien blijkt dat LC met een alkalische mobiele fase in combinatie met positieve ionisatie in de MS zéér bruikbaar is voor de analyse van vier verbindingen beschreven in dit proefschrift (KF, ABT-518, paclitaxel en elacridar). Dit analytisch systeem is mogelijk algemeen toepasbaar op basische geneesmiddelen. In het laatste hoofdstuk van dit proefschrift (**hoofdstuk 6.1**) wordt het gebruik van interne standaarden bij kwantitatieve LC-MS/MS methoden beschreven. SIL interne standaarden hebben de voorkeur. Hierbij zij opgemerkt dat bij deuterium gemerkte verbindingen op voorhand onverwachte fenomenen kunnen optreden, zoals verschillen in retentietijd of "recovery" met de te bepalen stof. Verder kan een SIL interne standaard met identieke chemische eigenschappen als die van de te bepalen stof problemen met stabiliteit, "recovery" en ionsuppressie verhullen.

Dit proefschrift beschrijft de ontwikkeling en validatie van LC-MS/MS methoden van

verschillende (nieuwe) oncolytica. De vraagstellingen die hiertoe hebben geleid waren voornamelijk afkomstig uit de kliniek en hadden tot doel ondersteuning te bieden aan het klinisch farmacologisch onderzoek. LC-MS/MS bleek hier bij uitstek geschikt voor te zijn. Het is te hopen dat met dit proefschrift een nuttige bijdrage wordt geleverd aan de medicamenteuze kankertherapie.

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Ellen

## Curriculum vitae

Ellen Stokvis werd geboren op 18 januari 1977 te Amersfoort. In 1995 behaalde zij het Gymnasium diploma aan het Corderius Lyceum te Amersfoort. In datzelfde jaar begon zij aan de studie Scheikunde aan de Universiteit Utrecht. Na o.a. een stage bij TNO Voeding te Zeist waar een LC-fluorescentie methode werd ontwikkeld voor de bepaling van een conserveermiddel in cosmetica, werd gestart met het doctoraal onderzoek. Er werd onderzoek gedaan naar het *E. coli* Glyoxalase I enzym en de non-covalente interacties van het enzym met inhibitors m.b.v. nanoES-TOF-MS onder supervisie van prof. dr A.J.R. Heck bij de vakgroep biomoleculaire massaspectrometrie. Na het behalen van het doctoraal examen in 2000 werd gestart met het in dit proefschrift beschreven promotie onderzoek in de apotheek van het Slotervaart Ziekenhuis te Amsterdam, onder begeleiding van prof. dr J.H. Beijnen, prof. dr J.H.M. Schellens en dr H. Rosing.

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