Development of practice-oriented LC-MS/MS methods for the determination of important drugs and their application for building PK/PD concepts

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Für meine Familie und Freunde

1. Introduction

1.1. The background of this thesis

Before the coupling of liquid chromatography (LC) to mass spectrometry, gas chromatography (GC), which became commercially available in 1967, was a widely used chromatographic method coupled to mass spectrometry. In GC the analytes are separated in the gas phase and enter directly into the mass spectrometer after leaving the GC column. Using GC as separation method requires sample volatility, extraction of aqueous samples, sometimes derivatization of analytes and solving the problem of thermal degradation of the samples in the GC oven [1]. With the idea of coupling LC to mass spectrometry the door was opened for substances that were not measurable with GC analysis, especially in field of life sciences.

However, a direct inlet of the LC eluent is not possible when it is coupled to a mass spectrometer. The eluent has to be removed or evaporated, otherwise the high vacuum in the mass spectrometer breaks down. Additionally, the analyte has to be ionized because only charged molecules can be analyzed. Therefore the use of an interface between the HPLC system and the mass spectrometer is necessary [2].

Various ionization sources (interfaces) are available among electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photo ionization (APPI) and matrix assisted laser desorption ionization (MALDI). ESI, APCI, APPI and MALDI are soft ionization techniques, which mean that they do not destroy the sample during the ionization process, and are therefore often used in bioanalytical analysis. It depends on the physical and chemical properties of the analyte as well as on the HPLC conditions which ionization source works best. ESI and APCI are the two most common soft ionization techniques and were used exclusively in this thesis.

Since the introduction of LC-MS/MS in routine analysis the technique underwent a rapid and continuous development. The first mass spectrometers needed enormous space and were extreamly heavy. Modern triple quadrupole mass spectrometers are easy transportable and are mostly bench top systems with a small footprint. Also the handling and maintenance became easier and made the whole LC-MS/MS technology accessible for the mass market.

The advantages of liquid chromatography coupled to mass spectrometry are perfectly clear. Small and large molecules can be investigated and quantitative and qualitative data can be obtained with a high reliability, speed and sensitivity. Through triple quadrupole technology high selectivity is achieved and a multiple component analysis, also with co-eluting analytes, is easy to handle. With today's ionization sources available on the market many substance classes can be analyzed with this technique. The continuous growing sensitivity enables easy sample handling especially for water or waste water samples ("dilute and shoot"). But also complex biological matrices can be analyzed after sample work up with low background using the multiple reaction mode. It is not surprising that in spite of the high acquisition costs the LC-MS/MS technology found and is still finding its way into many research laboratories. Old LC-UV or LC-FLUO systems are often replaced by modern LC-MS/MS systems. Especially when high sensitivity is required and a lot of samples have to be analyzed LC-MS/MS is the method of choice. Today the main applications of LC-MS/MS are in the field of pharmaceutical industry, contract research, food control and environmental analysis.

The following chapter gives a short overview of the quadrupole operating principles and the triple quadrupole technology.

1.2. Quadrupole technology

1.2.1. Quadrupole operating principles

Before the triple quadrupole can be discussed, the operating principle of the single quadrupole has to be considered. **Figure 1** shows a single quadrupole mass spectrometer with an ionization source, the quadrupole rods and the detection unit.

The ionization is performed under atmospheric pressure and the mass separation as well as the detection of the ions in the high vacuum.

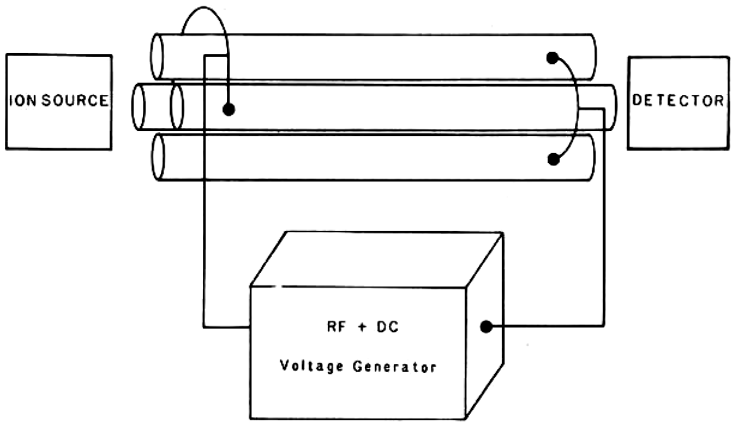

Figure 1: *Single quadrupole mass spectrometer [3]*

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On opposite metal rods of the quadrupole a voltage consisting of a fixed potential U (direct current DC) and an alternating radiofrequency RF is applied. **Scheme 1** shows the corresponding relation where V is the amplitude and ω the frequency of the radiofrequency.

$$\pm \underbrace{(U}_{DC} + \underbrace{V\cos(\omega t)}_{RF}$$

Scheme 1: *Potential between opposite rods*

Through alternating the DC and the RF values at a fixed DC/RF ratio only ions with a specific m/z value will have a stable trajectory through the quadrupole and all others will be discharged at the rods. For this reason the quadrupole is also known as “mass filter”.

The trajectories of the ions through the quadrupole can be described with the solutions of the Mathieu equation (**Scheme 2**), a complex differential equation, and are simplified helical:

$$\frac{d^2u}{d\xi^2} + (a_u - 2q_u\cos 2\xi)u = 0$$

Scheme 2: *Mathieu equation*

Where $\xi = W \cdot t/2$ (W=RF frequency, t=time), and u represents position along the coordinate axes x or y.

The Mathieu parameters a_u and q_u are defined as:

$$a_u = \frac{8eU}{mr_0^2\Omega^2} \quad \text{and} \quad q_u = \frac{4eV}{mr_0^2\Omega^2}$$

Scheme 3: *Mathieu parameters*

Where e is the charge on an electron, U is the applied DC voltage, V is the applied zero-to-peak RF voltage, m is the mass of the ion, and r is the effective radius between electrodes.

Only certain combinations of a and q give stable solutions to the Mathieu equation, that is ions passing through the quadrupole. Moreover, only a/q combinations that give stable solutions for both the x and y directions will be useful.

To become familiar with sensitivity and resolution in quadrupole mass spectrometry a graphical solution of the Mathieu equation can be performed. The solutions can be plotted as stability diagrams as shown exemplary in **Figure 2 (A)** for the m/z values 28, 69 and 219.

For each m/z value a triangle is received and whenever the ratio of DC/RF is within the boundary, the specific m/z value is allowed to pass the quadrupole. The two straight lines in **Figure 2 (A)** represent working lines of the quadrupole and operate at a constant DC/RF ratio. Operation with constant resolution of the quadrupole is represented by the continuous straight line. The effect can be seen in **Figure 2(B)**.

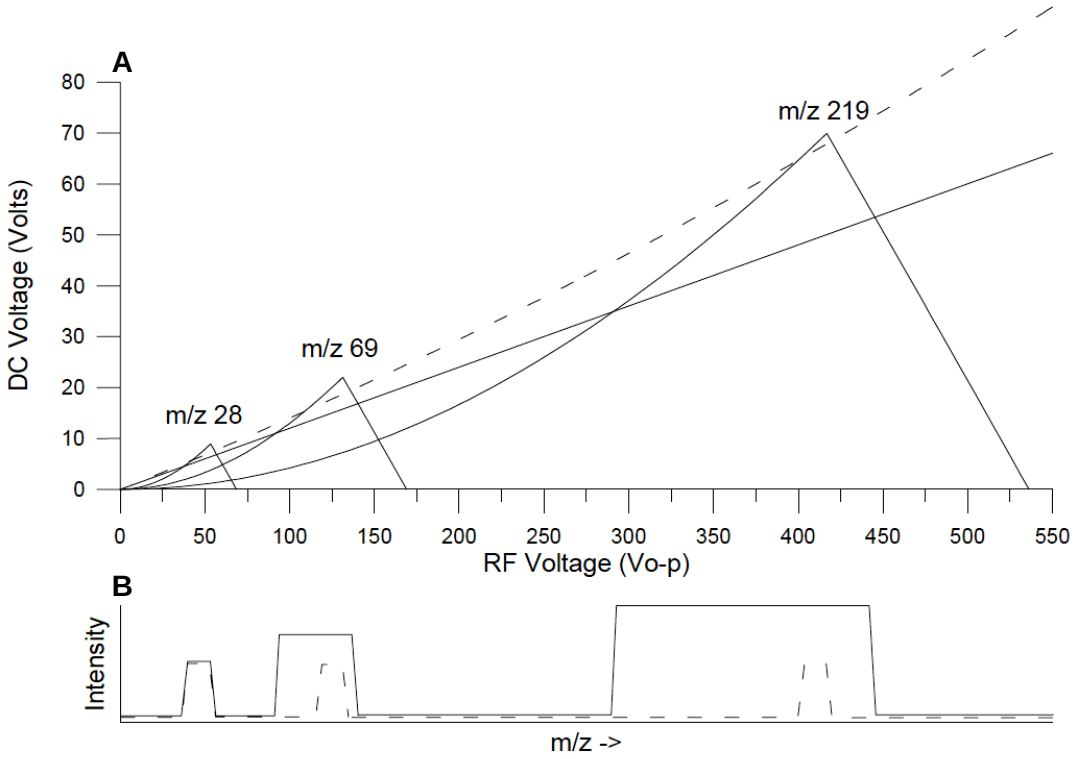


Figure 2: *Graphical approach of the Mathieu equation [4]*

Figure is provided courtesy of Extrel CMS, LCC

Working with constant resolution results in increasing peak width with increasing m/z values, which means higher sensitivity but lower mass resolution. Normally the quadrupole is operated, as shown by the dotted straight line, in the unit resolution mode, which means that the straight line goes through the origin and close to the maximum of each triangle of the respective m/z value. As shown in **Figure 2 (B)** working with unit resolution produces constant peak widths and therefore a constant resolution as well as constant sensitivity across the whole mass range [4-5].

1.2.2. Triple quadrupole

The roots of triple quadrupole technology go back to the late 1970´s when Enke and Yost [6] developed the first triple quadrupole mass spectrometer at the Michigan State University to explore selected ion fragmentation applications in analyzing mixtures and chemical structures.

In a triple quadrupole mass spectrometer three quadrupoles are connected in series, normally named as Q1, Q2 and Q3. **Figure 3** shows the ion path of the API 3000™. The Q1 and the Q3 act as mass selective quadrupoles as described above. The Q2, located between the Q1 and the Q3, is a RF only quadrupole and acts as the collision cell, which is filled with an inert gas as N_2 or Ar. In the collision cell (Q2) ions selected in the Q1 (precursor ions), collide with the inert gas and produce fragments (product ions), which are analyzed in Q3. This process is called *collision induced dissociation* (CID).

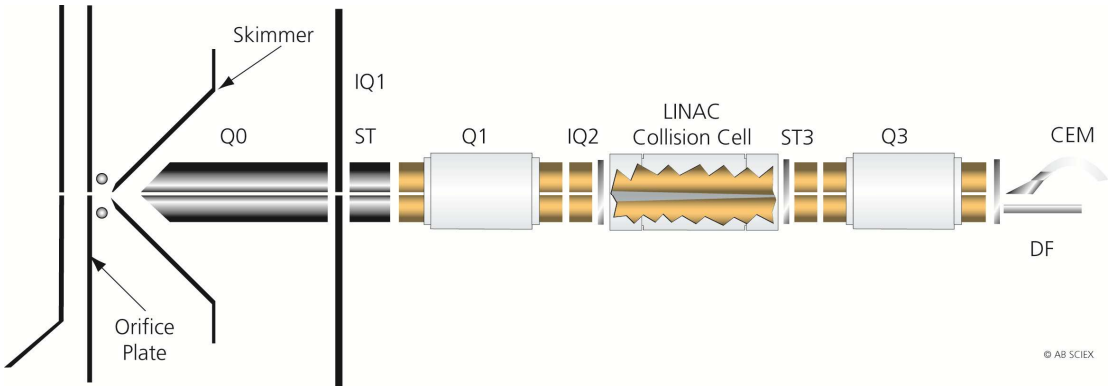


Figure 3: *Ion Path API 3000™*

Figure is provided courtesy of AB Sciex Pte.Ltd.

The high vacuum avoids collision of the ionized molecules with “air molecules” and a system of electric lenses between the quadrupoles makes sure that the ion beam keeps on the path.
In some cases partially fragmentation of the analyte in the change-over from atmospheric pressure into the vacuum can be observed which is known as *In-Source-CID*. This may happen to very fragile analytes due to their acceleration into the mass spectrometer.

The API 5000™ is a further development of the API 3000™ with a time gap of nearly ten years. **Figure 4** shows the ion path of the API 5000™.
Main difference in the ion path is that the skimmer, a metal cone behind the orifice, used in the API 3000™ was replaced by the QJet™ Ion Guide in the API 5000™. In comparison with the skimmer, that skims statically ions from the overall ion beam, the QJet™ is not only a static instrument component but also a small quadrupole which allows efficient separation of ions from neutrals. Therefore, the orifice of the API 5000™ could be enlarged, resulting in an increased sensitivity and better signal-to-noise ratio [7-8]. The required high vacuum was assured by using more powerful turbomolecular pumps.

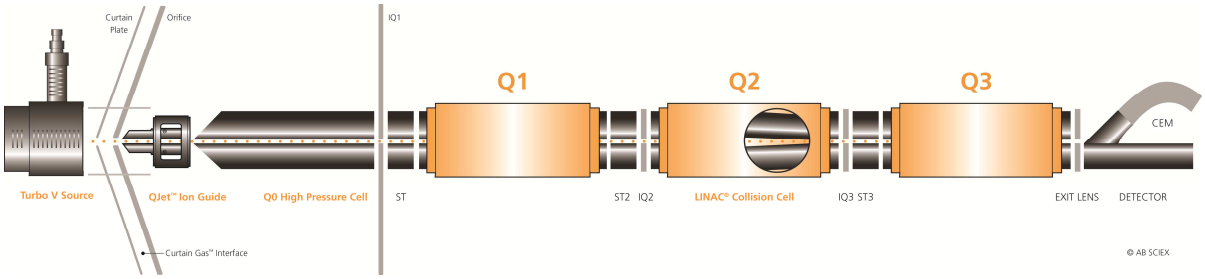


Figure 4: *Ion Path API 5000*

Figure is provided courtesy of AB Sciex Pte.Ltd.

Table 1 shows four typical scan modes of the triple quadrupole mass spectrometer. Moreover, the triple quadrupole mass spectrometer can be used as single quadrupole mass spectrometer when the Q1 and Q3 select the same ion and the collision gas is deactivated. Depending on the scan mode, qualitative and quantitative data can be generated, ranging from structural information and metabolite identification up to highly sensitive quantitative analysis.

Scan mode	Q1	Q2	Q3	Application
Product Ion	selection of a specific ion	collision	analysis of all product ions	structural information of the molecule
Precursor Ion	analysis of all ions	collision	selection of a specific product ion	searching for specific fragments (e.g. metabolite identification)
Neutral Loss	scanning between Q1 and Q3 with a specific m/z difference			information about substance classes
Multiple Reaction Monitoring	selection of specific precursor ion	collision	selection of specific product ion	for quantitative analysis

Table 1: *Triple quadrupole scan modes*

Mostly the triple quadrupole mass spectrometer is operated in the multiple reaction monitoring (MRM) mode and used for quantitative measurements whereas it is coupled to liquid chromatography.

1.3. ESI and APCI – Two common ionization techniques for LC-MS/MS

1.3.1. ESI – Electrospray ionization

ESI works well for substances that can be easily charged and analytes that are already charged in solution. As shown in **Figure 5** the eluent of the LC column flows into a capillary on which a high potential (up to ± 5kV, depending on the polarization) is applied. At the end of the capillary droplets are produced. Nebulizer gas (N_2) flowing conically around the capillary outlet supports the spraying process. The droplets contain positive and negative charges whereas the absolute charge is that of the analyte in the solution [9]. During evaporation of the solvent, the radius of the droplet decreases and the charges within the droplet move together, leading to an increase of the charge density on the droplet surface. When the radius of the droplets falls below the Rayleigh-Limit (the point on which the droplet contains the maximum number of identical charges) the droplets decay due to electrostatic repulsion into smaller droplets.
Different models for the generation of free ions in the gas phase are discussed:

1. The Charged Residue Model (CRM) of Röllgen et al. [10] assumes generation of tiny droplets with about 1nm in diameter that finally contain only one charged analyte molecule.

2. Iribarne and Thomson postulate in their Ion Evaporation Model (IEM) the emission of free ions in the gas phase from charged droplets [11-12] as shown in **Figure 5**.

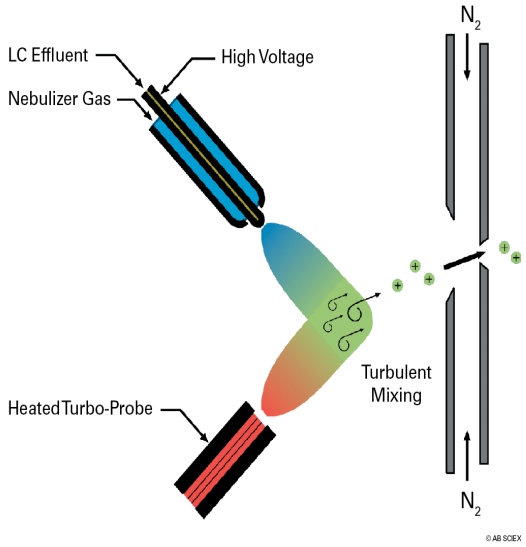


Figure 5: *ESI - Electrospray Ionization*

Figure is provided courtesy of AB Sciex Pte.Ltd.

The ions are accelerated into the mass spectrometer due to the applied potential and vacuum in the analyzer. Remaining clusters are broken by a N_2 curtain (curtain gas) which also prevents the high vacuum from solvent molecules.

The ESI technique is very gentle and therefore an ideal ionization technique for thermal labile compounds. For thermal stable compounds the desolvation of the droplets can be supported by a hot nitrogen stream (TurboIonSpray®) what normally produces higher signal intensities than without temperature.

One characteristic property of ESI is the ability to generate multiple charged ions like $[M+nH]^{n+}$ or $[M-nH]^{n-}$. In this manner molecules exceeding the mass range of the mass spectrometer can be analyzed because of m/z detection. ESI is one of the most effective and successful interface that was developed for LC-MS/MS analysis [5]. The ESI technique was established by J.B. Fenn who was awarded with the Noble Prize in 2002 for it.

1.3.2. APCI – Atmospheric pressure chemical ionization

In contrast to ESI, which requires charged ions in the mobile phase, ion generation in APCI is performed not until the gas phase. Therefore APCI is suitable for less polar or non polar compounds taking into account that the analyte is thermal stable. As **Figure 6** shows, the eluent of the LC column is vaporized via a heated quartz or ceramic tube what is supported according to ESI by a nebulizer gas [13]. Ionization of the molecule is performed via reaction with primary ions, which are generated on the corona discharge needle (current of 1 to 5 µA). Primary ions can be generated from the air (N_2^+, O_2^+, O_2^-) as well as from the used solvents (CH_3CNH^+, H_3O^+, OH^-, $CH_3OH_2^+$, CH_3O^-) or buffers (NH_4^+, CH_3COO^-) on the corona discharge needle [14].

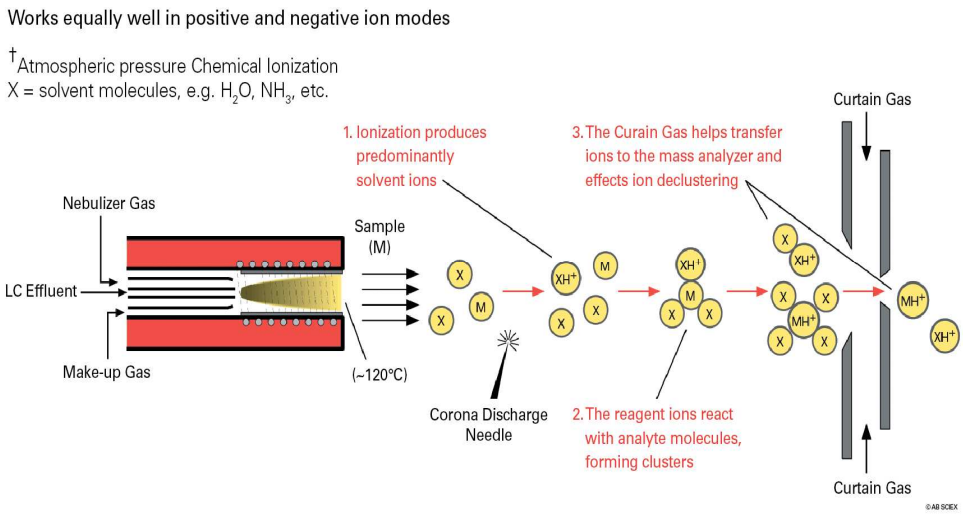
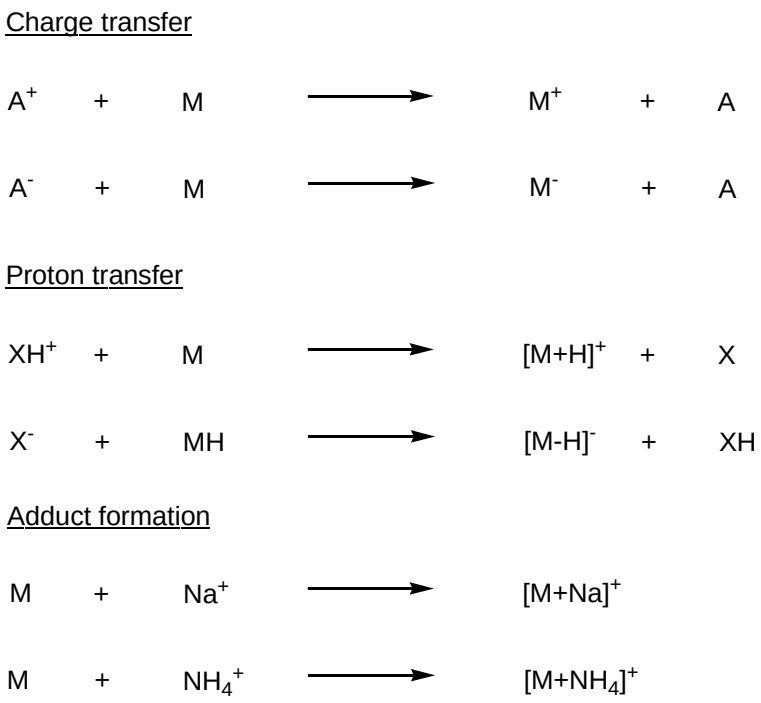


Figure 6: *APCI – Atmospheric Pressure Chemical Ionization*

Figure is provided courtesy of AB Sciex Pte.Ltd.

Ionization of the analyte happens via charge transfer or proton transfer as shown in **Scheme 4**. During charge-transfer a positive or negative charge is transferred from the primary ion onto the gaseous analyte. Via a proton transfer a proton is transferred from the positively charged primary ion onto the gaseous analyte what produces a protonated molecule with a molecular mass of +1 amu. On the other hand a proton can be abstracted by a negatively charged primary ion what produces a deprotonated molecule with a molecular mass of -1 amu.



Scheme 4: *Ionization processes with APCI*

Additionally in the positive mode formation of adducts like $[M+Na]^+$ or $[M+NH_4]^+$ can be observed. The APCI interface can be operated with higher LC flow rates than the ESI interface. Compared to ESI multiple charged ions are not generated with APCI.
Table 2 summarizes the main properties of the ESI and the APCI interface

Parameter	ESI	APCI
Temperature	low to moderate	moderate to high
Charge	single and multiple charged	single charged
Compounds	small polar molecules, large biomolecules, thermal labile compounds	small and less polar to non polar molecules, thermal stable compounds
Flow rate	low to medium	medium to high

Table 2: *ESI and APCI comparison*

It has to be noted that only volatile solvents, buffers (NH_4CH_3COO, NH_4HCOO, etc.) and additives should be used for ESI or APCI.

During LC-MS/MS method development it has to be investigated which interface provides the best ionization for the analyte. However, there are some other important points as compound information, expected concentration range of the samples, liquid chromatography, sample preparation, special requirements and sensitivity as well as reproducibility that should be considered during development of an LC-MS/MS method. They are specified in the following section.

1.4. Points that should be considered for LC-MS/MS method development

1.4.1. Compound information

The physical and chemical properties of the compound to be analyzed as pKa-value, solubility, thermal and light stability in solution are absolutely essential before starting the development. The molecular structure might give hints regarding the possible ionization mode(s) (positive or negative) and ionization source. However, it is absolutely mandatory to test the theoretical expectations because in practice sometimes unexpected results may occur.

1.4.2. Expected concentration range

When analyzing samples from clinical trials, the concentration range depends on the dose, the formulation and the bioavailability of the drug as well as on the sample matrix and the last sample collection point. For some methods also active metabolites were considered. In these cases literature often gives useful information.

The concentration ranges of a different dose of a drug may be estimated assuming a linear pharmacokinetic profile. Even if this is not true it is helpful information to estimate the expected concentration range.

1.4.3. Ionization source

For the sake of completeness, the ionization source is mentioned again at this point. Selection of the appropriate ionization technique plays an important role in an analytical method, because only vaporized and ionized analytes can be detected with the mass spectrometer. ESI and APCI were available and tested. Decision criteria whether to use ESI or APCI were summarized in **Table 2**. Additionally, sensitivity and reproducibility need to be consulted for finding a decision.

1.4.4. Liquid Chromatography

Whereas today the mass spectrometers are relatively easy to handle, the liquid chromatography part of the LC-MS/MS method is normally much more challenging. The two main parameters influencing the analyte´s chromatography are the analytical column and the composition of the mobile phase.

Today most of the LC-MS/MS methods employ reversed phase chromatography using a non-polar stationary phase and a polar mobile phase. The columns are silica based and their surfaces are modified with hydrophobic groups as for example C18-, C8- and hexyl-phenyl-chains or functional groups such as -CN or -NH_2. The columns are available in different lengths, diameters and with different particle sizes. Simplified can be said that the longer the column and the smaller the particle size the better is the resolution and the selectivity of the column. However, this may cause high system pressure, longer analytical run times and higher risk of plugging the analytical column due to sample particles. Therefore, the focus of the method has to be carefully defined and especially when it comes to multi component chromatography there often has to be made a compromise between resolution, selectivity and sensitivity.
Due to the selectivity of the triple quadrupole, often short columns with a length of about 50 to 100 mm and a particle size of 5 µm or 3 µm are used for LC-MS/MS methods.

The second main factor influencing the chromatography of the analyte is the composition of the mobile phase. Typically the mobile phase of an LC-MS/MS method contains a volatile buffer or acid (acetate, formate) and an organic modifier like acetonitrile or methanol. The exact composition has to be investigated throughout method development. The pH-value of the mobile phase has also an important role. Adjusting the wrong pH-value may cause no or too long retention, double peaks, peak tailing, bad ionization or in the worst case instability of the analyte. The selected pH-value has to be compatible with the possible pH working range of the analytical column.

1.4.5. Sample preparation

Sample work up depends on the sample matrix, the required sensitivity and the physical and chemical properties of the analyte. It is essential to remove matrix compounds for chromatography and mass spectrometry detection. Three sample preparation methods including protein precipitation, liquid-liquid extraction and solid phase extraction are possible for plasma samples.

Protein precipitation is the method of choice because it is a fast and cheap method for sample preparation. For protein precipitation sample and precipitation reagent are mixed at a ratio of 1:2 (v/v) and then centrifuged. Polson et al. [15] show an overview about protein precipitation methods. Often acetonitrile or methanol is used. The precipitation with methanol has to be performed at low temperature because the reaction is reversible. Acetonitrile may be mixed with organic acid (formic acid, acetic acid, trifluoro acetic acid) to improve the precipitation process. Sometimes it may be necessary to adjust a special pH-value before the precipitation step, which results in an overall dilution of the sample.

If the sample needs to be concentrated because the analyte of interest is present in very low concentrations a liquid-liquid extraction can be performed. Liquid-liquid extraction of plasma samples requires one part of sample and an excess (mostly 10-fold) of extraction solvent. After mixing the organic layer is transferred and evaporated.

The residue is dissolved in a small amount of mobile phase. Depending on the physical and chemical properties of the analyte extraction solvents as hexane, diethyl ether, ethyl acetate or dichloromethane may be used. The pH-value has a strong influence on the extraction process. Therefore it is necessary to test different pH-values as well as different extraction solvents to find out the optimal conditions. Due to the distinctive physiological pH-value of the blood strong buffers (about 1 molar), acids or bases are required to adjust the right pH-value. During method development of extraction assays, the pH-value should always be checked and adjusted.

As an alternative sample concentration method solid phase extraction can be used for sample work up. Hereby the sample is concentrated and purified via a stationary phase. The analyte binds to the stationary phase and most of the matrix components can be removed from the stationary phase with a washing step. After elution of the analyte from the stationary phase a pure extract for injection is yielded. Also for the solid phase extraction the correct pH-value needs to be adjusted.

In comparison to the solid phase extraction the liquid-liquid extraction is often preferred because it is cheaper, less contamination susceptible and easier to handle. Moreover, Bonfiglio et al. [16] showed that liquid-liquid extraction gives the purest extracts with the lowest noise (background) in the chromatogram. Especially when working in the low pg/mL-range and close to the limit of the mass spectrometer this might be an important advantage.

1.4.6. Special requirements

For analytes that are instable because of exposure to light, room temperature or remaining matrix components, special requirements are necessary during the sample preparation process.
Temperature instability can be avoided by using water cooling baths (approximately +4 °C) during the whole sample preparation process.
Light instability may occur from daylight and/ or from neon light. Daylight can easily be excluded from the lab. When there is a neon light instability other color spectra e.g. yellow fluorescence light have to be used.

For some analytes enzyme inhibitors have to be added to inhibit enzymes that are still active in plasma, e.g. addition of sodium fluoride to inhibit carboxyl esterases in rat plasma. It is important that the inhibitor is added in excess to ensure deactivation of all enzymes.
It is always helpful to check the stability with regard to temperature and light. Useful information such as start and the rate of the decay can be obtained.

1.4.7. Sensitivity and reproducibility

One major challenge during method development is the combination of high sensitivity with high reproducibility. The most sensitive method is useless when its lower limit of quantification can not be measured reproducible. Usually the reproducibility is lower when working at or close to the quantification limit of a method. This is especially the case when high sensitivity assays are operated at the limits of the mass spectrometer.

Regulatory authorities claim a defined signal-to-noise ratio together with a defined precision at the lower limit of quantification (LLOQ) when clinical study samples are measured. Already during the method development the precision of the method should be checked and if necessary chromatographic conditions, sample work up or mass spectrometer settings have to be changed. When the required precision and signal-to-noise value at the LLOQ can not be fulfilled the LLOQ must be shifted upwards.

At the end of the method development a ruggedness test, containing calibration curves and spiked quality control samples, is performed. Normally this is the last step before method validation which tests the suitability of the method for daily use.

1.5. Full, Partial or Cross Validation ?

Each method should be validated to ensure the generation of accurate, precise and reproducible data during routine sample analysis.

During a methods “lifetime” it may undergo changes and modifications as addition of a metabolite or lowering the LLOQ. After the changes it has to be demonstrated that the method works still with the required performance. Different levels of method validations, *Full Validation*, *Partial Validation*, and *Cross Validation* are characterized and defined [17].

Full Validation is necessary when a method is developed or implemented in an analytical process for the first time. Addition of new analytes, e.g. metabolites requires a *Full Validation* for all analytes measured.

A *Partial Validation* has to be performed when the bioanalytical method is changed but does not require a full revalidation. Depending on the extend of the *Partial Validation* it may come close to a *Full Validation*. Typical changes that require *Partial Validation* are method transfer (between laboratories or analysts), change of instrument and/or software platform, changes in species within matrix (e.g. from rat plasma to mice plasma), changes in matrix within a species (e.g. from human plasma to human urine), changes in analytical methodology (e.g. change of the detection system) or changes in sample processing procedures.

A *Cross Validation* is necessary when two bioanalytical methods are used within the same study (e.g. LC-MS/MS vs. ELISA) or when study samples are analyzed at more than one site. One method should act as “reference” and the other as “comparator”. The comparisons should be done both ways.

In this thesis *Full Validation* was performed for all drugs studied. Moreover, *Partial Validation* with regard to a system comparison of the API 3000™ and the API 5000™ was performed for some of the compounds.

1.6. Pharmacokinetic analysis

The pharmacokinetic parameters were determined from true (actual) sample collection times and assayed plasma concentrations at these times. Concentration values below the lower limit of quantification were set to zero.

For comparing the bioequivalence of different oral administered drug formulations the FDA recommends to report the maximum plasma concentration (C_{max}), the time of maximum plasma concentration (t_{max}), the area under the plasma concentration-time curve from time zero to the last quantifiable concentration ($AUC_{0\rightarrow last}$), the total area under the plasma concentration-time curve from time zero to infinity ($AUC_{0\rightarrow\infty}$) and the terminal half-life $t_{1/2}$ [18].

C_{max} and t_{max} were directly taken from the measured plasma concentration-time curves. $AUC_{0\rightarrow last}$ was calculated using the trapezoidal rule and $AUC_{0\rightarrow\infty}$ was calculated by the sum of $AUC_{0\rightarrow last}$ and AUC_{extra}: $AUC_{0\rightarrow\infty} = AUC_{0\rightarrow last} + AUC_{extra}$.

AUC_{extra} was calculated by the assumption of a mono-exponential decline of the plasma concentration curve after the last quantifiable data point. This area can be extrapolated with the following formula: $AUC_{extra} = C_{last} / k_{el}$, where C_{last} is the last quantifiable concentration and k_{el} the elimination rate constant which was derived by linear regression of the log-transformed plasma concentration versus time.

The terminal half-life $t_{1/2}$ was calculated using the following formula: $t_{1/2} = \ln 2 / k_{el}$.

1.7. The aim of this thesis

Aim of this thesis was to analyze human plasma samples from clinical trials with the drugs atorvastatin, clopidogrel, furosemide, itraconazole, loratadine, naproxen, nisoldipine and sunitinib, to calculate their pharmacokinetic parameters and to compare them with previously reported data in the literature. As the active metabolites are also of interest during clinical trial analysis 2-hydroxyatorvastatin, 4-hydroxyatorvastatin, hydroxyitraconazole, descarboethoxyloratadine, 4-hydroxynisoldipine and N-desethylsunitinib were included in the corresponding methods. Therefore sensitive, precise, accurate and robust LC-MS/MS methods were developed and validated. The sample preparation procedures were kept as easy as possible to reduce costs and allow an accurate and efficient workflow during routine sample analysis. As all measurements were carried out in a regulatory environment and the clinical trials were part of drug approval processes, method validation (*Full Validation*) was performed according to guidelines from regulatory authorities (FDA, EMA). The validation results and the analyzed and calculated pharmacokinetic parameters will be presented in this thesis.

Each of the drugs studied belongs to a different substance class and so a wide range of chemical and physical as well as pharmacokinetic properties had to be covered in the different methods. Furosemide, itraconazole, naproxen and sunitinib can be found in the µg/mL or upper ng/mL range in human plasma due to high bioavailability or a high drug dose. For these drugs simple and fast protein precipitation methods were investigated.
On the other hand methods were developed for atorvastatin, clopidogrel, loratadine and nisoldipine which require due to low a dose, high first pass effect and low bioavailability a sensitive detection in human plasma (lower ng/mL or pg/mL). For these drugs liquid-liquid extractions were investigated.

Moreover, for atorvastatin, 2-hydroxyatorvastatin, clopidogrel, loratadine and descarboethoxyloratadine a mass spectrometer system comparison (*Partial Validation*) was performed. The sensitivity and the performance of the two types of mass spectrometers (API 3000™ vs. API 5000™) was compared.
Moreover, atorvastatin, 2-hydroxyatorvastatin, clopidogrel, loratadine and descarboethoxyloratadine were used to investigate the sensitivity and performance of two types of mass spectrometers (API 3000™ vs. API 5000™).

2. Materials and Methods

2.1. Chemicals and reagents

Acetonitrile and Methanol were of HPLC-grade and from Fisher Chemical (Fisher Scientific GmbH, Schwerte, Germany). Ammonium acetate, ammonium formate, acetic acid and formic acid were of analytical grade and from VWR International GmbH (Darmstadt, Germany). Extraction solvents (diethyl ether and n-hexane) were of HPLC grade and from Chromatographie Handel Müller (Haag, Fridolfing, Germany). Ultra pure water was obtained using a Milli-Q purification system (Millipore Corporation, Bedford, MA, USA).

ATO was provided by Hexal AG (Holzkirchen, Germany), 2-HYD-ATO, 4-HYD-ATO and the internal standard d5-ATO were purchased from Syncom (Groningen, The Netherlands).

CLP bisulfate was provided by Hexal AG (Holzkirchen, Germany) and the internal standard diltiazem (DIL) was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany).

FUR was provided by Hexal AG (Holzkirchen, Germany) and the internal standard probenecid (PRO) was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany).

ITR and HYD-ITR were provided by Biochemie GmbH (Kundl, Austria) and the internal standard (CLA) was provided by Hexal AG (Holzkirchen, Germany).

LOR and DCL were provided by Sandoz Private Ltd. (Thane, India), the internal standards d4-LOR and d4-DCL were purchased from Toronto Research Chemicals Inc. (Ontario, Canada).

NAP was purchased from Roche Diagnostics GmbH (Mannheim, Germany) and the internal standard ketoprofen (KEP) was purchased from Procter&Gamble (Ohio, USA).

NIS was provided by Sandoz Private Ltd. (Thane, India) and the internal standard d4-NIS was purchased from Medical Isotopes Inc. (Pelham, USA). 4-HYD-NIS and the internal standard d6-4-HYD-NIS were purchased from Toronto Research Chemicals Inc. (Toronto, Canada).

SUN was provided from LC Laboratories (Woburn, MA, USA). DES-SUN and the internal standard d5-SUN were synthesized by P.W. Elsinghorst and A. Lindauer (Department of Clinical Pharmacy, Pharmaceutical Chemistry I, University Bonn, Germany).

2.2. Stock solutions

For all analytes and internal standards stock solutions were prepared. An accurately amount of the compound was weighed and then transferred with solvent into a volumetric flask. After complete dissolution the stock solutions were aliquoted and stored at -70 ℃ until use. **Table 3** shows the individual concentration and the used solvents.

Compound	Solvent	Stock solution concentration (mg/mL)
ATO	Acetonitril : Milli-Q-water, 9:1 (v/v)	1.0
2-HYD-ATO	Acetonitril : Milli-Q-water, 9:1 (v/v)	1.0
4-HYD-ATO	Acetonitril : Milli-Q-water, 9:1 (v/v)	1.0
d5-ATO	Acetonitril : Milli-Q-water, 9:1 (v/v)	1.0
CLP	Methanol	0.1
DIL	Milli-Q-Water	0.5
FUR	Methanol	0.5
PRO	Ethanol	1.0
ITR	Methanol	1.0
HYD-ITR	Methanol	1.0
CLA	Acetonitrile	1.0
LOR	Acetonitrile	0.1
DCL	Acetonitrile	0.1
d4-LOR	Acetonitrile	0.1
d4-DCL	Acetonitrile	0.1
NAP	Methanol	5.0
KEP	Methanol	1.0
NIS	Methanol	0.1
4-HYD-NIS	Methanol	0.1
d4-NIS	Methanol	0.1
d6-4-HYD-NIS	Methanol	0.1
SUN	Methanol	0.1
DES-SUN	Methanol	0.1
d5-SUN	Methanol	0.1

Table 3: *Stock solutions of analytes and internal standards*

2.3. Apparatus

LC-Pumps:	L-6200A HPLC Pump (Merck KGaA, Darmstadt, Germany) Agilent 1200 Series Binary Pump SL (Agilent, Waldbronn, Germany)
Autosampler:	CTC Combi Pal Autosampler (CTC Analytics, Switzerland)
Detectors:	AB Sciex API 3000™ and API 5000™ Mass Spectrometer (AB Sciex Concorde, Ontario, Canada) with TurboIonSpray® or APCI Interface
Centrifuges:	Allegra 6R Centrifuge (Beckmann Instruments, Inc., Palo Alto, CA, USA) Biofuge primo R (Heraeus Sepatech, Osterode, Germany)
Mixer:	Vortex Shaker Heidolph REAX 2000 (Heidolph-Elektro GmbH, Kelheim, Germany) Overhead Shaker Heidolph REAX 2 (Heidolph-Elektro GmbH, Kelheim, Germany)
Analytical balance:	Mettler AT261 Delta Range® FACT (Mettler-Toledo GmbH, Gießen, Germany)
Evaporator:	TurboVap® LV Evaporator (Zymark Center, Hopkinton, USA)
pH Meter:	WTW pH 521 (WTW GmbH Weilheim, Germany)
Pipettes:	Eppendorf Reference® 10-100 µL (Eppendorf, Hamburg, Germany) Eppendorf Reference® 100-1000 µL (Eppendorf, Hamburg, Germany) Eppendorf Reference® 500-2500 µL (Eppendorf, Hamburg, Germany) Eppendorf Reference Multipette® plus (Eppendorf, Hamburg, Germany)
Pipette Apparatus:	Multimek™ Automated 96-Channel Pipettor (Beckmann Coulter GmbH, Unterschleißheim, Germany)
Microplates:	Microplates: Microplate 96 Deep-Well (Porvair Sciences Ltd, Shepperton, United Kingdom).

2.4. Data acquisition and processing

Data acquisition on the API 3000™ was performed with Sample Control version 1.4, data processing was performed with LC2Tune version 1.4 and with MacQuan version 1.6.
Data acquisition and processing on the API 5000™ was performed with Analyst® Version 1.4.2 (AB Sciex, Concorde, Ontario, Canada).
Calculations were performed with Microsoft Excel 2000 from Microsoft Co. (Redmond, WA, USA, 1985-2000).

2.5. Recording MS/MS spectra

For each analyte a solution, containing 100 - 1000 ng/mL, was prepared in mobile phase and infused with a syringe pump that was coupled with a T-piece to the HPLC pump and the interface (TurboIonSpray® or APCI) of the mass spectrometer. The flow rate of the syringe pump was 0.6 mL/h and the flow rate of the HPLC pump was 0.8 mL/min. The individual mass spectra recorded for the Q1 and the corresponding product ion spectra recorded for the Q3 can be found in the Appendix. The fragmentation is shown for the used product ions. For itraconazole the same molecular ion was used in the Q1 and the Q3, therefore only the Q1 spectrum is shown.

2.6. Validation procedure – Full Validation

2.6.1. Determination of specificity

The specificity of the analytes was determined by screening at least six different batches (three males and three females) of control drug-free human plasma. The samples were prepared with and without addition of the internal standard. There should be no co-eluting peaks with areas of more than 20% of the analyte peak area at the LLOQ. **Table 4** shows the used anticoagulants and the number of different batches that were tested for each analyte.

Analyte	Anticoagulant	No. of drug free batches tested	
		male human plasma	female human plasma
ATO	Ammonium heparinate	3	3
2-HYD-ATO	Ammonium heparinate	3	3
4-HYD-ATO	Ammonium heparinate	3	3
CLP	Ammonium heparinate	3	3
FUR	Ammonium heparinate	3	3
ITR	Ammonium heparinate	3	3
HYD-ITR	Ammonium heparinate	3	3
LOR	Ammonium heparinate	6	6
DCL	Ammonium heparinate	6	6
NAP	Ammonium heparinate	3	3
NIS	Ammonium heparinate	6	6
4-HYD-NIS	Ammonium heparinate	6	6
SUN	Ethylenediaminetetraacetate	6	6
DES-SUN	Ethylenediaminetetraacetate	6	6

Table 4: *Determination of specificity*

2.6.2. Evaluation of linearity and lower limit of quantification

For determination of linearity on five different days a calibration curve in human plasma with calibration standards was prepared, in each case including a blank sample, which was not used for calculation of linear regression. For evaluation of the calibration standards a weighted linear regression was performed with theoretical concentrations of calibration standards and measured peak area ratios (peak area analyte/peak area internal standard) by MacQuan or Analyst®. The calibration curves were evaluated individually by linear regression and the concentrations of the calibration standards were back-calculated.

The slope, intercept and the correlation coefficient of the corresponding individual curve was calculated. The calibration curves were accepted if there were not more than two outliers. If there were two outliers they had not to be adjacent. A calibration standard was defined as an outlier if the back-calculated concentration deviated more than 15 % from the theoretical concentration at all concentrations except for the lowest concentration (LLOQ), where a deviation of 20 % was accepted.

The accuracy and precision as shown in equation **(1)** and **(2)** of all five validation days were determined as relative error (RE, %) and coefficient of variation (CV, %), respectively.

$$\text{CV (\%)} = \frac{\text{standard deviation}}{\text{mean}} \bullet 100 \qquad \textbf{(1)}$$

Where standard deviation is defined as:

$$\sigma_x = \sqrt{\text{Var}(x)}$$

$$\text{RE (\%)} = \frac{\text{mean assayed conc.} - \text{theoretical conc.}}{\text{theoretical conc.}} \bullet 100 \qquad \textbf{(2)}$$

The statistical calculations were performed in an MS Excel 2000 spreadsheet.

Table 5 shows the individual concentrations of the calibration standards from the prepared calibration curves. All concentrations were prepared by serial dilution.

On each validation day the signal-to-noise ratio is determined for the analytes at the LLOQ. The mean value must be ≥5.

	Concentration [ng/mL]												
Analyte	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**	**L9**	**L10**	**L11**	**L12**	**L13**
ATO*	50.0	40.0	20.0	10.0	5.00	1.00	0.40	0.20	0.10	-	-	-	-
2-HYD-ATO*	50.0	40.0	20.0	10.0	5.00	1.00	0.40	0.20	0.10	-	-	-	-
4-HYD-ATO*	50.0	40.0	20.0	10.0	5.00	1.00	0.40	0.20	0.10	-	-	-	-
CLP*	10.0	8.00	1.00	0.500	0.250	0.100	0.0500	0.0200	-	-	-	-	-
FUR*	5000	4000	1000	500.0	100.0	50.00	20.00	10.00	5.000	-	-	-	-
ITR*	1000	750.0	200.0	100.0	50.00	20.00	10.00	5.000	3.000	-	-	-	-
HYD-ITR*	1000	750.0	200.0	100.0	50.00	20.00	10.00	5.000	-	-	-	-	-
LOR**	15.0	11.2	5.00	1.00	0.100	0.0500	0.0250	0.0125	0.0100	-	-	-	-
DCL**	15.0	11.3	5.00	1.00	0.100	0.0500	0.0250	0.0125	0.0100	-	-	-	-
NAP*	50000	45000	25000	12500	5000.0	2500.0	1000.0	400.00	200.00	100.00	-	-	-
NIS**	10.2	8.15	6.12	3.04	1.52	1.22	0.608	0.302	0.151	0.0514	0.0206	0.0104	0.00520
4-HYD-NIS**	10.2	8.15	6.12	3.04	1.52	1.22	0.608	0.302	0.151	0.0513	0.0206	0.0104	0.00520
SUN**	100	75.0	50.0	25.0	10.0	5.00	1.00	0.500	0.150	0.0600	-	-	-
DES-SUN**	100	75.0	50.0	25.0	10.0	5.00	1.00	0.500	0.150	0.0600	-	-	-

*API 3000™, **API 5000™, -: not performed , L: level (calibration standard)

Table 5: *Concentrations of the calibration standards (levels)*

2.6.3. Evaluation of intra-day and inter-day precision and accuracy

For determination of the intra-day and inter-day precision and accuracy five sets of spiked quality control samples (SQCs) in human plasma were analyzed on each of the five validation days. The SQCs were prepared across the whole calibration range of the analyte, including the highest and the lowest calibration standard, and were calculated by the corresponding calibration curve. Means, standard deviations, coefficients of variation (%) and accuracy (%) were calculated of each SQC. **Table 6** shows the individual concentrations of the SQCs for all analytes.

	Concentration [ng/mL]						
Analyte	**SQC 1**	**SQC 2**	**SQC 3**	**SQC 4**	**SQC 5**	**SQC 6**	**SQC 7**
ATO	50.0	40.0	1.00	0.250	0.100	-	-
2-HYD-ATO	50.0	40.0	1.00	0.250	0.100	-	-
4-HYD-ATO	50.0	40.0	1.00	0.250	0.100	-	-
CLP	10.0	8.00	0.800	0.0500	0.0200	-	-
FUR	5000	3000	20.00	5.000	-	-	-
ITR	1000	500.0	100.0	10.00	5.000	3.000	-
HYD-ITR	1000	500.0	100.0	10.00	5.000	-	-
LOR	15.0	11.3	1.00	0.0250	0.0100	-	-
DCL	15.0	11.3	1.00	0.0250	0.0100	-	-
NAP	50000	25000	3000	300	100	-	-
NIS	9.88	7.96	1.53	1.23	0.154	0.0151	0.00511
4-HYD-NIS	9.87	7.95	1.53	1.23	0.154	0.0151	0.00511
SUN	100	75.0	10.0	0.150	0.0600	-	-
DES-SUN	100	75.0	10.0	0.150	0.0600	-	-

-: not performed

Table 6: *Concentrations of the spiked quality control standards*

The inter- and intra-day CVs for the spiked quality control samples as well as the accuracies should be within ±15 %, except at the LLOQ where a value of ±20 % is accepted.

2.6.4. Determination of absolute recovery

For the determination of the recovery across the calibration range of each analyte and the corresponding internal standard, spiked quality control samples in human plasma and spiked quality controls in processed blank human plasma were prepared. Each sample was analysed five times.

The recovery of the analyte and the internal standard was evaluated as shown in equation **(3)**.

$$\text{Recovery}\,(\%) = \frac{\text{Mean peak area of analyte in spiked and processed human plasma samples}}{\text{Mean peak area of analyte added to processed blank human plasma}} \bullet 100 \qquad \textbf{(3)}$$

2.6.5. Evaluation of stability

Stability of the analytes in human plasma was assessed by analyzing spiked quality control samples at two concentrations (high and low), exposed to different conditions of time and temperature. The results were compared with those for freshly prepared spiked quality control samples. According to the guidelines the “high” concentration was set by the highest calibration point and the “low” concentration was set as a factor 2 to 4 higher than the LLOQ. Each sample was prepared and analyzed five times.

The short-term stability was evaluated after exposure of the plasma samples to room temperature for 2 h and 4 h.

The long-term stability was assessed after storage of the test samples at -20 °C and -70 °C. The stability of the samples was measured a fter defined time intervals after the start of the stability test.

The freeze-thaw stability was determined after three freeze-thaw cycles (-70 °C to room temperature).

The post-preparative storage stability of the analytes was assessed at approximately +4 °C (autosampler temperature) and approximately -70 °C after defined time intervals after preparation.

The stability of the stock solutions (analyte and internal standard) was tested at room temperature for 6 h. The stock solutions standing for 6 hours at room temperature were analyzed and compared with stock solutions that were prepared freshly. **Table 7** summarizes the performed stability experiments.

Analyte	Conc. high [ng/mL]	Conc. low [ng/mL]	ST [h] RT	LT [d] -20 °C	LT [d] -70 °C	PP [h] +4 °C	PP [h] -70 °C	SL [h] R T	FT
ATO	50	0.25	2, 4	8, 30, 90	8, 30, 90	72	72	6	1-3
2-HYD-ATO	50	0.25	2, 4	8, 30, 90	8, 30, 90	72	72	6	1-3
4-HYD-ATO	50	0.25	2, 4	8, 30, 90	8, 30, 90	72	72	6	1-3
CLP	10	0.0500	2, 4	6, 30, 150	6, 30, 150	24, 96	24, 96	6	1-3
FUR	5000	20	2, 4	4, 7, 30	4, 7, 30	24, 48	24, 48	6	1-3
ITR	1000	10	2, 4	3, 30	3, 30	24, 48, 120	24, 48, 120	-	1-3
HYD-ITR	1000	10	2, 4	3, 30	3, 30	24, 48, 120	24, 48, 120	-	1-3
LOR	15.0	0.025	2, 4	5, 30, 90	5, 30, 90	24, 48, 120	24, 48, 120	6	1-3
DCL	15.0	0.025	2, 4	5, 30, 90	5, 30, 90	24, 48, 120	24, 48, 120	6	1-3
NAP	50000	300	2, 4	2, 4, 20	2, 4, 20	24, 48, 72	24, 48, 72	6	1-3
NIS	10	0.0151	2, 4	2, 13, 40	2, 13, 40	48	48	6	1-3
4-HYD-NIS	10	0.0150	2, 4	2, 13, 40	2, 13, 40	48	48	6	1-3
SUN	100	0.150	2, 4	150	150	24, 48	-	-	1-3
DES-SUN	100	0.150	2, 4	150	150	24, 48	-	-	1-3

ST: short term, RT: room temperature, LT: long term, PP: post preparative, SL: stock solution, FT: freeze-thaw, -: not performed

Table 7: *Stability experiments*

Statistical evaluation was performed by calculating 95 % ANOVA based confidence intervals for the ratios between the concentrations measured after given periods of time or after repeated thawing/freezing and the respective control (to allow for any contribution of assay imprecision). Instability was concluded if both the upper and lower limit of the confidence interval were greater than -10 %.

2.6.6. Influence of dilution

The influence of dilution on the determination of the analytes in human plasma was investigated by measuring five samples of spiked quality control standards in human plasma which were diluted (1:5) with drug-free human plasma prior to sample preparation. The CV should be ≤15 %. The mean value should be within ±15 % of the nominal value.

2.6.7. Influence of hemolyzed plasma

Hemolyzed plasma was prepared by adding 1 % of frozen and thawed drug-free blood to drug-free human plasma.
The influence of hemolyzed plasma on the determination of the analytes was performed by measuring five samples of spiked quality control standards at a high and a low concentration in hemolyzed human plasma. The spiked quality control standards in hemolyzed human plasma were analyzed together in the same run with the spiked quality control standards prepared in non-hemolyzed human plasma. The CVs should be ≤15 %. The mean value should be within ±15 % of the nominal value.

2.6.8. Influence of lipemic plasma

The influence of lipemic plasma on the determination of the analytes was determined by measuring five samples of spiked quality control standards at a high and a low concentration in lipemic human plasma. The spiked quality control standards in lipemic human plasma were analyzed together with the human non-lipemic plasma spiked quality control standards in the same run. The CVs should be ≤15 %. The mean value should be within ±15 % of the nominal value.

2.6.9. Influence of different batches of human plasma

The influence of six different batches of human plasma on the determination of the analytes was investigated by measuring three samples of each spiked quality control standard at a high and a low concentration in six different batches of human plasma. The CVs should be ≤15 %. The mean value should be within ±15 % of the nominal value.

Table 8 shows the influences that were tested for the individual analytes.

Analyte	Conc. high [ng/mL]	Conc. low [ng/mL]	Human plasma			
			hemolyzed	lipemic	6 different bachtes	dilution
ATO	50	0.25	x	x	x	1:5
2-HYD-ATO	50	0.25	x	x	x	1:5
4-HYD-ATO	50	0.25	x	x	x	1:5
CLP	10.0	0.0500	x	x	x	1:5
FUR	5000	20	x	-	x	-
ITR	1000	10	x	-	-	-
HYD-ITR	1000	10	x	-	-	-
LOR	15.0	0.025	x	x	x	1:5
DCL	15.0	0.025	x	x	x	1:5
NAP	50000	300	x	-	x	1:5
NIS	10	0.0151	x	x	x	1:5
4-HYD-NIS	10	0.0151	x	x	x	1:5
SUN	100	0.150	x	x	x	1:5
DES-SUN	100	0.150	x	x	x	1:5

x: performed, -: not performed

Table 8: *Investigated influences on the determination of the analytes*

2.6.10. Precision in incurred human plasma samples

For the determination of the precision of the analytes in incurred human plasma samples, samples from clinical trials were used. Incurred sample analysis was performed for CLP, LOR and DCL, NIS and 4-HYD-NIS, SUN and DES-SUN.
The samples were analyzed and compared to the results of the first analysis. The absolute differences in percent of the first to second analysis were calculated and the mean of these absolute differences in percent was calculated. The mean percental deviation should be lower than 20 % of the absolute percental difference of the first and second analysis.

2.6.11. Matrix Effect

The matrix effect was investigated with quantitative determination of the matrix factor (MF). To determine the MF the analytes and internal standards were added to mobile phase and six different drug free processed blank human plasma samples. Each sample was measured trice. The area ratios of analyte/ internal standard were calculated in processed blank human plasma and mobile phase. The MF was calculated as shown in equation **(4)**.

$$MF = \frac{\text{mean area ratio of analyte in processed blank human plasma}}{\text{mean area ratio of analyte in mobile phase}} \qquad \textbf{(4)}$$

The variability of the matrix factor, as measured by the CV should be less than 15 %. A matrix factor greater or smaller than one suggests analyte ion enhancement or supression, respectively, due to matrix components. A value of one signifies no matrix effect. In the *Full Validation* the MF value was determined for LOR and DCL, NIS and 4-HYD-NIS, SUN and DES-SUN.

2.7. Method application to clinical trials

The LC-MS/MS procedures developed and validated were used to analyse human plasma samples from clinical trials. All administrations were performed under fasting conditions except for ITR. **Tables 9-11** show information about the study design, concentrations of calibration standards and spiked quality control samples. An adequate number of calibration standards and SQCs was prepared for each study before the beginning of analysis and stored at -70 ℃ before usage.

Samples of subjects were measured together with calibration standards and spiked quality control samples.
A calibration standard was defined as an outlier if the back-calculated concentration deviated more than ±15 % from the theoretical concentration at all concentrations. The calibration curve was accepted if there were not more than two outliers. The number of quality control samples per sequence was defined by the 10 % rule: At least 10 % of the measured samples must be spiked quality control samples. The determined concentration of spiked quality control standards was compared to the theoretical concentration for accuracy. At least 2/3 of the same SQC concentration must be within ±15 %, otherwise the sequence can not be accepted.

Analyte	Subjects/ gender	Study design	Dose	Sample collection times after administration [h]
ATO	24/ m, f	2-way crossover	40 mg	Pre, 0.33, 0.50, 0.67, 0.75, 1.00, 1.33, 1.67, 2.00, 2.50, 3.00, 3.50, 4.00, 6.00, 8.00, 16.00, 24.00, 36.00 and 48.00
CLP	24/ m	2-way crossover	75 mg	Pre, 0.25, 0.50, 0.67, 0.83, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 4.00, 8.00, 12.00, 16.00 and 24.00
FUR	39/ m, f	2-way crossover	40 mg	Pre, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 4.00, 5.00, 6.00, 8.00, 10.00 and 12.00
ITR	40/ m, f	2-way crossover	100 mg	Pre, 0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 8.00, 12.00. 16.00, 24.00, 36.00, 48.00, 72.00 and 96.00
LOR	24/ m, f	2-way crossover	10 mg	Pre, 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 4.00, 8.00, 12.00, 24.00, 36.00, 48.00 and 72.00
NAP	30/ m	2-way crossover	220 mg	Pre, 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 48.00 and 72.00
NIS	24/ m, f	3-way crossover	10 mg	Pre, 0.5, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00, 12.00. 14.00, 16.00, 20.00, 24.00, 36.00, 48.00 and 72.00
SUN	12/ m	explorative study	50 mg	Pre, 1, 2, 4, 6, 8, 10, 12, 24, 25, 36, 48, 49, 60, 72, 96, 120, 240, 336 and 384 (Subj. 1-4) Pre, 0.5, 24.5, 48.5, 72.5, 73, 96.5, 97, 98, 100, 102, 104, 106, 108, 144, 168 and 432 (Subj. 8-12)

m: male, f: female

Table 9: *Clinical trials studied*

	Concentrations [ng/mL]									
Analyte	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**	**L7**	**L8**	**L9**	**L10**
ATO	50.6	40.5	20.2	10.1	5.06	1.01	0.405	0.202	0.0995	-
2-HYD-ATO	50.7	40.6	20.3	10.1	5.07	1.01	0.405	0.203	0.0997	-
CLP	2.09	1.04	0.517	0.103	0.0515	0.0206	0.0103	0.00411	0.00205	0.000998
FUR	5047	4044	1082	529.3	104.3	51.31	20.76	10.32	4.698	-
ITR	977.9	730.4	195.3	98.03	47.84	19.15	9.564	4.783	2.910	-
HYD-ITR	984.1	735.1	196.5	98.66	48.14	19.27	9.625	4.814	-	-
LOR	15.1	11.3	5.02	1.01	0.100	0.0501	0.0250	0.0125	0.00999	-
DCL	15.1	11.3	5.03	1.01	0.100	0.0501	0.0250	0.0125	0.0100	-
NAP	50166	45156	25084	12542	5016.8	2508.8	1003.8	403.55	201.65	99.860
NIS	1.56	1.25	0.639	0.307	0.153	0.0519	0.0206	0.0104	0.00521	-
4-HYD-NIS	1.56	1.25	0.639	0.307	0.153	0.0519	0.0206	0.0105	0.00521	-
SUN	102	76.0	50.0	25.1	9.81	4.97	0.963	0.490	0.145	0.0572
DES-SUN	111	82.1	54.0	27.1	10.6	5.37	1.04	0.529	0.157	0.0618

-: not performed, L: level (calibration standard)

Table 10: *Concentrations of the calibrations standards during PK analysis*

Analyte	Concentration [ng/mL] SQC 1	SQC 2	SQC 3	SQC 4	No. of sequences	No. of SQC sets per sequence
ATO	40.0	32.0	0.799	0.250	12	3
2-HYD-ATO	40.1	32.1	0.803	0.251	12	3
CLP	1.61	0.403	0.0301	0.00298	9	3
FUR	4988	4008	103.2	10.06	15	3
ITR	927.2	461.1	93.81	9.069	15	3
HYD-ITR	933.3	464.2	94.43	9.129	-	-
LOR	11.2	4.98	0.994	0.0248	9	3
DCL	11.2	4.98	0.995	0.0248	-	-
NAP	40400	20200	2430.0	243.00	11	3
NIS	1.20	0.150	0.0150	-	12	5
4-HYD-NIS	1.20	0.150	0.0150	-	-	-
SUN	101	74.6	9.62	0.148	5	3
DES-SUN	110	81.2	10.5	0.162	-	-

Table 11: *Concentrations of the spiked quality control samples during PK analysis*

2.8. Validation Procedure – Partial Validation

A system comparison that was performed as a *Partial Validation* was conducted for ATO, 2-HYD-ATO, CLP, LOR and DCL. The fully validated methods on the API 3000™ were transferred and partially validated on the API 5000™ and vice versa. The chromatographic conditions and sample preparation were not changed but the LLOQs were adjusted to the new system. In this way the two generations of mass spectrometers could be compared regarding their sensitivity, precision and accuracy as well as the matrix effect.
In the *Partial Validation* specificity, linearity, lower limit of quantification (LLOQ), intra-day and inter-day precision and accuracy of the substances were evaluated. Furthermore the matrix effect was investigated to characterise the different ionization sources of the instruments.

2.8.1. Determination of specificity

The specificity of the analytes was determined by screening six different batches (three males and three females) of control drug-free human plasma. The samples were prepared with and without addition of the internal standard. There should be no co-eluting peaks with areas of more than 20 % of the analyte peak area at the LLOQ.

2.8.2. Evaluation of linearity, lower limit of quantification, intra-day and inter-day precision and accuracy

Linearity, lower limit of quantification, intra-day and inter-day precision and accuracy were performed as described for *Full Validation*, with the exception that three days instead of five days were validated. **Table 12** and **Table 13** show the concentrations of the calibration standards and spiked quality control samples, respectively. Additionally the used mass spectrometers are listed.

2.8.3. Matrix Effect

The matrix effect was determined for CLP, LOR and DCL as described for *Full Validation.*

Analyte	Mass Spectrometer	Concentration [ng/mL] L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11
ATO	API 5000™	50.0	40.0	20.0	9.99	5.00	0.999	0.400	0.200	0.0999	0.0500	0.0250
2-HYD-ATO	API 5000™	50.0	40.0	20.0	10.0	5.00	1.00	0.400	0.200	0.100	0.0500	0.0250
CLP	API 5000™	2.06	1.03	0.515	0.102	0.0508	0.0202	0.0101	0.00404	0.00202	0.000993	-
LOR	API 3000™	20.0	15.0	11.2	5.00	1.000	0.500	0.250	0.150	0.100	-	-
DCL	API 3000™	20.0	15.0	11.3	5.00	1.00	0.500	0.250	0.150	-	-	-

-: not performed, L: level (calibration standard)

Table 12: *Concentrations of the calibration standards (system comparison)*

Analyte	Mass Spectrometer	Concentration [ng/mL] SQC 1	SQC 2	SQC 3	SQC 4	SQC 5	SQC 6
ATO	API 5000	50.0	5.00	0.200	0.0250	-	-
2-HYD-ATO	API 5000	50.0	5.00	0.200	0.0250	-	-
CLP	API 5000	2.02	1.00	0.100	0.00501	0.00199	0.00100
LOR	API 3000	20.0	5.00	0.250	0.150	-	-
DCL	API 3000	20.0	5.00	0.250	0.150	-	-

-: not performed

Table 13: *Concentrations of the spiked quality control standards (system comparison)*

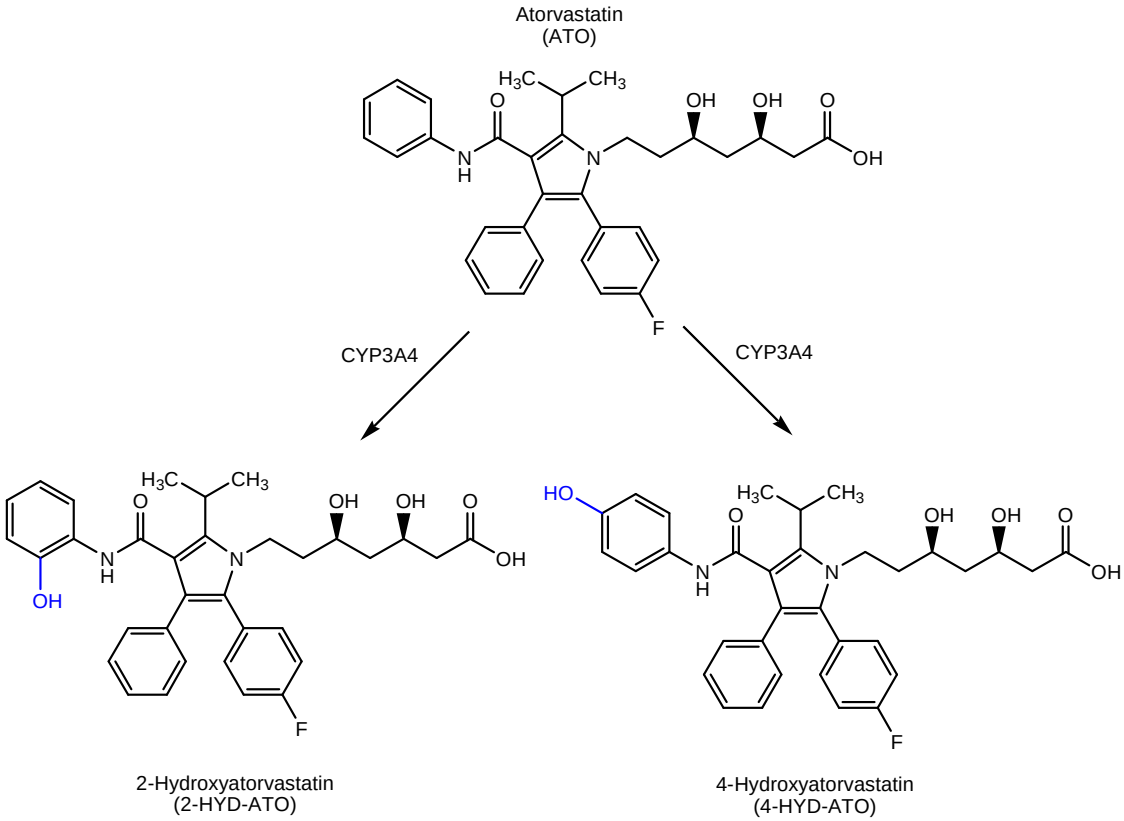
3. Results and Discussion

3.1. Atorvastatin, 2-Hydroxyatorvastatin and 4-Hydroxyatorvastatin

The synthetic compound atorvastatin (ATO) is a competitive inhibitor of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which transforms HMG-CoA into mevalonate, a precursor of cholesterol synthesis. Inhibition of this early rate-limiting step of cholesterol biosynthesis lowers the intrahepatic cholesterol concentration and causes an increased amount of LDL receptors on the cell surfaces thereby leading to lesser LDL concentrations and a decrease of the cholesterol concentration in the blood. Therefore, ATO is used for the treatment and prevention of atherosclerotic disease [19-22].

ATO is quickly and completely absorbed after oral administration and maximal concentrations are reached after one to two hours. Food decreases the absorption rate after oral administration and causes decreased peak concentrations and a later peak concentration [23].
The drug undergoes an extensive first pass metabolism in the gut wall and in the liver, causing a relatively low bioavailability of about 14 %. ATO is metabolized via CYP3A4 into the active metabolites 2-hydroxyatorvastatin (2-HYD-ATO) and 4-hydroxyatorvastatin (4-HYD-ATO) **(Scheme 5)** [19].

High ATO doses and concentrations are toxic in muscles and may even cause rhabdomyolysis [24-25]. This is especially the case when it is co-administrated with CYP3A4 inhibitors such as as calcium channel blockers, macrolides, cimetidine or grape fruit juice. On the other hand CYP-3A4 inducers as St John´s wort, rifampicin or troglitazone cause lower ATO blood levels as expected and influence the LDL cholesterol lowering effect negatively.
Several clinical trials are reported about drug co-administration with ATO including CYP3A4 inhibitors [26-30] or inducers [30-31]. Additionally, the influence of grape fruit juice was extensively studied [32-34]



Scheme 5: *Atorvastatin and active metabolites*

Due to the low ATO doses and the low bioavailability very sensitive analytical methods are required to determine ATO and active metabolite concentrations in human plasma. Several bioanalytical methods have been published in the last years including LC-UV, LC-MS and LC-MS/MS. The LC-UV methods reported by Bahrami et al. [35] and Altuntas et al. [36] determine only atorvastatin and none of the metabolites in human plasma with a LLOQ of 4 ng/mL and 18 ng/mL, respectively. More often LC-UV methods are used for determination of ATO in drug products [37-39].

For quantitation in the pg/mL range about 10 to 100 times higher sensitivity is required and can be achieved by using LC-MS [40] or LC-MS/MS methods [41-47]. All LC-MS/MS methods detect ATO and the two active hydroxy-metabolites with exception of the method of Borek et al. [45], which does not detect 4-HYD-ATO. Furthermore, the LC-MS/MS methods [42, 44, 46-47] detect additionally the lactone form of ATO and its active metabolites. The LC-MS method reported by Ma et al. [40] does only detect ATO.

Protein precipitation does not provide enough sensitivity for determination in the pg/mL range [36] and therefore all methods operating in this range use liquid-liquid or solid phase extraction with at least 0.5 mL of human plasma.

3.1.1. Method development and optimization

Based on the reported LC-MS/MS methods in the literature [41-47] for ATO and its hydroxy-metabolites mass spectra were recorded using the TurboIonSpray® source. The positive ionization mode showed more intensive signals than the negative mode. The protonated molecular ions $[M+H]^+$ for ATO, 2-HYD-ATO and 4-HYD-ATO were found at m/z 559, m/z 576 and m/z 576, respectively. For the internal standard d5-ATO $[M+H]^+$ was found at m/z 564. Product ion spectra were recorded of all four compounds and most abundant product ion was m/z 440. The individual MS settings are shown in **Table 14**.

Analyte	IS (V)	Temp. (°C)	OR (V)	CE (eV)	Q1 m/z	Q3 m/z
Atorvastatin	+5000	350	+56	30	559	440
2-Hydroxyatorvastatin	+5000	350	+36	30	576	440
4-Hydroxyatorvastatin	+5000	350	+36	30	576	440
d5-Atorvastatin	+5000	350	+56	30	564	440

IS: ionspray voltage, Temp.: temperature, OR: orifice voltage, CE: collision energy

Table 14: *API 3000™ system parameters of atorvastatin, metabolites and internal standard*

The orifice voltage was set to 56 V for ATO and d5-ATO and to 36 V for 2-HYD-ATO and 4-HYD-ATO. The optimum collision energy was 30 eV for all four compounds and the optimum temperature was found to be 350 °C. The recorded pr ecursor and product ion spectra as well as the proposed fragmentation can be found in the Appendix.

According to the chemical structure of ATO, 2-HYD-ATO, 4-HYD-ATO and d5-ATO a phenyl column (YMC-Pack Phenyl, 50 x 4.6 mm, 3µ, YMC Europe GmbH, Dinslaken, Germany) and a C18 column (Thermo Betasil C18, 50 x 4.6 mm, 3µ, Thermo Scientific, Dreieich, Germany) were tested for liquid chromatography. The isocratic starting conditions were 50 % 1 mM ammonium formate and 50 % acetonitrile at a flow rate of 0.8 mL/min.

On the phenyl column the analytes eluted very late (>5min) and increasing the amount of acetonitrile did not significantly move the peaks forward to lower retention times. Therefore a strong π-π interaction between the stationary phase and the analytes can be concluded.
Further experiments were performed on the C18 phase. Analyte injection with the above described conditions did only show poor retention or separation of the compounds. Therefore the pH of the mobile phase was set to 3.0. At this pH the carboxylic acid function of all analytes should be protonated (pKa values ~4.3). Adjusting the pH value of the mobile phase (50 % 1 mM ammonium formate and 50 % acetonitrile) to 3.0 yielded retention times of approximately 1.4, 2.0 and 2.8 min for 4-HYD-ATO, 2-HYD-ATO and ATO, respectively. The acetonitrile amount was stepwise increased until the optimal retention time for all compounds was found. The best result was achieved when the acetonitrile amount was set to 60 %. Additionally the buffer strength of the aqueous phase was investigated. Buffers of ammonium formate between 1 mM and 10 mM were investigated. The optimal buffer strength was found to be 5 mM. Higher buffer concentrations showed decreased signals for the analytes. Compared to the lower buffer concentrations the peak shapes were sharper when using the 5 mM ammonium formate buffer. Also the retention times seemed to be more stable at the higher buffer concentration. The chromatographic conditions can be summarized as follows: mobile phase: 5 mM ammonium formate buffer : MeCN (40/60, v/v, pH 3.0), analytical column: Thermo Betasil C18 (50 x 4.6 mm, 3µ), flow rate: 0.8 mL/min.

Using the described chromatographic conditions the retentions time for 4-HYD-ATO, 2-HYD-ATO and ATO were approximately 0.9, 1.6 and 1.9 min, respectively. The deuterated internal standard d5-ATO showed the same retention time as ATO. Representative chromatograms can be found in the Appendix.

The LC-MS/MS methods reported in the literature [41-47] use LLEx or SPE for sample preparation. Therefore protein precipitation was not investigated during method development. As SPE is contamination susceptible and the cartridges are expensive the sample preparation was tested first with LLEx.

During sample preparation ATO and its metabolites may undergo interconversion. The hydroxy function and carboxylic acid may react to the corresponding lactones. This reaction is favoured at pH values below the pka value. On the other hand inactive lactone metabolites are present in the human plasma samples from clinical trials. If the pH value is too high (pH >6), the lactone could be converted into the corresponding protonated acid [47]. Therefore a pH value of 5.0 was ensured during the whole sample preparation procedure.
The human plasma samples (750 µL) were mixed with 50 µL of d5-ATO internal standard solution (400 ng/mL in acetonitrile) and buffered with 750 µL 0.1M sodium acetate buffer (pH 5.0). For the extraction 4.5 mL extraction solvent were added. Diethyl ether, ethylacetate and *tert*-methylbutlyether were tested as extraction solvents. After thorough mixing for 10 minutes the samples were centrifuged for another 10 minutes at 3000 rpm (2095 g) at +4 °C. The organic layer was separated and evaporated to dryness at room temperature under a gentle nitrogen stream. The residual was redissolved in 150 µL of 1.6 mM ammonium acetate buffer and acetonitrile (6:4, v:v, pH 5.0). After centrifugation (11,000 rpm) 25 µL of each sample were injected into the LC-MS/MS system. The best extraction efficiency was yielded with diethyl ether. Samples extracted with ethylacetate and *tert*-methylbutlyether showed a clearly lower signal intensity. Therefore diethyl ether was used as extraction solvent.

The limit of determination of the method is about 0.025 ng/mL for all analytes. A signal-to-noise ratio of five was required at the limit of quantification. Therefore the calibration range of the method was set from 0.1 to 50 ng/mL.

3.1.2. Method validation

Based on the analysis of drug-free human plasma, matrix components did not interfere with ATO, 2-HYD-ATO, 4-HYD-ATO and the internal standard d5-HYD-ATO near or at their retention times and over the concentration range (0.1 - 50 ng/mL) described herein. In the Appendix typical chromatograms of blank plasma samples, calibration standards and samples from healthy volunteers are presented.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration ranges of the analytes in human plasma. The mean linear regression equations of the calibration curves generated during the validation were:

y = 0.0001 (±0.0002) + 0.0881 (±0.0243) x for ATO, $r^2 \geq 0.999$

y = 0.0001 (±0.0002) + 0.0283 (±0.0036) x for 2-HYD-ATO, $r^2 \geq 0.999$

y = 0.0001 (±0.0002) + 0.0232 (±0.0040) x for 4-HYD-ATO, $r^2 > 0.998$

where y represents the ratio of the analyte peak area to that of the internal standard, and x represents the plasma concentration of the analyte. The mean correlation coefficients were equal or better than 0.998 and demonstrate the excellent linearity of the validated method. The validated calibration range covers $5 \cdot 10^2$ orders of magnitude or each analyte.

Table 15 summarizes the inter-day precision and accuracy ranges across the calibration range as well as the precision and accuracy of the individual LLOQs.

For all analytes inter-day CV and RE were ≤8 % and <4 %, respectively. At the LLOQs the inter-day CV and RE were <4 % and <3 %, respectively. The mean signal-to-noise values at the LLOQs were ≥5 for all analytes.

The intra-day CV and RE of the spiked quality control samples were ≤10 % except for 4-HYD-ATO which showed intra-day CV <15 %. The inter-day CV and RE were <12 % and <7 %, respectively. Therefore, the obtained results were clearly within the acceptance criteria [48] of no more than 20 % deviation at LLOQ and no more than 15 % deviation for spiked quality control standards above LLOQ. Percental ranges for intra-day as well as inter-day CV and RE are summarized in **Table 15**.

	CV [%]	**RE [%]**	**CV [%]**	**RE [%]**
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
ATO	0.3 to 3.8	-1.6 to 2.5	1.4	-0.1
2-HYD-ATO	1.5 to 8.0	-2.4 to 3.2	3.9	-2.4
4-HYD-ATO	1.7 to 6.8	-1.2 to 0.9	3.6	-0.3
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
ATO	2.5 to 6.6*	-2.2 to 0.1*	2.9 to 8.4*	-5.1* to -0.6
2-HYD-ATO	4.0 to 11.0*	-4.6 to -1.6*	1.4 to 9.6	-3.9 to 3.9*
4-HYD-ATO	4.8 to 11.7*	-6.7* to -4.4	1.6 to 14.7*	-9.9 to -0.5

*: value at LLOQ

Table 15: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for atorvastatin and metabolites*

The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits. In consideration of an assay with three compounds and the used LLEx the intra- and inter-day precision and relative errors are very good and demonstrate the robustness of the validated method.

The mean absolute extraction recoveries of ATO, 2-HYD-ATO, 4-HYD-ATO and d5-ATO are shown in **Table 16**. The values were evaluated across whole concentration range.

Analyte	Mean [%]	SD [%]	CV [%]
ATO	72.5	6.1	8.4
2-HYD-ATO	78.9	3.0	3.7
4-HYD-ATO	78.0	7.3	9.4
d5-ATO	73.9	3.6	4.8

Table 16: *Mean absolute extraction recoveries for atorvastatin, metabolites and internal standard*

The mean absolute recoveries of ATO and the internal standard d5-ATO were 72.5 % and 73.9 % respectively. For 2-HYD-ATO and 4-HYD-ATO mean absolute recoveries were 78.9 % and 78.0 %, respectively.
The yielded recoveries were sufficient to achieve the required limits of quantification of 0.1 ng/mL for each analyte. As can be seen from **Table 16** the recoveries for all analytes show a relative standard deviation (CV) <10 % and were therefore to be judged acceptable. No concentration dependency of the recovery was observed..

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for all analytes except the long term stability of 4-HYD-ATO. After 3 months a significant decrease of 4-HYD–ATO stored at -20 ℃ and -70 ℃ was observed. All stati stical evaluations of the stability experiments can be found in the Appendix.

Dilution, hemolyzed and lipemic human plasma as well as the use of different batches of human plasma did not influence the determination of ATO, 2-HYD-ATO and 4-HYD-ATO. Except for dilution all influences were tested at a low and a high concentration of the respective analytes. **Table 17** shows the RE and the CV of the individual experiments.

Analyte	Dilution	Hemolyzed		Lipemic		Different batches	
		high	low	high	low	high	low
RE (%)							
ATO	-3.7	-2.9	-2.8	4.7	3.5	-4.3	-0.6
2-HYD-ATO	-12.6	-11.2	-9.5	-8.3	-4.8	-1.6	-2.4
4-HYD-ATO	-10.4	-10.6	-7.6	-5.8	-4.0	9.3	6.6
CV (%)							
ATO	1.0	3.8	5.9	2.5	1.8	1.6	3.4
2-HYD-ATO	2.1	2.2	6.7	1.5	6.2	1.6	5.1
4-HYD-ATO	1.8	3.7	5.0	5.4	6.5	2.5	5.6

Table 17: *Relative errors (%) and precisions (%) of the investigated influences for atorvastatin and metabolites*

The relative errors and precisions were <13 % and <7 % for all analytes, respectively. Therefore any influence of dilution, hemolyzed, lipemic and different batches of human plasma on the determination of the analytes can be excluded.

3.1.3. Comparison of the developed method with existent methods

In **Table 18** the developed method is compared with previously published methods for the determination of ATO and metabolites in human plasma.

The presented LC-UV methods [35-36] have high LLOQs and determine ATO only. They are not suitable for the analysis of ATO and its metabolites during pharmacokinetic studies and are more useful for the determination in drug products [37-39]. For the low concentrations (<1 ng/mL) MS or MS/MS analysis is the preferable detection technique connected with a sample preparation involving analyte concentration via liquid-liquid extraction or solid phase extraction [40-43, 45-47].

The developed method has a short run time of 3 min and uses only 0.750 mL of human plasma achieving an LLOQ of 0.1 ng/mL. The lowest calibration point of Nirogi et al. [43] and Borek-Dohalsky et al. [45] is also 0.1 ng/mL but requires a higher sample volume for the first one and a longer run time for the latter one, respectively.

Moreover, Borek-Dohalsky et al. [45] do not detect 4-HYD-ATO. Novakova et al. [47] have a comparable LLOQ for ATO but a longer run time and a higher sample volume (0.9 mL) is required. With respect to ATO the lowest calibration points for 2-HYD-ATO and 4-HYD-ATO are twice or thrice higher.

The developed method uses diethyl ether as extraction solvent and shows sufficient recovery (70 - 80 %) for all analytes which was adequate to achieve a LLOQ of 0.1 ng/mL. Bullen et al. [41] also using diethyl ether as extraction solvent report higher recoveries (about 100 %) for ATO and 2-HYD-ATO. However, a lower recovery (64 - 72 %) compared to the developed method is reported for 4-HYD-ATO. Other extraction solvents as ethylacetate [35, 40], *tert*-methylbutylether [42] or mixtures of diethyl ether and dichloromethane [43, 45] were used with satisfactory recovery (>50%).
In comparison with methods involving solid phase extraction [44, 46-47] the developed method shows better precisions and lower relative errors, except for the online solid phase extraction method of van Pelt et al. [44] that has precisions and relative errors <10 % but a LLOQ of 5 ng/mL.

Moreover, during method validation the influence of hemolyzed and lipemic human plasma on the determination of atorvastatin and its metabolites was investigated, which has to the authors' knowledge not been reported in the literature.

3.1.4. Pharmacokinetic analysis

The validated method was used to analyze ATO and 2-HYD-ATO in human plasma samples from a clinical trial. In one period of the 2-way crossover study a 40 mg ATO reference tablet was administrated to 24 male and female subjects under fasting conditions. Blood samples were taken before administration and after 0.33, 0.50, 0.67, 0.75, 1.00, 1.33, 1.67, 2.00, 2.50, 3.00, 3.50, 4.00, 6.00, 8.00, 16.00, 24.00, 36.00 and 48.00 hours. Collected blood samples were centrifuged for 10 min at 3000 rpm (2095 g) at +4 °C and two aliquots were stored at -70 °C until sample analysis.

Method	Linearity range [ng/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Preparation	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experiments*
LC-MS/MS current thesis	0.1 - 50	3.0	0.75	LLEx	2.5 to 6.6 (ATO) 4.0 to 11.0 (2-HYD) 4.8 to 11.7 (4-HYD)	-2.2 and 0.1 % (ATO) -4.6 and -1.6 (2-HYD) -6.7 and -3.0 (4-HYD)	72.5 ± 6.1 (ATO) 78.9 ± 3.0 (2-HYD) 78.0 ± 7.3 (4-HYD) 73.9 ± 3.6 (d5-ATO)	>0.999 (ATO, 2-HYD) >0.998 (4-HYD)	d5-ATO	1, 2a-e, 3, 4a, b, 5
Bahrami et al. [35] LC-UV	4 - 256	<4	1.0	LLEx	1.9 to 11.7	0.3 to 2.7	95 ± 4 (ATO) 80 ± 8 (IS)	0.9965	diclofenc	1, 2b, d, e
Altuntas et al. [36] LC-UV	500 - 86000	3.0	0.5	PP	1.0 - 1.3	-2 to 2.3	99.0	0.998	ibuprofen	1, 2e
Ma et al. [40] LC-MS	0.25 - 20	5.5	1.0	LLEx	≤7.4	≤ 15	55.5 - 60.0	≥ 0.9996	pitavastatin	1, 2a-e, 6
Bullen et al. [41] LC-MS/MS	0.250 - 25.0	6.0	1.0	LLEx	4.5 to 11.3 (ATO) 2.8 to 12.8 (2-HYD) 10.5 to 12.7 (4-HYD)	-13.6 to -3-4 (ATO) -1.3 to 2.9 (2-HYD) -2.1 to 11.9 (4-HYD)	101 - 102 (ATO) 101 - 104 (2-HYD) 63.6 - 71.6 (4-HYD) 98.4 (d5-ATO) 97.9 (d5-2-HYD)	0.998 (ATO) 0.993 (2-HYD) 0.979 (4-HYD)	d5-ATO d5-2-HYD	1, 2a-e
Jemal et al. [42] LC-MS/MS	0.5 - 200	3.5	0.5	LLEx	≤15.0 (ATO, 2-HYD, 4-HYD)	≤15.0 (ATO, 2-HYD, 4-HYD)	60 - 100 (ATO, 2-HYD, 4-HYD)	n.r.	d5-ATO d5-2-HYD d5-4-HYD	1, 2a, b, d, e, 3
Nirogi et al. [43] LC-MS/MS	0.1 - 20	2.5	1.0	LLEx	0.3 to 4.4 (ATO) 0.7 to 8.0 (2-HYD) 0.4 to 5.4 (4-HYD)	-1.2 to 5.2 (ATO) -2.4 to 5.7 (2-HYD) -3.3 to 2.4 (4-HYD)	54.2 ± 3.2 (ATO) 50.1 ± 3.8 (2-HYD) 65.2 ± 3.6 (4-HYD) 71.7 ± 2.7 (IS)	≥0.999	rosuvastatin	1, 2a-e, 3
Van Pelt. [44] LC-MS/MS	5 - 100	1.7	0.1	SPE	1.8 to 2.4 (ATO) 3.1 to 6.9 (2-HYD) 1.9 to 4.6 (4-HYD)	-3.1 to -8.3 (ATO) -3.4 to -9.6 (2-HYD) -6.3 to -7.3 (4-HYD)	n.r.	>0.99	d5-ATO d5-2-HYD d5-4-HYD	n.r.
Borek-Dohalsky et al. [45] LC-MS/MS	0.1 - 40	8.0	0.5	LLEx	3.0 to 7.3 (ATO) 3.4 to 7.7 (2-HYD)	0.3 to 2.0 (ATO) 0.8 to 1.7 (2-HYD)	89.6 - 92.5 (ATO) 81.3 - 85.3 (2-HYD) 82.4(IS)	>0.999	clindamycin	1, 2a-e, 5, 6
Hermann et al. [46] LC-MS/MS	0.2 – 30 (ATO, 4-HYD) 0.5 - 30 (2-HYD)	20.0	0.75	SPE	6.5 to 18.0 (ATO) 3.9 to 15.2 (2-HYD) 14.3 to 21.4 (4-HYD	<0.1 to -15.0 (ATO) <0.1 to 17.3 (2-HYD) <0.1 to -12.0 (4-HYD	53 to 78 (ATO, 2-HYD, 4-HYD)	≥0.99	methaqualone	1, 2c, 6
Nováková et al. [47] UPLC-MS/MS	0.08 - 56 (ATO) 0.19 - 56 (2-HYD) 0.32 - 56 (4-HYD)	5.0	0.9	SPE	10.6 to 13.7 3.9 to 11.2 2.5 to 9.2	n.r.	84.6 - 86.0 (ATO) 65.3 - 86.0 (2-HYD) 78.8 - 89.3 (4-HYD)	0.9999 (ATO) 0.9996 (2-HYD) 0.9997 (4-HYD)	d5-ATO	1, 6

n.r.: not reported, PP: protein precipitation, LLEx: liquid-liquid extraction, SPE: solid phase extraction
*1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e)), 3: influence of dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: influence of different individuals, 6: matrix effect

Table 18: *Atorvastatin and metabolites method comparison*

All collected blood samples were analyzed in a total of 13 sequences. Concentrations were calculated by the calibration curve that was measured at the beginning of each sequence and quality was ensured by measuring spiked quality control samples within each sequence. Calibration was performed by weighted (1/concentration^2) linear regression for both compounds.

Atorvastatin

The standard curve was linear between 0.0995 and 50.6 ng/mL. The lower limit of quantification was 0.0995 ng/mL. The inter-day precision and the analytical recovery of the spiked quality control standards of ATO in human plasma ranged from 4.2 to 5.4 % and were 101.8 % (40.0 ng/mL), 99.7 % (32.0 ng/mL), 98.2 % (0.799 ng/mL) and 97.8 % (0.250 ng/mL), respectively.

Within the set of SQC samples (n = 39) analyzed with the batches of study samples, all SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

2-Hydroxyatorvastatin

The standard curve was linear between 0.0997 and 50.7 ng/mL. The lower limit of quantification was 0.0997 ng/mL.

The inter-day precision and the analytical recovery of the spiked quality control standards of 2-HYD-ATO in human plasma ranged from 4.7 to 7.9 % and were 102.7 % (40.1 ng/mL), 101.0 % (32.1 ng/mL), 99.4 % (0.803 ng/mL) and 98.8 % (0.251 ng/mL), respectively.

Within the set of SQC samples (n = 39) analyzed with the batches of study samples, 152 out of 156 SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

Figure 7 shows as example the mean plasma concentration profiles of ATO and 2-HYD-ATO after oral administration of a 40 mg atorvastatin reference tablet to 24 healthy male and female volunteers. The mean peak concentration (C_{max}) of 22.4 ng/mL for atorvastatin was attained at 1.03 h after administration of the product. The mean peak concentration (C_{max}) of 26.0 ng/mL for 2-HYD-ATO was attained at 1.3 h after administration of the product.

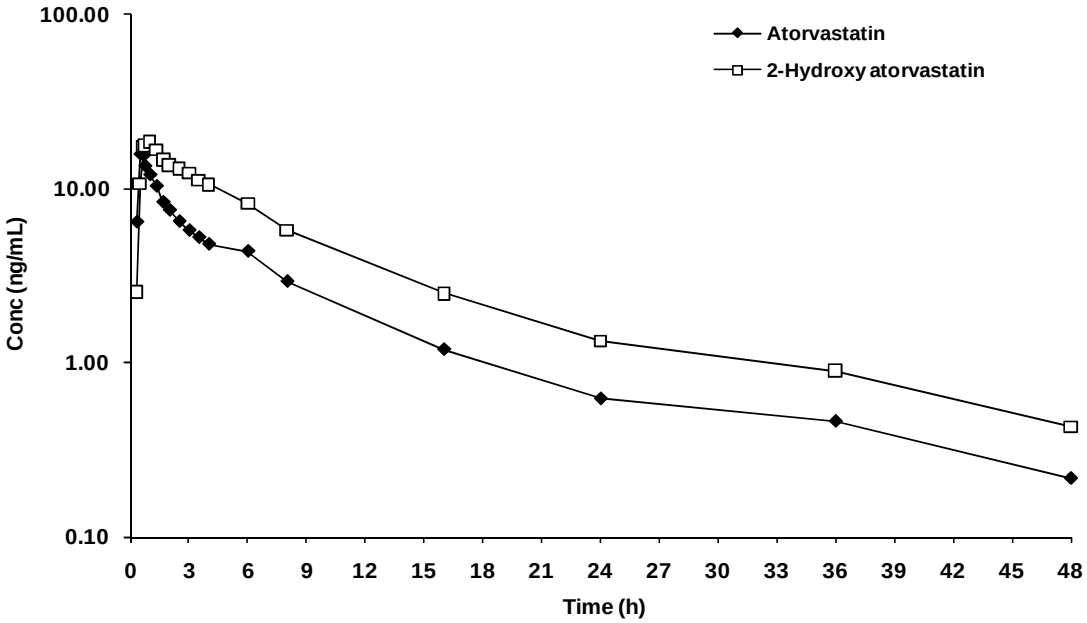


Figure 7: *Mean plasma profile of atorvastatin and 2-hydroxyatorvastatin concentration vs. time following a 40 mg oral dose of reference atorvastatin tablet to healthy volunteers*

Table 19 shows the pharmacokinetic parameters of ATO and 2-HYD-ATO observed in this thesis and from previously reported clinical trials. Different ATO doses are shown but due to the high variability of the drug and its metabolites they were not normalized.

For 40 mg administration of ATO tablet the $AUC_{0\rightarrow\infty}$ values for ATO and 2-HYD-ATO were calculated as 82.9 and 161.0 ng*h/mL, respectively. This is clearly higher than in previously reported literature for a 40 mg dose by Kantola et al. [49], Lilja et al. [34] and Mendoza et al. [50] as shown in **Table 19**.

Also the C_{max} for ATO and 2-HYD ATO determined in this thesis are clearly higher. However, the calculated t_{max} and $t_{1/2}$ values for ATO and 2-HYD-ATO are comparable to the other studies.

Mazzu et al. [29] published pharmacokinetic parameters for an ATO dose of 20 mg and reports ATO AUC values that are close to the values in this thesis as shown in **Table 19**. However, the metabolite values (AUC and C_{max}) are lower than the parent compound concentrations which is in all other published papers exactly vice versa.

Reference	No. of subjects / gender	Last time point [h]	Dose / Formulation	Analyte	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current thesis	24/ m, f	48	40 mg/ tablet	ATO	80.5 ± 27.7	82.9 ± 30.4	22.4 ± 13.4	1.0 ± 0.8	11.8 ± 3.5
				2-HYD-ATO	147.4 ± 39.7	161.0 ± 42.9	26.0 ± 14.7	1.3 ± 0.8	12.4 ± 3.3
Mazzu et al. [29]	18/ m, f	60	20 mg/ tablet	ATO	79.1 (56.5)*	98.7 (49.4)*	6.9 (53.1)*	1.8 (54.8)*	13.8 (46.5)*
				2-HYD-ATO	37.6 (35.4)*	36.1 (51.0)*	3.6 (67.4)*	2.2 (26.8)*	n.r.
Kantola et al. [49]	10/ 5m, 5f	72	40 mg/ tablet	ATO	50.6 ± 21.9	54.2 ± 24.2	13.4 ± 9.5	1.0 (0.5-3)	7.0 ± 3.7
				2-HYD-ATO	77.2 ± 30.1	86.6 ± 30.3	9.8 ± 6.1	2.2 (0.5-4)	10.8 ± 3.5
Lilja et al. [34]	12/ 6m, 6f	72	40 mg/ tablet	ATO	58.1 ± 34.7	61.4 ± 36.2	12.7 ± 7.8	1.0 (0.5-3)	7.8 ± 3.6
				2-HYD-ATO	71.3 ± 23.2	77.5 ± 24.0	7.7 ± 2.4	1.5 (1-3)	9.7 ± 2.8
Mendoza et al. [50]	52/ m	72	40 mg/ tablet	ATO	54.07	57.7	14.5	0.7 ± 0.4	9.1 ± 2.7
				2-HYD-ATO	65.73	69.6	10.5	1.27 ± 1.0	9.1 ± 1.9
Borek-Dohalsky et al. [45]	44/ n.r.	72	80 mg/ tablet	ATO	91.5 ± 42.9	95.1 ± 43.8	23.2 ± 10.4	0.5 (0.25-3)	12.4 ± 7.0
				2-HYD-ATO	124.6 ± 67.6	134.1 ± 68.2	21.6 ± 9.2	1.0 (0.5-4)	12.7 ± 5.5
Siedlik et al. [27][a]	12/ n.r.	96	10 mg/ tablet	ATO	n.r.	126	5.81	2.0	45.6
Ma et al. [40]	18/ n.r.	48	20 mg/ tablet	ATO	54.77 ± 21.82	58.3 ± 23.1	8.5 ± 5.1	1.34 ± 0.7	8.5 ± 2.7
Radulovic et al. [23][a]	16 / 10m, 6f	72	80 mg/ capsule	ATO	868	n.r.	78.4	2.6	35.7

m: male, f: female, n.r.: not reported, * Geometric mean (CV, %), a: mean equivalent pharmacokinetic parameters

Table 19: *Reported values for atorvastatin and 2-hydroxyatorvastatin pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after overnight fasting and administration of a single oral dose*

Reference	No. of subjects / gender	Last time point [h]	Dose / Formulation	Analyte	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]
Posvar et al. [51][a]	22 / 14m, 8f	72	2.5 mg/ capsule	ATO	17.9	n.r.	1.62	4.0	n.r.
			5 mg / capsule	ATO	88.4	n.r.	6.80	6.0	16.3
			10 mg/ capsule	ATO	232	n.r.	6.58	4.0	n.r.
			20 mg/ capsule	ATO	282	n.r.	12.0	3.0	44.3
			40 mg / capsule	ATO	382	n.r.	27.4	3.0	22.6
			80 mg/ capsule	ATO	335	n.r.	33.1	2.8	19.2
			120 mg / capsule	ATO	2130	n.r.	302	2.0	n.r.

m: male, f: female, n.r. not reported, a: mean equivalent pharmacokinetic parameters

Table 19: *continued*

Comparing the pharmacokinetic parameters in **Table 19** observed from the different ATO doses, no dose proportionality can be concluded. Even in the presented data of Posvar et al. [51] who investigated the pharmacokinetic parameters in an dose proportionality study, their proportional increase with the dose is not obvious. Especially, when administering 120 mg ATO, the AUC and the C_{max} showed an over proportional increase.

Finally it has to be noted that Posvar et al. [51], Radulovic et al. [23]. and Siedlik et al. [27] report the calculated AUCs in ng·eq·h/mL and the concentration in ng·eq/mL due to determination with an radioimmuno assay (RIA) (Posvar, Radulovic) and an enzyme inhibition assay (EIA) (Siedlik), respectively. Not only the reported AUCs and C_{max} values are higher than the other reported values (**Table 19**) but also the t_{max} and $t_{1/2}$ values are significantly higher. Siedlik et al. [27] contribute this to the ATO metabolites that are also detected with the unspecific EIA. However, Radulovic et al. [23] and Posvar et al. [51] report an influence of only 11% of the ATO metabolites to the assay. If this is the case other factors must be responsible for this strong increase and deviation.

3.1.5. Method comparison on API 5000™ (*Partial Validation*)

For a comparison of ATO and 2-HYD-ATO of the validated method on an API 5000™ spectra were recoded in the first step. This was performed as described above on the API 3000™. The optimal parameters for ATO and 2-HYD-ATO on the API 5000™ are shown in **Table 20**.

Analyte	IS (V)	Temp. (°C)	DP (V)	CE (eV)	Q1 m/z	Q3 m/z
Atorvastatin	+5500	400	+80	80	559	440
2-Hydroxyatorvastatin	+5500	400	+60	80	576	440
d5-Atorvastatin	+5500	400	+80	80	564	440

IS: ionspray voltage, Temp.: temperature, DP: declustering potential, CE: collision energy

Table 20: *API 5000™ system parameters of atorvastatin and metabolite*

The IS was set to 5500 V and the best signal intensity was obtained at a temperature of 400 °C for all analytes. The declustering potential (DP) and collision energy (CE) for ATO and d5-ATO were set to 80 V and 80 eV, respectively.

The collision energy (CE) for 2-HYD-ATO was also set to 80 eV and the declustering potential was with 60 V slightly lower than for ATO. For ATO, 2-HYD- ATO and d5-ATO the same precursor and product ions as on the API 3000™ were optimized on the API 5000™.
For system comparison neither the chromatographic conditions nor the sample preparation procedure was changed. Therefore the mass spectrometers can be compared directly.
Table 21 shows the results of the calibration standards of the *Partial Validation* (system comparison) in comparison to the calibration standards of the *Full Validation* method.

Method comparison						
Analyte	**MS**	**Linearity [ng/mL]**	**Inter-day CV [%]**	**Inter-day RE [%]**	**Inter-day CV [%] at LLOQ**	**Inter-day RE [%] at LLOQ**
ATO	API 5000™	0.0250 - 50.0	0.7 to 5.7	-5.2 to 4.5	2.8	2.8
ATO	API 3000™	0.100 - 50.0	0.3 to 3.8	-1.6 to 2.5	1.4	-0.1
2-HYD-ATO	API 5000™	0.0250 - 50.0	0.5 to 9.0	-5.3 to 6.8	2.8	0.0
2-HYD-ATO	API 3000™	0.100 - 50.0	1.5 to 8.0	-2.4 to 3.2	3.9	-2.4

Table 21: *Method comparison – Calibration Standards for atorvastatin and metabolites*

For ATO and 2-HYD-ATO the LLOQ achieved on the API 5000™ was better by a factor of 4. However, the inter-day precision and relative error of the calibration standards were better on the API 3000™.
In **Table 22** the intra-day and inter-day precision and relative error measured for ATO and 2-HYD-ATO on the API 3000™ and API 5000™ are shown.

Method comparison					
Analyte	**Mass Spectrometer**	**Intra-day CV [%]**	**Intra-day RE [%]**	**Inter-day CV [%]**	**Inter-day RE [%]**
ATO	API 5000™	1.1. to 6.3*	-4.5 to 1.6	1.6 to 6.0*	-5.1 to 0.6
ATO	API 3000™	2.9 to 8.4*	-5.1 to -0.6*	2.5 to 6.6*	-2.2 to 0.1*
2-HYD-ATO	API 5000™	2.0 to 6.6*	-7.9 to 3.8	2.9 to 8.3*	-7.9 to 2.4
2-HYD-ATO	API 3000™	1.4 to 9.6	-3.9* to 0.2	4.0 to 11.0*	-4.6 to -1.6*

*: at LLOQ

Table 22: *Method comparison – Quality Control Samples for atorvastatin and metabolites*

On the API 5000™ the intra-day and inter-day CVs and REs for ATO and 2-HYD-ATO were within ±10 %. **Figure 8** illustrates the inter-day REs of the standard quality control samples including the *Full Validation* method as well as the *Partial Validation* method.

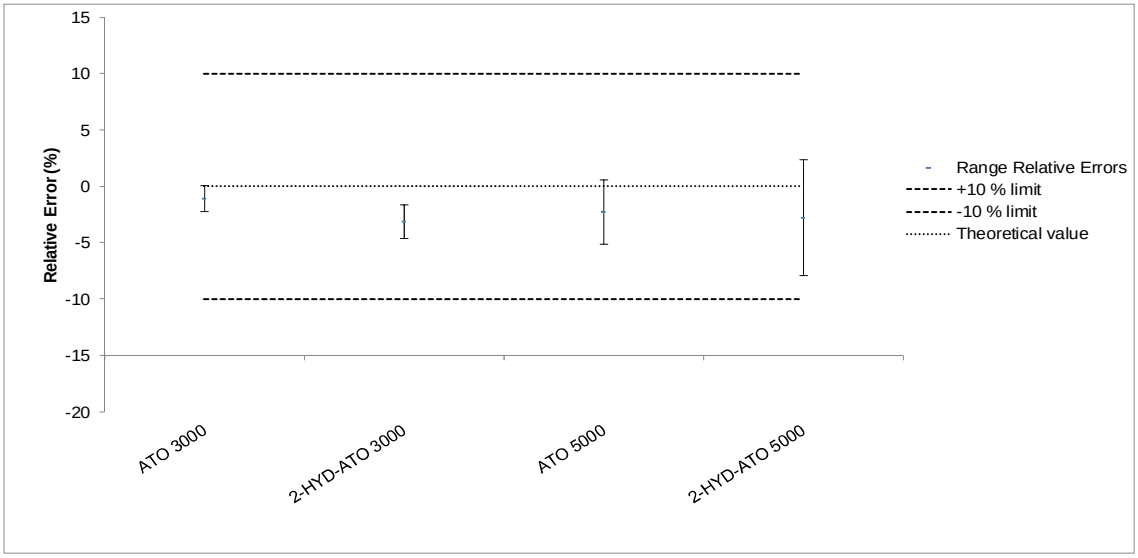


Figure 8: *Inter-day relative error ranges API 3000™ and API 5000™ for atorvastatin and metabolite*

Comparing both mass spectrometers it can be seen from **Figure 8** that for ATO and 2-HYD-ATO the REs were determined predominantly below the expected theoretical value when measurements are performed with the API 3000™. The REs enter the positive deviation from the nominal value when measurements were performed with the API 5000™. However, the intervals of the REs are smaller, which means a better precision, when measurements were performed on the API 3000™. In this experiment ATO and 2-HYD-ATO could be determined with clearly better precision on the API 3000™ than on the API 5000™.

3.2. Clopidogrel

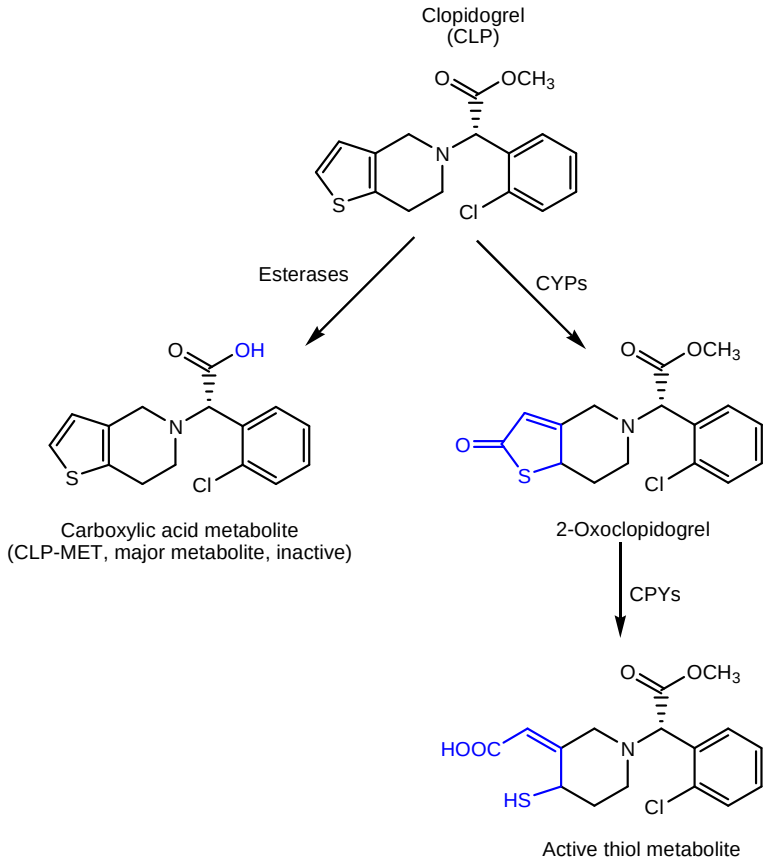
Clopidogrel (CLP), a thienopyridine derivative, is a potent platelet aggregation inhibitor and is used for the treatment of strokes, acute coronary syndromes, after coronary artery stenting, and for the treatment of peripheral vascular disease. Therapy with CLP may be an alternative for patients unable to tolerate aspirin due to gastrointestinale haemorrhage [52-55].

As a prodrug CLP needs hepatic biotransformation. Hydrolysis through esterases primarily forms the inactive carboxylic acid metabolite (CLP-MET). To a minor amount different enzymes of the P450 family metabolize CLP into the intermediate 2-oxoclopidogrel, which is further transformed into the active thiol metabolite. The active metabolite binds selectively and irreversibly through a disulfide bridge to the P2Y12 receptor on the platelet surface. This blocks the ADP induced platelet aggregation via the GP-IIb/IIIa receptor complex and results in inhibition of platelet aggregation [52-55]. CLP and metabolism pathways are shown in **Scheme 6**.

After oral administration in humans CLP is rapidly and extensively metabolized as described above and plasma concentrations of the parent drug and the thiol metabolite are very low (pg/mL) [56]. Another problem is the instability of the thiol metabolite, which has to be stabilized when blood samples are collected [57].

For these reasons, in several studies the major circulation carboxylic acid metabolite of CLP has been used to determine indirectly the pharmacokinetic profile of CLP [56, 58-60].

Several analytical methods including LC-UV, LC-MS, LC-MS/MS and GC-MS have been reported for the determination of inactive CLP-MET [60-63]. However, the actual plasma concentration of parent drug and/ or active metabolite(s) are of major interest in pharmacokinetic studies. Methods for the simultaneous determination of CLP and CLP-MET using liquid chromatography coupled with mass spectrometry have been reported by Mani et al. [59], Patel et al. [64] and Reddy et al. [65].



Scheme 6: *Clopidogrel and metabolites*

All three methods have runtimes greater than 5 min and show less sensitivity for CLP with a LLOQ of ≥100 pg/mL. To generate a complete pharmacokinetic profile for CLP, analytical methods with higher sensitivity are required, especially when the elimination phase has to be considered. Analytical methods determining only CLP [66-69] show better sensitivity with LLOQs lower than 20 pg/mL (down to 5 pg/mL), than methods determining CLP and its inactive metabolite simultaneously, whereas LC-MS/MS is the method of choice.

3.2.1. Method development and optimization

CLP spectra for *Full Validation* were recorded on the API 3000™. Initial tests showed higher sensitivity for CLP with TurboIonSpray® than with APCI. Therefore optimization of the spectra was performed with TurboIonSpray®.

In the negative ionization mode only a poor signal for $[M-H]^-$ was detected. The positive ionization mode showed a clearly strong signal of the protonated molecular ion at m/z 322. The IS was set to 5000 V. Lower IS voltages decreased the signal for m/z 322, but higher values than 5000 V did not significantly increase the $[M+H]^+$ signal. The temperature was set to 350 °C and in the precursor ion spectrum m/z 322 was maximal at a OR voltage of 45 V. The most abundant signal in the product ion spectrum was m/z 212 which showed maximal intensity at a CE of 26 eV. For the internal standard DIL the same MS parameters were used. For DIL the precursor ion spectrum in Q1 showed the $[M+H]^+$ ion at m/z 415. After collision in Q2 the most abundant fragment was found at m/z 178 in the product ion spectrum. **Table 23** summarizes the MS settings for CLP and DIL, respectively.

Analyte	IS (V)	Temp. (°C)	OR (V)	CE (eV)	Q1 m/z	Q3 m/z
Clopidogrel	+5000	350	+45	26	322	212
Diltiazem	+5000	350	+45	26	415	178

IS: ionspray voltage, Temp.: temperature, OR: orifice voltage, CE: collision energy

Table 23: *API 3000™ system parameters for clopidogrel and diltiazem*

The corresponding mass spectra and the proposed fragmentation of CLP and DIL can be found in the Appendix.

Based on the molecular structure of CLP a C18 column (Thermo Betasil C18, 50 x 4.6 mm, 3µ, Thermo Scientific, Dreieich, Germany) was used for the first chromatography tests. Different concentrations of ammonium formate and ammonium acetate buffers at a pH of 3.0 were investigated.

Compared to the ammonium acetate buffer better sensitivities were achieved with the ammonium formate buffer. The amount of acetonitrile was found to be optimal at 80 %. Using 80 % acetonitrile and 20 % 5 mM ammonium formate buffer (pH 3.0) clopidogrel eluted after about 2.3 min from the C18 column. The sensitivity could be increased by replacing the ammonium formate buffer by 0.01 % formic acid. Finally it was found out that the addition of some drops of ammonia, shifting the pH of the mobile phase to about 9.0, increased the intensity of the CLP signal by a factor of about 2-3. Under these conditions CLP eluted form the C18 column after about 1.9 min. The chromatographic conditions can be summarized as follows: mobile phase: 0.01% formic acid (pH 9.0) : acetonitrile (20/80, v/v), analytical column: Thermo Betasil C18 (50 x 4.6 mm, 5µ), flow rate: 1.0 mL/min.

Clopidogrel-d4 should be used as the internal standard. However, the reference compound from TRC (Toronto Research Chemicals Inc., Ontario Canada) contained residuals of undeuterated CLP. Deuterated internal standards from other manufacturers were not considered. Diltiazem was found to be a good alternative. Under the conditions described above DIL eluted after about 1.0 min from the C18 column.

Preparing human plasma samples with protein precipitation showed only poor sensitivity for CLP. Therefore a LLEx method was developed. The challenge here was to find the right conditions to optimize the extraction efficiency (recovery) on the one hand and on the other hand to avoid the cleavage of the ester function of CLP. The optimal pH value during the extraction procedure was found to be 4. Strong alkaline or acidic conditions produced the inactive carboxylic acid metabolite during sample preparation. Diethyl ether and ethyl acetate were tested as extraction solvents. Under the above described conditions diethyl ether showed the best recovery results. For the final sample preparation procedure 500 µL of human plasma were mixed with 50 µL of acetonitrile containing DIL (2.5 ng/mL) and with 100 µL of 1 M ammonium formate buffer (pH 4.0).

Extraction was performed by adding 4 mL diethyl ether. After thorough mixing for 10 minutes the samples were centrifuged for 10 minutes at 3000 rpm (2095 g) at +4 °C. The organic layer was evaporated to dryness at room temperature and the residual was redissolved in 150 µL of 0.001 M ammonium formate buffer (pH 3.0) and acetonitrile (1:5, v:v). After centrifugation (11,000 rpm) for 3 minutes 15 µL of each sample were injected into the LC-MS/MS system.

3.2.2. Method validation

Based on the analysis of drug-free human plasma, matrix components did not interfere with CLP and the internal standard DIL near or at their retention times and over the concentration range (0.02 - 10 ng/mL) described herein. In the Appendix typical chromatograms of blank plasma samples, calibration standards and samples from healthy volunteers are presented.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range of CLP in human plasma. The mean linear regression equation of the calibration curves generated during the validation was:

$y = 0.0016\ (\pm 0.0019) + 0.2879\ (\pm 0.0511)\ x$ for CLP, $r^2 \geq 0.997$

where *y* represents the ratio of the CLP peak area to that of DIL, and *x* represents the plasma concentration of CLP. The mean correlation coefficient was equal or better than 0.997 and demonstrates the excellent linearity of the validated method. The validated calibration range covers an order of magnitude of $5 \cdot 10^2$.

Inter-day CV and RE of the calibration standards are summarized in **Table 24** and were <7 % and <4 %, respectively. At the LLOQ the inter-day CV and RE error were even better with 2.0 % and -0.4 % respectively. The mean signal-to-noise value at the LLOQ was ≥5 for CLP.

	CV [%]	**RE [%]**	**CV [%]**	**RE [%]**
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
CLP	2.0 to 6.7	-2.5 to 3.8	2.0	-0.4
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
CLP	5.1 to 10.0*	-4.9* to -2.2	2.7 to 5.0*	-2.3 to 8.3*

*: value at LLOQ

Table 24: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for clopidogrel*

The spiked quality control samples also showed the excellent performance of the assay. The intra-day CV and RE were ≤5 % and <9 %, respectively. The inter-day CV and RE were ≤10 % and <5 %, respectively and therefore judged to be acceptable as all results were within the acceptance criteria of no more than 20 % deviation at the LLOQ and 15 % above the LLOQ. All quality data of the spiked quality control samples are summarized in **Table 24**. The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits.

The recovery data shown in **Table 25** demonstrate the overall very good sample preparation procedure and that the appropriate conditions (pH value and extraction solvent) were selected. The mean absolute extraction recovery of CLP was determined with 100.9 % which indicates that no CLP is lost during the extraction procedure. The recovery of DIL was determined with 81.4 %. For CLP no concentration dependency was detected which is indicated by the good SD (<7 %) and CV (<7 %).

Analyte	Mean [%]	SD [%]	CV [%]
CLP	100.9	6.4	6.3
DIL	81.4	4.6	5.7

Table 25: *Mean absolute extraction recoveries for clopidogrel and diltiazem*

The recovery was sufficient to achieve the required limit of quantification of 0.02 ng/mL for CLP.

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for CLP. All statistical evaluations of the stability experiments can be found in the Appendix.

Dilution, hemolyzed and lipemic human plasma as well as the use of different batches of human plasma did not influence the determination of CLP. Except for dilution all influences were tested at a low and a high concentration. **Table 26** shows the RE and the CV of the individual experiments.

Analyte		Dilution	Hemolyzed		Lipemic		Different batches	
			high	low	high	low	high	low
	RE (%)							
CLP		-4.8	-8.5	-0.5	5.0	8.0	-8.9	-9.0
	CV (%)							
CLP		3.9	2.3	5.8	4.8	2.7	4.2	5.5

Table 26: *Relative errors (%) and precisions (%) of the investigated influences for clopidogrel*

For all experiments the RE and CV were <10 % and <6 %, respectively. Therefore any influence of dilution, hemolyzed, lipemic and different batches of human plasma on the determination of CLP can be excluded.

In the incurred samples reanalysis experiment 45 CLP samples received from a clinical trial and previously analyzed with the developed method were analyzed again. The batch of reanalyzed samples contained blank samples as well as samples with low and high concentrations to proof the performance of the method over the whole validation range. The result of this experiment is summarized in **Table 27**.

Analyte	No. of reanalyzed samples	% of blanks	Percentual samples within ≤5 %	5 - 10 %	10 - 15 %	15 - 20 %	>20 %
CLP	45	35.6	15.6	8.9	24.4	13.3	2.2

Table 27: *Incurred samples reanalysis for clopidogrel*

84.5 % of the reanalyzed samples were within the ±15 % range. 13.2 % of the reanalyzed samples showed a deviation within 15-20 %. Only 2.2 % showed a deviation of more than 20 % compared to the first analysis. A deviation below 5 % was observed for 15.6 % of the reanalyzed samples. The mean absolute difference of the 1st to 2nd analysis was 6.9 % which demonstrates the excellent performance and reproducibility of the developed method.

3.2.3. Comparison of the developed method with existent methods

In **Table 28** the developed method is compared with previously published methods for the determination of CLP in human plasma.

The developed method has a short run time of 2 min, uses only 0.5 mL of human plasma and has a LLOQ of 0.02 ng/mL.

Robinson et al. and Shin et al. [68-69] report a LLOQ of 0.01 ng/mL requiring 0.3 mL and 0.5 mL of human plasma, respectively. The lowest LLOQ is reported by Nirogi et al. [67] with 0.005 ng/mL. This method uses a diethyl ether -hexane mixture as extraction solvent and MS/MS detection is performed with an API 4000™.

Method	Linearity range [ng/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Preparation	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experiments*
LC-MS/MS current thesis	0.02 - 10	2.0	0.5	LLEx	5.1 to 10.0	-4.9 to -2.2	100.9 ± 6.4	≥0.997	diltiazem	1, 2a-e, 3-5, 7
Lainesse et al. [66] LC-MS/MS	0.02 - 10	3.5	n.r.	LLEx	<4.8	3.1 to 9.3	>83.3	0.9980	n.r.	n.r.
Mani et al. [59] LC-MS/MS	0.25 - n.r.	12.0	0.5	automated SPE	3.6 to 9.2	n.r.	n.r.	n.r.	n.r.	n.r
Nirogi et al. [67] LC-MS/MS	0.005 - 6	2.5	0.5	LLEx	0.4 to 7.0	-8.3 to 0.5	76.8 ± 1.9	0.9997[a]	ticlopidine	1, 2a-e, 3, 4a-b, 5
Patel et al. [64] LC-MS/MS	0.25 - 25	5.0	0.3	SPE	2.1 to 10.2	0.0 to 2.4	98.1	≥0.9989	glimepiride	1, 2a-e, 3, 4a-b, 6
Reddy et al. [65] LC-MS/MS	0.1 - 8	7.5	0.5	online sample extraction	0.6 to 12.2	-8.5 to 1.8	94	>0.995	ticlopidine	1, 2a-d
Robinson et al. [68] LC-MS/MS	0.01 - 12	3.0	0.3	LLEx	2.2 to 7.2	-0.7 to 3.0	61.5 - 68.6	0.9993[a]	$^{2}H_{3}$-clopidogrel	2a- e, 3, 6
Shin et al. [69] LC-MS/MS	0.01 - 10	3.0	0.5	LLEx	4.4 to 8.1	-1.6 to 3.5	83.9 - 90.6	>0.999	ticlopidine	2a-e

a: mean, n.r.: not reported, LLEx: liquid-liquid extraction, SPE: solid phase extraction
*1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e)), 3: influence of dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: influence of different individuals, 6: matrix effect, 7: incurred samples

Table 28: *Clopidogrel method comparison*

However, the developed method in this thesis has the highest recovery compared to the reported liquid-liquid extraction methods in **Table 28**. The solid phase extractions of Mani et al. [59] and Patel et al. [64] as well as the online sample extraction of Reddy et al. [65] show less sensitivity than methods employing a liquid-liquid extraction. However, it should be noted that these methods also determine CLP-MET in the same run. The developed methods of Lainesse et al. [66] and Mani et al. [59] do not report any additional validation experiments beyond linearity or recovery. Beside the methods of Nirogi et al. [67] and Patel et al. [64] the method validated in this thesis method reports comprehensive validation experiments, including incurred sample reanalysis that demonstrated the excellent performance of the method and which is not reported to the authors' knowledge in literature.

3.2.4. Pharmacokinetic analysis

CLP was analyzed in human plasma samples received from a clinical trial. In one period of the 2-way crossover study a 75 mg CLP reference tablet was administrated to 24 male subjects under fasting conditions. Blood samples were taken before the administration and after 0.25, 0.50, 0.67, 0.83, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 4.00, 8.00, 12.00, 16.00 and 24.00 hours. Collected blood samples were centrifuged for 10 min at 3000 rpm (2095 g) at +4 °C and two aliquots were stored at -70 °C until sample analysis.

For sensitivity reasons the CLP method developed on the API 3000™ was transferred to the API 5000™ in a *Partial Validation* (see next sections). On the API 5000™ a LLOQ of 1 pg/mL could be achieved enabling the detection of CLP concentrations in the late elimination phase. The study samples were analyzed on the API 5000™.

All collected blood samples were measured in a total of 9 sequences. A standard curve and spiked quality control samples prepared in human plasma were analyzed in each sequence together with the study samples. Calibration was performed by weight ($1/concentration^2$) linear regression. The standard curve was linear between 0.000998 ng/mL and 2.09 ng/mL. The limit of quantification was 0.000998 ng/mL. The coefficient of correlation of resulting linear regressions was at least 0.997.

The inter-day precision and accuracy of the spiked quality control standards of CLP in human plasma analyzed with the batches of study samples ranged from 3.2 to 7.8 % and were

104.3 % (1.61 ng/mL), 105.3 % (0.403 ng/mL), 105.1 % (0.0301 ng/mL) and 103.8 % (0.00298 ng/mL), respectively. Within the set of SQC samples (n = 27) analyzed with the batches of study samples, 107 out of 108 were within ± 15 % of their respective nominal value.

Figure 9 shows as example the mean plasma concentration profile of CLP after oral administration of a 75 mg CLP reference tablet (Plavix®) to 24 healthy volunteers. The mean peak concentration (C_{max}) of 0.910 ng/mL for CLP was attained at 2.38 h after administration of the product.

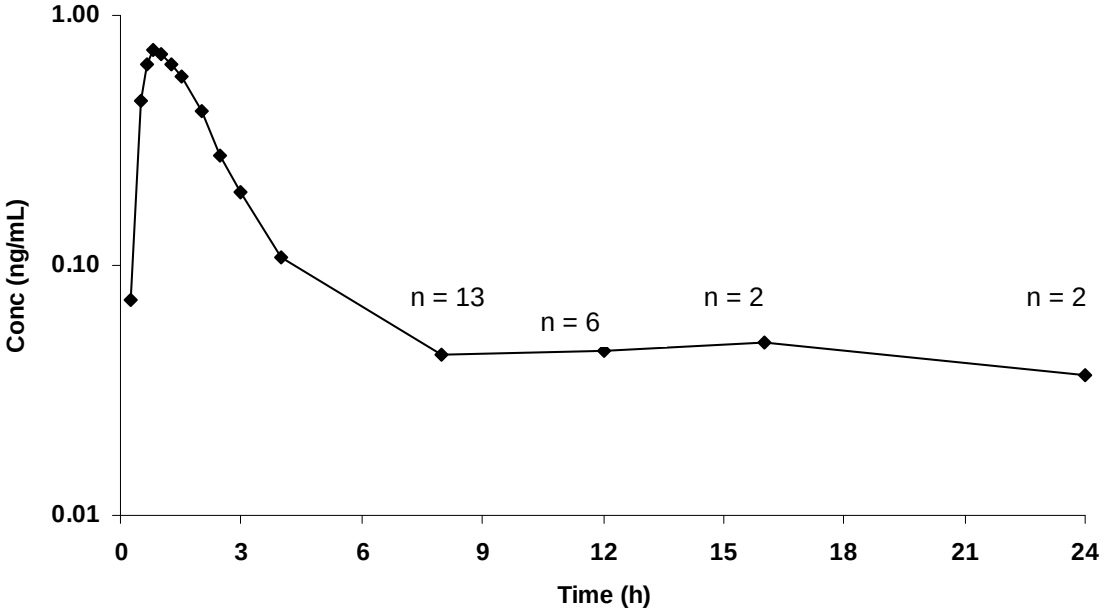


Figure 9: *Mean plasma profile of clopidogrel concentration vs. time following a 75 mg oral dose of reference clopidogrel tablet (Plavix®) to healthy volunteers.*

Table 29 shows the pharmacokinetic parameters of CLP after oral administration of 75 mg CLP in comparison to pharmacokinetic parameters reported in previously published studies. All shown values are normalized to a 75 mg CLP dose. The calculated AUC, C_{max} and t_{max} from this thesis match well with the reported values in literature except for Lainesse et al. [66] who report clearly higher values.

The calculated half-lifes in **Table 29** show high variability what may be due to the number of concentrations estimated in the elimination phase. With higher sensitivity, more points can be detected in the elimination phase and a more reliable half-life can be determinated. With the developed method that has a LLOQ of 1 pg/mL, concentrations in the late elimination phase can be measured reliable and therefore it is excellent suitable for CLP half-life calculation.

Reference	No. of subjects/ gender	Last time point [h]	Dose/ Formulation	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current thesis	24/ m	24	75 mg/ film-coated tablet	1.72 ± 1.42	1.82 ± 1.46	0.91 ± 0.81	1.12 ± 0.73	2.38 ± 2.33
Filipe et al. [70]	64/ 32m, 32f	24	75 mg/ film-coated tablet	1.79 ± 2.31	1.92 ± 2.40	1.28 ± 1.72	0.89 ± 0.82	5.71 ± 4.21
Lainesse et al. [66]	36/ m, f	48	2x75 mg/ film-coated tablets	14.42 ± 6.54	14.97 ± 6.55	3.96 ± 1.96	1.25 ± 0.50[a]	7.26 ± 1.49
Nirogi et al. [71]	12/ m	24	75mg/tablet	1.76 ± 0.28	1.88 ± 0.30	1.02 ± 0.14	1.08 ± 0.2	2.51 ± 1.18
Pawlowska et al. [72]	48/ m	48	2x75 mg/ film-coated tablets	1.43 ± 1.36	1.47 ± 1.37	0.84 ± 0.96	1.01 ± 0.54	8.66 ± 3.96
Richter et al. [73]	48/ m	24	2x75 mg/ film-coated tablets	1.89 ± 3.56	2.01 ± 3.58	0.88 ± 1.71	1.13 ± 0.64	3.03 ± 1.70

m: male, f: female, a: median and interquartile ranges

Table 29: *Reported values for CLP pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after overnight fasting and administration of an oral dose. Values are normalized to a 75 mg clopidogrel dose*

3.2.5. Method comparison on API 5000™ (*Partial Validation*)

As mentioned in the last section, for sensitivity reasons the CLP method was transferred to an API 5000™. Therefore spectra were recorded in the first step. The optimal system parameters for CLP and DIL are shown in **Table 30**.

Analyte	IS (V)	Temp. (°C)	DP (V)	CE (eV)	Q1 m/z	Q3 m/z
Clopidogrel	+5500	425	+120	24	322	212
Diltiazem	+5500	425	+121	35	415	178

IS: ionspray voltage, Temp.: temperature, DP: declustering potential, CE: collision energy

Table 30: *API 5000™ system parameters of clopidogrel*

The IS was set to +5500 V and the best signal intensity for CLP was obtained at a temperature of 425 °C. For CLP and DIL almost the same DP was set with 120 V and 121 V, respectively. The CE was set to 24 eV and 35 eV for CLP and DIL, respectively, and the same fragments were obtained as on the API 3000™. To compare the CLP on the API 3000™ and API 5000™ neither the sample preparation procedure nor the chromatographic conditions were changed.

In **Table 31** the results of the calibration standards of the *Partial Validation* (system comparison) in comparison to the calibration standards of the *Full Validation* method are shown.

Method Comparison						
Analyte	**MS**	**Linearity [ng/mL]**	**Inter-day CV [%]**	**Inter-day RE [%]**	**Inter-day CV [%] at LLOQ**	**Inter-day RE [%] at LLOQ**
CLP	API 5000™	0.00993 - 2.06	1.3 to 7.3	-7.2 to 5.4	2.3	0.3
CLP	API 3000™	0.0200 - 20.0	2.0 to 6.7	-2.5 to 3.8	2.0	-0.4

Table 31: *Method comparison – Calibration Standards for clopidogrel*

Comparing the sensitivity of both instruments the LLOQ on the API 5000™ was clearly lower by a factor of 20 than on the API 3000™.

This enables the determination of low CLP concentrations in the late elimination phase. On the other hand the highest calibration point on the API 5000™ had to be adjusted to 2 ng/mL. Higher concentrations for the highest calibration points resulted in the loss of linearity. Comparing the linearity range the API 5000™ covers with three orders of magnitude ($2 \cdot 10^3$) a clearly larger calibration range than the API 3000™ ($5 \cdot 10^2$). Regarding this aspect the quality data of the calibration standards (**Table 31**) measured on the API 5000™ are excellent as they are almost identical to the data obtained on the API 3000™.

Table 32 compares the intra-day and inter-day precision and relative errors for both detectors. For the API 5000™ the intra-day CV and RE were <9 and <5 %, respectively.

Method Comparison					
Analyte	**Mass Spectrometer**	**Intra-day CV [%]**	**Intra-day RE [%]**	**Inter-day CV [%]**	**Inter-day RE [%]**
CLP	API 5000™	1.9 to 8.6*	-1.8 to 4.7	4.6 to 9.7*	-0.9 to 2.8*
CLP	API 3000™	2.7 to 5.0*	-2.3 to 8.3*	5.1 to 10.0*	-4.9* to -2.2

*: at LLOQ

Table 32: *Partial and Full Validation – Quality Control Samples for clopidogrel*

Figure 10 illustrates the inter-day RE of the standard quality control samples including the *Full Validation* method as well as the *Partial Validation* method.

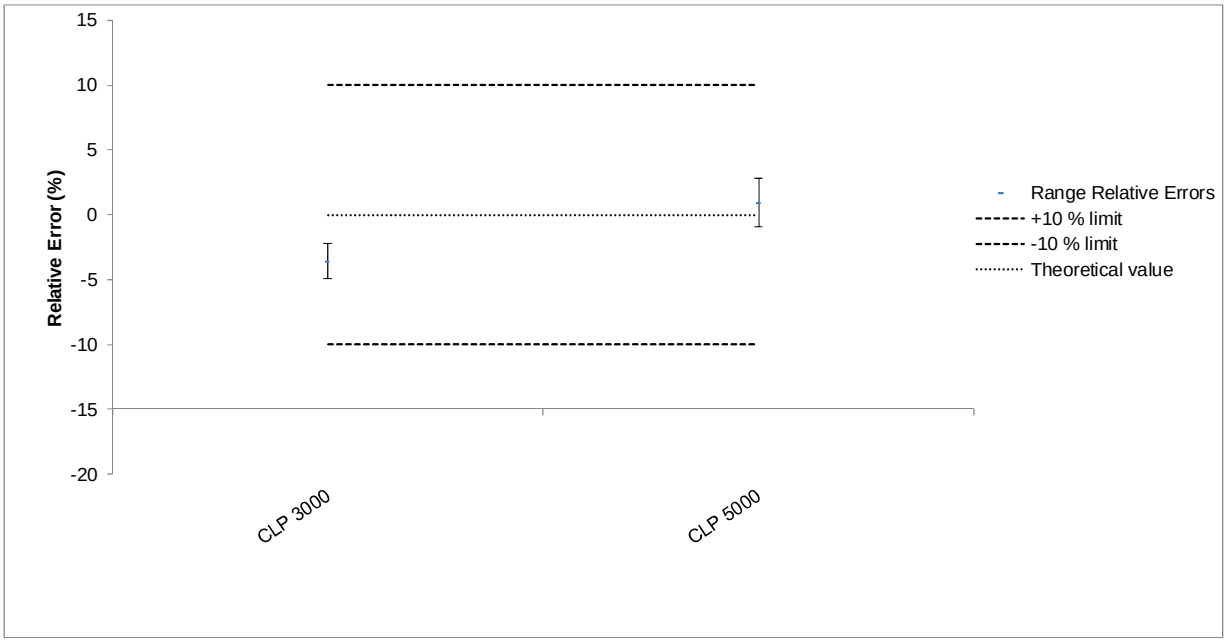


Figure 10: *Inter-day relative error ranges API 3000™ and API 5000™ for clopidogrel*

The inter-day range of the relative error of CLP was lower on the API 3000™ than on the API 5000™ indicating a better precision on the API 3000™. However, all values determined on the API 3000™ were below the expected theoretical concentrations. The values were shifted to positive deviations from the theoretical concentration when measurements were performed ion the API 5000™.

In **Table 33** the matrix factor (MF) values of CLP and DIL are shown. The values indicate signal suppression on the API 3000™ and the API 5000™ as they are below 1. The values of 0.845 and 0.708 measured on the API 3000™ indicate a stronger suppression for DIL than for CLP, respectively.

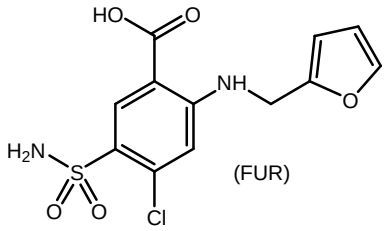
Analyte	Mass Spectrometer	Matrix Factor mean	CV (%)	Matrix Factor IS normalized	CV (%)
CLP	API 5000™	0.761	11.1	0.986	7.5
DIL	API 5000™	0.775	12.0	-	-
CLP	API 3000™	0.845	8.1	1.195	3.5
DIL	API 3000™	0.708	7.9	-	-

Table 33: *Matrix effect measured on API 5000™ and API 3000™ for clopidogrel*

On the API 5000™ CLP and DIL are suppressed to a comparable extend which is expressed by the IS normalized MF value of 0.986. But the situation is different on the API 3000™. Due to the fact that DIL is much more suppressed than CLP the IS normalized MF value shows overall signal enhancement of about 20 %. However, the enhancement is reproducible constant, which is indicated by the low CVs, and therefore an effect of human plasma on CLP determination on the API 3000™ could be excluded. This is in accordance with the guidelines [74]. Due to the fact that the matrix effect appears before the ions enter the mass spectrometer, it has to be concluded that the difference is attributed to the different ionization sources (TurboIonSpray® on the API 3000™ and Turbo V™ source on the API 5000™) of the mass spectrometers.

3.3. Furosemide

Furosemide (FUR) as shown in **Scheme 7** is a short-acting sulphonamide-type loop diuretic, which is widely used for the treatment of oedema associated with congestive heart failure, renal disease, cirrhosis of the liver and hypertension [75-78].



Scheme 7: *Furosemide*

FUR is administered both orally and intravenously. Haegeli et al. [79] reports a study in which sublingual administration of FUR was investigated and compared to intravenous and oral administration. Compared to the intravenous administration, both the oral and sublingual administration showed a slower onset of action. However, the elimination half-life was longer than after intravenous administration. Compared with oral administration, sublingual administration shows a significant increase in maximal plasma concentration of furosemide, AUC and bioavailability.

Following oral administration, FUR is rapidly but incompletely absorbed with a bioavailability of 60-70%. It is extensively bound to plasma proteins [80] and cleared predominantly by the kidneys (85%), where about half is metabolised and half is actively secreted unchanged by the organic acid transporter (OAT1) in the proximal tubules [81].
FUR acts on the [Na^+-K^+-$2Cl^-$] symporter at the intra-luminal side of the thick ascending limb of the loop of Henle. This inhibits the reabsorption of sodium and chloride in the ascending limb of the loop of Henle and also in the proximal and distal tubules. The inability to reabsorb salts therefore results in a higher osmolality and a decreased ability of the kidney to re-absorb water [22, 76].

FUR belongs to the class of high ceiling diuretics. This class of diuretics is highly active on the [Na^+-K^+-$2Cl^-$] symporter and has a dose/ concentration linearity over a wide concentration range. Linear pharmacokinetics of FUR were reported by Waller et al. [82] and Cutler et al. [83] over the dose range of 20-80 mg and 40-120 mg, respectively.

However, bioavailability studies show a wide inter- and intra-subject variability in the absorption, which is influenced by the dosage form, disease progress and the presence of food, and excretion of FUR from oral dosage forms [84-85].

An accurate and precise determination of FUR in biological fluids is important not only in therapeutic drug monitoring and pharmacokinetic studies but also in the field of doping control, since FUR is a diuretic banned in sports by the World Anti Doping Agency:

(The 2006 Prohibited List: www.wadaama.org/rtecontent/document/2006_LIST.pdf.)

For the determination of FUR and other diuretics in human urine screening and quantification methods have been published [86-91]. Except of Brunelli et al. [87] who evaporate the sample to dryness followed by derivatization and Politi et al. [88] who inject the urine sample directly, Zhang et al. [86], Deventer at al. [89], Morra et al. [90] and Sanz-Nebot et al. [91] use LLEx for sample preparation. Determination is performed with UV [86], GC-MS [87, 90] or LC-MS/(MS) [88-89, 91]

Several analytical methods, including thin-layer chromatography (TLC) [92], gas chromatography-mass spectrometry (GC-MS) with electron ionization [93] and liquid chromatography with spectrophotometric (LC-UV) [94-97] and spectrofluorometric (LC-FLUO) [98-104] detection have been reported for the determination of FUR in human serum and plasma. Many of these approaches have been applied in pharmacokinetic studies [85, 95-96, 99-100, 103, 105-115].

Although the latter methods provide acceptable LLOQs in the range of 5 to 30 ng/mL, the majority of them present a series of limitations like the need for a derivatization step in the case of GC-MS analysis due to the polar nature of FUR, time-consuming and for the analysis of large batches of samples not suitable LLEx (Liquid-Liquid Extraction) procedures and long chromatographic run times (≥5.3 min) needed in most cases to achieve good chromatographic resolution and to avoid problems with the differentiation between FUR and potential interferences from endogenous components and FUR metabolites or degradation products. Walshe et al. [94] compared LLEx and a column-switching technique for the extraction of FUR from human plasma. The column-switching method had the advantage of being faster than the LLEx method, but it yielded inferior reproducibility and recovery and an interfering peak was observed in the chromatograms.

Huclova et al. [101] published a clean-up procedure based on sequential injection analysis-restricted access material (SIA-RAM) combined with fluorescence detection, achieving a total analysis time of 20 min per sample and excellent precision and accuracy due to minimum sample manipulation, however the sensitivity of the method was very poor with a LLOQ of 10 µg/mL. Wenk et al. [102] published a monolithic silica rod LC method with fluorescence detection for the determination of FUR in human plasma and urine with a total run time of 8 min. Although the method is relatively fast, sensitive (lowest calibration point: 7.8 ng/mL) and reliable, the available selection of monolithic stationary phases is quite restricted so far, and therefore its application in pharmacokinetic studies limited. In order to provide a specific and fast determination LC-MS is particularly useful.

A LC-MS method to quantify FUR in human plasma using a mass spectrometer operated in the negative single ion monitoring (SIM) mode with APCI as ionization process [116] was reported. The method was robust, reliable and sensitive with a limit of quantitation of 10 ng/mL and a run time of approximately 6 min. Although LC-MS working in SIM mode generally offers adequate specificity and sensitivity, LC -MS/MS is considered the preferred technique for the determination of drugs and their metabolites in biological samples. In particular, MS/MS triple quadrupole mass spectrometry used in the multiple reaction monitoring mode (MRM) provides surpassing speed, sensitivity and selectivity. However, very few LC-MS/MS methods have been up to now reported for the determination of FUR in biological matrices [89, 117] and to the authors' knowledge there is hitherto no LC-MS/MS method published for the determination of FUR in human plasma.

3.3.1. Method development and optimization

FUR spectra were recorded on the API 3000™. Compared to APCI the ESI spectra showed higher signal intensities for the ionized molecule. During optimization of the compound dependant parameters it was found out that the deprotonated molecular ion $[M-H]^-$ showed a higher signal compared to the protonated $[M+H]^+$ ion. For that reason further optimization for FUR was performed in negative ionization mode using ESI. The best signal for FUR was achieved by setting the ion spray voltage to -5 kV, temperature to 350 °C and the orifice voltage to -36 V. With these parameters the $[M-H]^-$ ion in the Q1 spectrum was observed at m/z 329 for FUR. The best fragmentation was achieved setting the collision energy to 32 eV. Most intensive fragment in the product ion spectrum was m/z 205.

For the internal standard PRO the same settings were applied. This produced in the Q1 spectrum the corresponding $[M-H]^-$ ion at m/z 284. Most intensive fragment in the product ion spectrum was found at m/z 198. The corresponding spectra for FUR and PRO as well as a proposed fragmentation can be found in the Appendix. **Table 34** summarizes the MS settings for FUR and PRO.

Analyte	IS (V)	Temp. (°C)	OR (V)	CE (eV)	Q1 m/z	Q3 m/z
Furosemide	-5000	350	-36	32	329	205
Probenecid	-5000	350	-36	32	284	198

IS: ionspray voltage, Temp.: temperature, OR: orifice voltage, CE: collision energy

Table 34: *API 3000™ system parameters of furosemide and probenecid*

The challenge for the chromatography conditions for FUR laid in the fact of the zwitterionic nature of the molecule. Initially different stationary phases (C18, phenyl, CN) were tested for the chromatography of FUR. The CN phase (YMC-Pack Cyano, 50 x 4.6 mm, 3µ, YMC Europe GmbH, Dinslaken, Germany) produced the best peak shape and showed under similar chromatographic conditions the most appropriate retention time compared to the other columns. Therefore chromatography optimization was performed with the CN column. In addition to hydrophobic interactions the cyano column offers polar interactions with the analyte molecule. For that reason acidic pH values of the buffers were investigated. Comparing ammonium formate and ammonium acetate buffers, FUR signal intensity was better with the latter one.

Acetonitrile was chosen as organic modifier as reaction of the carboxy function with MeOH should be prevented. Finally a 5 mM ammonium acetate buffer with a pH of 3.5 was chosen. Using 55 % of the buffer and 45 % of ACN at a flow rate of 0.7 mL/min FUR eluted from the CN column after about 1.2 min. A molecule with a similar chemical structure and properties should be used as the internal standard. The ideal candidate was found to be probenecid. Under the above described conditions PRO eluted after approximately 1.5 min.

Example chromatograms can be found in the Appendix. To ensure acidic conditions during the sample p reparation process the precipitation regent ACN was acidified. Therefore a solution of 10 % of acetic acid was prepared in ACN. A concentration of ca. 1 µg/mL of the internal standard PRO was spiked into that mixture. Moreover, the acidic condition increased the precipitation process and the overall sensitivity of the assay.

To prevent FUR decomposition all steps were carried out under light protection. For sample preparation 100 µL of human plasma were deproteinized by addition of 200 µL of 10 % acetic acid in acetonitrile containing ca. 1 µg/mL PRO. After thorough mixing, the samples were centrifuged for 10 min at 3600 rpm (3016 g) at approximately +4 °C. 100 µL of the supernatant were mixed with 200 µL 0.005 M ammonium acetate buffer (pH 3.5). After mixing, 25 µL of each sample were injected into the LC-MS/MS system.

3.3.2. Method validation

The specificity of the assay was shown by injecting drug free prepared human plasma samples. The matrix components did not interfere with FUR and the internal standard PRO at their retention times and over the concentration range (5 - 5000 ng/mL) described herein. Typical chromatograms of blank samples, standards and samples from human volunteers can be found in the Appendix.

The peak area ratios versus concentrations were fitted with linear regression over the concentration range of FUR in human plasma. The mean linear regression equation of the calibration curves generated during the validation was:

$y = 0.0017\ (\pm 0.0024) + 0.0030\ (\pm 0.0007)\ x$ for FUR, $r^2 \geq 0.997$

where *y* represents the ratio of the FUR peak area to that of the internal standard PRO, and *x* represents the plasma concentration of the analyte. Excellent linearity is shown by the mean correlation coefficient that was equal or better than 0.997. The validated concentration range covers an order of magnitude of 10^3.

Table 35 summarizes the inter-day CV and RE ranges across the calibration range as well as the CV and RE at the LLOQ. Inter-day CV and RE were <6 % and 7 %, respectively. At the LLOQ the inter-day CV and RE were <4 % and <1 %, respectively.

	CV [%]	RE [%]	CV [%]	RE [%]
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
FUR	2.0 to 5.9	-6.1 to 5.1	3.7	-0.8
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
FUR	4.8 to 9.5	-5.0 to 5.6*	2.7 to 7.8*	-1.5* to 7.7

*: value at LLOQ

Table 35: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for furosemide*

The intra-day and inter-day CV and RE of the spiked quality control samples that were obtained on five consecutive days are shown in **Table 35**. The intra-day CV was better than 8 % and the intra-day RE showed no more deviation than 8 % from the theoretical value. Between the individual validation days the CV was better than 10 % and the deviation from the theoretical value was lower than 6 %. The determined quality data are very well within the acceptance criteria [48] and were therefore judged to be acceptable. The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits.

The precipitation assay showed complete recovery for FUR. With the acidified precipitation step a mean absolute recovery of 100 % for FUR could be determined. A mean absolute extraction recovery of 98.6 % was observed for PRO. This value also illustrates that PRO is an ideal internal standard for this method as it shows under the assay conditions almost identical behavior compared to the analyte molecule FUR.

Analyte	Mean [%]	SD [%]	CV [%]
FUR	100.0	2.1	2.1
PRO	98.6	4.0	4.0

Table 36: *Mean absolute extraction recoveries for furosemide and probenecid*

Table 36 summarizes the recovery data and shows also very low SD and CV values that

indicate the absence of any concentration dependency over the validated concentration range.

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for all analytes. All statistical evaluations of the stability experiments can be found in the Appendix.

Hemolyzed human plasma and different batches of human plasma showed no effect on the determination of FUR. All RE values were below 6 % and the CV values were below 10 %. **Table 37** shows the individual data.

Analyte		**Hemolyzed**		**Different batches**	
		high	**low**	**high**	**low**
	RE (%)				
FUR		-2.6	-5.3	-2.1	0.5
	CV (%)				
FUR		5.9	3.0	9.6	6.1

Table 37: *Relative errors (%) and precisions (%) of the investigated influences for furosemide*

3.3.3. Comparison of the developed method with existent methods

In **Table 38** the developed method is compared with previously published methods for the determination of FUR in human plasma.
All previously reported methods showed less sensitivity except for the method of Abou-Auda et al. [100] and Okuda et al. [97], who also reported a LLOQ of 5 ng/mL but with longer run times of 10 min and 17 min, respectively. Huclova et al. [101], who used direct determination of FUR in serum comprising on-line sample preparation based on SIA-RAM in combination with fluorescence detection achieved a total analysis time of 20 min per sample and showed good inter-day precision and accuracy due to the minimum manipulation of the biological sample.

Method	Linearity range [ng/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Preparation	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experiments*
LC-MS/MS current thesis	5 - 5000	2.5	0.1	PP	5.5 to 9.5	-5.0 to 5.6	100.0	≥0.997	probenecid	1, 2a-e, 3, 4
Abdel-Hamid et al. [116] LC-MS	50 - 2000	6.0	1.0	LLEx	2.7 to 11.5	1.3 to 3.0	93.0	0.9979	diclofenac	1, 2b, 5
Ptacek et al. [93] GC-MS	10 - 4000 4000 - 100000	9.0	0.4	LLEx	1.3 to 9.7	-4.1 to 10.1	n.r.	≥0.9999	bumetanide	n.r.
Walshe et al. [94] LC-UV	25 - 300	15.0	0.125	LLEx	5.0 to 19.0	-9.0 to 16.0	90.8	≥0.995	pindolol	5
	25 - 200	15.0	0.125	Column switching	7.0 to 24.0	-5.0 to 16.0	76.3	≥0.995	pindolol	5
Jankowski et al. [95] LC-UV	30 - 3000	12.0	1.0	LLEx	7.4 to 8.2	1.4 to 4.3	80.2	≥0.9892	nitrazepam	2b, c
Okuda et al. [97] LC-UV	5 - 1000	17.0	0.17	oline SPE	1.7 to 2.6	0.5 to 2.0	n.r.	≥0.9999	exernal standard	n.r.
Galaon et al. [104] LC-FD	25 - 4000	5.3	0.2	Prec.	2.3 to 5.2	n.r.	100.0	≥0.9994	norfloxacin	2a-e
Najib et al. [99] LC-FD	20 - 1200	10.0	0.5	LLEx	5.9 to 7.6	-1.5 to -0.4	86.9	n.r.	naproxen	2b
Abou-Auda et al. [100] LC-FD	50 - 2000	10.0	0.5	LLEx	4.3 to 10.8	-0.6 to 5.5	90.1	≥0.99	warfarin	1, 2b, 5
Wenk et al. [102] LC-FD	7.8 - 1000	8.0	0.5	LLEx	6.3 to 7.0	-3.8 to 9.0	76.5	≥0.998	naproxen	5
Huclova et al. [101] LC-FD	10000 - 80000	20.0	0.1	SIA-RAM	5.5 to 6.6	n.r.	101.4 - 103.4	≥0.999	n.r.	1
Vree et al. [98] LC-FD	7 - n.r.	30.0	0.1	Prec.	1.2 to 3.0	n.r.	91.5	≥0.9998	n.r.	n.r.

n.r.: not reported, PP: protein precipitation, LLEx: liquid-liquid extraction, SPE: solid phase extraction, SIA-RAM: sequential injection analysis restricted access material
*1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e)), 3: influence of hemolyzed plasma, 4: influence of different individuals, 5: interference of drugs

Table 38: *Method comparison for furosemide*

However, this method provides very low sensitivity with a LLOQ of 10 µg/mL, which is ascribed to the diffusion of the zones in the column of high diameter and particle size and partly to the construction of the detection cell used in SIA. Moreover the SIA-RAM extraction procedure is convenient for the determination of one drug, not for separation of complicated mixtures and simultaneous determination of a number of substances due to the limited separation efficiency of the RAM (restricted access material) column.

The online SPE method of Okuda et al [97] shows very good precision and low inter-day relative error. However, no recovery data or further validation experiments are reported in the paper and the use of an internal standard was not possible.

All protein precipitation methods in **Table 38** show equal or better recovery than liquid-liquid extraction or online preparation methods for the determination of FUR.

In comparison to the LC-MS method reported by Abdel-Hamid [116], the LC-MS/MS method in this thesis is more sensitive and faster. Although the reported LC-MS method was specific and proven to be free of interferences from endogenous compounds or drugs that may be co-administered with FUR, a LC-MS/MS method is more reliable, since it selects the compound of choice by a specific ion transition (precursor $\rightarrow$ product ion) characteristic of the analyte of interest. FUR is a small molecule with a molecular mass in the lower mass region, which is an additional negative influence on the specificity in a LC-MS method since interferences are more likely to occur.

The developed method shows excellent performance with simple sample preparation, has a fast run time and provides highest sensitivity. Moreover, in this thesis validation experiments on the stability of FUR were performed and the influence of hemolyzed human plasma as well as the influence of different batches of plasma from different individuals was studied.

3.3.4. Pharmacokinetic analysis

The developed and validated method was used to analyze FUR in human plasma samples from a clinical trial. In one period of the 2-way crossover study a 40 mg FUR reference tablet was administrated to 39 male and female subjects under fasting conditions. Blood samples were taken before administration and after 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 4.00, 5.00, 6.00, 8.00, 10.00 and 12.00 hours. Collected blood samples were centrifuged for 10 min at 3000 rpm (2095 g) at +4 °C and two aliquots were stored at -70 ℃ until sample analysis.

In the analyzed study FUR concentrations of more than 1300 human plasma samples were measured for the bioequivalence evaluation. Concentrations were calculated by the calibration curve that was measured at the beginning of each sequence and quality was ensured by measuring spiked quality control samples within each sequence. Calibration was performed by weighted (1/concentration^2) linear regression. The coefficient of correlation of the resulting linear regressions was at least 0.998. The inter-day precision and accuracy of the spiked quality control standards of FUR in human plasma analyzed with the batches of study samples ranged from 4.9 % to 8.2 % and from 93.9 % to 101.3 %, respectively.

Figure 11 shows as example the mean plasma concentration profile, including the standard deviations, of FUR after oral administration of a 40 mg reference formulation tablet to 39 healthy volunteers. The mean peak concentration (C_{max}) of 1200 ng/mL for FUR was attained at 1.61 h after administration of the reference product and then declined rapidly and remained detectable up until 12 h.

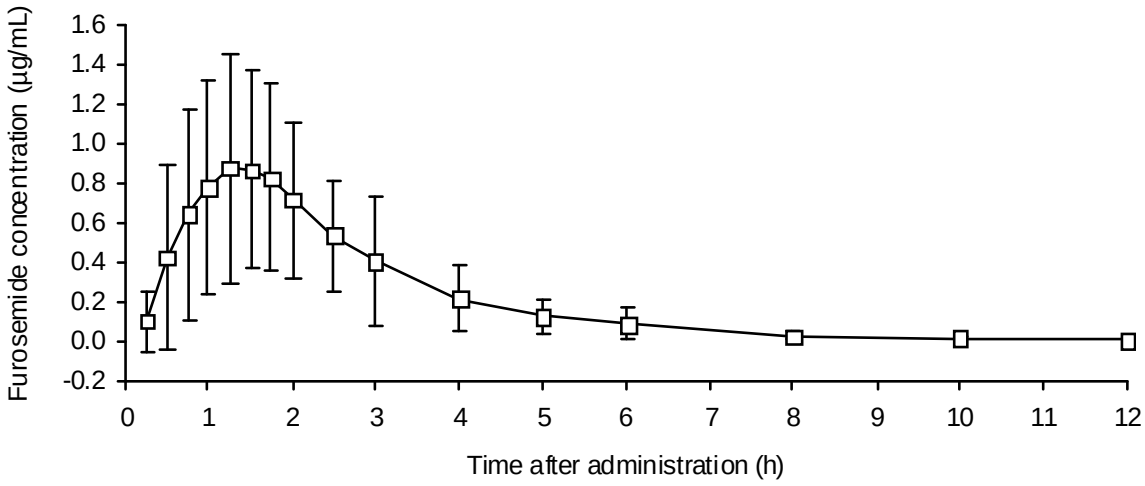


Figure 11: *Mean plasma profile of furosemide concentration vs. time following a 40 mg oral dose of reference furosemide tablet to healthy volunteers (n=39)*

Table 39 shows the pharmacokinetic parameters of FUR reference tablet in comparison to pharmacokinetic parameters reported in previously published studies, where the same dose (40 mg FUR) and study design was used.

Reference	No. of subjects/ gender	Last time point [h]	Formulation	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current thesis	39/ m, f	12	Tablet	2370 ± 574	2450 ± 569	1200 ± 494	1.61 ± 0.90	3.21 ± 1.05
Awad et al. [96]	20/ m	8	Tablet	2345 ± 215 *		1004 ± 112	2.03 ± 0.30	n.r.
Najib et al. [99]	24/ m	12	Tablet	2516 ± 959.0	2609 ± 953.5	1109 ± 309.1	1.36 ± 0.59	2.47 ± 0.92
Abou-Auda et al. [100]	6/ m	12	Tablet, Brand A	n.r.	2216.2 ± 146.9	1058.0 ± 188.1	1.38 ± 0.54	1.84 ± 0.31
			Tablet, Brand B	n.r.	2038.5 ± 466.9	763.5 ± 242.9	1.60 ± 0.58	1.66 ± 0.21
			Tablet, Brand C	n.r.	2101.9 ± 642.7	858.2 ± 433.9	2.2 ±1.00	1.71 ± 0.41
Yagi et al. [106]	4/ m	24	Tablet	n.r.	2530.5 ± 224.5	1038.7 ± 412.5	1.13 ± 0.40	n.r.
Bindschedler et al. [107]	12/ m	24	Tablet	2850 ± 1000	n.r.	1260 ± 620	1.50 (0.5-3.0)$^{\Delta}$	n.r.
Martin et al. [110]	12/ m	10	Reference tablet	2977 ± 981	3066 ± 959	1658 ± 585	1.1 ± 0.40	2.55 ± 0.70
Straughn et al. [113]	14/ m	16	Tablet, Brand A	3000 ± 660	3040 ± 669	1070 ± 289	1.89 ± 1.10	n.r.
			Tablet, Brand B	3490 ± 838	3510 ± 842	1160 ± 267	1.38 ± 0.77	n.r.
			Tablet, Brand C	3320 ± 930	3330 ± 932	1130 ± 328	1.31 ± 0.88	n.r.
			Tablet, Brand D	3480 ± 1044	3500 ± 1050	1290 ± 297	1.29 ± 0.89	n.r.
			Tablet, Brand E	2990 ± 1044	3040 ± 638	1010 ± 212	2.14 ± 1.24	n.r.
			Tablet, Brand F	3340 ± 635	3350 ± 670	1140 ± 274	1.94 ± 1.00	n.r.
			Tablet, Brand G	3100 ± 1302	3120 ± 1217	1040 ± 426	1.21± 0.56	n.r.
Kaojarern et al. [114]	8/ m	6	Tablet, Brand A	2760 ± 620	n.r.	640 ± 160	2.00 ± 0.54	n.r.
			Tablet, Brand B	1940 ± 180	n.r.	610 ± 140	1.64 ± 0.24	n.r.
			Tablet, Brand C	3130 ± 490	n.r.	1120 ± 310	1.63 ± 0.30	n.r.
			Tablet, Brand D	2620 ± 290	n.r.	830 ± 120	1.85 ± 0.33	n.r.

m: male, f: female, *: value reported as mean AUC, Δ: expressed as median (range), n.r.: not reported

Table 39: *Reported values for furosemide pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after overnight fasting (single oral dose = 40 mg furosemide, standard release)*

Maximum peak concentrations were reached within 1 to 2 hours whereas the t_{max} of this thesis is in good agreement with the literature. Values for half-lifes are reported within 1.7 and 3.2 hours. The calculated half life in this thesis is clearly higher than the values reported in the other papers. However, the present study had the highest number of participants and included also female volunteers.

As shown in **Table 39** the mean peak concentrations measured in the current thesis is in good agreement with the published literature. However, it has to be noted that Abou-Auda et al. [100] and Kaojarern et al. [114] report for some of their investigated reference formulations significantly lower mean peak concentrations. Kaojarern et al. [114] investigated the drug in Thai volunteers but this was not the case for the study of Abou-Auda [100]. On the other hand Martin et al. [110] report clearly higher mean C_{max} values of about 1700 ng/mL.

The AUC values calculated in this thesis are comparable to the values reported by Najib et al., Abou-Auda et al. and Yagi et al. [99-100, 106]. Higher values are reported by the other authors listed in **Table 39**.

3.4. Itraconazole and Hydroxyitraconazole

Itraconazole (ITR) is a first generation synthetic antifungal agent of the azol group and is used for prophylaxis and treatment of systemic fungal infections [118]. Azoles inhibit the 14-alpha-demethylase (a P450 enzyme) which catalyzes the synthesis of ergosterol, a major compound of cell membrane of yeast and fungal cells causing inhibition of fungal cell growth replication [119].

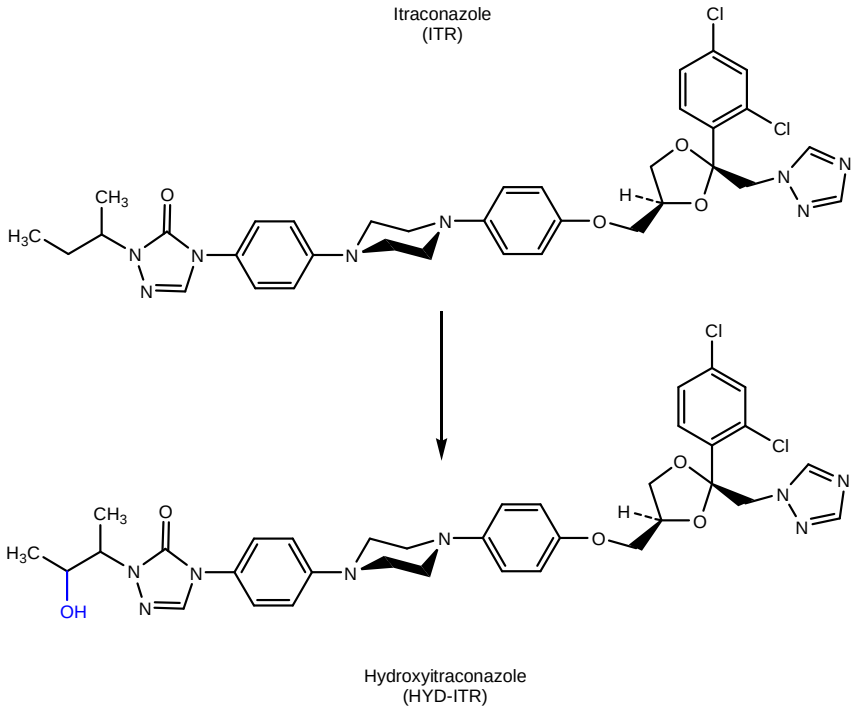
After oral administration ITR is good absorbed and the drug has a bioavailability of about 55%. The absorption is enhanced by food in the stomach and higher serum concentrations are shown after intake of the drug with an acidic beverage [120-121]. Solubility of oral or intravenous solutions can be enhanced by hydroxypropyl-ß-cyclodextrin [122].

The drug is highly bound to plasma proteins (96 %) and strongly penetrates due to its high lipophilicity into tissue (lung, kidney, liver, bone, stomach, spleen, muscle and fat) whereas tissue concentrations 2 to 10 times higher than the plasma concentrations were found.

Itraconazole is extensively metabolized in the liver into various metabolites among hydroxyitraconazole (HYD-ITR) (**Scheme 8**) which shows about 2 to 3 times higher concentrations than the parent compound and antifungal activity as the parent compound [123]. ITR is a potent inhibitor of CYP3A4 and can therefore change the pharmacokinetics of other drugs [123].

Various analytical methods have been published for the determination of ITR and HYD-ITR including liquid chromatography coupled with MS, MS/MS, UV or fluorescence detection [124-149]. With exception of the methods reported by Yao et al. [125] and Rhim et al. [129], that apply protein precipitation, all other mass spectrometry methods require liquid-liquid or solid phase extraction. However, Yao et al. [125] and Rhim et al. [129] did not determine the active hydroxy-metabolite.

The most sensitive MS method determining ITR and HYD-ITR uses SPE and is reported by Bharathi et al. [128]. The method has a short run time of only 3 min while achieving a LLOQ of 0.5 ng/mL for ITR and HYD-ITR, respectively.



Scheme 8: *Itraconazole and active metabolite*

UV- or FLUO-detection methods provide also good LLOQs (1.0 to 5.0 ng/mL) for the determination of ITR and HYD-ITR [135, 138, 147-148]. However, these methods require at least 0.5 mL of human plasma and have a run time of at least 12 min.
Moreover, analytical procedures as bioassays or micellar electrokinetic chromatography have been reported [136, 141, 149].

3.4.1. Method development and optimization

Solutions of ITR and HYD-ITR were prepared in mobile phase and infused with a syringe pump on the API 3000™. Ionization with the heated nebulizer showed clearly better sensitivity than with the ESI interface. Abundant peaks correspondent to the protonated molecules $[M+H]^+$ with m/z 705 and m/z 721 were observed in the positive ion mass spectrum of ITR and HYD-ITR recorded by scanning Q1. In Q1 and in Q3 the same molecular ions were selected in order to have an overall higher sensitivity. The API 3000™ was operated in the MRM mode with the following transitions m/z 705 → m/z 705 and m/z 721 → m/z 721 for ITR and HYD-ITR, respectively. This reduced the specificity of the assay but increased the overall sensitivity. The mass spectrometer settings can be found in **Table 40**.

Analyte	NC (µA)	Temp. (°C)	OR (V)	CE (eV)	Q1 m/z	Q3 m/z
Itraconazole	+5	500	+101	56	705	705
Hydroxyitraconazole	+5	500	+101	56	721	721
Clarithromycin	+5	500	+101	56	749	749

NC: nebulizer current, Temp.: temperature, OR: orifice voltage, CE: collision energy

Table 40: *API 3000™ system parameters for itraconazole, metabolite and clarithromycin*

The best signal intensities for ITR and HYD-ITR were obtained with a NC of +5 µA and setting the temperature to 500 °C. Orifice voltage was set to 101 V and the collision energy to 56 eV. The collision gas was set to zero to avoid fragmentation. For the internal standard CLA the same settings were used and the transition m/z 749 → m/z 749 was monitored.
Both ITR and HYD-ITR both have basic nitrogen atoms that may interact in their protonated state with free silanol groups on the stationary phase thereby causing peak tailing. For that reason neutral to alkaline conditions were investigated.

Due to the high lipophilicity of ITR a C18 should be able to separate the drug and its metabolite. Based on the above described problem with the silanol groups an endcapped C18 phase (Symmetry C18, 50 x 4.6 mm, 3.5 µ, Eschborn, Germany) was used.
A mixture of 50 mM ammonium acetate (30 %) and acetonitrile (70 %) was found to produce good chromatographic separation of ITR and HYD-ITR. However, there was still peak tailing for the analytes observed. This could have been solved by increasing the pH value of the mobile phase or by addition of an additive such as triethylamine to the mobile phase that would interact with the free silanol groups of the column. As a sufficient protonation of ITR and HYD-ITR in the gas phase should be ensured the pH value of the mobile phase was not increased. Also additives such as triethylamine were not used as they are known to contaminate the mass spectrometer. This may cause ion suppression and impact the performance of other assays on the system [150]. For that reason NaOH was added to the samples prior to precipitation which also produced a very good peak shape. Under the conditions described above ITR and HYD-ITR eluted after approximately 1.3 min and 0.9 min, respectively. CLA was found to elute after approximately 1.5 min under these conditions and was therefore used as internal standard. The chromatographic conditions can be summarized as follows: mobile phase: 50 mM ammonium acetate buffer : MeCN (30/70, v/v), analytical column: Waters Symmetry C18 (50 x 4.6 mm, 3.5µ), flow rate: 1.0 mL/min.

For preparation of the human plasma samples 100 µL sample were mixed with 25 µL 2 M NaOH and deproteinized by addition of 0.25 mL of acetonitrile containing CLA (ca. 400 ng/mL) as internal standard. After thorough mixing the samples were centrifuged for approximately 15 minutes at 3600 rpm (3016 g) at approximately +4 °C. Thirty µL of the supernatant were injected into the LC-MS/MS system.

3.4.2. Method validation

Injection of prepared blank human plasma samples showed no interferences near or at the retention times of ITR, HYD-ITR and the internals standard CLA. Therefore any interference of the matrix compounds with the analytes over the validated concentration range (3 ng/mL (ITR), 5 ng/mL (HYD-ITR) - 1000 ng/mL) can be excluded. Typical chromatograms can be found in the Appendix.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration ranges of ITR and HYD-ITR in human plasma. The mean linear regression equations of the calibration curves generated during the validation were:

y = -0.0001 (±0.0009) + 0.0016 (±0.0004) x for ITR, $r^2 \geq 0.996$

y = 0.0041 (±0.0031) + 0.0137 (±0.0009) x for HYD-ITR, $r^2 \geq 0.997$

where y represents the ratio of the analyte peak area to that of the internal standard CLA, and x represents the plasma concentration of the analyte. The mean correlation coefficients were equal or better than 0.996 and 0.997 for ITR and HYD-ITR, respectively.

The inter-day CV of the calibration rows for ITR and HYD-ITR is shown in **Table 41** and was better than 6 % and 7 %, respectively. Also the RE which was below 7 % for ITR and below 5 % for HYD-ITR represents the excellent linearity for both analytes. At the LLOQ all CV and RE were below 4 % and 5 %, respectively.

	CV [%]	**RE [%]**	**CV [%]**	**RE [%]**
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
ITR	1.2 to 5.9	-6.6 to 6.9	2.6	4.7
HYD-ITR	1.2 to 6.5	-4.6 to 4.5	3.7	0.0
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
ITR	2.2 to 7.1*	0.3 to 6.5	1.3 to 5.9	-0.5 to 5.9
HYD-ITR	3.1 to 6.6*	-1.5 to 8.5*	2.2 to 8.0*	-0.9 to 3.1*

*: value at LLOQ

Table 41: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for itraconazole and metabolite*

The results of the spiked quality control samples that were analyzed with the calibration standards on five consecutive days are shown in **Table 41**. ITR and HYD-ITR could be determined with high precision and low relative errors. The intra-day CV of the spiked quality control samples was better than 6 % and 8 % for ITR and HYD-ITR, respectively. Intra-day RE was below 6 % for ITR and below 4 % for HYD-ITR. The quality data between the validation days showed similar results. The inter-day CV was <8 % and <7 % for ITR and HYD-ITR, respectively.

The inter-day RE for both compounds was below 7 % and 9 % for ITR and HYD-ITR, respectively. The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits.

The mean absolute extraction recovery for ITR in human plasma was determined with 82 %. HYD-ITR showed an about 4 % lower mean absolute extraction recovery which was determined with 78 %. Both recoveries showed low standard deviations and CV values across the validated concentration ranges. Therefore any concentration dependence of the recovery can be excluded. The recovery results are shown in **Table 42**. Mean absolute extraction recovery of CLA was 64 % which is not very high for a precipitation assay but sufficient as CLA is the internal standard.

Analyte	Mean [%]	SD [%]	CV [%]
ITR	82.1	4.9	6.0
HYD-ITR	78.2	5.8	7.4
CLA	64.3	1.3	1.9

Table 42: *Mean absolute extraction recoveries for itraconazole and metabolite*

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for all analytes. All statistical evaluations of the stability experiments can be found in the Appendix.

The influence of hemolyzed human plasma on the determination of ITR and HYD-ITR was investigated. As shown in **Table 43** no influence was observed from hemolyzed human plasma.

Analyte	CV [%]		RE [%]	
	high	low	high	low
ITR	1.6	4.1	-3.9	-2.7
HYD-ITR	2.2	6.4	-2.6	3.0

Table 43: *Influence of hemolyzed human plasma for itraconazole and metabolite*

The CV was better than 5 % and 7 % for ITR and HYD-ITR, respectively. All determined relative errors showed no more than 4 % deviation from the theoretical value.

3.4.3. Comparison of the developed method with existent methods

In **Table 44** the developed method is compared with previously published methods for the determination of ITR and HYD-ITR in plasma and serum of human [126-144] , rat [125], dog [124] and bovine [149] species in comparison to the present LC-MS/MS method.

All previously published LC-MS and LC-MS/MS methods make use of electrospray ionization. During method development APCI showed the highest sensitivity and was therefore used instead of the electrospray interface. The mass spectrometer was operated in the MRM mode but the same molecular ion was used in Q1 and Q3. This procedure provided the best sensitivity. None of the shown methods with MS detection determine ITR and HYD-ITR simultaneously with a simple protein precipitation, except the method presented here. The direct comparison with the work of Kousoulos et al. [127], who also used the API 3000™ MS-System, shows that the developed method has a better recovery and needs less sample volume with almost the same sensitivity.

Although most of the published analytical methods use UV or FLUO detection, LC-MS methods have the advantages of short run times which are useful during high sample load e.g. during measurement of bioequivalence studies, of requiring low sample volumes and of achieving lower LLOQs of 0.5 ng/mL [128].

However, it has to be noted that also with UV-detection and less sample volume a lower limit of quantification of 7.0 ng/mL can be reached which is close to the LC-MS LLOQ [139].
In the case of ITR and HYD-ITR the achieved recoveries with protein precipitation are similar to those achieved by LLEx, whereas some of the published methods require two or three time consuming extraction steps. Very good recoveries were produced with solid phase extraction, especially when performed online.

Beside the commonly used analytical methods for the determination of ITR and HYD-ITR Warnock et al. [136] and Hülsewede et al. [141] report microbiological bioassays.

Method	Linearity [ng/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Prepa-ration	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experi-ments*
LC-MS/MS current thesis	3 - 1000 (ITR) 5 - 1000 (HYD-ITR)	2.5	0.1	PP	2.2 to 7.1 (ITR) 3.1 to 6.6 (HYD-ITR)	0.3 to 6.5 (ITR) 1.5 to 8.5 (HYD-ITR)	82.1 ± 4.9 (ITR) 78.2 ± 5.8 (HYD-ITR) 64.3 ± 1.3 (IS)	≥0.996 (ITR) ≥0.997(HYD-ITR)	clarithromycin	1, 2a-d, 4
Carrier at al. [124] LC-MS	2.5 - 2000	3.5	0.5	LLEx	10.5 (ITR)[a] 11.9 (HYD-ITR)[a]	n.r.	70.0 (ITR) 82.0 (HYD-ITR)	>0.99	miconazole	1, 2a, c, d
Yao et al. [125] LC-MS	4 - 1000	4.0	0.10	PP	0.0 to 7.14 (ITR)	1.8 to 9.7 (ITR)	91.6 (ITR)	≥0.994	nefazodone	1, 2c
Vogeser et al. [126] LC-MS/MS	10 - 10000	5.0	0.05	SPE	4.8 to 5.1 (ITR) 4.9 to 5.2 (HYD-ITR)	n.r.	92.3 ± 1.2 (ITR) 94.0 ± 1.4 (HYD-ITR) 92.7 ± 1.7 (IS)	>0.999	n.r.	5, 6
Kousoulos et al. [127] LC-MS/MS	2 - 500 (ITR) 4 - 1000 (HYD-ITR)	2.0	0.15	LLEx	1.4 to 10.1 (ITR) 2.5 to 13.3 (HYD-ITR)	0.6 to 5.7 (ITR) -3.6 to 2.5 (HYD-ITR)	54.5 to 66.3 (ITR) 57.5 to 59.3 (HYD-ITR)	>0.998	R51012	1, 2a-e, 5
Bharathi et al. [128] LC-MS/MS	0.5 - 263	3.0	0.5	SPE	3.9 to 6.8 (ITR) 2.1 to 11.5 (HYD-ITR)	-9.8 to 3.0 (ITR) -8.5 to 1.0 (HYD-ITR)	79.8 ± 4.4 (ITR) 87.2 ± 6.3 (HYD-ITR) 86.7 ± 3.0 (IS)	≥0.998	fluconazole	1, 2a-e, 5
Rhim et al [129] LC-MS/MS	1 - 500	1.5	1.00	PP	3.7 to 10.9 (ITR)	-13.4 to 17.5 (ITR)	n.r.	≥0.9952	clebopride	1
Compas et al. [130] LC-UV	100 - 3200	8.0	1.0	LLEx	3.7 - 7.9 (ITR) 3.4 - 6.0 (HYD-ITR)	n.r.	77.0 ± 5.0 (ITR) 83.0 ± 3.0 (HYD-ITR) 79.0 ± 3.0 (IS)	≥0.9999	ketoconazole	2b, e
Darouiche et al. [131] LC-UV	5 - 20000	8.0	0.25	PP	3.3 to 4.2	n.r.	80.0 to 85.0 (ITR)	≥0.99	n.r.	1, 7
Gordien et al. [132] LC-UV	100 - 40000	19.0	0.3	SPE	6.4 to 11.7 (ITR) 6.7 to 11.0 (HYD-ITR)	-6.2 to 6.0 (ITR) -3.7 to 8.7 (HYD-ITR)	81.2 to 84.5 (ITR) 81.5 to 86.2 (HYD-ITR)	≥0.99	linezolid	1, 2a,-e, 3, 7
Gubbins et al. [133] LC UV	25 - 1500	22.0	0.25	PP	n.r.	≤ 3.5 (ITR) ≤ -7.2 (HYD-ITR)	93.3 (ITR) 92.9 (HYD-ITR)	≥0.999	sapraconazole	1, 7
Rifai et al. [134] LC-UV	250 - 1000	4.0	0.10	SPE	5.8 to 12.0 (ITR)	n.r.	99.0 to 102.0	1.00	R051012	1, 2b, 7
Uno et al. [135] LC UV	3 - 500 (ITR) 3 - 1000 (HYD-ITR)	20.0	1.00	LLEx	2.3 to 5.0 (ITR) 2.4 to 4.9 (HYD-ITR)	-4.7 to 2.5 (ITR) -0.7 to 2.6 (HYD-ITR)	57.3 to 61.3 (ITR) 62.3 to 66.8 (HYD-ITR)	≥0.9997 (ITR) ≥0.9996 (HYD-ITR)	R051012	1, 2b, c, e, 7
Warnock et all [136] LC-UV	10 - 10000	10.0	1.00	LLEx	2.2 to 13.2 (ITR)	n.r.	100.0	n.r.	R51012	1, 7
	100 - 1600	-	-	Bioassay	11.0 to 17.1 (ITR)	n.r.	-	-	-	n.r.

a) calculated by CR slopes, b) calibration row, PP: protein precipitation, LLEx: liquid-liquid extraction, SPE: solid phase extraction, n.r.: not reported
*1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e)), 3: influence of dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: matrix effect, 6: incurred samples, 7: interference with drugs

Table 44: *Method comparison for itraconazole and metabolite*

Method	Linearity [ng/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Prepa-ration	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional VAL-Experi-ments*
Kubalec et al. [137] LC-UV	12 - 2700 15 - 2700	12.0	0.10	SPE	1.3 to 6.8 (ITR) 1.8 to 5.3 (HYD-ITR)	n.r.	97.6 to 101.8 (ITR) 98.5 to 102.3 (HYD-ITR)	0.991 (ITR) 0.995 (HYD-ITR)	n.r.	n.r.
Woestenborgh et al. [138] LC-UV	1 - 500	n.r.	1.00	LLEx	0.3 to 13.9	-5.3 to 9.0	71.5 ± 2.3 (ITR)	0.9999	R51012	1
Badcock [139] LC-UV	7 - 4200	12.0	0.10	PP	6.2	-	95.0 to 101.0	-	R51012	1, 7
Ohkubo and Osanai [140] LC-UV	10 - 500	20.0	1.00	SPE	3.9 to 6.9	n.r.	89.1 to 98.2	≥0.999	bifonazole	n.r.
Hülsewede et al. [141] LC-UV	125 - 4000	20.0	0.50	LLEx	4.1 to 15.5[b]	n.r.	n.r.	n.r.	R51012	n.r.
	250 - 4000	-	0.20	Bioassay	9.8 to 36.9[b]	n.r.	n.r.	n.r.	R51012	n.r.
Khoschsorur [142] LC-UV	100 - 8000	20.0	1.00	LLEx	2.4 to 9.9 (ITR) 1.6 to 4.3 (HYD-ITR)	-4.0 to -3.0 (ITR) -2.8 to 1.7 (HYD-ITR)	95.7 (ITR) 97.7 (HYD-ITR)	>0.996	ketoconazole	1, 2b-d
Koks et et. [143] LC-FLUO	10 - 1000	20.0	0.50	SPE	4.0 to 9.0 (ITR) 6.0 to 8.0 (HYD-ITR)	4 to 13 (ITR) -9 to 103 (HYD-ITR)	85.0 (ITR) 86.0 (HYD-ITR)	n.r.	R051012	1, 2a, c, d, 7
Poirier et al. [144] LC-FLUO	20 - 4000	30.0	0.50	LLEx	1.6 to 10.9 (ITR) 2.6 to 8.5 (HYD-ITR)	n.r.	81.2 ± 4.4 (ITR) 83.0 ± 4.0 (HYD-ITR) 67.9 ± 3.1 (IS)	>0.999	n.r.	1, 2a, c, 7
Redman et al. [145] LC-FLUO	50 - 2000	15.0	0.10	PP	6.3 to 16.6 (ITR) 7.0 to 18.4 (HYD-ITR)	-14.7 to -7.4 (ITR) -9.7 to -0.1(HYD-ITR)	87.1 to 96.7	>0.99	R051012	1, 7
Al-Rawithi et al. [146] LC-FLUO	10 -10000	13.0	0.1	PP	6.3 to 6.7 (ITR) 4.1 to 5.8 (HYD-ITR)	-7.5 to -4.1 (ITR) -6.1 to -4.6 (HYD-ITR)	89.0 to 97.0 (ITR) 92.0 to 95.0 (HYD-ITR)	≥0.9991 (ITR) ≥0.9986 (HYD-ITR)	ketoconazole	1, 7
Srivatsan et al. [147] LC-FLUO	5 - 500	22.0	1.00	LLEx	5.8 to 12.1 (ITR) 9.4 to 14.7 (HYD-ITR)	-5.2 to 10.7 (ITR) 0.8 to 10.1 (HYD-ITR)	76.0 to 84.5 (ITR) 70.5 to 73.3 (HYD-ITR) 91.4 (IS)	1.00	loratadine	1, 2a-e
J.W. Wong [148] LC-FLUO	2.8 - 720 (ITR) 5.6 - 720 (HYD-ITR)	12.0	0.50	LLEx	4.1 to 10.6 (ITR) 0.4 to 12.7 (HYD-ITR)	-7.1 to 5.2 (ITR) -7.1 to 7.9 (HYD-ITR)	93.2 (HYD-ITR) 85.4 (ITR) 79.5 (IS)	0.999 0.999	R051012	1, 2b, e
Breadmore et al. [149] MEKC	100 - 4000	9.0	0.125	PP	3.4 to 6.4 (ITR) 1.9 to 6.05 (HYD-ITR)	-4.75 to 3.00 (ITR) -5.71 to 4.28 (HYD-ITR)	n.r.	≥0.9967 (ITR) ≥0.9995 (HYD-ITR)	R051012	1

a) calculated by CR slopes, b) calibration row, PP: protein precipitation, LLEx: liquid-liquid extraction, SPE: solid phase extraction, n.r.: not reported
*1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e)), 3: influence of dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: matrix effect, 6: incurred samples, 7: interference with drugs

Table 44: *Method comparison for itraconazole and metabolite (continued)*

These methods have large coefficients of variation and Warnock et al. [136] reported much higher concentrations compared to the same sample measured with LC-UV.
Breadmore at al. [149] developed a micellar electrokinetic chromatography method with UV detection and reported comparable results to HPLC measurements.

3.4.4. Pharmacokinetic analysis

The developed and validated method was used to analyze ITR and HYD-ITR in human plasma samples from a clinical trial. In one period of the 2-way crossover study a 100 mg ITR reference tablet was administrated to 40 male and female subjects. Blood samples were taken before administration and after 0.50, 1.00, 2.00, 3.00, 4.00, 5.00, 6.00, 8.00, 12.00. 16.00, 24.00, 36.00, 48.00, 72.00 and 96.00 hours. Collected blood samples were centrifuged for 10 min at 3000 rpm (2095 g) at +4 °C and two aliquots were stored at -70 ℃ until sample analysis.

In the analyzed study ITR and HYD-ITR concentrations of 1280 human plasma samples were measured for the bioequivalence evaluation. Concentrations were calculated by the calibration curve that was measured at the beginning of each sequence and quality was ensured by measuring spiked quality control samples within each sequence. Calibration was performed by weighted (1/concentration2) linear regression for both analytes.

Itraconazole
The standard curve was linear between 2.910 and 977.9 ng/mL. The lower limit of quantification was 2.910 ng/mL. The inter-day precision and the analytical recovery of the spiked quality control standards of ITR in human plasma ranged from 3.6 to 5.5 % and were 99.9 % (927.2 ng/mL), 99.1 % (461.1 ng/mL), 99.2 % (93.81 ng/mL) and 99.5 % (9.069 ng/mL), respectively.

Within the set of SQC samples (n = 45) analyzed with the batches of study samples, all SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

Hydroxyitraconazole

The standard curve was linear between 4.814 and 984.1 ng/mL. The lower limit of quantification was 4.814 ng/mL.

The inter-day precision and the analytical recovery of the spiked quality control standards of HYD-ITR in human plasma ranged from 4.0 to 10.9 % and were 99.4 % (933.3 ng/mL), 99.8 % (464.2 ng/mL), 101.0 % (94.43 ng/mL) and 99.1 % (9.129 ng/mL), respectively.

Within the set of SQC samples (n = 45) analyzed with the batches of study samples, 175 out of 180 SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

Figure 12 shows the mean plasma concentration profiles of ITR and HYD-ITR after oral administration of a 100 mg ITR reference capsule to 40 healthy male volunteers. The mean peak concentration (C_{max}) of 67 ng/mL for ITR was attained at 4.2 h after administration of the product. The mean peak concentration (C_{max}) of 122 ng/mL for HYD-ITR was attained at 5.0 h after administration of the product.

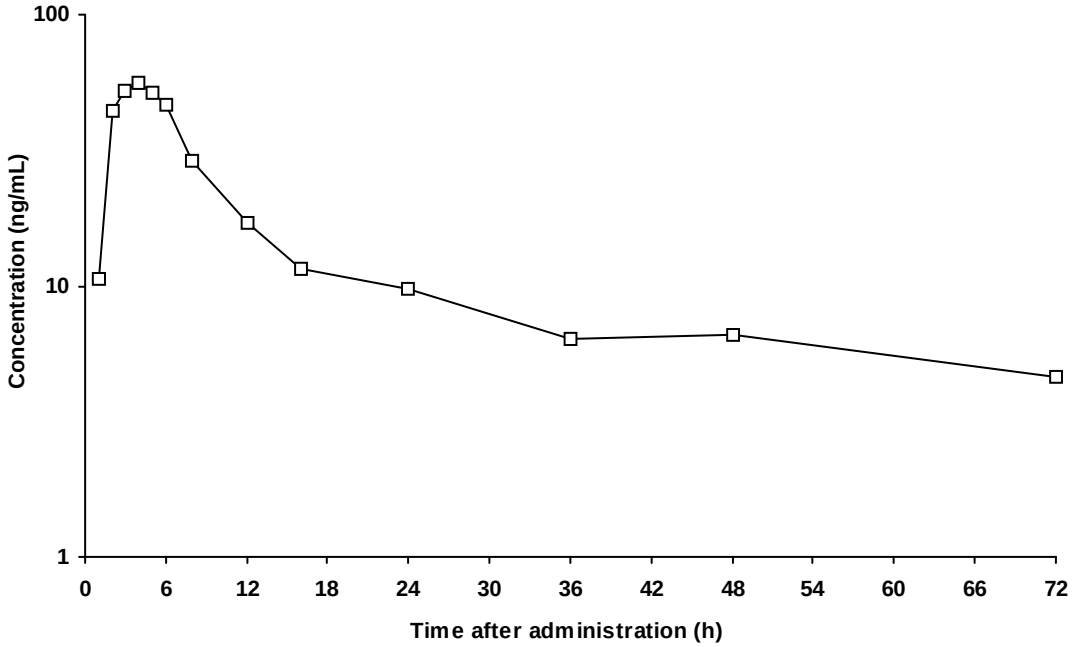
Table 45 shows the pharmacokinetic parameters of ITR and HYD-ITR after oral administration of a 100 mg capsule in comparison to pharmacokinetic parameters reported in previously published studies [121, 128, 151-154] under fasting or non-fasting conditions.

The calculated mean AUCs, C_{max}, and t_{max} values in this thesis for ITR are comparable to the values published by Kawakami et al. [152]. However, the $t_{1/2}$ calculated in this thesis was about twice as long. Bharathi et al. [128] report a comparable mean AUC but a lower mean C_{max} and a clearly higher $t_{1/2}$ of about 29 hours.

The mean C_{max} for HYD-ITR calcluated in this thesis is comparable to Bharathi et al. [128], but the mean AUC, C_{max} and $t_{1/2}$ was clearly lower in this thesis.

Yun et al. [154] investigated the influence of rice meal and bread meal in a multicentre study. A decrease of the systemic bioavailability was observed when ITR was administrated with rice meal. According to the authors this might be contributed to an increased gastric pH value after consumption of rice meal.

Itraconazole



Hydroxyitraconazole

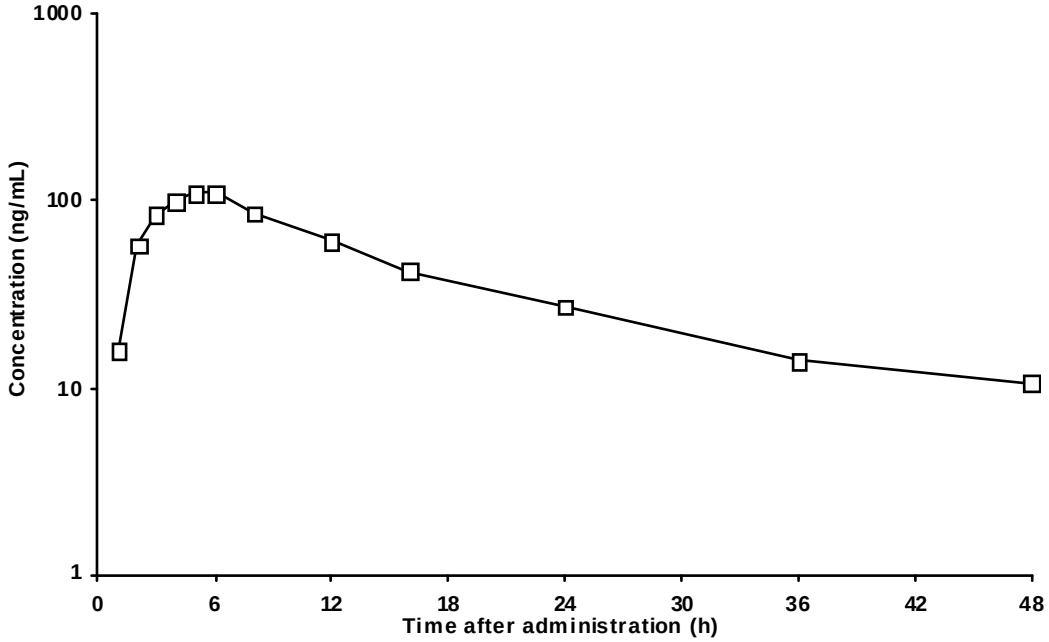


Figure 12: *Mean plasma profile of itraconazole and hydroxyitraconazole concentration vs. time following a 100 mg oral dose of itraconazole (reference formulation) to healthy volunteers (n=40)*

Reference	No. of subjects/ gender	Last time point [h]	Dose/ Formulation	Analyte	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]	Food
Current thesis	36/ m	96	100 mg/ capsule	ITR	661 ± 480	775 ± 525	67 ± 44	4.2 ± 1.6	16.3 ± 7.75	yes
				HYD-ITR	1607 ± 1120	1742 ± 1201	122 ± 58	5.0 ± 1.4	10.9 ± 11.7	
Bharathi et al. [128]	24/ n.r.	72	100 mg/ capsule	ITR	n.r.	798	50.0	ca. 4	ca. 29	n.r.
				HYD-ITR	n.r.	2347	117	ca. 6	ca. 14	n.r.
Hardin et al. [151]	5/ m	72	100 mg/ capsules	ITR	n.r.	1320 ± 651	110 ± 58	2.8 ± 1.1	15.0 ± 5.7 [a]	yes
Jaruratanasirikul et al. [121]	8/ m	24	100 mg/ capsule with water	ITR	1120 ± 109	n.r.	140 ± 900	2.6 ± 0.6	n.r.	no
			100 mg/ capsule with coca-cola	ITR	2020 ± 141	n.r.	310 ± 180	3.4 ± 0.8	n.r.	
Kawakami et al. [152]	22/ m	24	100 mg/ capsule	ITR	n.r.	770 ± 310	79 ± 33	4.7 ± 1.0	8.9 ± 2.6	yes
Van Peer et al. [153]	6/ m		100 mg/ capsules	ITR	n.r.	722 ± 289	38 ± 20	3.3 ± 1.0	24.0 ± 9.0	no
			100 mg/ capsules	ITR	n.r.	1900 ± 838	132 ± 67	4.0 ± 1.1	17.0 ± 3.0	yes
Yun et al. [154]	20/ m	48	100 mg/ tablet	ITR	n.r.	1704 ± 806	130 ± 58	2.9 ± 1.2	n.r.	no
	40/ m	72	100 mg/ tablet	ITR	n.r.	452 ± 316	31 ± 20	2.7 ± 1.2	n.r.	no
	24/ 23m, 1f	72	100 mg / capsule	ITR	n.r.	1122 ± 542	82 ± 36	3.2 ± 1.0	n.r.	no

m: male, f: female, n.r.: not reported, a: values are harmonic mean

Table 45: *Reported values for ITR and HYD-ITR pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after administration of a single oral dose after a standardized breakfast or overnight fasting*

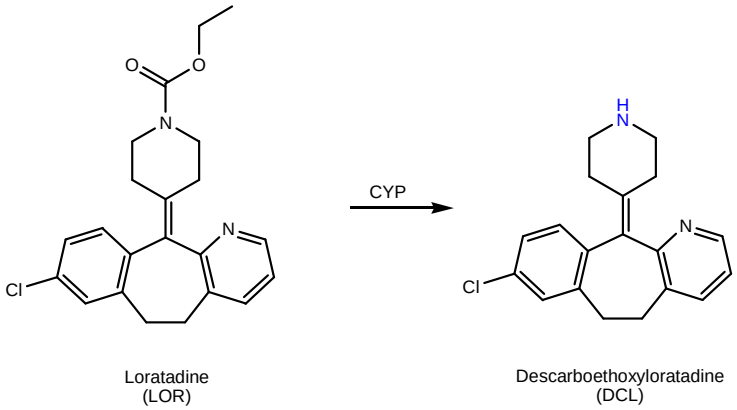
A dose dependant clinical trial (50mg, 100mg, 200 mg) with food was investigated by Van Peer et al. [153]. In their study no linear kinetics were found for ITR. The AUC and C_{max} values increased more than proportionally with ITR dose. They ascribed that to a possible saturated metabolism which means that all ITR metabolizing enzymes are allocated, resulting in higher than dose proportional AUCs and C_{max} values. Van Peer et al. [153] also investigated the pharmacokinetics of ITR with and without food. Food increases the bioavailability because of causing a lower gastric pH value. An analogue result was observed by Jaruratanasirikul et al. [121] during coadministration of coca-cola beverage and ITR capsules.

3.5. Loratadine and Descarboethoxyloratadine

Loratadine (LOR), a tricyclic H1 antihistamine of the second generation, is used for the treatment of allergic diseases such as rhinitis or urticaria. The advantage of the second generation H1 antihistamines is their non-sedating effect on the central nervous system which is observed with first generation H1 antihistamines because of crossing the blood-brain barrier due to their high lipophilicity. LOR is available as tablet, oral suspension and syrup [22].

Following oral administration LOR is well and readily absorbed from the gastro intestinal tract and peak plasma concentrations are achieved within 1-3 hours. LOR undergoes an extensive first pass metabolism to its active metabolite descarboethoxyloratadine (DCL) (**Scheme 9**) by CYP enzymes and is mainly excreted in the urine in its unchanged form [22]. Linear pharmacokinetics for LOR and DCL were shown by Hilbert et al. [155] for 10, 20 and 40 mg doses. As LOR is a long acting drug it has to be taken only once a day.

It has long time been assumed that H1 antihistamines compete with histamine at the receptor binding site. Today it is known that H1-receptors are G-protein-coupled-receptors (GPCRs), the inactive and active conformations of which coexist in equilibrium. LOR binds on the inactive conformation and shifts the equilibrium towards the inactive site therefore reducing the typical allergic appearance. For this reason the H1 antihistamines act as inverse agonists and not as antagonists [156].



Scheme 9: *Loratadine and active metabolite*

Several analytical methods for the determination of LOR or DCL in separate assays or both drugs in one assay have been published in the literature.

The UV- or FLUO-detection methods of El-Enany et al. [157], Kunicki et al. [158] and Amini et al. [159] determine either LOR or DCL and have runtimes longer than 10 min. Simultaneous determination of LOR and DCL extends the runtime up to 20 min [160-161]. Moreover, these methods have relatively low sensitivity (> 0.2 ng/mL) and are more suitable for analyzing LOR dosage forms or plasma samples after multiple LOR dosages.

Gas chromatography was used by Martens et al. [162] and Johnson et al. [163] employing MS and nitrogen phosphorus detection (NPD), respectively. The GC-MS method provides low sensitivity (>0.5 ng/mL) and has a runtime of 18 minutes. With the GC-NPD method, LOR and DCL can be determined with a LLOQ of 0.1 ng/mL. This method employs a double extraction procedure and has a runtime of 6 min.

Ghoneim et al. [164] studied the polarographic behaviour and the concentration of LOR by cathodic adsorptive stripping voltammetry. A radioimmunoassay was used to determine LOR during a dose proportionality study in human plasma by Hilbert et al. [155].

For routine analysis and high sensitivity, especially at low doses of LOR, LC-MS/MS is the method of choice. Several methods determining LOR [165-166], DCL [167-168] or both analytes in one run [169-175] have been published in the literature.

Different sample preparation procedures including PP [173] LLEx [169-170, 172, 175], and SPE [171, 174], depending up on the required sensitivity, are reported.
Naidong et al. [169] reports a highly sensitive LC-MS/MS method for the determination of LOR and DCL with a LLOQ of 0.010 and 0.025 ng/mL, respectively.

3.5.1. Method development and optimization

For LOR and DCL as well as for their deuterated internal standards (d4-LOR and d4-DCL) precursor and product ion spectra were recorded on the API 5000™. All spectra were recorded in the positive ionization mode as the negative mode showed only poor sensitivity for the analytes. Solutions containing ca. 100 ng/mL of each compound were infused into the API 5000™ and the individual parameters were optimized for each compound. At a IS voltage of +5 kV and a temperature of 350 °C a peak in the intensity was observed for LOR and DCL as well as for their internal standards. The DP was optimal for all compounds at a value of 84 V. With this settings the protonated molecular ions $[M+H]^+$ were observed a m/z 383 and m/z 311 for LOR and DCL, respectively. Precursor ion spectra of d4-LOR and d4-DCL showed at m/z 387 and m/z 315 the protonated molecular ions $[M+H]^+$. Product ion spectra of LOR and d4-LOR were obtained by setting CE to 33 eV. This produced the most intensive fragments for LOR and d4-LOR at m/z 337 and m/z 341, respectively. Best fragmentation for DCL and d4-DCL was obtained at a value of 27 eV. The most intensive fragments for DCL and d4-DCL were m/z 259 and m/z 263, respectively. The MS settings for all compounds are summarized in **Table 46**.

Analyte	IS (V)	Temp. (°C)	DP (V)	CE (eV)	Q1 m/z	Q3 m/z
Loratadine	+5000	350	+84	33	383	337
Descarboethoxyloratadine	+5000	350	+84	27	311	259
d4-Loratadine	+5000	350	+84	33	387	341
d4-Descarboethoxyloratadine	+5000	350	+84	27	315	263

IS: ionspray voltage, Temp.: temperature, DP: declustering potential, CE: collision energy

Table 46: *API 5000™ system parameters of loratadine, metabolite and internal standards*

For development of the liquid chromatography method two different columns were tested. Due to the number of pi electrons of LOR and DCL a phenyl column (YMC-Pack Phenyl, 50 x 4.6 mm, 3µ, YMC Europe GmbH, Dinslaken, Germany) was tested.
Additionally a cyano column (YMC-Pack Cyano, 50 x 4.6 mm, 3µ, YMC Europe GmbH, Dinslaken, Germany) was tested because of the polarity of the compounds. The first tests were made with 5 mM ammonium acetate buffer and acetonitrile with different ratios at a flowrate of 0.8 mL/min. Comparing the cyano and the phenyl column the latter one retains LOR and DCL much stronger. Therefore LOR and DCL eluted very late on the phenyl column. Even at high acetonitrile ratios the runtime was too long to be used for high throughput sample analysis. On the cyano column the retention time of DCL was lower than 0.8 min which was too early. This could be fixed by adjusting the pH value of the buffer to 5.0 and the amount of acetonitrile to 45 %. Under these conditions DCL eluted after approximately 1.0 min. The retention time of LOR was approximately 1.7 min. The chromatographic conditions can be summarized as follows: mobile phase: 5 mM ammonium acetate (pH 5.0) : MeCN (55/45, v/v), analytical column: YMC-Pack Cyano (50 x 4.6 mm, 3µ), flow rate: 0.8 mL/min.

As a LLOQ of 10 pg/mL should be reached with the assay, protein precipitation could not be used for sample preparation. Limit of detection for precipitated samples was in the upper ng/mL range.
For liquid-liquid extraction the basic functional groups in LOR and DCL had to be considered. As a extraction solvent with low polarity should be used, the basic nitrogens must not be protonated. Therefore during the extraction process an alkaline pH value had to be ensured. This was performed by adding 3M NaOH solution to the sample before the extraction procedure. Dichloromethane, Diethyl ether and n-hexane were tested as extraction solvents. The worst extraction efficiency was obtained with dichloromethane. Diethyl ether showed better extraction of LOR and DCL than with dichloromethane but still not as good as with n-hexane. Using n-hexane DCL showed compared to LOR a clearly lower extraction efficiency but due to a better signal-to-noise ratio for DCL a LLOQ of 10 pg/mL could be achieved for both compounds.
For the final sample preparation 600 µL of human plasma were mixed with 50 µL 3M sodium hydroxide solution and 100 µL internal standard solution (containing 10 ng/mL d4-LOR and 10 ng/mL d4-DCL). For the extraction 3.0 mL n-hexane were added. After mixing for 10 minutes, the samples were centrifuged at 3000 rpm (2095 g) for 10 minutes at +4 °C. The organic layer was transferred into a new tube and evaporated to dryness using nitrogen at a temperature of 40 °C.

The residual was redissolved with 200 µL 5 mM ammonium acetate/ acetonitrile (40:60, v:v, pH 5). Thirty µL of each sample were injected into the LC-MS/MS system.

3.5.2. Method validation

After the injection of prepared drug free human plasma, matrix components did not interfere with LOR or DCL and their internal standards near or at their retention times over the concentration range (0.01 ng/mL - 15 ng/mL) described herein. Typical chromatograms of blank plasma samples, standards and samples from healthy volunteers can be found in the Appendix.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration ranges of the analytes in human plasma. The mean linear regression equations of the calibration curves generated during the validation were:

$y = 0.0002\ (\pm 0.0001) + 0.4506\ (\pm 0.0234)\ x$ for LOR, $r^2 > 0.998$

$y = 0.0013\ (\pm 0.0006) + 0.5670\ (\pm 0.0222)\ x$ for DCL, $r^2 > 0.999$

where *y* represents the ratio of the analyte peak area to that of the internal standard, and *x* represents the plasma concentration of the analyte.

As **Table 47** shows, inter-day CV of the calibration rows was <5 % and <3 % for LOR and DCL, respectively. Inter-day RE was <6 % and <7 % for LOR and DCL, respectively. At the LLOQ CV and RE were <7 % for both compounds.

	CV [%]	**RE [%]**	**CV [%]**	**RE [%]**
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
LOR	0.8 to 4.9	-5.8 to 4.3	4.4	-4.0
DCL	0.5 to 2.5	-6.2 to 5.5	1.6	-6.2
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
LOR	1.4 to 5.5*	-4.3 to 1.3	1.0 to 9.6*	-2.8 to 2.0*
DCL	1.2 to 9.9*	-1.9 to 1.4	1.3 to 6.6	-1.6 to 7.3*

*: value at LLOQ

Table 47: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for loratadine and metabolite*

On five consecutive days of the validation spiked quality control samples were analyzed. The intra-day CV was <10 % and <7 % for LOR and DCL, respectively. The intra-day RE was <3 % and <8 % for LOR and DCL, respectively. The inter-day CV and RE was <6 % and <10 % for LOR and DCL, respectively. The inter-day RE was <5 % and < 2% for LOR and DCL, respectively. The excellent CV and RE values represent the excellent performance of the developed method. Percental ranges for intra-day as well as inter-day precision and relative error are summarized in **Table 47**. The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits.

The results of the recovery experiment are shown in **Table 48**. LOR and d4-LOR show mean absolute extraction recoveries of 79 % and 76 %, respectively. For DCL and d4-DCL mean absolute extraction recoveries were 38 % and 33 %, respectively.

Analyte	Mean [%]	SD [%]	CV [%]
LOR	79.3	4.3	5.4
DCL	37.9	3.3	8.8
d4-LOR	76.2	1.5	2.0
d4-DCL	32.9	1.8	5.5

Table 48: *Mean absolute extraction recoveries for loratadine and metabolite*

A very low absolute mean extraction recovery was obtained for DCL. However, the recovery was sufficient for the detection of 10 pg/mL DCL in human plasma. According to the recommended FDA-guideline [48, 74] the recovery of an analytical method needs not to be very high but consistent, precise and reproducible. As can be seen from **Table 48**, the recoveries for all LOR and DCL show a relative standard deviation (CV) <10 % and were therefore judged to be acceptable. No concentration dependency of the recovery was observed for any of the analytes.

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for all analytes. All statistical evaluations of the stability experiments can be found in the Appendix.

Dilution of samples prior to analysis did not affect LOR and DCL determination. Hemolyzed and lipemic human plasma as well as different batches of human plasma did not influence the determination of LOR and DCL. **Table 49** summarizes the CVs and the REs of the investigated influences.

Analyte	Dilution	Hemolyzed		Lipemic		Different batches	
		high	low	high	low	high	low
RE (%)							
LOR	-1.4	-10.3	-4.5	-9.5	-3.8	-0.6	4.8
DCL	1.7	-7.3	-2.2	-5.3	3.6	3.2	3.7
CV (%)							
LOR	1.7	0.6	3.8	0.9	3.2	1.0	2.1
DCL	1.0	0.9	2.3	1.8	6.1	1.0	4.4

Table 49: *Relative errors (%) and precisions (%) of the investigated influences for loratadine and metabolite*

All RE values were below 11 % for LOR and DCL, respectively. The CV values were low and did not exceed 4 % for LOR and 7 % for DCL, respectively. Therefore any influence on the determination of LOR and DCL can be excluded.

In the incurred samples reanalysis experiment 100 human plasma samples containing LOR and DCL that were previously analyzed in a clinical trial with the developed method were analyzed again. With the exception of one observed peak interference all reanalyzed blanks did not show any signal at the retention time of LOR or DCL, respectively. 99 % and 98 % of the reanalyzed samples were within the ±15 % range for LOR and DCL, respectively. For LOR only one sample showed a greater deviation than 15 % compared to the first analysis and no sample was reanalyzed with a deviation greater than 20 %. For DCL the 15 % limit and the 20 % limit were exceeded by one sample, respectively. All results are shown in **Table 50.**

The mean absolute difference of the 1st to the 2nd analysis was 2.6 % and 5.2 % for LOR and DCL, respectively. Both results represent the excellent performance of the developed method.

Analyte	No. of reanalyzed samples	% of blanks	Percentual samples within ≤5 %	5 - 10 %	10 - 15 %	15 - 20 %	>20 %
LOR	100	29.0	62.0	7.0	1.0	1.0	none
DCL	100	25.0	53.0	10.0	10.0	1.0	1.0

Table 50: *Incurred samples reanalysis for loratadine and metabolite*

The influence of the sample matrix on the determination of LOR and DCL as well as their internal standards d4-LOR and d4-DCL were investigated. All determined MF vales shown in **Table 51** are below 1 and therefore indicate a low ion suppression of the corresponding signals. Comparing LOR and DCL the latter one is suppressed to a lower extend.

Analyte	Matrix Factor mean	CV [%]	Matrix Factor IS normalized	CV [%]
LOR	0.889	0.5	1.042	0.7
d4-LOR	0.853	0.7	-	-
DCL	0.960	4.5	0.998	0.6
d4-DCL	0.962	4.6	-	-

Table 51: *Matrix Factors (MF) and IS normalized MF values for loratadine and metabolite*

The same result is observed for d4-LOR and d4-DCL. Analytes and internal standards are suppressed to the same extend. Therefore the IS normalized matrix factors are 1.042 and 0.998 for LOR and DCL, respectively. This indicates the absence of any matrix influences on the determination of LOR or DCL, respectively.

3.5.3. Comparison of the developed method with existent methods

In **Table 52** the developed method is compared with previously published methods for the determination of LOR and DCL.

Method	Linearity range [pg/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Preparation	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experiments *
LC-MS/MS current thesis	0.010 - 15.0	3.0	0.6	LLEx	1.4 to 5.5 (LOR) 1.2 to 9.9 (DCL)	-4.3 to 1.3 (LOR) -1.9 to 1.4 (DCL)	79.3 ± 4.3 (LOR) 37.9 ± 3.3 (DCL)	≥0.998 (LOR) ≥0.999 (DCL)	d4-loratadine d4-desloratadine	1, 2a-e, 3, 4a,b, 5, 6, 7
Naidong et al. [169] LC-MS/MS	0.010 - 1.0 (LOR) 0.025 - 2.5 (DCL)	3.0	1.0	LLEx	4.8 to 5.7 (LOR) 3.5 to 9.4 (DCL)	-5.1 to 0.4 (LOR) 4.0 to 7.9 (DCL)	41.4 to 66.1 (LOR) 44.8 to 68.0 (DCL)	>0.995 (LOR) >0.999 (DCL)	d3-loratadine d3-desloratadine	1, 2a-d, 3
Patel at al. [170] LC-MS/MS	0.050 – 15.0	3.0	0.5	LLEx	6.0 to 8.6 (LOR) 4.3 to 7.7 (DCL)	-5.3 to 3.5 (LOR) -6.8 to 2.0 (DCL)	89.6 to 95.3 (LOR) 37.8 to 42.5 (DCL)	0.9984 (LOR) 0.9979 (DCL)	desipramine	1, 2a-e, 3, 6
Srinubabu et al. [171] LC-MS/MS	0.400 - 20.0	3.5	0.5	online sample extraction	4.4 to 15.2	-2.6 to 9.1	73.2 (LOR) 57.1 (DCL)	0.99	cetirizine	1, 2a-e,
Sutherland et al. [172] LC-MS/MS	0.100 - 20.0	6.4	1.0	LLEx	7.3 to 12.6 (LOR) 8.9 to 14.3 (DCL)	3.0 to 11.0 (LOR) -3.0 to 1.0 (DCL)	61.0 (LOR) 100.0 (DCL)	n.r.	external standards fluspirilene and fluoxetine	1, 2c, 6
Vlase et al. [173] LC-MS/MS	0.520 - 52.0	8.0	0.3	PP	2.9 to 19.2 (LOR) 3.0 to 5.7 (DCL)	-0.3 to 0.6 (LOR) 1.2 to 3.0 (DCL)	90.3 to 116.6 (LOR) 107.0 to 113.5 (DCL)	>0.998	metoclopramide	1, 2a-d
Yang et al. [174] LC-MS/MS	1.00 - 10^3	3.5	0.5	SPE	0.97 to 16 (LOR) 0.68 to 11.0 (DCL)	-8.4 to 10.5 (LOR) 6.4 to - 9.1 (DCL)	n.r.	>0.99	d4-loratadine d4-desloratadine	1
Zhang et al. [175] LC-MS/MS	0.200 - 20.0	n.r.	1.0	LLEx	< 14.2	< 1.2 (LOR) < 4.6 (DCL)	n.r.	0.9992 (LOR) 0.9995 (DCL)	diphenhydramine	n.r.
Sora et al. [160] LC-FLUO	0.500 - 20.0	20.0	1.0	LLEx	2.4 to 12.4	n.r.	75.7 ± 4.4 (LOR) 72.2 ± 4.4 (DCL)	around 0.9996	8-chloroazatadine	1, 2a-e
Yin et al. [161] HPLC-FLUO	0.500 - 16.0	20.0	1.0	LLEx	4.3 to 5.4 (LOR) 4.1 to 8.3 (DCL)	-0.2 to 4 (LOR) -1.7 to 0.5 (DCL)	80.4 to 81.8 (LOR) 67.2 to 69.8 (DCL)	0.998 (LOR)[a] 0.996 (DCL)[a]	propanolol HCl	1, 2c,d, 3,
Johnson et al. [163] GC-NPD	0.100 - 30.0	6.0	1.0	LLEx	5.0 to 8.2 (LOR) 3.5 to 6.3 (DCL)	-0.4 to -5.0 (LOR) 7.2 to 10.7 (DCL)	n.r.	≥0.998	SCH 37370 (LOR) SCH 40129 (DCL)	n.r.

a: mean, n.r.: not reported, PP: protein precipitation, LLEx: liquid-liquid extraction, SPE: solid phase extraction
*1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e)), 3: influence of dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: influence of different individuals, 6: matrix effect, 7: incurred samples

Table 52: *Method comparison for loratadine and metabolite*

The analytical methods in **Table 52** include LC-MS/MS, LC-FLUO and GC-NPD. The LC-FLUO methods [160-161] provide sensitivity down to 0.5 ng/mL but have very long runtimes of 20 min and require high sample volumes which might be a problem when sample reanalysis has to be performed.

The gas chromatography method with nitrogen-phosphorous detection of Johnson et al. [163] has a LLOQ of 100 pg/mL and shows good quality data but does not report further additional validation experiments which are essential for pharmacokinetic analysis of clinical trials. All LC-MS/MS methods have shorter runtimes and with the exception of Naidong et al. [169] and Sutherland et al. [172] need less sample volume than the other methods.
Solid phase extraction, protein precipitation and liquid-liquid extraction can be used for sample work up, but highest sensitivities are achieved with liquid-liquid extraction whereas recoveries are strongly dependent on the used extraction solvent.
By now the most sensitive LC-MS/MS method was published by Naidong et al. [169] with a LLOQ for LOR and DCL of 0.01 and 0.025 ng/mL, respectively. However, the QC-samples in the validation were three times higher than the LLOQ. Beyond specificity, stability experiments and influence of dilution no additional validation experiments are reported. The assay shows high variation in recovery as coefficients of variation between 15 % and 25 % were determined.

For LOR the developed method on the AB Sciex API 5000™ shows the same sensitivity as the method of Naidong et al. [169], but reaches a factor 2.5 higher sensitivity for DCL. Key factors for LC-MS/MS methods as influence of different individuals and the matrix effect were determined. The excellent assay over the whole calibration range is demonstrated by the quality data and the incurred samples reanalysis. Patel et al. [170] also investigated the matrix effect but the method shows a five times higher LLOQ for both analytes.

3.5.4. Pharmacokinetic analysis

The developed method was used to determine the bioequivalence of two LOR formulations. In one of the two study periods a 10 mg LOR reference tablet was administered to 24 male and female subjects under fasting conditions.

Blood samples were taken before the administration and after 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 4.00, 8.00, 12.00, 24.00, 36.00, 48.00 and 72.00 hours. Collected blood samples were centrifuged for 10 min at 3000 rpm (2095 g) at +4 °C and two aliquots were stored at -70 °C until sample analysis.

All samples of the clinical trial were analyzed in nine sequences. At the beginning of each sequence a calibration row was measured and spiked quality control samples were analyzed within the sequence. Calibration was performed by weighted ($1/concentration^2$) linear regression for both compounds. The standard curves for LOR and DCL were linear between 0.0100 and 15.0 ng/mL. The lower limit of quantification was 0.0100 ng/mL.

Loratadine

The inter-day precision and the analytical recovery of the spiked quality control standards of LOR in human plasma ranged from 2.0 to 3.7 % and were 93.5 % (11.0 ng/mL), 96.7 % (5.00 ng/mL), 98.6 % (1.00 ng/mL) and 96.8 % (0.0250 ng/mL), respectively.

Within the set of spiked quality control samples (n = 27) analyzed with the batches of study samples, all of the 108 spiked quality control samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

Descarboethoxyloratadine

The inter-day precision and the analytical recovery of the spiked quality control standards of DCL in human plasma ranged from 1.8 to 5.1 % and were 94.8 % (11.0 ng/mL), 95.2 % (5.00 ng/mL), 95.2 % (1.00 ng/mL) and 93.7 % (0.0250 ng/mL), respectively.

Within the set of spiked quality control samples (n = 27) analyzed with the batches of study samples, 106 out of 108 spiked quality control samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

Figure 13 shows as example the mean plasma concentration profile of LOR and DCL after oral administration of a 10 mg LOR reference tablet to 24 healthy volunteers.

The mean peak concentration (C_{max}) of 3.7 ng/mL for LOR was attained at 1.2 h after administration of the product. The mean peak concentration (C_{max}) of 2.3 ng/mL for DCL was attained at 1.5 h after administration.

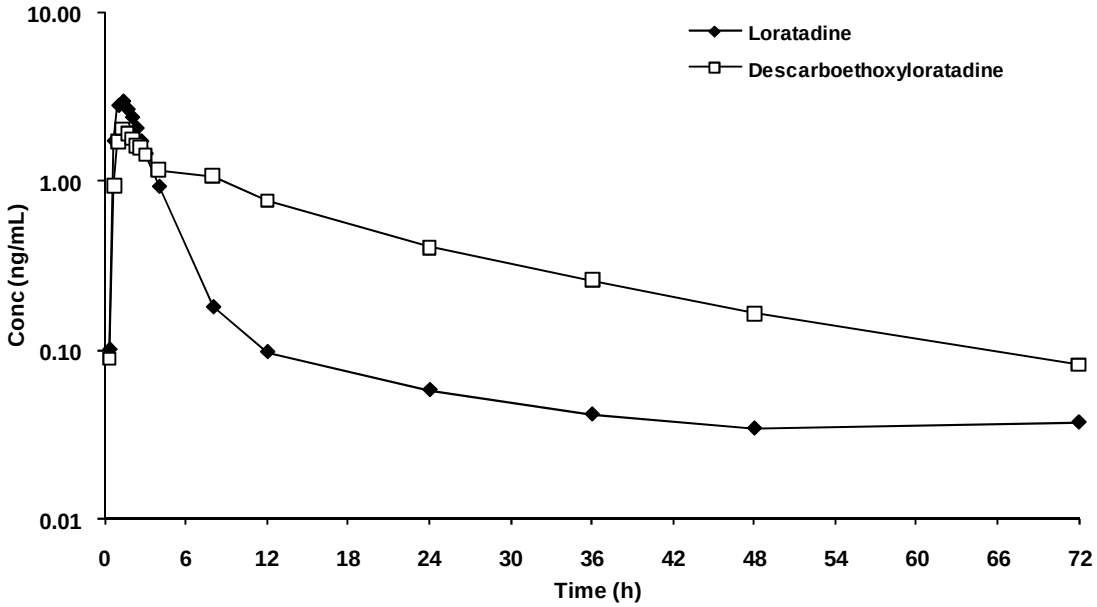


Figure 13: *Mean plasma profile of loratadine and descarboethoxyloratadine concentration vs. time following a 10 mg oral dose of loratadine to healthy volunteers (n=24)*

Table 53 shows the pharmacokinetic parameters of LOR and DCL after oral administration a LOR tablet in comparison to pharmacokinetic parameters reported in previously published studies [155, 160, 170, 175-176]. The presented data were obtained from Caucasian [155, 160, 176], Chinese [175] and Indian subjects [170].
In order to compare the pharmacokinetics of LOR and DCL, given in different doses, the calculations of C_{max}, $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\infty}$ were dose-corrected to a 10 mg loratadine dose.

The observed t_{max} values for LOR and DCL are comparable to previously reported t_{max} values except for Hilbert et al. [155] and Radwanski et al. [176] who report clearly higher t_{max} values for DCL. The calculated AUCs and C_{max} for LOR are comparable to the values published by Sora et al. [160]. However, for DCL these two pharmacokinetic parameters were calculated lower in this thesis than in the literature shown in **Table 53**.

The determined half-lifes for LOR and DCL fit best with the values reported by Sora et al. [160]. The high inter-study differences for Caucasian subjects may be due to different study conditions and the extensive first pass effect of loratadine.

Reference	No. of subjects/ gender	Last time point [h]	Dose/ Formulation	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current thesis	24/ m, f	72	10 mg/ tablet	11.6 ± 12.6 (LOR) 30.2 ± 11.4 (DCL)	12.5 ± 13.8 (LOR) 32.8 ± 13.0 (DCL)	3.7 ± 3.9 (LOR) 2.3 ± 0.8 (DCL)	1.2 ± 0.5 (LOR) 1.5 ± 0.5 (DCL)	20.8 ± 14.8 (LOR) 20.6 ± 3.1 (DCL)
Hilbert et al. [155]	12/ m	96	10 mg/ capsule	n.r. n.r.	14.0 ± 9.5 (LOR) 68.6 ± 43.5 (DCL)	4.7 ± 2.7 (LOR) 4.0 ± 1.7 (DCL)	1.5 ± 1.0 (LOR) 3.7 ± 3.1 (DCL)	n.r. n.r.
	12/ m	96	20 mg/ capsule	n.r. n.r.	17.2 ± 11.1 (LOR) 70.9 ± 26.8 (DCL)	5.4 ± 2.2 (LOR) 5.0 ± 1.5 (DCL)	1.0 ± 0.3 (LOR) 1.5 ± 0.7 (DCL)	11.0 ± 9.4 (LOR) 17.3 ± 6.9 (DCL)
	12/ m	96	40 mg/ capsule	n.r. n.r.	18.1 ± 8.5 (LOR) 72.2 ± 24.5 (DCL)	6.5 ± 3.2 (LOR) 4.0 ± 1.5 (DCL)	1.2 ± 0.3 (LOR) 2.0 ± 1.3 (DCL)	7.8 ± 4.2 (LOR) 24.0 ± 9.5 (DCL)
Radwanski et al. [176]	12/ m	24	40 mg/ capsule	17.5 ± 23.2 (LOR) 44.3 ± 22.6 (DCL)	n.r. n.r.	5.3 ± 5.7 (LOR) 4.4 ± 1.7 (DCL)	1.6 ± 0.7(LOR) 2.9 ± 1.8 (DCL)	n.r. n.r.
Zhang et al. [175]	20/ m	36	20 mg/ tablets	n.r.	23.5 ± 24.5 (LOR) 90.5 ± 61 (DCL)	8.5 ± 7.0 (LOR) 8.0 ± 4.5 (DCL)	1.2 ± 0.6 (LOR) 1.5 ± 0.5 (DCL)	6.0 ± 4.0 (LOR) 13.4 ± 2.6 (DCL)
Patel et al. [170]	28/ m	144	10 mg/ tablet	19.6 ± 31.0 (LOR) 42.7 ± 17.1 (DCL)	21.0 ± 31.5(LOR) 45.1 ± 7.3 (DCL)	5.5 ± 6.7(LOR) 3.1 ± 0.8 (DCL)	1.1 ± 0.3 (LOR) 1.5 ± 0.4 (DCL)	12.4 ± 21.0(LOR) 22.2 ± 6.7 (DCL)
Sora et al. [160]	23/ m, f	96	20 mg/ tablet	12.5 ± 6.2 (LOR) 53.7 ± 7.7 (DCL)	13.8 ± 6.6 (LOR) 57.4 ± 8.1 (DCL)	3.3 ± 1.4 (LOR) 4.4 ± 0.8 (DCL)	1.2 ± 0.3 (LOR) 1.6 ± 0.3 (DCL)	18.1 ± 8.5 (LOR) 17.0 ± 3.4 (DCL)

m: male, f: female, n.r.: not reported

Table 53: *Reported values for loratadine and descarboethoxyloratadine pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after overnight fasting and administration of a single oral dose. Presented values are normalized to a 10 mg dose*

Regarding the AUCs and C_{max} obtained for LOR and DCL from the Indian subjects the calculated values in this thesis are lower. A high difference can be seen when the data are compared with Zhang et al. [175], which are silhouetted out in **Table 53** and were obtained from Chinese subjects. The C_{max} and AUC values are clearly higher than the reported values from Indian or Caucasian subjects.
Yin et al. [177] found out that loratadine pharmacokinetics is affected by CYP 2D6 polymorphism prevalent in the Chinese population.

3.5.5. Method comparison on API 3000™ (*Partial Validation*)

For a comparison of LOR and DCL of the validated method on an API 3000™ spectra were recorded. This was performed as described above for the API 5000™. The optimal parameters for LOR and DCL as well as for their internal standards are shown in **Table 54**.

Analyte	IS (V)	Temp. (°C)	OR (V)	CE (eV)	Q1 m/z	Q3 m/z
Loratadine	+5000	350	+51	34	383	337
Descarboethoxyloratadine	+5000	350	+51	30	311	259
d4-Loratadine	+5000	350	+51	34	387	341
d4-Descarboethoxyloratadine	+5000	350	+51	30	315	263

IS: ionspray voltage, Temp.: temperature, OR: orifice voltage, CE: collision energy

Table 54: *API 3000™ system parameters for loratadine, metabolite and internal standards*

The best signal intensity for all compounds was obtained at a IS voltage of 5 kV and a temperature of 350 °C. The OR was optimal for all compounds at a value of 51 V. In the precursor ion spectrum the same m/z values as described above for the API 5000™ were obtained. The protonated molecular ions of LOR and DCL were obtained at m/z 383 and 311, respectively. Their d4-deuterated internals standards showed the corresponding signals at m/z 387 and 315, respectively. The most intensive fragments for LOR and d4-LOR were obtained at a CE of 34 eV and were m/z 337 and m/z 341, respectively. DCL and d4-DCL produced the most intensive fragments at a collision energy of 30 eV. The corresponding fragments were m/z 259 and m/z 263 for DCL and d4-DCL, respectively.

For system comparison neither the chromatographic conditions nor the sample preparation procedure was changed. Therefore the mass spectrometers can be compared directly.

Table 55 shows the results of the calibration standards of the *Partial Validation* (system comparison) in comparison to the calibration standards of the *Full Validation*.

Method comparison						
Analyte	**MS**	**Linearity [ng/mL]**	**Inter-day CV [%]**	**Inter-day RE [%]**	**Inter-day CV [%] at LLOQ**	**Inter-day RE [%] at LLOQ**
LOR	API 3000™	0.100 - 20.0	0.1 to 9.3	-2.3 to 2.5	4.2	1.2
LOR	API 5000™	0.0100 - 15.0	0.8 to 4.9	-5.8 to 4.3	4.4	-4.0
DCL	API 3000™	0.150 - 20.0	1.3 to 10.5	-3.7 to 10.0	5.6	-3.7
DCL	API 5000™	0.0100 - 15.0	0.5 to 2.5	-6.2 to 5.5	1.6	-6.2

Table 55: *Method comparison – Calibration Standards for loratadine and metabolite*

For LOR and DCL the LLOQ achieved on the API 5000™ was better by a factor of 10 and 15, respectively. Inter-day CV and RE for the calibration rows were better on the API 5000™ for both compounds.

In **Table 56** the intra-day and inter-day CV and RE of the spiked quality control samples measured for LOR and DCL on the API 3000™ and API 5000™ are shown. On the API 5000™ the intra-day and inter-day CV for LOR and DCL were <10 %. For LOR the measured precision was slightly better on the API 3000™ than on the API 5000™. For DCL the precision measured on the API 5000™ was better compared to the API 3000™.

Method comparison					
Analyte	**Mass Spectrometer**	**Intra-day CV [%]**	**Intra-day RE [%]**	**Inter-day CV [%]**	**Inter-day RE [%]**
LOR	API 3000™	2.2 to 6.3*	-6.0 to -1.2	2.5 to 7.0*	-6.7 to -1.9
LOR	API 5000™	1.0 to 9.6*	-2.8 to 2.0*	1.4 to 5.5*	-4.3 to 1.3
DCL	API 3000™	3.6 to 13.9*	-13.5 to 4.2*	8.1 to 14.8*	-5.8 to 0.5*
DCL	API 5000™	1.3 to 6.6	-1.6 to 7.3*	1.2 to 9.9*	-1.9 to 1.4

*: at LLOQ

Table 56: *Method comparison – Quality Control Samples for loratadine and metabolite*

Figure 14 illustrates the inter-day RE of the spiked quality control samples including the *Full Validation* method as well as the *Partial Validation* method for LOR and DCL.

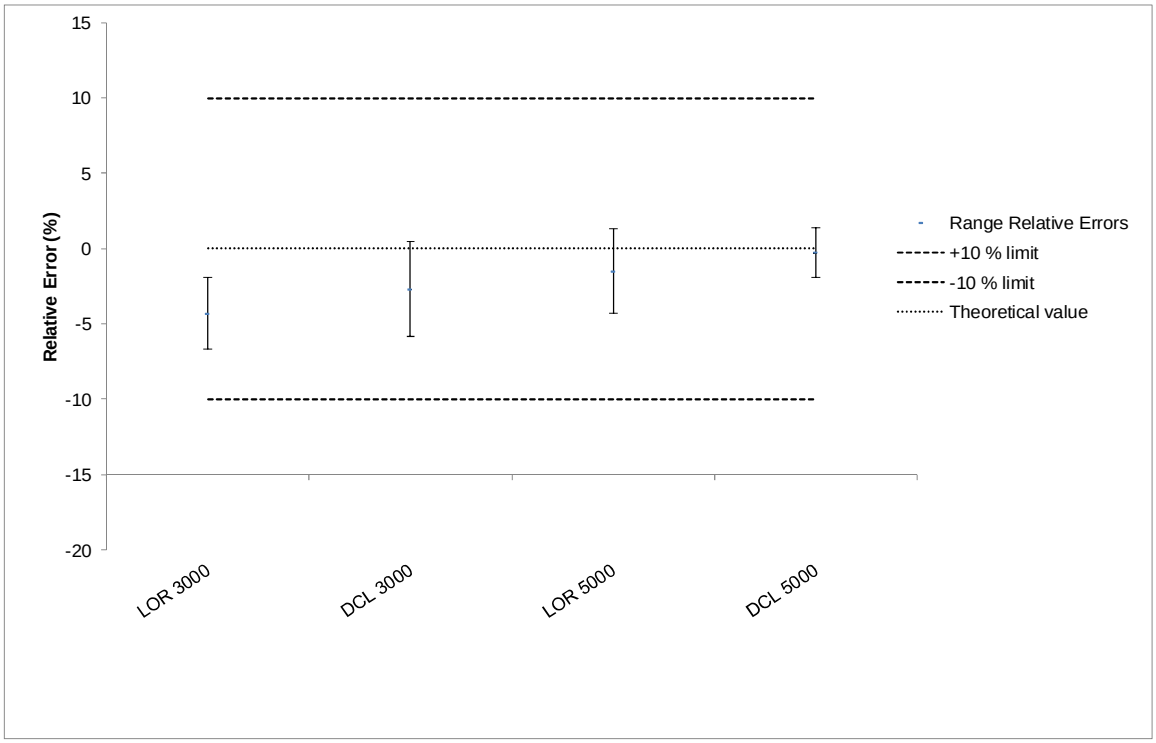
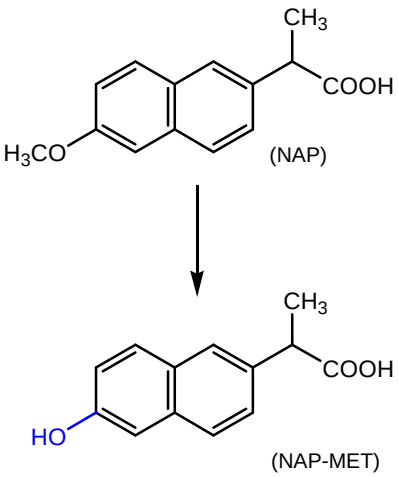


Figure 14: *Inter-day relative error ranges API 3000™ and API 5000™ loratadine and metabolite*

Comparing the relative errors determined on both instruments it can be seen that the values measured on the API 3000™ were mainly below their theoretical value. On the API 5000™ the relative errors enter clearly the positive deviation from the theoretical value. As **Figure 14** shows the intervals of the relative errors were smaller for LOR on the API 3000™ which means a better precision than on the API 5000™. For DCL the precision was better on the API 5000™ compared to the API 3000™ as indicated by the smaller interval of the RE.

3.6. Naproxen

Naproxen (NAP) as shown in **Scheme 10** is a member of the aryl propionic acid group of non-steroidal anti-inflammatory drugs (NSAIDs), which is largely used to treat rheumatoid arthritis and other inflammatory rheumatic diseases [178].



Scheme 10: *Naproxen and metabolite*

The mechanism of action of NAP, like that of other NSAIDs, is associated with the inhibition of cyclooxygenase (COX) activity which was first proposed by Vane in 1971 [179]. Today it is a fact that the COX exists in at least two isoforms, COX-1 and COX-2. COX-1 catalyzes the biosynthesis of prostaglandins that are important for maintaining physiological functions, e.g. prostacyclin which is cytoprotective when released by the gastric mucosa or anti-thrombogenic when released by the vascular endothelium. COX-2 produces prostaglandins which are involved in inflammation, fever and pain. Adverse events as gastrointestinal bleeding and ulceration of the NSAIDs are based on their inhibition of the COX-1 isoenzyme [180]. Therefore selective COX-2 inhibitors have been developed. However, a clinical trial [181] comparing NAP and the selective COX-2 inhibitor rofecoxib showed fewer serious gastrointestinal adverse events for rofecoxib but an increased risk of heart attack and rofecoxib was withdrawn from the market in 2004 [180].

NAP is administered as suppository formulation or orally as suspension or tablet. When administered orally, NAP is completely absorbed; peak plasma concentrations are achieved between one and two hours [182-183]. At therapeutic levels it is greater than 99% albumin-bound [184]. NAP is extensively metabolized by phase I metabolism to 6-O-desmethylnaproxen (NAP-MET). Both compounds are conjugated by phase II metabolism into NAP acyl glucuronide and 6-O-desmethylnaproxen acyl glucuronide [185].

NAP has a chiral centre and the pharmacological activity resides mainly in the (S)-naproxen. While other non-steroidal anti-inflammatory drugs are marketed as racemates, such as ketoprofen and flurbiprofen, NAP is sold only as S-NAP [186].

Methods to quantify NAP in human blood, serum or plasma include gas chromatography mass spectrometry (GC-MS) [187], gas chromatography with flame ionization detection (GC-FID) [188], liquid chromatography with spectrophotometric (LC-UV) [188-193] and spectrofluorometric (LC-FLUO) [189, 194] detection, micellar electrokinetic capillary chromatography (MECC) with simultaneous absorbance and fluorescence detection [195] and chip-based immunoaffinity capillary electrophoresis (IACE) [196]. Many of these approaches have been applied in pharmacokinetic studies [191-192, 197-205].

Although the majority of the latter methods provide acceptable lower limits of quantitation (LLOQs), such as 5 ng/mL (GC-MS) and 100 to 8000 ng/mL (LC-UV, LC-FLUO, IACE), they present a series of limitations like the need for a derivatization step in the case of GC-MS analysis due to the polar nature of NAP [187], time-consuming and for the analysis of large batches of samples not suitable liquid-liquid extraction procedures (LLEx) [187-189, 191-194] or ultrafiltration with following adjustment of the protein concentration [196], and long chromatographic run times (≥10 min) [188-191, 193-195, 206] to achieve good chromatographic resolution and to avoid problems with the differentiation between NAP and potential interferences from endogenous components and NAP metabolites or degradation products.

In order to provide a specific and fast determination LC-MS and LC coupled to tandem mass spectrometry (MS/MS) are particularly useful. Sultan et al. [207] and Miksa et al. [208] described two methods to simultaneously quantify and identify NAP and other NSAIDs in human plasma, employing LC-MS/MS in full-scan MS mode. Both methods were robust and reliable, however their run times were quite long (20 min and 17 min, respectively) and they presented rather high reproducible LLOQ values of 2 μg/mL and 20 μg/mL, respectively. Although LC-MS/MS working in full-scan mode may offer adequate specificity and sensitivity, MS/MS triple quadrupole mass spectrometry used in the multiple reaction monitoring mode (MRM) provides surpassing speed, sensitivity and selectivity in quantitative analysis.

3.6.1. Method development and optimization

Spectra for NAP were recorded on the API 3000™. Having a carboxy function NAP was planned to be analyzed in the negative ionization mode. Compared to ionization with the heated nebulizer ESI showed less sensitivity during initial tests. For that reason the method development and optimization was performed with the heated nebulizer. For optimization of the mass spectrometer parameters a solution containing approximately 10 µg/mL NAP was infused into the API 3000™. All optimized parameters can be found in **Table 57**. The deprotonated molecular ion $[M-H]^-$ was observed in the precursor ion spectrum at m/z 229 which showed maximal intensity at a NC of -3 µA and a temperature of 500 °C. The corresponding OR voltage was -6 V. For optimizing the fragmentation product ion spectra were recorded. Most abundant fragment was m/z 185. The fragment was maximized by setting the collision energy to 10 eV.

Analyte	NC (µA)	Temp. (°C)	OR (V)	CE (eV)	Q1 m/z	Q3 m/z
Naproxen	-3	500	-6	10	229	185
Ketoprofen	-3	500	-16	12	253	209

NC: nebulizer current, Temp.: temperature, OR: orifice voltage, CE: collision energy

Table 57: *API 3000™ system parameters of naproxen and ketoprofen*

For the internal standard ketoprofen (KEP) the NC and TEMP were set to the same values as for NAP. Setting the OR voltage to -16 V the deprotonated molecular ion in the precursor ion spectrum was found at m/z 253. The most abundant fragment in the product ion spectrum was m/z 209 which showed maximal intensity at a CE of 12 eV.

Based on a method published by Miksa et al. [208] NAP should be analyzed on a C18 (Thermo Betasil C18, 50 x 4.6 mm, 3µ, Thermo Scientific, Dreieich, Germany) column. As the drug has a pKa of 4.8 the pH value of the buffer was set to 4.0. At this pH NAP should be sufficient protonated to interact with the C18 reversed phase column. Ammonium acetate and ammonium formate were tested as buffer salts. Compared to ammonium formate the acetate salt showed better signal intensity. Only poor sensitivity was obtained when diluted acetic acid or formic acid were used. Therefore the method was optimized with ammonium acetate buffer. Different buffer concentrations with a pH value of 4.0 were tested.

The best combination of retention and sensitivity was found with a 20 mM ammonium acetate buffer (pH 4.0) and acetonitrile at a ratio of 30:70 and a low rate of 1.0 mL/min. Under these conditions NAP eluted after approximately 0.9 min from the analytical column. Example chromatograms can be found in the Appendix.
KEP and ibuprofen (IBU) were tested to be used as internal standards. IBU showed due its higher lipophilicity stronger interaction with the C18 phase and eluted therefore beyond 2 min runtime. Due to a high number of samples the 2 min runtime should not be exceeded. Therefore KEP was selected as internal standard which eluted under the above described conditions after approximately 0.9 min from the C18 column.
Protein precipitation should be used for sample preparation as the expected NAP concentrations in human plasma were in the µg/mL range. As the method should be used for sample analysis from several clinical trials it was automated using a Multimek™ automated 96-channel pipettor (Beckmann Coulter GmbH, Unterschleißheim, Germany). All pipetting steps were carried out using the automated pipettor. For sample preparation 100 µL human plasma samples were deproteinized by addition of 200 µL acetonitrile containing the internal standard (2 µg/mL KEP). After thorough mixing, the samples were centrifuged for 10 minutes at 3600 rpm (3016 g) at approximately +4 °C. Forty µL of the supernatant were mixed with 360 µL 20 mM ammonium acetate buffer (pH 4.0). After mixing, 10 µL of each sample were injected into the LC-MS/MS system.

3.6.2. Method validation

After injection of prepared human blank plasma samples the matrix components did not interfere with NAP and its internal standard KEP near or at their retention times and over the concentration range (100 - 50000 ng/mL) described herein.

In the Appendix typical chromatograms of blank plasma samples, calibration standards and samples from healthy volunteers are presented.

Peak ratios versus concentrations were fitted by linear regression over the concentration range of NAP. The mean linear regression equation of the calibration curves generated during the validation was:

$y = 0.002\ (\pm 0.001) + 0.073\ (\pm 0.004)\ x$ for NAP, $r^2 \geq 0.998$

where *y* represents the ratio of the NAP peak area to that of the internal standard KEP, and *x* represents the plasma concentration of the analyte. The mean correlation coefficients are equal or better than 0.998 and demonstrate the excellent linearity of the validated method. The validated calibration range covers an order of magnitude of $5 \cdot 10^2$.

Table 58 shows the quality data of the calibration standards that were measured on five consecutive days during the validation of the NAP assay.

	CV [%]	**RE [%]**	**CV [%]**	**RE [%]**
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
NAP	1.6 to 5.7	-2.9 to 5.0	3.5	-1.6
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
NAP	4.4 to 9.4*	-5.6 to 3.1	0.9 to 8.4	-2.5 to 5.2

*: value at LLOQ

Table 58: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for naproxen*

Inter-day CV and RE were <6 % and ≤5 %, respectively. At the LLOQ the inter-day CV was 3.5 % and the inter-day RE was -1.6 %. The mean signal-to-noise values at the LLOQs were ≥5 for all analytes.

The CV and RE of the spiked quality control samples are shown in **Table 58**. Intra-day CV and Intra-day RE were <9 % and <6 %, respectively. Between the days the CV was better than 10 % and the RE was better than 6 %. The data were clearly within the acceptance criteria [48] of no more than 20 % deviation at LLOQ and no more than 15 % deviation for spiked quality control standards above LLOQ. The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits.

The mean absolute extraction recovery of NAP was determined with 90 %. Low values for SD (≤3.6 %) and CV (≤4.0 %) indicate the absence of a concentration dependency of the recovery over the validated concentration range. For KEP the mean absolute extraction recovery was determined with 81 %. The recovery for NAP was sufficient to achieve the required LLOQ of 100 ng/mL. **Table 59** summarizes the recovery results.

Analyte	Mean [%]	SD [%]	CV [%]
NAP	90.0	3.6	4.0
KEP	80.6	1.3	1.6

Table 59: *Mean absolute extraction recoveries for naproxen and ketoprofen*

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for NAP. All statistical evaluations of the stability experiments can be found in the Appendix.

Different influences on the determination of NAP were investigated. Dilution of human plasma samples prior to analysis, hemolyzed human plasma and different batches of human plasma did not influence the determination of NAP represented by CV and RE values that were all <10 %. **Table 60** summarizes the RE and CV of the investigated influences.

Analyte	Dilution	Hemolyzed		Different batches	
		high	low	high	low
RE (%)					
NAP	-5.4	2.3	2.2	-9.2	1.3
CV (%)					
NAP	1.8	1.1	3.8	3.5	6.7

Table 60: *Relative errors (%) and precisions (%) of the investigated influences for naproxen*

3.6.3. Comparison of the developed method with existent methods

In **Table 61** the developed method is compared with previously published methods for the determination of NAP in human plasma.

All previously reported LC-UV and LC-MS/MS methods showed less sensitivity with LLOQs at least 5 times higher than the LLOQ value achieved in the present method.

Method	Linearity range [µg/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Preparation	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experiments*
LC-MS/MS current thesis	0.1 - 50	2.0	0.1	PP	4.4 to 9.4	-5.6 to 3.1	90	≥0.9980	ketoprofen	1-5
Sultan et al. [207] LC-MS/MS	0.5 - 15	20.0	0.5	LLEx	3.98 to 5.94[c]	n.r.	98.16 ± 0.85	0.992	n.r.	n.r.
Miksa et al. [208] LC-MS/MS	20[a]	17.0	0.4	PP	≤ 10	n.r.	> 79	>0.9930	n.r.	n.r.
Larsen et al. [187] GC-MS	0.005[a]	n.r.	1.0	LLEx	< 10[b]	< 10[b]	n.r.	n.r.	d3-naproxen	n.r.
Blagbrough et al. [191] LC-UV	5.0 - 100	10.0	0.5	LLEx	n.r.	n.r.	100	>0.995	diphenyl-acetic acid	n.r.
Marzo et al. [192] LC-UV	1.0 - 100	5.0	0.5	LLEx	≤ 2.3	n.r.	ca. 100	n.r.	n.r.	n.r.
Slattery et al. [193] LC-UV	8.0 - 80	12.0	0.5	LLEx	2.4 to 5.1	-5.3 to 5.0	96.4 ± 4	n.r.	p-chloro-warfarin	1
Van Loenhout et al. [189] LC-UV	1.0 - 100	15.0	0.5	LLEx	0.71 to 2.40	-1.1 to 15.2	95	0.9999	diflunisal	1
Van Loenhout et al. [189] LC-FLUO	0.1 - 10	15.0	0.5	LLEx	1.2 to 3.9	-3.2 to 1.4	95	0.9997	ethyl-naproxen	1
Vree et al. [190] LC-UV	1.5[a]	>20.0	0.1	PP	2.3 to 10.3	n.r.	n.r	n.r.	n.r.	n.r.
Shimek et al. [194] LC-FLUO	2.5 - 70	>30.0	0.1	LLEx	n.r.	n.r.	66.6	0.9913	n.r.	1, 6
Phillips et al. [196] IACE	0.1 - 100	5.0	n.r.	Ultra-filtration	5.81	n.r	n.r.	n.r.	n.r.	n.r.

n.r.: not reported, a: LLOQ, b: for 20 µg/L, c: intra-day precision, PP: protein precipitation, LLEx: liquid-liquid extraction
* 1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock sulution (e)), 3: dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: influence of different individuals, 6: interference of drugs

Table 61: *Naproxen method comparison*

The GC-MS method [187] and both LC-FLUO methods [189, 194] showed good sensitivity with LLOQs of 0.005 µg/mL, 0.1 µg/mL and 0.5 µg/mL, respectively. The IACE (Immunoaffinity Capillary Electrophoresis) method of Phillips et al. [196] also provides good sensitivity with a LLOQ of 0.1 µg/mL. However, compared with the actual LC-MS/MS method the previously reported methods are more time-consuming in terms of sample preparation and run time. The developed method requires only 0.1 mL of sample and has an excellent recovery, which is comparable to the recoveries achieved by LLEx.

Moreover, no additional validation experiments beyond inter-day, intra-day and recovery are provided by most of the shown methods in **Table 61**. The developed method provides full information about quality and recovery data of the developed assay, stability of the analyte pre and post sample work up and under different storing conditions as well as the influence of hemolyzed and lipemic plasma on the determination of NAP. The validated assay is conform to GLP and guidelines from regulatory authorities.

In comparison to the LC-MS/MS methods in full-scan mode reported by Sultan et al. [207] and Miksa et al. [208], the present LC-MS/MS method is more sensitive and faster. The better sensitivity can be ascribed among other factors to the scan mode used (MRM). In general, the selected reaction monitoring mode (SRM) maximizes signal intensity for selected product ions because it increases the duty cycle, the quadrupole remains parked on that one ion - a duty cycle of close to 100%, resulting in a greater number of scans across a chromatographic peak. In the full-scan mode, triple quadrupoles scan by passing one m/z value to the detector at any one time. Generating a scan means changing parameters with respect to time and recording a signal for each m/z value. It is an inherently inefficient process and most ions never reach the detector, i.e. as one m/z is being counted, others are being lost. Therefore better LOQ values and better precision for quantitative experiments are achieved with a triple quadrupole working in the SRM mode in comparison to full-scan mode.

3.6.4. Pharmacokinetic analysis

With the developed and validated method human plasma samples from a clinical trial comparing the bioequivalence of two NAP formulations were analyzed. In one period of the 2-way crossover study a 220 mg NAP formulation was administered to 30 male subjects under fasting conditions.

Samples were collected before the administration and after 0.17, 0.33, 0.50, 0.67, 0.83, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 48.00 and 72.00 hours. Collected blood samples were centrifuged for 10 min at 3000 rpm (2095 g) at +4 ℃ and two aliquots were stored at -70 ℃ until sample analysis.

The blood samples were analyzed in a total of eleven sequences. Each sequence contained at the beginning a calibration row for calculation of the results and within spiked quality control samples to ensure quality during the analysis. Calibration was performed by weighted ($1/concentration^2$) linear regression. The coefficient of correlation of all measured calibration rows was at least 0.998. The inter-day precision and accuracy of the spiked quality control standards of NAP in human plasma analyzed with the batches of study samples ranged from 4.4 to 6.7 % and from 96.1 to 100.9%, respectively.

Figure 15 shows as example the mean plasma concentration profile of NAP after oral administration of a 220 mg reference formulation tablet to 30 healthy volunteers. The mean peak concentration (C_{max}) of 43.70 µg/mL for NAP was attained at 1.09 h after administration of the reference product. The half-time was 18.54 h.

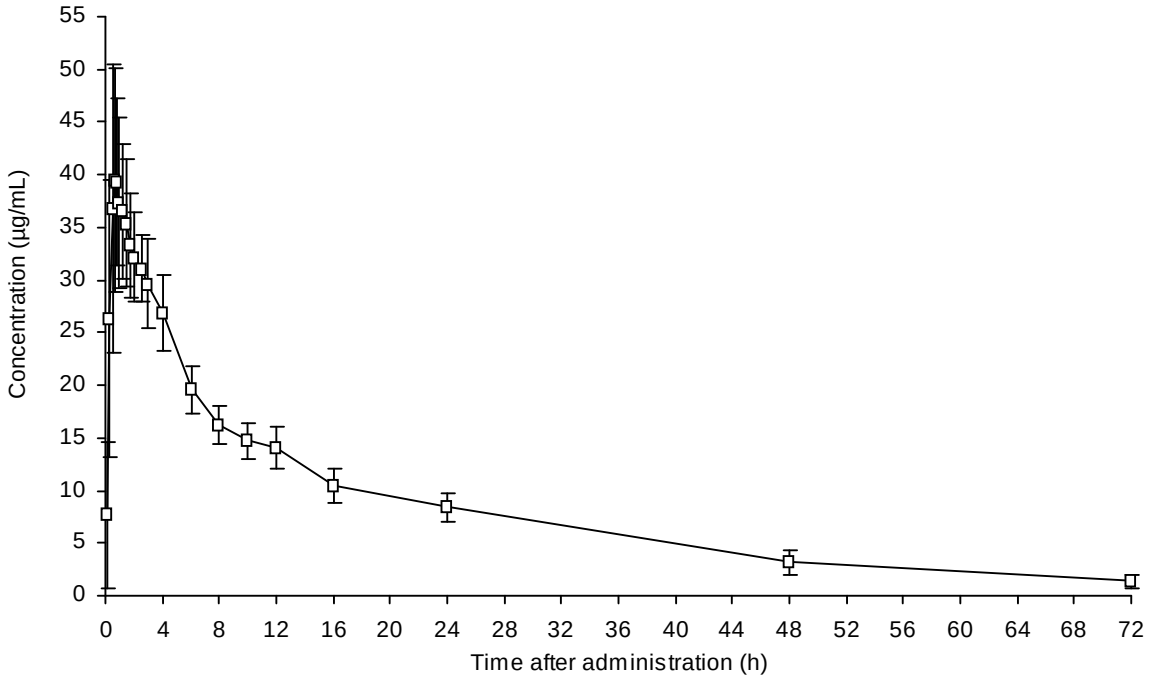


Figure 15: *Mean plasma profile of naproxen concentration vs. time following a 220 mg oral dose of reference naproxen sodium tablet to healthy volunteers (n=30)*

Table 62 shows the pharmacokinetic parameters of NAP after administration of the reference tablet (220 mg) in comparison to pharmacokinetic parameters reported in previously published studies. Dose dependant studies of NAP in healthy volunteers after single and multiple doses observed a non-linear relationship between NAP and plasma concentrations at higher doses [209-210]. Segre et al. [211] reported a linear relationship of NAP dose and plasma concentration within 100-300 mg single dose and a non linear relationship at multiple doses of 375 to 750 mg NAP. However, Niazi et al. [201] observed a non-linear relationship between NAP dose and plasma concentrations at 250 and 500 mg single dose NAP.

The non-linearity in the reported studies is expressed in a less than proportional increase of the AUC and the C_{max} regarding the dose. The plasma protein binding sites are assumed to be saturated at high NAP doses which results in a higher concentration of unbound NAP and leads to a higher excretion rate and clearance [201]. This unbound NAP concentration was shown to be proportional to NAP concentration at 500, 1000 and 1500 mg doses [209]. For this reason the PK parameters are not shown dose corrected in **Table 62**.

The reported mean t_{max} and $t_{1/2}$ values in **Table 62** range from 1.09 - 2.86 h and from 12.27 - 24.7 h, respectively. Administration of the NAP sodium formulation leads to a shorter t_{max} due to the better solubility than the free acid [192] and [this thesis]. The half-life calculated in this thesis is similar to the half-lifes reported by Anttila et al. [200], Ling et al. [203], Strocchi et al. [202] and Zhou et al. [205]. Due to non-linear pharmacokinetics the other pharmacokinetic parameters could not be compared.

Reference	No. of subjects / gender	Last time point [h]	Dose / Formulation	$AUC_{0\rightarrow last}$ [µg*h /mL]	$AUC_{0\rightarrow\infty}$ [µg*h /mL]	C_{max} [µg/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current paper	30/ m,f	72	220 mg/ tablet	585 ± 81	626 ± 102	43.7 ± 7.3	1.1 ±0.8	18.5 ± 3.3
Anttila et al. [200]	8/ 4m, 4f	72	250 mg/ tablet	n.r.	797 ± 191	n.r.	n.r.	17.7 ± 3.0
Caillé et al. [198]	6/ 6m	48	500 mg/ tablet	n.r.	1624 ± 99	95.6 ± 5.8	2.2 ± 0.5	15.9 ± 0.6
Caillé et al. [197]	12/ n.r.	48	500 mg/ tablet	n.r.	1310 ± 79	82.7 ± 3.4	1.4 ± 0.2	15.5 ± 1.0
Charles et al. [206]	14/ 13m, 1f	60	500 mg/ tablet	n.r.	1211	71.4	1.5	n.r.
Ling et al.[203]	6/ 6m	48	750 mg/ tablets	n.r.	1435 (15)[a]	93.2 (7)[a]	1.7 (31)[a]	17.2 (11)[a]
Marzo et al. [192]	12/ 6m,6f	24	502 mg/ tablet	710.3	1024.0	63.8	1.1	14.1
Niazi et al. [201]	28/ n.r.	72	250 mg/ tablet	n.r.	561 ± 30	35.5 ± 1.5	2.9 ± 0.3	12.3 ± 0.6
Niazi et al. [201]	28/ n.r.	72	500 mg/tablet	n.r.	942 ± 42	64.1 ± 2.1	2.3 ± 0.3	13.3 ± 1.3
Ryley et al. [204]	12/ 12m	60	750 mg/ tablets	1393 ± 346	1488 ± 387	97.3 ± 26.5	2.4 ± 1.2	16.1
Ryley et al. [204]	12/ 12m	60	750 mg/ tablets	1391 ± 312	1491 ± 343	98.6 ± 25.2	2.3 ± 1.1	16.5
Strocchi et al. [202]	12/ 12m	48	750 mg/ tablet	n.r.	1547 ± 235	88.9 ± 11.9	1.8 ± 1.0	17.8 ± 2.6
Vree et al. [199]	9/ 4m, 5f	120	500 mg/ tablet	n.r.	1140 ± 171	62.2 ± 11.1	1.5 ± 0.7	24.7 ± 6.4
Zhou et al. [205]	10/ 10m	48	500 mg/ tablets	1207 ± 122	1428 ± 193	87.3 ± 15.5	2.6 ± 1.5	17.7 ± 3.0

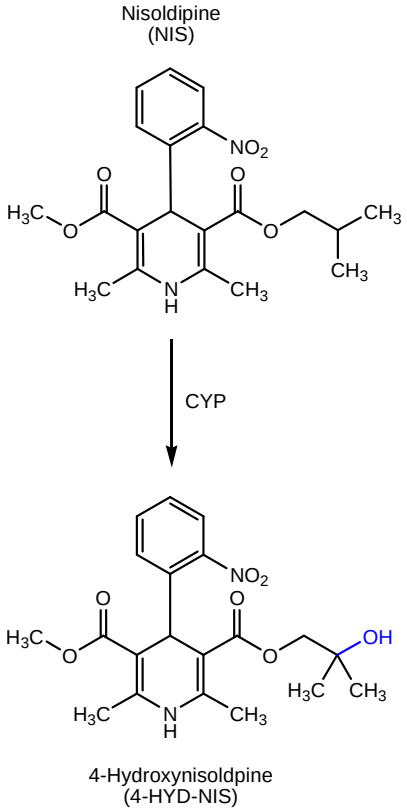
a: mean (% CV), m: male, f: female, n.r.: not reported

Table 62: *Naproxen pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after overnight fasting and administration of a tablet under fasting conditions*

3.7. Nisoldipine and 4-Hydroxynisoldipine

Nisoldipine (NIS) is a second generation 1,4-dihydropyridine calcium channel blocker which is used for treating hypertension and coronary heart disease (angina pectoris). It selectively seems to dilate arterioles with little or no effect on other blood vessels or the heart and reduces vascular resistance and blood pressure by inhibiting the calcium uptake of myocardial and muscle cells [212-213].

After oral absorption NIS is almost completely absorbed but due to its high first pass effect in the gut and liver its bioavailability is about 5 % only. Therefore the concentrations in human plasma are low and analytical methods with high sensitivity are required. Metabolism pathways are dehydrogenation of the dihydropyridine ring to the corresponding pyridine, ester cleavage and hydroxylation of the isobutyl ester (**Scheme 11**). The latter generates the active metabolite 4-HYD-NIS having about 10% of the activity of the parent compound [212-213].



Scheme 11: *Nisoldipine and active metabolite*

NIS has two different ester functions at the dihydropyridine nucleus and is therefore a chiral drug. Some analytical papers determine the NIS enantiomers in a two step analysis [214-216]. In the first step the enantiomers are separated with chiral HPLC. In the second step the enantiomers are analyzed with GC-MS. These methods are extremely time consuming and are not practicable when a high number of samples must be analyzed.
Louagie et al. [217] describes a LC method with UV detection which is only suitable at very high NIS concentrations. GC or LC coupled with MS are the methods of choice to quantify therapeutic drug concentrations in the low pg/mL range [214-216, 218-221]. Most of the reported methods require a time consuming LLEx to achieve high sensitivity. The latest analytical LC-MS/MS method by Kang [219] describes a simple protein precipitation step with a methanol/zinc sulfate solution for the determination of NIS and achieves a LLOQ of 0.5 ng/mL.

3.7.1. Method development and optimization

NIS and 4-HYD-NIS are unstable under UV light and daylight and therefore method development and optimization as well as method validation and sample measurement were performed under yellow fluorescent light.
NIS and 4-HYD-NIS spectra were recorded on the API 5000™ with the ESI ion source in the negative ionization mode. **Table 63** shows the optimized parameters. The best intensities for NIS and 4-HYD-NIS were found by setting the IS to -4.5 kV and the TEMP to 500 °C. The signals of the deprotonated molecular ions were found to optimal at DP -90 V and -50 V for NIS and 4-HYD-NIS, respectively. The corresponding signals in the precursor ion spectrum were found at m/z 387 and m/z 403 for NIS and 4-HYD-NIS, respectively.

Analyte	IS (V)	Temp. (°C)	DP (V)	CE (eV)	Q1 m/z	Q3 m/z
Nisoldipine	-4500	500	-90	18	387	122
4-Hydroxynisoldipine	-4500	500	-50	20	403	208
d4-Nisoldipine	-4500	500	-90	18	391	126
d6-4-Hydroxynisoldipine	-4500	500	-50	36	409	164

IS: ionspray voltage, Temp.: temperature, DP: declustering potential, CE: collision energy

Table 63: *API 5000™ system parameters for nisoldipine, metabolite and internal standards*

For d4-NIS and d6-4-HYD-NIS the same settings were used and their signals in the precursor ion spectrum were found at m/z 391 and m/z 409, respectively. In the product ion spectrum the most abundant ions for NIS and 4-HYD-NIS were m/z 122 and m/z 208, respectively. Their signal intensities were maximal at collision energies of 18 eV and 20 eV for NIS and 4-HYD-NIS, respectively. For d4-NIS the CE was also set to 18 eV which showed the most abundant product ion at m/z 126. However, d6-4-HYD-NIS required a higher collision energy (36 eV) than the undeuterated compound as a smaller fragment with m/z 164 was found to be the optimal production ion. All spectra with proposed fragmentations can be found in the Appendix.
Based on the molecular structure of NIS and 4-HYD-NIS a C18 column (Thermo Betasil C18, 50 x 4.6 mm, 3µ, Thermo Scientific, Dreieich, Germany) and a phenyl column (YMC-Pack Phenyl, 50 x 4.6 mm, 3µ, YMC Europe GmbH, Dinslaken, Germany) were considered during method development. Under the same liquid chromatography conditions the phenyl column produced sharper peaks and a better signal intensity for NIS and 4-HYD-NIS. Therefore further method development was performed with the phenyl column. Comparing ammonium acetate and ammonium formate the acetate salt produced better sensitivity. The sensitivity was optimized by replacing the ammonium acetate buffer by diluted acetic acid. The signal was maximized by using 0.1 % acetic acid and acetonitrile at a ratio of 50:50. Setting the flow rate to 1.0 mL/min NIS and 4-HYD-NIS eluted after approximately 2.4 and 1.0 min, respectively. The corresponding deuterated internal standards d4-NIS and d4-4-HYD-NIS showed identical retention behaviour.

Sample preparation with protein precipitation produced enough sensitivity to achieve a LLOQ of 0.005 ng/mL for NIS and 4-HYD-NIS, respectively. Therefore 100 µL human plasma sample were deproteinized by addition of 0.200 mL of acetonitrile containing the internal standards (3 ng/mL d4-NIS and d6-4-HYD-NIS). After mixing, the samples were centrifuged at 3600 rpm (3016 g) for 5 minutes at approximately +4 °C. Thirty µL of each sample were injected into the LC-MS/MS system.

3.7.2. Method validation

Injection of prepared human blank plasma samples showed no interferences of matrix components with NIS, 4-HYD-NIS and their internal standards at or near their retention times over the concentration range (0.005 ng/mL - 10 ng/mL) described herein.

In the Appendix typical chromatograms of prepared blanks, standards and samples from healthy volunteers are shown.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration ranges of the analytes in human plasma. The mean linear regression equations of the calibration curves generated during the validation were:

$y = 0.0000\ (\pm 0.0001) + 0.2144\ (\pm 0.0167)\ x$ for NIS, $r^2 \geq 0.999$

$y = 0.0001\ (\pm 0.0002) + 0.4224\ (\pm 0.0240)\ x$ for 4-HYD-NIS, $r^2 \geq 0.998$

where *y* represents the ratio of the analyte peak area to that of the internal standard, and *x* represents the plasma concentration of the analyte. The mean correlation coefficients were equal or better than 0.999 and 0.998 for NIS and 4-HYD-NIS, respectively and demonstrate the excellent linearity of the validated method. The validated calibration range covers an order of magnitude of $2 \cdot 10^3$ for each analyte.

In **Table 64** the measured quality data of the calibration rows are shown. Inter-day CV was <7 % and <6 % for NIS and 4-HYD-NIS, respectively. The inter-day RE was <5 % and <6 %, respectively.

	CV [%]	**RE [%]**	**CV [%]**	**RE [%]**
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
NIS	0.3 to 6.5	-3.9 to 4.4	3.6	0.8
4-HYD-NIS	0.7 to 5.6	-5.3 to 4.7	2.3	-0.6
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
NIS	3.1 to 8.3*	-2.5 to 2.7	1.2 to 8.4	-6.5 to 8.0*
4-HYD-NIS	4.0 to 8.5*	-4.7 to 0.0	3.1 to 12.1	-7.8 to -2.1*

*: value at LLOQ

Table 64: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for nisoldipine and metabolite*

For both compounds an excellent precision and relative error was obtained at the lowest calibration point.

Intra-day and inter-day CV and RE of the spiked quality control samples are shown in **Table 64**. Intra-day CV for NIS and 4-HYD-NIS was below 9 % and 13 %, respectively. The RE within the validation days was ≤8 % for NIS and 4-HYD-NIS, respectively. The inter-day CV was better than 9 % for both analytes. The RE between the five consecutive validation days was <3 % and <5 % for NIS and 4-HYD-NIS, respectively. The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits.

All quality data were within the required range (within ±20 % at the LLOQ and within ±15 % above the LLOQ) and were therefore judged to be acceptable. With the simple and easy protein precipitation assay the LLOQ (0.005 ng/mL) could be measured with high precision and low relative error.

The mean absolute extraction recovery of NIS and 4-HYD-NIS was 84 % and 82 %, respectively Up to nearly 20% of the compounds were lost during the sample preparation procedure. However, the recoveries were sufficient to achieve a LLOQ of 0.005 ng/mL for both analytes. **Table 65** shows the individual values and also the determined recoveries of the corresponding internal standards.

Analyte	Mean [%]	SD [%]	CV [%]
NIS	84.2	2.6	3.1
4-HYD-NIS	81.8	3.8	4.7
d4-NIS	77.6	4.5	5.8
d6-4-HYD-NIS	69.3	2.6	3.7

Table 65: *Mean absolute extraction recoveries for nisoldipine, metabolite and internal standards*

The mean absolute extraction recovery of d4-NIS and d6-4-HYD-NIS was 78 % and 69 %, respectively. Compared to their undeuterated compounds the recoveries of the internal standards were 7 % and 13 % lower, respectively.

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for all analytes. All statistical evaluations of the stability experiments can be found in the Appendix.

Dilution, hemolyzed and lipemic human plasma as well as different batches of human plasma did not influence the determination of NIS and 4-HYD-NIS, respectively. **Table 66** shows the measured CV and RE values of the individual experiments. All RE and CV values were better than 8 %.

Analyte	Dilution	Hemolyzed		Lipemic		Different batches	
		high	low	high	low	high	low
RE (%)							
NIS	-1.8	4.1	5.4	4.7	5.9	2.3	-1.9
4-HYD-NIS	-3.6	4.8	-2.6	1.5	7.7	-5.1	-0.6
CV (%)							
NIS	1.1	2.5	4.5	1.3	3.7	3.3	7.8
4-HYD-NIS	1.8	2.3	4.5	1.9	5.8	2.9	5.3

Table 66: *Relative errors (%) and precisions (%) of the investigated influences for nisoldipine and metabolite*

With an acceptable ±15 % range for CV and RE all individual values were very well within the required acceptance criteria.

The excellent reproducibility of the developed method was demonstrated by reanalyzing human plasma samples received from a clinical trial that were measured earlier with the same method. The reanalyzed samples can be seen as biological quality control samples.
In total a number of 40 samples were reanalyzed and compared to the concentration measured during the first analysis. 85 % and 90 % of the reanalyzed samples were within the ±15 % range for NIS and 4-HYD-NIS, respectively. 15 % and 10 % of the reanalyzed samples showed a deviation of more than 15 % for NIS and 4-HYD-NIS, respectively. The individual values are shown in **Table 67**.

Analyte	No. of reanalyzed samples	% of blanks	Percentual samples within				
			≤5 %	5 - 10 %	10 - 15 %	15 - 20 %	>20 %
NIS	40	25.0	25.0	22.5	12.5	7.5	7.5
4-HYD-NIS	40	37.5	25.0	25.0	2.5	5.0	5.0

Table 67: *Incurred samples reanalysis for nisoldipine and metabolite*

The mean absolute difference of the 1st to 2nd analysis was 9.9 % and 7.6 % for NIS and 4-HYD-NIS, respectively. In consideration of the large concentration range of the method and the low LLOQ that can be achieved with simple protein precipitation the values are very good and demonstrate the high reproducibility not only in spiked quality control samples but also in biological human plasma samples.

In this method the influence of the matrix on the NIS and 4-HYD-NIS signal was of special interest as a very low LLOQ could be achieved with protein precipitation. The MF values in **Table 68** show suppression of NIS and d4-NIS of around 19 % and 25 %, respectively.

Analyte	Matrix Factor mean	CV [%]	Matrix Factor IS normalized	CV [%]
NIS	0.813	2.1	1.078	1.9
d4-NIS	0.754	2.6	-	-
4-HYD-NIS	1.201	2.7	0.905	1.3
d6-4-HYD-NIS	1.327	4.0	-	-

Table 68: *Matrix Factors (MF) and IS normalized MF values for nisoldipine and metabolite*

This is exactly vice versa for 4-HYD-NIS and d6-4-HYD-NIS. Signal enhancements of about 20 % and 33 % were observed, respectively.
As the analyte and the corresponding internal standard is suppressed or enhanced approximately to the same extend there is no overall influence of the matrix on the determination of NIS and 4-HYD-NIS, respectively. The IS normalized MF value for NIS and 4-HYD-NIS were 1.078 and 0.902, respectively. The low values for the CV indicate the high reproducibility of the determined MF and IS normalized MF values.

3.7.3. Comparison of the developed method with existent methods

In **Table 69** the developed method is compared with previously published methods for the determination of NIS and 4-HYD-NIS in human [214-215, 217-220] as well as in dog, rat and mouse [216, 221] plasma.

Method	Linearity range [ng/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Preparation	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experiments*
LC-MS/MS current thesis	0.005 - 10.0	3.0	0.2	PP	3.1 to 8.3 (NIS) 4.0 to 8.5 (4-HYD-NIS)	-2.5 to 2.7 (NIS) -4.7 to 0.0 (4-HYD-NIS)	84.2 (NIS) 81.8 (4-HYD-NIS)	>0.999 (NIS) >0.998 (4-HYD-NIS)	d_4-NIS d_6-4-HYD-NIS	1, 2a-e, 3, 4a,b, 5, 7, 10
Zhang et al. [214] LC-MS	0.5 - 20.0	8.0	1.0	LLEx	11.1 to 4.1	-6.0 to -0.2	89.31 to 94.92	>0.9986	nimodipine	1, 2b
van Harten et al. [218] GC-MS	0.100 - 50.0	10.0	1.0	LLEx	<10.0	n.r.	78.5 (NIS) 92.8 (4-HYD-NIS)	>0.999	nitrendipine	8
Marques et al. [215] HPLC-GC/MS	0.05 - 50.0[a]	20.0 (LC) 9.0 (GC)	2.0	LLEx	11.6 to 14.1 (+) NIS 14.1 to 14.7 (-) NIS	-10 to -3.1 (+) NIS -8.8 to -3.1 (-) NIS	55.6 (+) NIS 51.8 (-) NIS	>0.9912 (+) NIS >0.9839 (-) NIS	nitrendipine	1, 6
Louagie et al. [217] LC-UV	20.0 - n.r.	5.0	2.0	LLEx	n.r.	n.r.	102.6 to 104.9	>0.999	nifedipine	1
Zimmer et al. [216] HPLC-GC/MS	0.5 - 100[a]	20.5 (LC) 9.0 (GC)	1.0	LLEx	<15.0	< 15.0	91 - 98	n.r.	$^{13}C_4$-NIS	9
Kang et al. [219] LC-MS/MS	0.5 - 20.0	2.5	0.2	PP	6.3 to 9.8	-14.5 to 4.2	n.r.	>0.9999	felodipine	1
Heinig et al. [220] HPLC-GC/MS	0.1 - 5.0[a]	17.0 (LC) 15.0 (GC)	1.0	LLEx	8.4 to 17.1 (+) NIS 5.6 to 9.7 (-) NIS	-16 to -7 (+) NIS -15 to 1 (-) NIS	n.r.	non linear regression	d_9-NIS	1, 2a, b
Wang et al. [221] LC-MS/MS	0.2 - 20.0	3.0	0.1	PP	3.21 to 8.40	2.0 to 8.6	104.8 - 112.3	≥0.998	clonazepam	1, 2a, c, e

a: for each enantiomer, n.r.: not reported, PP: protein precipitation, LLEx: liquid-liquid extraction
* 1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), room temperature (e)), 3: dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: influence of different individuals, 6: interference of drugs, 7: incurred samples, 8: photo stability, 9: rat, mouse and dog plasma, 10: matrix effect

Table 69: *Method comparison for nisoldipine and metabolite*

The GC-MS method of van Harten et al. [218] that also detects 4-HYD-NIS needs a fivefold higher sample volume than the method in this thesis because of LLEx (1.0 mL), has a runtime of 10 min and twenty fold less sensitivity than the developed method.

Marques at al. [215] and Heinig et al. [220] present methods that determine both NIS enantiomeres. Both methods perform a chiral LC with separation of the enantiomers followed by separate detection via GC-MS detection. However, 4-HYD-NIS is not detected and both methods are very time consuming.

Table 69 shows that all PP methods require less sample volume and use LC-MS/MS. To the authors' knowledge this is the first PP method that also determines the 4-HYD-NIS metabolite with high sensitivity. Wang et al. [221] observed a higher signal and sensitivity for both analytes in the negative mode which was also observed during method development in this thesis. Kang et al. [219] and Wang et al. [221] use both the API 4000™ but the latter one needs less sample volume and achieves a higher sensitivity due to the negative ionization mode. Therefore, it is the most suitable precursor ion when quantification limits in the low pg/mL range for NIS and 4-HYD-NIS must be achieved.

During method validation of the developed method the influence of dilution and hemolyzed human plasma as well as the influence of different batches on the determination of NIS and 4-HYD-NIS was investigated. In none of the papers in **Table 69** any of these experiments were reported. Moreover, incurred samples reanalysis was performed for both analytes and the results demonstrated the excellent performance of the assay. Incurred samples reanalysis is also not reported by any of the other papers.

Due to the use of deuterated internal standards and the overall robustness inter-day CVs and REs of the developed method are equal of mostly better than the CVs or REs of the other methods.

3.7.4. Pharmacokinetic analysis

The validated method was used to analyze NIS and 4-HYD-NIS in human plasma samples from a clinical trial. In one period of the 3-way crossover study a 10 mg NIS reference tablet was administrated to 24 male and female subjects under fasting conditions.

Blood samples were taken before administration and after 0.5, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00, 12.00, 14.00, 16.00, 20.00, 24.00, 36.00, 48.00 and 72.00 hours. Collected blood samples were centrifuged for 10 min at 3000 rpm (2095 g) at +4 °C and aliquoted samples were stored at -70 °C until sample analysis.

All collected blood samples were analyzed in a total of 12 sequences. Concentrations were calculated by the calibration curve that was measured at the beginning of each sequence and quality was ensured by measuring spiked quality control samples within each sequence. Calibration was performed by weighted (1/concentration^2) linear regression for NIS and 4-HYD-NIS. The lower limit of quantification was 0.00521 ng/mL for both analytes.

Nisoldipine

The inter-day precision and the analytical recovery of the spiked quality control standards of NIS in human plasma ranged from 3.9 to 5.0 % and were 104.1 % (1.20 ng/mL), 99.3 % (0.154 ng/mL) and 99.2 % (0.0154 ng/mL), respectively.

Within the set of SQC samples (n = 60) analyzed with the batches of study samples, 179 out of 180 SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

4-Hydroxynisoldipine

The inter-day precision and the analytical recovery of the spiked quality control standards of 4-HYD-NIS in human plasma ranged from 3.8 to 6.2 % and were 102.5 % (1.20 ng/mL), 99.6 % (0.154 ng/mL) and 99.0 % (0.0154 ng/mL), respectively.

Within the set of SQC samples (n = 60) analyzed with the batches of study samples, 178 out of 180 SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

The mean peak concentration (C_{max}) of 0.722 ng/mL for NIS was attained at 6.8 h after administration of the product. The mean peak concentration (C_{max}) of 0.777 ng/mL for 4-HYD-NIS was attained at 5.6 h after administration of the product. **Table 70** shows the pharmacokinetic parameters for NIS and 4-HYD-NIS after oral administration of a 10 mg tablet in comparison to pharmacokinetic parameters reported in previously published studies.

Reference	No. of subjects / gender	Last time point [h]	Dose	Analyte	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current thesis	24 / m, f	72	10 mg /	NIS	11.4 ± 4.76	12.1 ± 5.41	0.72 ± 0.38	6.8 ± 2.6	15.6 ± 5.3
				4-HYD-NIS	11.4 ± 6.35	11.8 ± 6.67	0.78 ± 0.39	5.6 ± 2.4	12.0± 5.1
Baksi et al. [222]	young group 9 / m (mean age 24 ± 3)	24	10 mg	NIS	7.0 ± 3.1	n.r.	1.8 ± 0.8	1.7 ± 1.3	4.3 ± 5.0
	young group 12 / m, f (mean age 69 ± 3)	24	10 mg	NIS	15.0 ± 9.3	n.r.	5.0 ± 3.2	1.6 ± 1.1	4.3 ± 3.6
van Harten et al. [223]	young 10 / m, f (mean age 22 ± 1)	32	10 mg	NIS	n.r.	6.9 ± 3.4	1.9 ± 0.8	1.7 ± 1.8	8.9 ± 2.5
				4-HYD-NIS	n.r.	10.2 ± 3.2	n.r.	n.r.	n.r.
	middle-aged 8 / m, f (mean age 53 ± 3)	32	10 mg	NIS	n.r.	9.8 ± 6.1	2.7 ± 2.3	1.3 ± 0.5	9.3 ± 4.1
				4-HYD-NIS	n.r.	15.1 ± 8.1	n.r.	n.r.	n.r.
	elderly 6 / m, f (mean age 78 ± 3)	32	10 mg	NIS	n.r.	8.9 ± 5.3	3.2 ± 2.2 NIS	1.1 ± 0.8 NIS	8.0 ± 5.9 NIS
				4-HYD-NIS	n.r.	13.3 ± 4.4	n.r.	n.r.	n.r.
Ohtani et al. [224]	8 / m	24	10 mg	NIS	9.2 ± 3.8	n.r.	3.2 ± 1.8	1.2 ± 0.8	n.r.
				4-HYD-NIS	9.8 ± 3.6	n.r.	2.7 ± 1.1	1.5 ± 1.0	n.r.

a: mean (% CV), m: male, f: female, n.r.: not reported

Table 70: *Reported values for nisoldipine and 4-hydroxynisoldipine pharmacokinetic parameters (mean ± standard deviation) in healthy subjects after overnight fasting and administration of a single oral dose*

The calculated AUCs for NIS and 4-HYD-NIS in this thesis fit best with the AUC values reported by van Harten et al. [235] for middle aged and young healthy volunteers, respectively. The observed peak concentrations for NIS and 4-HYD-NIS were clearly lower than reported in the literature listed in **Table 70**. The reason for that is the extended release formulation that was administered in the present clinical trial.

Therefore, also the t_{max} occurs clearly later for both compounds than in the other studies, where peak concentrations for NIS are reported within 1.8 - 5.0 ng/mL and as 2.7 ng/mL and 5.0 ng/mL for 4-HYD-NIS, respectively. An obviously higher $t_{1/2}$ value was calculated in the present study. Values for $t_{1/2}$ are not reported for 4-HYD-NIS in the papers listed in **Table 70**.

Regarding the age effect for NIS and 4-HYD-NIS pharmacokinetic parameters, two different studies are presented. Baksi et al. [222] observed an effect of age and found double AUC and more than double C_{max} when comparing a young group with an elderly one. On the other hand van Harten et al. [223] observed no effect of age on NIS and 4-HYD-NIS pharmacokinetic parameters.

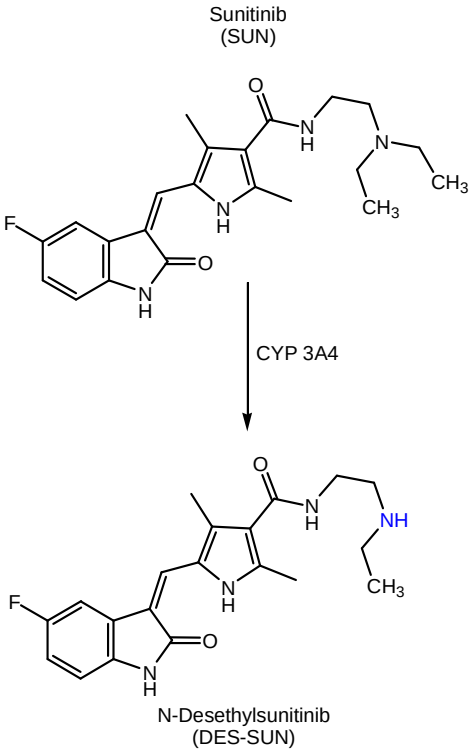
3.8. Sunitinib and N-Desethylsunitinib

Sunitinib (SUN) is a small-molecule inhibitor of multiple tyrosine kinases with antitumor and antiangiogenetic effect, which is approved for the treatment of gastrointestinal stromal tumor (GIST) after disease progression or intolerance of imatinib therapy and for advanced renal cell carcinoma (RCC). The activity of SUN is based on the inhibition of vascular endothelial growth factor receptors 1-3 (VEGFR1 - VEGFR3), platelet-derived growth factor receptors (PDGFRα and PDGFRβ), steam cell factor receptor (KIT), fsm related tyrosine kinase-3 (FLT3), and colony-stimulating factor-1 receptor (CSF-1R). Targeting the protein kinases has a greater specificity and fewer side effects than the traditional cytotoxic therapy (“magic bullet” concept of Paul Ehrlich) [225].

After oral administration SUN is completely absorbed from the gastrointestinal tract and maximum peak concentrations in plasma are reached within 6 to 12 h after dosing. The AUC and the C_{max} increase proportionally with increasing dose within the dose range of 25 to 100 mg and are not affected by food.

The drug is metabolized by CYP3A4 to DES-SUN (**Scheme 12**) which is equipotent to the parent compound. Terminal half-lifes of SUN and DES-SUN are 40 to 60 h and 80 to 110 h, respectively [226-229].

Analytical methods for the determination of SUN and DES-SUN in human plasma and monkey tissue include LC-UV and LC-MS/MS. The LC-UV methods published by Blanchet et al. [230] and Etienne-Grimaldi et al. [231] have a runtime of at least 10 min and use a liquid-liquid extraction for sample preparation. Therefore at least 0.5 mL of human plasma is required. Moreover, Blanchet et al. [230] determines only SUN and not the active metabolite. The highest sensitivity with LC-UV detection is reported by Etienne-Grimaldi et al. [231] with a LLOQ of 5.0 and 2.5 ng/mL for SUN and DES-SUN, respectively.



Scheme 12: *Sunitinib and active metabolite*

The LC-MS/MS methods of Haouala et al. [232] and Minkin et al. [233] show lower LLOQs and require considerably less sample volume. However, both methods do not determine DES-SUN. In addition the method of Haouala et al. [232] has a unusual long run time for LC-MS/MS methods of 14 min. Bello et al. [229] describes a method for determination of SUN and DES-SUN whereas sample preparation is performed by liquid-liquid extraction.

Hereby a LLOQ of 0.1 ng/mL for both analytes is achieved. Also de Bruijn et al. [234] detect SUN and DES-SUN with LLEx and achieve a LLOQ of 0.2 ng/mL for both analytes. Zhou et al. [235] and Honeywell at al. [236] report LC-MS/MS methods for the determination of SUN when less sample material is available.
Because just a few LC-MS/MS methods have been reported for the determination of SUN and its active metabolite, the method of Baratte et al. [237] determining both analytes in monkey tissue should be noted.

3.8.1. Method development and optimization

SUN and DES-SUN spectra were recorded on the API 5000™. Therefore a solution containing ca. 10 ng/mL SUN and DES-SUN was prepared. In the negative mode no deprotonated molecular ions with m/z 397 and m/z 369 were observed for SUN and DES-SUN, respectively. In the positive mode the protonated molecular ions $[M+H]^+$ at m/z 399 and 371 were observed in the precursor ion spectrum. The signals showed the best abundance at a IS voltage of 2 kV and 350 °C. Higher IS voltages decreased the signal for both compounds, probably due to in source fragmentation. Temperatures higher than 350 °C did not enhance the signals for SUN and DES-SUN, respectively. The optimal DP voltages were 70 V and 40 V for SUN and DES-SUN, respectively. In the product ion spectra the ion with the highest abundance was m/z 283 for both analytes. The collision energies were optimized and finally set to 33 eV and 26 eV for SUN and DES-SUN, respectively. All parameters are summarized in **Table 71**.

Analyte	IS (V)	Temp. (°C)	DP (V)	CE (eV)	Q1 m/z	Q3 m/z
Sunitinib	+2000	350	+70	33	399	283
N-Desethylsunitinib	+2000	350	+40	26	371	283
d5-Sunitinib	+2000	350	+70	33	404	283

IS: ionspray voltage, Temp.: temperature, DP: declustering potential, CE: collision energy

Table 71: *API 5000™ system parameters for sunitinib, metabolite and internal standard*

For d5-SUN the same parameters as for SUN were selected. Therefore in the Q1 and the Q3 m/z 404 and m/z 283 were monitored, respectively.

Reversed phase C18 and C8 columns were used for liquid chromatography by de Bruijn et al. [234] and Haouala et al. [232], respectively. Based on the molecular structures of SUN and DES-SUN, a phenyl column (YMC-Pack Phenyl, 50 x 4.6 mm, 3µ, YMC Europe GmbH, Dinslaken, Germany) should be tested at first in this thesis. As reported by de Bruijn et al. [234] and Haouala et al. [232] a mobile phase containing diluted formic acid and acetonitrile was used and the phenyl column showed good retention of the analytes. However, at any ratio of 0.1 % formic acid and acetonitrile for each analyte a second peak was observed in the chromatogram. According to de Bruijn et al. [234] and Haouala et al. [232] SUN and DES-SUN are susceptible to E/Z-isomerism. That's why two peaks were observed on each MRM transition. Several approaches including the replacement of formic acid by acetic acid, the use of buffer instead of diluted acid, the replacement of acetonitrile by methanol and the change of the pH value were performed to shift the equilibrium to one side. During the experiments it was observed that increasing the pH value of the mobile phase caused a change in the peak ratio of booth isomers. As the phenyl column allowed a maximum pH value of 7.5 an alternative column allowing alkaline or strong alkaline conditions should be used. A similar stationary phase to the phenyl column was found to be the PLRP-S column (Agilent PLRP-S, 50 × 4.6 mm I.D., 5 µm, Agilent Technologies, Waldbronn, Germany). This column is based on polymeric media and provides full chemical stability of the stationary phase across the whole pH range. Using a mobile phase of 50 mM ammonium formate buffer (pH 11, adjusted with 25 % liquid NH_3) and acetonitrile in the ratio of 57:43 and a flow rate of 1.0 mL/min resulted in one peak per MRM transition on the PRLP-S column. Under these conditions SUN and DES-SUN eluted after 3.5 min and 1.4 min, respectively. The deuterated internal standard d5-SUN eluted after approximately 3.3 min from the column.

For sample preparation daylight was excluded and artificial neon light was used. Working under yellow fluorescent light was not necessary to prevent E/Z-isomerism. Protein precipitation showed sufficient sensitivity to achieve a LLOQ of 0.06 ng/mL. For sample preparation 100 µL of human plasma sample were deproteinized by addition of 200 µL of acetonitrile containing the internal standard (1 ng/mL d5-SUN). After thorough mixing, the samples were centrifuged at 3600 rpm (3016 g) for 5 minutes at approximately +4 °C. Following centrifugation, 20 µL of each sample supernatant were injected onto the LC-MS/MS system.

3.8.2. Method validation

After injection of prepared human blank plasma samples the matrix components did not interfere with SUN, DES-SUN and the internal d5-SUN at or near their retention times over the concentration range (0.06 - 100 ng/mL) described herein. In the Appendix typical chromatograms of blank samples, standards and samples from healthy human volunteers are shown.

The peak area ratios versus concentrations were fitted with linear regression over the concentration range of the analytes in human plasma. The mean linear regression equations of the calibration curves generated during the validation were:

y = -0.0039 (±0.00146) + 0.3910 (±0.1125) x for SUN, r^2>0.999

y = -0.0017 (±0.0012) + 0.2948 (±0.0564) x for DES-SUN, r^2>0.999

where y represents the ratio of the analyte peak area to that of the internal standard, and x represents the plasma concentration of the analyte. Correlation coefficients were better than 0.999 for both compounds and represent the excellent linearity of the assay. The validated concentration range covers an order of magnitude of $1.7 \cdot 10^3$.

Table 72 summarizes the inter-day CV and RE ranges across the calibration range as well as the precision and accuracy of the individual LLOQs. The inter-day CV was below 6 % and 5 % for SUN and DES-SUN, respectively. At the lowest calibration points the inter-day CVs were with 2 % for SUN and 1 % for DES-SUN even better. The RE of the calibration rows were below 3 % for both analytes and did not exceed 1 % at the LLOQ.

	CV [%]	**RE [%]**	**CV [%]**	**RE [%]**
Calibration Rows	**Inter-day**		**Inter-day at LLOQ**	
SUN	0.9 to 5.8	-2.5 to 2.1	2.0	1.0
DES-SUN	0.5 to 4.9	-2.4 to 1.9	1.1	0.7
Spiked Quality Control Samples	**Inter-day**		**Intra-day**	
SUN	1.6 to 6.1*	0.2 to 9.1*	0.4 to 6.4*	0.0 to 7.9*
DES-SUN	1.1 to 5.3*	-0.1 to 6.2*	1.1 to 2.4	-0.7 to 8.8*

*: value at LLOQ

Table 72: *Inter-day and Intra-day precision and relative error of the calibration rows and spiked quality control samples for sunitinib and metabolite*

The quality data of the spiked quality control samples are shown in **Table 72**. The intra-day CV was below 7 % and 3 % for SUN and DES-SUN, respectively. The RE within the days did not exceed 8 % and 9 % for SUN and DES-SUN respectively. The (*) values indicate the percental value at the LLOQ. If there is no (*), the percental value at the LLOQ is better than the presented limits.
Between the days the CV was better than 7 % and 6 % for SUN and DES-SUN, respectively. The inter-day RE was better than 10 % and 7 % for SUN and DES-SUN, respectively. All determined quality data were below 10 % and represent the excellent performance and reproducibility of the developed method.

The precipitation assay showed very good recoveries for SUN and DES-SUN, respectively. The mean absolute extraction recovery of SUN and DES-SUN was 86 % and 85 %, respectively. The recoveries were sufficient to achieve a LLOQ of 0.06 ng/mL for both analytes. The mean absolute extraction recovery of d5-SUN was 85 %, which was almost identical to the recovery of the undeuterated compound.

Analyte	Mean [%]	SD [%]	CV [%]
SUN	86.2	6.4	7.4
DES-SUN	84.8	5.0	5.9
d5-SUN	85.3	0.8	0.9

Table 73: *Mean absolute extraction recoveries for sunitinib, metabolite and internal standard*

Table 73 lists the determined recoveries and shows also the measured SDs and CVs. All SDs and CVs were <10 % and therefore a concentration dependency of the recovery could be excluded.

The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes for all analytes. All statistical evaluations of the stability experiments can be found in the Appendix.

None of the investigated influences did affect the determination of SUN and DES-SUN, respectively. **Table 74** shows the individual experiments and the corresponding REs and CVs.

Analyte	Dilution	Hemolyzed		Lipemic		Different batches	
		high	low	high	low	high	low
RE (%)							
SUN	-2.0	-0.5	1.3	-6.7	-6.4	-6.3	-5.8
DES-SUN	2.9	7.3	11.3	1.2	4.8	2.4	4.4
CV (%)							
SUN	1.1	2.1	9.3	1.6	5.4	4.7	5.1
DES-SUN	1.6	3.7	6.8	5.4	8.0	6.0	3.9

Table 74: *Relative errors (%) and precisions (%) of the investigated influences for sunitinib and metabolite*

The REs of all experiments were below 8 % except for “Hemolyzed low” where a RE of 11 % was obtained. However, this value is well below the acceptance criteria of the allowed 15 % range. The CVs (**Table 74**) of the investigated influences were below 10%.

All determined values were within the allowed acceptance criteria (±15 %) and therefore any influence of dilution, hemolyzed and lipemic human plasma as well as different batches of human plasma on the determination of SUN and DES-SUN could be excluded.
During the incurred samples reanalysis experiment 42 samples that were previously analyzed with the developed method were measured again. For DES-SUN around 95 % of the samples were reanalyzed with a percentual deviation of less than 5 % compared to the first analysis. The remaining 5 % did not exceed a deviation of 10 % with respect to the first analysis. For SUN around 98 % of the reanalyzed samples showed less than 10 % deviation to their first analysis. 2 % of the reanalyzed samples had a deviation of more than 10 % but did not exceed a deviation of 15 % compared to the first analysis. 81 % and 91 % of the reanalyzed samples showed equal or less deviation of 5 % compared to the first analysis for SUN and DES-SUN, respectively. The individual data are shown in **Table 75**.

Analyte	No. of reanalyzed samples	% of blanks	Percentual samples within ≤5 %	5 - 10 %	10 - 15 %	15 - 20 %	>20 %
SUN	42	4.8	81.0	11.9	2.4	none	none
DES-SUN	42	4.8	90.5	4.8	none	none	none

Table 75: *Incurred samples reanalysis for sunitinib and metabolite*

The mean absolute difference was 2.8 % and 2.3 % for SUN and DES-SUN, respectively. The biological quality control samples demonstrate the excellent performance of the developed method.

The influence of the matrix on the ionization of SUN and DES-SUN was investigated by comparing the behaviour of the analytes in prepared human blank plasma and mobile phase during ionization. The mean MF values of SUN and DES-SUN were 1.012 and 0.992, respectively. Both values indicate the absence of any matrix effects during the ionization process in the API 5000™ source. The MF value of d5-SUN was close to SUN and had a value of 1.009.

Analyte	Matrix Factor mean	CV [%]	Matrix Factor IS normalized	CV [%]
SUN	1.012	0.3	1.003	0.2
d5-SUN	1.009	0.3	-	-
DES-SUN	0.992	0.3	0.982	0.5

Table 76: *Matrix Factors (MF) and IS normalized MF values for sunitinib and metabolite*

Table 76 shows also the IS normalized MF values both analytes, which were 1.003 and 0.982 for SUN and DES-SUN, respectively. For SUN the IS normalization improved the MF value as the internal standard showed almost identical behavior during the ionization process. For DES-SUN the IS normalized MF value is slightly lower than the MF value. This is due to the fact that d5-SUN and DES-SUN show slightly different behavior during the ionization process. The determined values from **Table 76** indicate the absence of any influence of matrix in the ionization of SUN and DES-SUN, respectively. All calculated CVs were <1 % and indicate the high reproducibility of the experiment.

3.8.3. Comparison of the developed method with existent methods

In **Table 77** the developed method is compared with previously published methods for the determination of SUN and DES-SUN in human plasma and monkey tissue.

All methods with MS- or MS/MS-detection apply electrospray ionization in the positive mode and have short run times. The method of Haouala et al. [232] is especially prepared for therapeutic drug monitoring and is able to quantify six tyrosine kinase inhibitors within 14 min. SUN elutes in this method after ca. 8 min. Hence this method becomes very time consuming in clinical trials where the interest is on one special tyrosine kinase inhibitor and lots of samples need to be analysed. A faster analytical method with a runtime of about 4 min for the determination of SUN ond three other tyrosine kinase inhibitors using PP is reported by Honeywell et al. [236]. In comparison to the analytical method of Bello et al. [229] the developed method provides a factor 1.7 more sensitive LLOQ and uses simple PP for sample preparation instead of LLEx. Honeywell at al. [236] and Zhou et al. [235] report LC-MS/MS methods that require less sample material. Using LLEx de Bruijn et al. [234] can detect SUN and DES-SUN with a LLOQ of 0.2 ng/mL.

Compared to the LC-MS and LC-MS/MS methods the LC-UV methods of Blanchet et al. [230] and Etienne-Grimaldi et al. [231] show less sensitivity and selectivity as well as longer run times. A sample volume of al least 0.5 mL is required and in both methods a LLEx has to be performed. Additionally no deuterated internal-standards can be used when UV detection is applied.

For therapeutic drug monitoring where multiple receptor tyrosin kinases are inhibited in concentrations of 50-100 ng/mL [227] the LC-UV methods provide enough sensitivity and are an alternative, especially when there is no LC-MS/MS system available.
However, LC-MS/MS is the analytical method of choice when sample volume is low e.g. when several measurements from one sample have to be performed or when SUN and DES-SUN have to be detected in complex matrices like tissues. Also the method is essential during high sample loads and when there has to be detailed knowledge of the elimination profile of SUN and DES-SUN at very distant times after drug administration.

Also the analytical methods of Britten et al. [238] and Fiedler et al. [239] which are not listed in the table should be noted. Both report LC-MS/MS measurements of SUN and DES-SUN with a LLOQ of ca. 0.1 ng/mL but without any further information about sample preparation procedures or quality data.

Method	Linearity range [ng/mL]	Approx. run time [min]	Sample Volume [mL]	Sample Prepa-ration	Inter-day CV [%]	Inter-day relative error [%]	Recovery [%]	Correlation coefficient	Internal Standard	Additional Validation Experiments*
LC-MS/MS current method	0.06 - 100	4.0	0.1	PP	1.6 to 6.1 (SUN) 1.1 to 5.3 (DES-SUN)	0.2 to 9.1 (SUN) -0.1 to 6.2 (DES-SUN)	86.2 ± 6.4 (SUN) 84.8 ± 5.0 (DES-SUN) 85.3 ± 0.8 (IS)	>0.999 (SUN) >0.999 (DES-SUN)	d5-SUN	1, 2a-d, 3, 4a, b, 5, 6, 7
de Bruijn et al. [234] LC-MS/MS	0.200 - 50.0	4.0	0.1	LLEx	1.14 - 6.52 (SUN) 1.15 - 5.95 (DES-SUN)	-9.5 - 0.7 (SUN) -6.0 - 106.8 (DES-SUN)	101 ± 7.9 (SUN) 102 ± 7.9 (DES-SUN) 105 ± 6.7 (IS)	≥ 0.9980	d_{10}-SUN	1, 2a, c, d, f, 6, 9, 10
Zhou et al. [235] LC-MS/MS	1.37 - 1000	3.2	0.01	PP	1.7 - 4.9 (SUN)	4.2 - 12.7 (SUN)	93.1 ± 96.1 (SUN) 93 (IS)	≥ 0.99	camptothecin	1, 2d
Honeywell et al [236]. LC-MS/MS	1 - 4000	< 4.0	0.02	PP	4.3 - 6.0 (SUN)	0.2 - 11.3 (SUN)	74.3 ± 78.7 (SUN)	> 0.99 (SUN)	n.r.	1, 2b
Bello et al. [229] LC-MS	0.1 - n.r.	-	-	LLEx	2.4 to 6.5 (SUN) 3.7 to 11.1 (DES-SUN)	-1.3 to 1.3 (SUN) -1.7 to 2.3 (DES-SUN)	-	-	d10-SUN	n.r.
Baratte et al. [237] LC-MS/MS	2 - 2000 ng/g	5.0	50 mg	LLEx	2.4 to 9.6 (SUN) 4.5 to 7.6 (DES-SUN)	-1.3 to 2.2 (SUN) -9.4 to 2.1 (DES-SUN)	-	>0.990 (SUN) >0.988 (DES-SUN)	d10-SUN	1, 2a, c, e, 3
Haouala et al. [232] LC-MS/MS	1 - 500	14.0	0.1	PP	1.3 - 6.1 (SUN)	-5.4 to -0.3 (SUN)	91.3 to 96.8	>0.99 (SUN)	d10-SUN	1, 2a, b, d, 6, 8
Minkin et al. [233] LC-MS/MS	0.2 - 500	3.0	0.2	LLEx	2.2 to 10.1 (SUN)	-3.3 to 5.8 (SUN)	39.2 to 46.1 (SUN) 63.0 (IS)	>0.995	clozapine	1, 2a-d, e
Blanchet et al. [230] LC-UV	20 - 200	10.0	1.0	LLEx	< 7	0.2 to 5.9	50.0 to 70.5 18.2 (IS)	>0.995	ranitidine	1, 2a-e, 9
Etienne-Grimaldi et al. [231] LC-UV	5.0 (SUN) 2.5 (DES-SUN) - 250	14.0	0.5	LLEx	6.4 to 14.5 (SUN) 8.3 to 10.0 (DES-SUN)	n.r.	59.0 to 63.2 (SUN) 80.4 to 92.8 (DES-SUN)	>0.993 (SUN) >0.998 (DES-SUN)	vandetanib	1, 2d, f

n.r.: not reported, PP: protein precipitation, LLEx: liquid-liquid extraction
*1: specificity, 2: stability (short-term (a), long-term (b), post-preparative (c), freeze-thaw (d), stock solution (e), light (f)), 3: influence of dilution, 4: influence of hemolyzed (a), lipemic (b) plasma, 5: influence of different individuals, 6: matrix effect, 7: incurred samples, 8: influence of anticoagulants, 9: influence of drugs in patient matrix, 10: interference of drugs in human EDTA plasma

Table 77: *Method comparison for sunitinib and metabolite*

3.8.4. Pharmacokinetic analysis

The LC-MS/MS procedure developed and validated here was used to generate the pharmacokinetic parameters of sunitinib and its active metabolite after administration of 50 mg sunitinib (Sutent™) once daily over 3 days (volunteers 1-4) and 5 days (volunteers 5-12) and has recently been reported [240].

Blood samples were collected before administration and after 1, 2, 4, 6, 8, 10, 12, 24, 25, 36, 48, 49, 60, 72, 96, 120, 240, 336 and 384 hours after the first dose for volunteers 1-4 and additionally after 0.5, 24.5, 48.5, 72.5, 73, 96.5, 97, 98, 100, 102, 104, 106, 108, 144, 168 and 432 hours after the first dose for volunteers 5-12.

Samples of subjects for measurement of SUN and DES-SUN were analyzed in a total of five sequences. Concentrations were calculated by the calibration curve that was measured at the beginning of each sequence and quality was ensured by measuring spiked quality control samples within each sequence. Calibration was performed by weighted ($1/concentration^2$) linear regression for both compounds. Calibration standards (low to high concentrations) and quality control standards were measured within 9 hours. The determined concentration of spiked quality control standards was compared to the theoretical concentration for accuracy.

The standard curves were linear between 0.0572-102.0 ng/mL and between 0.0618 - 111.0 ng/mL for SUN and DES-SUN, respectively. The coefficient of correlation of resulting linear regressions was at least 0.9996 for both analytes.

Sunitinib

The inter-day precision and the analytical recovery of the spiked quality control standards of SUN in human plasma ranged from 1.0 to 5.3 % and were 100.1 % (100.8 ng/mL), 100.9 % (74.59 ng/mL), 101.2 % (9.619 ng/mL) and 105.6 % (0.1484 ng/mL), respectively.

Within the set of SQC samples (n = 15) analyzed with the batches of study samples, 59 out of 60 SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

N-Desethylsunitinib

The inter-day precision and the analytical recovery of the spiked quality control standards of DES-SUN in human plasma ranged from 1.0 to 5.3 % and were 97.8 % (109.7 ng/mL), 102.4 % (103.9 ng/mL), 101.2 % (103.4 ng/mL) 105.6 % (0.1615 ng/mL) and, respectively.
Within the set of SQC samples (n = 15) analyzed with the batches of study samples, 59 out of 60 SQC samples were within ±15 % of their respective nominal value. Therefore, the accuracy and precision of the analysis of the study samples were judged acceptable.

Figure 16 shows as example the plasma concentration profile of SUN and DES-SUN after oral administration of 50 mg SUN once daily for three **(A)** and five **(B)** consecutive days to two healthy volunteers.

On the first day the mean peak concentration for SUN was 30.0 ng/mL and was attained at 7.0 h after administration of the product. Mean peak concentration for DES-SUN was 6.6 ng/mL and was attained at 6.0 h after administration of the product. C_{max} and t_{max} are comparable to values reported by Faivre et al. [241] and Khosravan et al. [242].

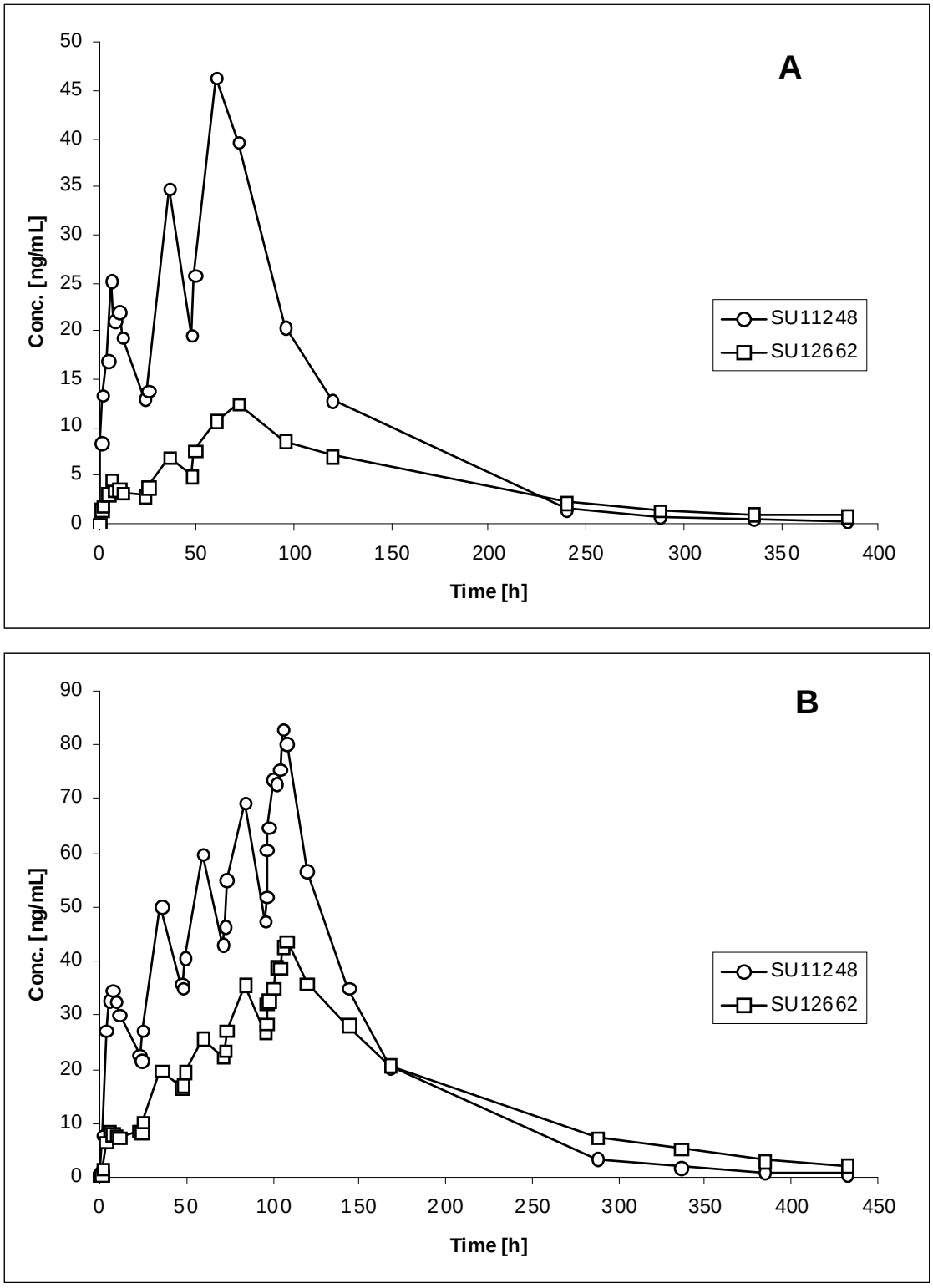


Figure 16: *Plasma profile of sunitinib and N-desethylsunitinib concentration vs. time following a 50 mg oral dose of sunitinib once daily for three (A) and five (B) consecutive days of healthy volunteers.*

4. Summary

In this thesis eight robust and reliable LC-MS/MS methods were developed and validated to analyze atorvastatin, clopidogrel, furosemide, itraconazole, loratadine, naproxen, nisoldipine and sunitinib in human plasma. The active metabolites 2-hydroxyatorvastatin, 4-hydroxyatorvastatin, hydroxyitraconazole, descarboethoxy-loratadine, 4-hydroxynisoldipine and N-desethylsunitinib were also included in the corresponding methods. Due to the different physical, chemical and pharmacokinetic properties of the analytes a wide spectrum regarding sample preparation techniques, chromatography and mass spectrometric detection was covered. Protein precipitation methods were developed for furosemide, itraconazole, naproxen, nisoldipine and sunitinib. Liquid-liquid extraction methods were developed for atorvastatin, clopidogrel and loratadine. Criteria to choose protein precipitation or liquid-liquid extraction were the final plasma concentrations of the drugs, which are mainly dependant on the dose, bioavailability and $t_{1/2}$ and of course cost-effectiveness.

Altogether, the methods have a concentration range from 0.001 ng/mL (LLOQ of clopidogrel) to 50000 ng/mL (highest calibration point for naproxen), covering 5 x 10^7 orders of magnitude. The runtime of the methods ranged from 2 to 4 minutes, facilitating a high sample throughput.

All developed methods were validated according to recent guidelines as they were used to analyze sampes from clinical trials. Excellent linearity, intra-day and inter-day precision and accuracy were observed in the validated calibration ranges. Hemolyzed, lipemic and different batches of human plasma as well as sample dilution did not affect the determiantion of the analytes.

Clopidogrel, loratadine, nisoldipine and sunitinib and if available their metabolites were subjected to a matrix effect test, resulting in no influence of different batches of human plasma on the analytical methods. Noteworthy is clopidogrel that shows a slight effect on one of the two used mass spectrometers. However, that effect was reproducible and did therefore not affect clopidogrel determination.

No evidence of instability during chromatography, extraction and sample storage processes for all analytes except 4-hydroxyatorvastatin was found, for which a significant decrease was observed after three months. During incurred sample reanalysis of study samples 95 % of the samples were within ±15 % with respect to the first analysis.

Moreover, the atorvastatin, loratadine and clopidogrel method were compared on two generations of triple quadrupole mass spectrometers, the API 3000™ and the API 5000™. The new ion source and the changes in the ion path of the API 5000™ provided higher sensitivity, the extend depending on the substance. However, the API 3000™ had very good precision in the performed system comparison.

The validated methods showed excellent performance and quality data during routine sample analysis of eight clinical trials. Moreover, they are suitable for high sample throughput due to their short run times.

Table 78 summarizes PK parameters of all analytes that were observed and calculated in this thesis. The analytes are sorted by peak concentration C_{max}. Other parameters shown are LLOQ and the sample preparation technique.

Analyte	Dose (mg)	Last time point [h]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	LLOQ [ng/mL]	Sample Prep.
NAP	200	72	43700±7300	1.1±0.8	18.5±3.3	5850±810	6260±1020	100	PP
FUR	40	12	1200±494	1.6±0.9	3.2±1.1	2370±574	2450±569	5	PP
HYD-ITR	-	96	122±58	5.0±1.4	10.9±11.7	1607±1120	1742±1201	5	PP
ITR	100	96	67±44	4.2±1.6	16.3±7.8	661±480	775±525	3	PP
SUN	50	*	30.0±6.7	7.0±1.4	-	-	-	0.06	PP
2-HYD-ATO	-	48	26.0±14.7	1.3±0.8	12.4±3.3	147.4±39. 7	161.0±42.9	0.01	LLEX
ATO	40	48	22.4±13.4	1.0±0.8	11.8±3.5	80.5±27.7	82.9± 30.4	0.01	LLEX
DES-SUN	-	*	6.6±2.8	6.0±0.0	-	-	-	0.06	PP
LOR	10	72	3.7±3.9	1.2±0.5	20.8±14.8	11.6±12.6	12.5±13.8	0.01	LLEX
DCL	-	72	2.3±0.8	1.5±0.5	20.6±3.1	30.2±11.4	32.8±13.0	0.01	LLEX
CLP	75	24	0.91±0.81	1.1±0.7	2.4±2.3	1.72±1.42	1.82±1.46	0.001	LLEX
NIS	10	72	0.722±0.375	6.8±2.6	15.6±5.3	11.4±4.76	12.1±5.41	0.005	PP
4-HYD-NIS	-	72	0.777±0.392	5.6±2.4	12.0±5.1	11.4±6.35	11.8±6.67	0.005	PP

PP: Protein Precipitation, LLEx: Liquid-Liquid Extraction, *: explorative study, -: not available

Table 78: *Summary PK parameters*

The point of interest lies in the direct correlation between the expected peak concentrations and the corresponding LLOQs. With exception for NIS and 4-HYD-NIS the LLOQs of the developed methods decrease with observed C_{max} concentrations. This correlation is also observed for AUC and the LLOQ of the developed methods.

Calculated and observed PK parameters of the investigated drugs were mostly in good agreement with previously published data in the literature. Reasons for deviation could be found in different study conditions, dose accuracy and drug formulation as well as subjects with different ethnic background.

5. Zusammenfassung

In dieser Dissertation wurden acht robuste und verlässliche LC-MS/MS-Methoden zur Analyse von Atorvastatin, Clopidogrel, Furosemid, Itraconazol, Loratadin, Naproxen Nisoldipin und Sunitinib in Humanplasma entwickelt und validiert. Außerdem enthalten die Methoden die aktiven Metaboliten 2-Hydroxyatorvastatin, 4-Hydroxyatorvastatin, Hydroxyitraconazol, Descarboethoxyloratadin, 4-Hydroxynisoldipin und N-Desethylsunitinib. Wegen der unterschiedlichen physikalischen, chemischen und pharmakokinetischen Eigenschaften der Analyten, deckt diese Arbeit ein weites Spektrum bezüglich Probenaufarbeitung, Chromatographie und Massenspektrometrie ab. Präzipitationsmethoden wurden für Furosemid, Itraconazol, Naproxen, Nisoldipin und Sunitinib entwickelt. Flüssig-flüssig-Extraktionen wurden für Atorvastatin, Clopidogrel und Loratadin entwickelt. Kriterien für die Auswahl von Präzipitation oder Extraktion waren die erwartete Plasmakonzentration, die im Wesentlichen von der Dosis, Bioverfügbarkeit und Halbwertszeit abhängig ist, und natürlich Kosteneffektivität.

Insgesamt erstrecken sich die Methoden über einen Kalibrierbereich von 0.001 ng/mL (LLOQ von Clopidogrel) bis zu 50000 ng/mL (HLOQ von Naproxen), das entspricht $5x10^7$ Größenordnungen. Die Laufzeiten pro Probe liegen im Bereich von zwei bis vier Minuten, was einen sehr hohen Probendurchsatz ermöglicht.
Alle in dieser Arbeit entwickelten Methoden wurden gemäß aktueller Richtlinien (FDA, GLP) validiert und verwendet um Proben aus Pharmakokinetikstudien zu analysieren. Ausgezeichnete Linearität, Präzision und Genauigkeit zeichnen diese Methoden aus. Hämolysiertes, lipämisches und verschiedene Batches von Humanplasma, sowie Vorverdünnung hatten bei keiner Methode Einfluss auf die Bestimmung der Analyten.

Clopidogrel, Loratadin, Nisoldipin und Sunitinib und gegebenenfalls deren Metabolite wurden einem Matrix-Effekt-Test unterzogen. Dabei wurde festgestellt, dass keine der Methoden durch die Probenmatrix beeinflusst wurde. Erwähnenswert ist Clopidogrel, da an einem der Massenspektrometer ein leichter Effekt beobachtet werden konnte, der sich auf alle untersuchten Matrices gleich auswirkte und somit keinen Einfluss auf die gesamte Methode hatte.
Weiterhin fand sich bei keiner der untersuchten Substanzen ein Hinweis auf Instabilität während der Probenlagerung, -aufarbeitung und -messung, außer bei 4-Hydroxyatorvastatin, dessen Konzentration nach drei Monaten signifikant abnahm.

Während der Reanalyse von Studienproben (incurred samples) lagen über 95 % der Proben innerhalb von ±15 % im Vergleich zur ersten Messung.

Außerdem wurden die Methoden zur Bestimmung von Atorvastatin, Loratadin und Clopidogrel an zwei Generationen von Massenspektrometern verglichen, nämlich dem API 3000™ und dem API 5000™. Die neue Ionenquelle und die Verbesserungen im Ionenpfad beim API 5000™ ermöglichten - abhängig von der analysierten Substanz - höhere Sensitivität. Allerdings konnte das API 3000™ bei den durchgeführten Experimenten mit einer hohen Präzision aufwarten.

Die validierten Methoden zeigten im Alltagbetrieb bei der Messung von acht klinischen Studien hervorragende Performance und Qualitätsdaten. Darüber hinaus sind die Methoden aufgrund ihrer kurzen Laufzeiten ideal für Messungen die einen hohen Probendurchsatz erfordern.

Tabelle 79 fasst die PK-Parameter die während der Messung der klinischen Studien erhalten und kalkuliert wurden zusammen. Die Analyten sind nach absteigender C_{max} sortiert. Weiterhin sind LLOQ und die Methode zur Probenaufarbeitung gezeigt.

Analyte	Dose (mg)	Last time point [h]	C_{max} [ng/mL]	t_{max} [h]	$t_{1/2}$ [h]	$AUC_{0\rightarrow last}$ [ng*h /mL]	$AUC_{0\rightarrow\infty}$ [ng*h /mL]	LLOQ [ng/mL]	Sample Prep.
NAP	200	72	43700±7300	1.1±0.8	18.5±3.3	5850±810	6260±1020	100	PP
FUR	40	12	1200±494	1.6±0.9	3.2±1.1	2370±574	2450±569	5	PP
HYD-ITR	-	96	122±58	5.0±1.4	10.9±11.7	1607±1120	1742±1201	5	PP
ITR	100	96	67±44	4.2±1.6	16.3±7.8	661±480	775±525	3	PP
SUN	50	*	30.0±6.7	7.0±1.4	-	-	-	0.06	PP
2-HYD-ATO	-	48	26.0±14.7	1.3±0.8	12.4±3.3	147.4±39. 7	161.0±42.9	0.01	LLEX
ATO	40	48	22.4±13.4	1.0±0.8	11.8±3.5	80.5±27.7	82.9± 30.4	0.01	LLEX
DES-SUN	-	*	6.6±2.8	6.0±0.0	-	-	-	0.06	PP
LOR	10	72	3.7±3.9	1.2±0.5	20.8±14.8	11.6±12.6	12.5±13.8	0.01	LLEX
DCL	-	72	2.3±0.8	1.5±0.5	20.6±3.1	30.2±11.4	32.8±13.0	0.01	LLEX
CLP	75	24	0.91±0.81	1.1±0.7	2.4±2.3	1.72±1.42	1.82±1.46	0.001	LLEX
NIS	10	72	0.722±0.375	6.8±2.6	15.6±5.3	11.4±4.76	12.1±5.41	0.005	PP
4-HYD-NIS	-	72	0.777±0.392	5.6±2.4	12.0±5.1	11.4±6.35	11.8±6.67	0.005	PP

PP: Protein Precipitation, LLEx: Liquid-Liquid Extraction, *: explorative study, -: not available

Table 79: *Zusammenfassung PK-Parameter*

Interessant ist hierbei die Korrelation zwischen der maximalen Plasmakonzentration und dem entsprechenden LLOQ. Mit Ausnahme von NIS und 4-HYD-NIS nehmen die LLOQ mit sinkender C_{max} ab. Eine entsprechende Korrelation findet sich auch für die AUC und den verschiedenen LLOQ der entwickelten Methoden.

Die gemessenen und kalkulierten PK Parameter stimmten meist gut mit den in der Literatur berichteten Daten überein. Eventuelle Abweichungen von den Literaturdaten können auf unterschiedliche Studienbedingungen, Genauigkeit bei der Dosierung, die Formulierung der verabreichten Tabletten und auf Probanden unterschiedlicher Herkunft zurückzuführen sein.

6. List of Abbreviations

AC	Alternating Current
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photoionization
AUC	Area Under the Curve
AUC_{0-t}	AUC from time of administration up to the time of the last quantifiable concentration
$AUC_{0-\infty}$	AUC from time of administration up to time infinity
ATO	Atorvastatin
2-HYD-ATO	2-Hydroxyatorvastatin
4-HYD-ATO	4-Hydroxyatorvastatin
d5-ATO	d5-Atorvastatin
CAD	Collisionally Activated Dissociation (MS)
CAS	Cathodic Adsorptive Stripping (voltammetry)
CEM	Channel Electron Multiplier (MS)
CID	Collision-Induced Dissociation (MS)
CLA	Clarithromycin
Clast	Last quantifiable plasma concentration
CLP	Clopidogrel
CLP-MET	Clopidogrel Carboxylic Acid
Cmax	Maximal observed Plasma Concentration
COX	Cyclooxygenase
CRM	Charged Residue Model
CV	Coefficient of Variation
CR	Calibration Row
DC	Direct Current
DCL	Descarboethoxyloratadine
d4-DCL	d4-Descarboethoxyloratadine
DIL	Diltiazem
EMA	European Medicines Agency
ESI	Electro Spray Ionization
Et_2O	Diethyl ether
FDA	US Food and Drug Administration
FUR	Furosemide

ELISA	Enzyme-linked Immunosorbent Assay
FT	Freeze-thaw stability
GC	Gas Chromatography
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
HLOQ	Highest Level of Quantification
HPLC	High Performance Liquid Chromatography
HYD-ITR	Hydroxyitraconazole
ICH	International Conference on Harmonization
IACE	Immunoaffinity Capillary Electrophoresis
IS	Internal Standard
ITR	Itraconazole
K_{el}	Elimination Rate Constant
KEP	Ketoprofen
L	Level (Calibration Stabdard)
ln	Natural Logarithm
log	Logarithm
LC	Liquid Chromatography
LC-FLUO	Liquid Chromatography with Spectrofluorometric Detection
LC-MS	Liquid Chromatography coupled to Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LC-UV	Liquid Chromatography with Spectrophotometric Detection
LLEx	Liquid-Liquid Extraction
LLOQ	Lower Limit of Quantification
LOR	Loratadine
LT	Long-term stability
d4-LOR	d4-Loratadine
MALDI	Matrix Assisted Laser Desorption Ionization
MeCN	Acetonitrile
MF	Matrix Factor
MRM	Multiple Reaction Monitoring
MS	mass spectrometry
m/z	Mass to Charge Ratio
NAP	Naproxen
NAP-MET	6-O-desmethylnaproxen

NPD	Nitrogen Phosphorus Detection
NIS	Nisoldipine
d4-NIS	d4-Nisoldipine
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
4-HYD-NIS	4-Hydroxynisoldipine
d6-4-HYD-NIS	d6-4-Hydroxynisoldipine
PD	Pharmacodynamics
PK	Pharmacokinetics
PRO	Probenecid
RE	Relative Error
RIA	Radioimmunoassay
RF	Radio Frequency
RT	Room Temperature
SL	Stock Solution
SPE	Solid Phase Extraction
ST	Short-term stability
SUN	Sunitinib
d5-SUN	d5-Sunitinib
DES-SUN	N-Desethylsunitinib
PP	Protein Precipitation
SD	Standard Deviation
SQC	Spiked Quality Control
$T_{1/2}$	Half-life
Tmax	Time to peak Concentration
VAL	Validation

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8. Curriculum Vitae

Name	Michael Rodamer
Date of birth	January 02, 1981
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Martial status	Married

Education

Oct 2006 - 2011	Ph.D. student at the Institute for Biomedical and Pharmaceutical Research (IBMP) in Nürnberg-Heroldsberg, Germany, supervisor: Professor Dr Fritz Sörgel, and the Department of Pharmaceutical Chemistry, University of Würzburg, Germany, supervisor: Professor Dr Ulrike Holzgrabe
Oct 2006	Diploma (MSc) in Chemistry
Nov 2001 - Oct 2006	Studies of Chemistry at the Friedrich-Alexander-University, Erlangen-Nürnberg, Germany
Jun 2001 - Oct 2006	Scientific co-worker at the Institute for Biomedical and Pharmaceutical Research (IBMP) in Nürnberg-Heroldsberg, Germany, supervisor: Professor Dr Fritz Sörgel
Sep 1991 - May 2000	Secondary school, Gymnasium Scheinfeld, Bavaria, Germany
Sep 1987 - Jul 1991	Primary school Geiselwind, Bavaria, Germany

9. Full Papers and Congress Presentations

Full Papers

1. Elsinghorst PW, Kinzig M, Rodamer M, Holzgrabe U, Sörgel F: An LC-MS/MS procedure for the quantification of naproxen in human plasma: Development, validation, comparison with other methods, and application to a pharmacokinetic study, J Chromatogr B Analyt Technol Biomed Life Sci, (2011).

2. Rodamer M, Elsinghorst PW, Kinzig M, Gutschow M, Sörgel F: Development and validation of a liquid chromatography/tandem mass spectrometry procedure for the quantification of sunitinib (SU11248) and its active metabolite, N-desethyl sunitinib (SU12662), in human plasma: Application to an explorative study, J Chromatogr B Analyt Technol Biomed Life Sci, 879 (2011) 695-706.

3. Lindauer A, Di Gion P, Kanefendt F, Tomalik-Scharte D, Kinzig M, Rodamer M, Dodos F, Sörgel F, Fuhr U, Jaehde U: Pharmacokinetic/Pharmacodynamic Modeling of Biomarker Response to Sunitinib in Healthy Volunteers, Clin Pharmacol Ther, 87 (2010) 601-608.

4. Landersdorfer CB, Kirkpatrick CM, Kinzig M, Bulitta JB, Holzgrabe U, Jaehde U, Reiter A, Naber KG, Rodamer M, Sörgel F: Competitive inhibition of renal tubular secretion of ciprofloxacin and metabolite by probenecid, Br J Clin Pharmacol, 69 (2010) 167-178.

5. Abduljalil K, Kinzig M, Bulitta J, Horkovics-Kovats S, Sörgel F, Rodamer M, Fuhr U: Modeling the autoinhibition of clarithromycin metabolism during repeated oral administration, Antimicrob Agents Chemother. 2009 Jul;53(7):2892-901.

6. Jetter A, Kinzig M, Rodamer M, Tomalik-Scharte D, Sörgel F, Fuhr U: Phenotyping of N-acetyltransferase type 2 and xanthine oxidase with caffeine: when should urine samples be collected?, Eur J Clin Pharmacol. 2009 Apr;65(4):411-7.

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Congress Presentations

1. Mees ST, Jakob AV, Rodamer M, Kinzig M, Haier J, Haupt W, Schellerer V, Müller C, Sörgel F: Tigecycline by LC-MS/MS in Seven Human Intestinal Tissues and Comparison with Rat and Porcine Tissue, European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, May 16 - 19, 2009.

2. Rodamer M, Sörgel F, Jakob AV, Kinzig M, Basel B, Krüger W, Peiffer M, Lodise T, Bulitta J: Epithelial Lining Fluid Concentrations of Antibiotics – Methodoligical Artifacts ?, European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, May 16 - 19, 2009.

3. Rodamer M, Jakob AV, Mees ST, Kinzig M, Hüttner S, Sörgel F: Development and Validation of a Liquid Chromatography-Tandem Mass Spectrometry Procedure for the Quantification of Tigecycline in Human Serum: Application to Drug Monitoring, European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, May 16 - 19, 2009.

4. Rodamer M, Strauss R, Kinzig M, Jakob AV, Ganslmayer M, Gleich C, Sörgel F: New Horizons to Modern Therapeutic Drug Monitoring – Use of Tandem Mass Spectrometry to Analyze the Forty most important Anti-Infectives, European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, May 16 - 19, 2009.

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6. Kinzig M, Sörgel F, Rodamer M, Jakob AV: Incurred Samples – Practical Experience from Four Drugs and Two Metabolites, 2ndWorld Conference on Magic Bullets, Nürnberg, Deutschland, October 03 - 05, 2008.
7. Bulitta JB, Kinzig M, Landersdorfer CB, Naber KG , Rodamer M, Jakob AV, Wagenlehner FM, Drusano GL, Holzgrabe U, Sörgel F: Pharmacokinetic (PK) / Pharmacodynamic (PD) Comparison of Ciprofloxacin (CIP) and Levofloxacin (LEV) by a Population PK Meta-Analysis and Monte Carlo Simulation (MCS), 2ndWorld Conference on Magic Bullets, Nürnberg, Deutschland, October 03 - 05, 2008.
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9. Kinzig M, Sörgel F, Hüttner S, Rodamer M: Validation of an ELISA for Recombinant Human Erythropoietin According to FDA Requirements, 2ndWorld Conference on Magic Bullets, Nürnberg, Deutschland, October 03 - 05, 2008.
10. Rodamer M, Kinzig M, Jakob AV, Hüttner S, Sörgel F: Determination of Loratadine and Desloratadine in human plasma by LC-MS/MS, 2ndWorld Conference on Magic Bullets, Nürnberg, Deutschland, October 03 - 05, 2008.
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12. Rodamer M, Kinzig-Schippers M, Clark T, Bauer W, Sörgel F: ESI/APCI-MS Library of Antibiotics for use in Drug Discovery, Bioanalytics / Pharmacokinetics and Pharmaceutical Analysis, Doktorandentagung der Deutschen Pharmazeutischen Gesellschaft (DPhG), Nürnberg-Heroldsberg, Deutschland, September 6 - 8, 2006.
13. Rodamer M, Jakob AV., Kinzig-Schippers M, Clörs CD, Ritter W, Sörgel F: Determination of the neurotoxin palytoxin in anemones with LC-MS/MS, Jahrestagung der Deutschen Pharmazeutischen Gesellschaft (DPhG); Mainz, Deutschland, Oktober 5 - 8, 2005.
14. Kinzig-Schippers M, Landersdorfer C, Jakob V, Rodamer M, Skott A, Holzgrabe U, Sörgel F: Sensitive Analysis of Roxithromycin in Human Plasma by LC-MS/MS, World Conference on Magic Bullets, Nürnberg, Germany, September 9 - 11, 2004.
15. Rodamer M, Fuhr U, Tomalik-Scharte D, Jetter A, Bulitta J, Kinzig-Schippers M, Sörgel F: Personalized Isoniazid (INH) Dosing Based on Genotyping for Arylamine N-Acetyltransferase Type 2 (NAT2), World Conference on Magic Bullets, Nürnberg, Germany, September 9 - 11, 2004.
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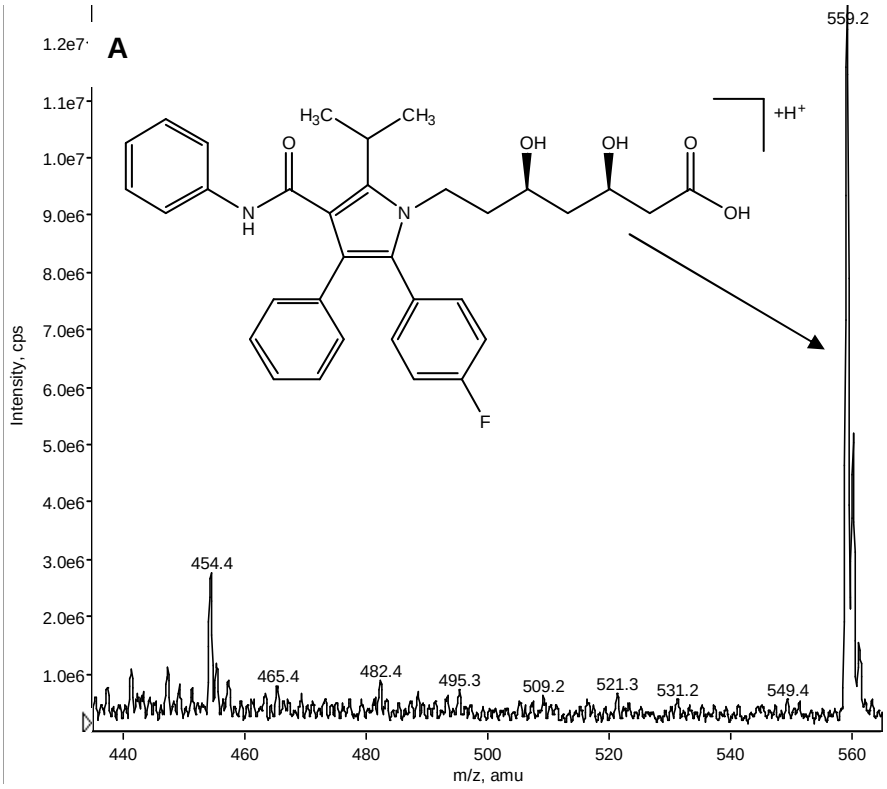
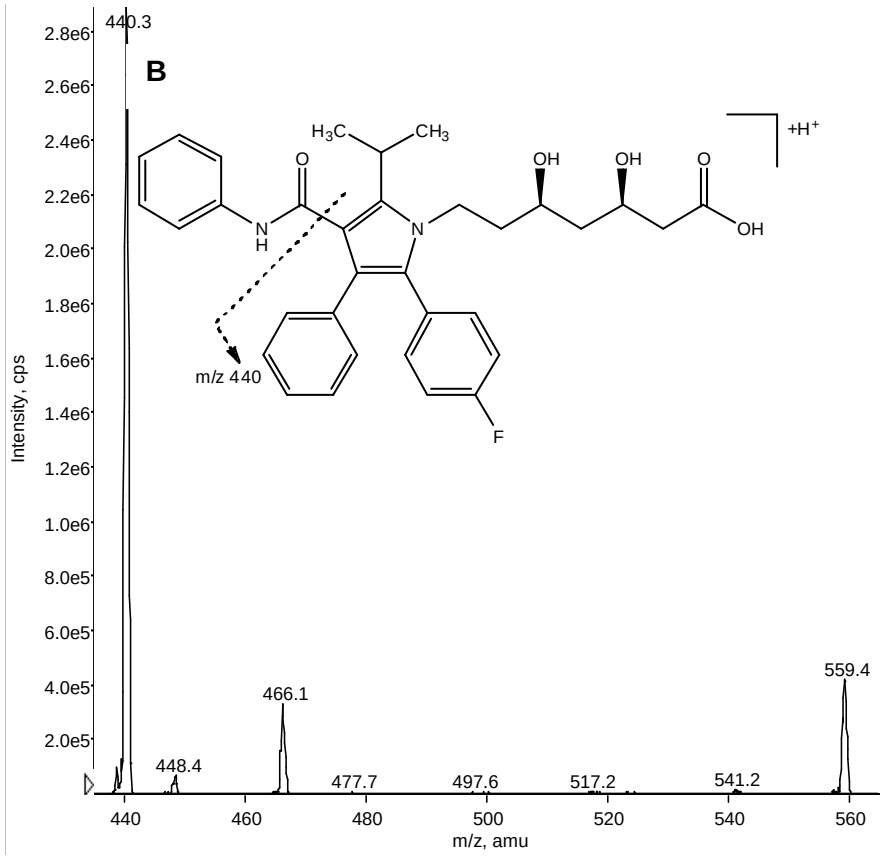
20. Bulitta J, Horkovics-Kovats S, Borek M, Skott A, Illauer M, Rodamer M, Kinzig-Schippers M, Sörgel F: Self-Inhibition of Clarithromycin's (CLA) Metabolism in Humans at Steady-State, 43rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Illinois/USA; September 14 - 17, 2003.
21. Bulitta J, Kinzig-Schippers M, Naber CK, Naber KG, Sauber C, Kleinschnitz M, Wahode H, Rodamer M, Sörgel F: Limitations in the use of drug cocktails (DC) to compare the pharmacokinetics (PK) of drugs: ciproflox bacin (CIP) vs. levofloxacin (LEV), AAPS Annual Meeting and Exposition, Denver, Colorado/USA; October 21 - 25, 2001.
22. Bulitta J, Kinzig-Schippers M, Naber CK, Naber KG, Sauber C, Kleinschnitz M, Wahode H, Rodamer M, Sörgel F: Limitations in the use of drug cocktails (DC) to compare the pharmacokinetics (PK) of drugs: ciprofloxacin (CIP) vs. levofloxacin (LEV), 40th Interscience Conference on Antimicrobial Agents and Chemotherapy; Toronto/Canada, September 17 - 20, 2000.

10. Appendix

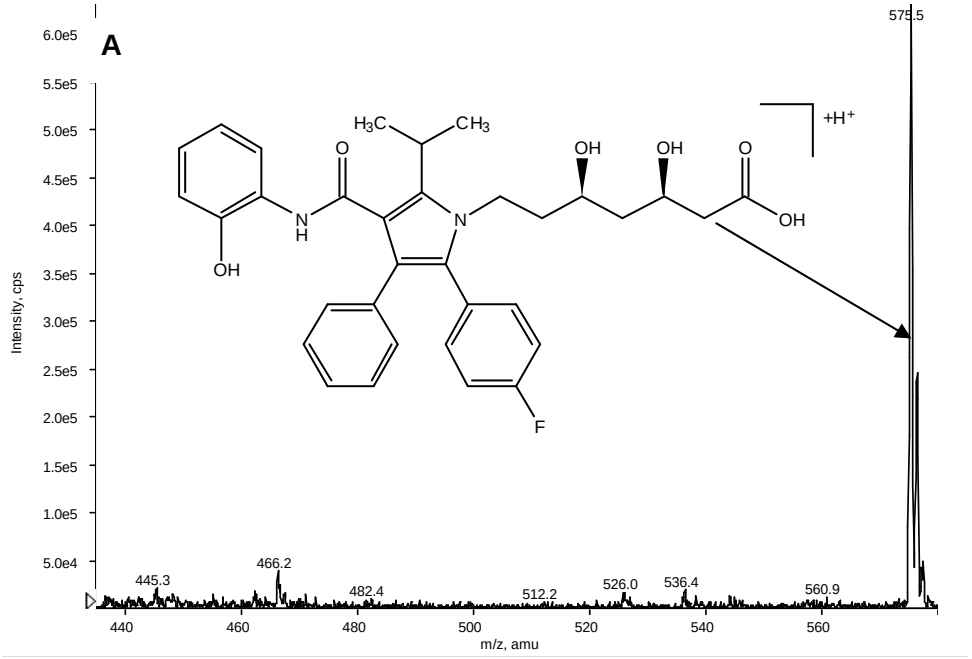
10.1. Mass spectra

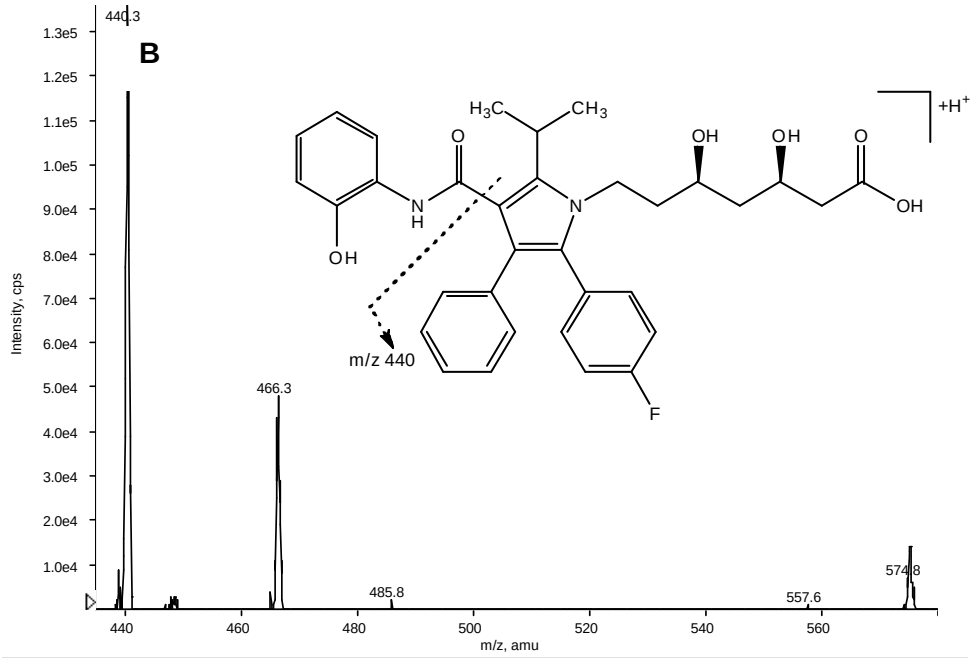
Atorvastatin: Full-scan (A) and product ion mass spectra (B)



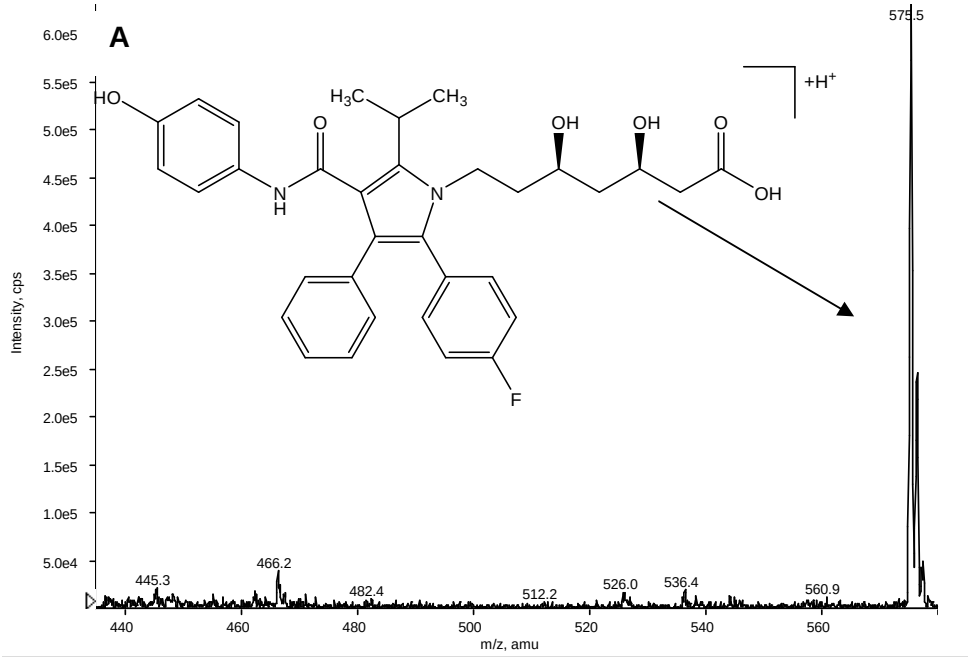


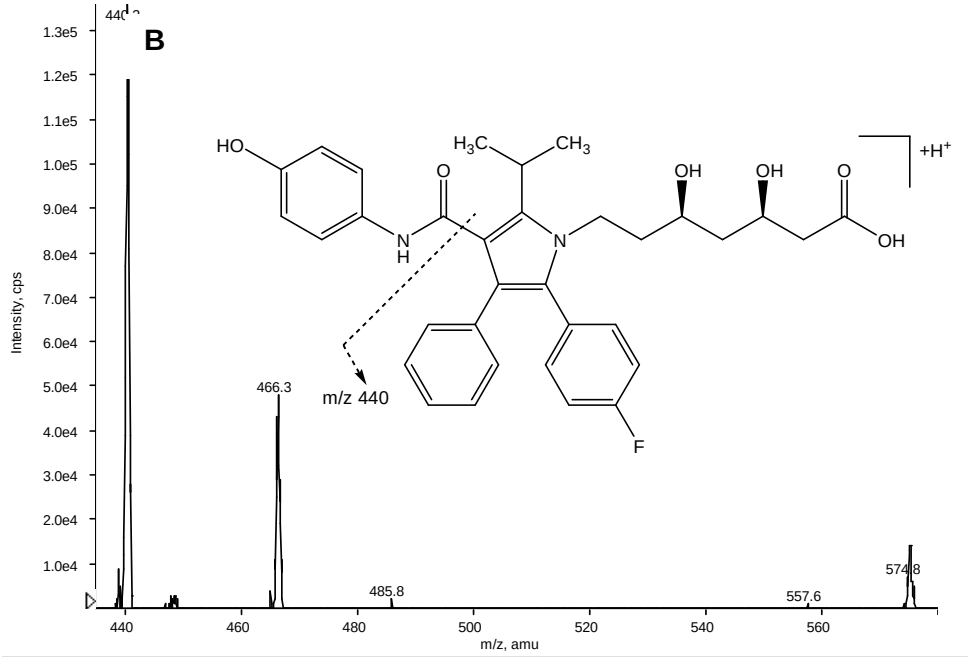
2-Hydroxyatorvastatin: Full-scan (A) and product ion mass spectra (B)



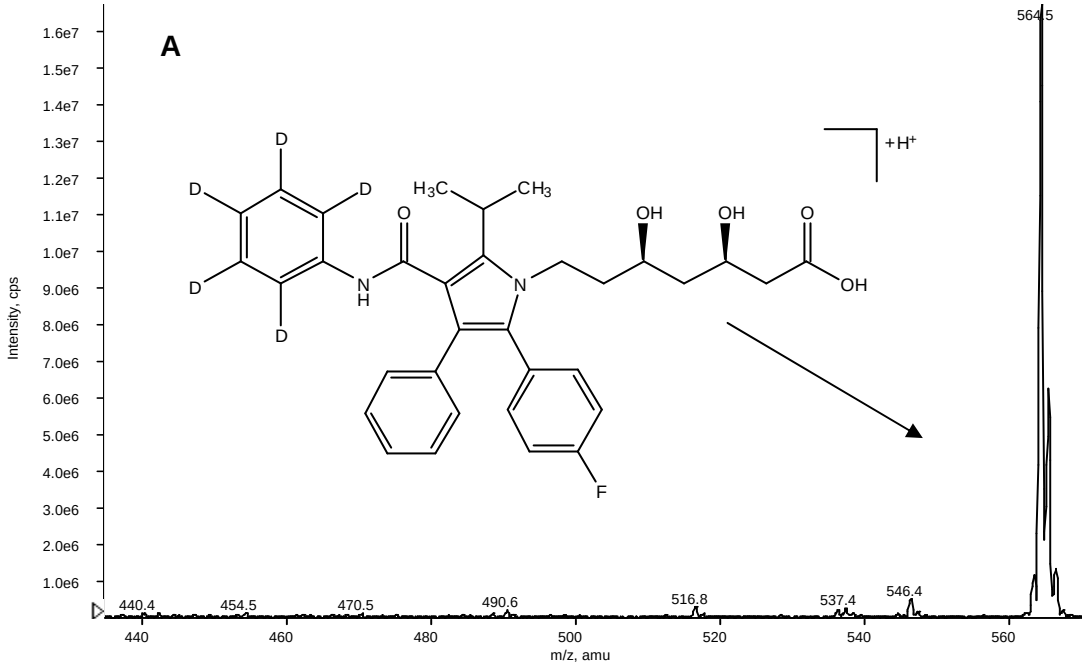


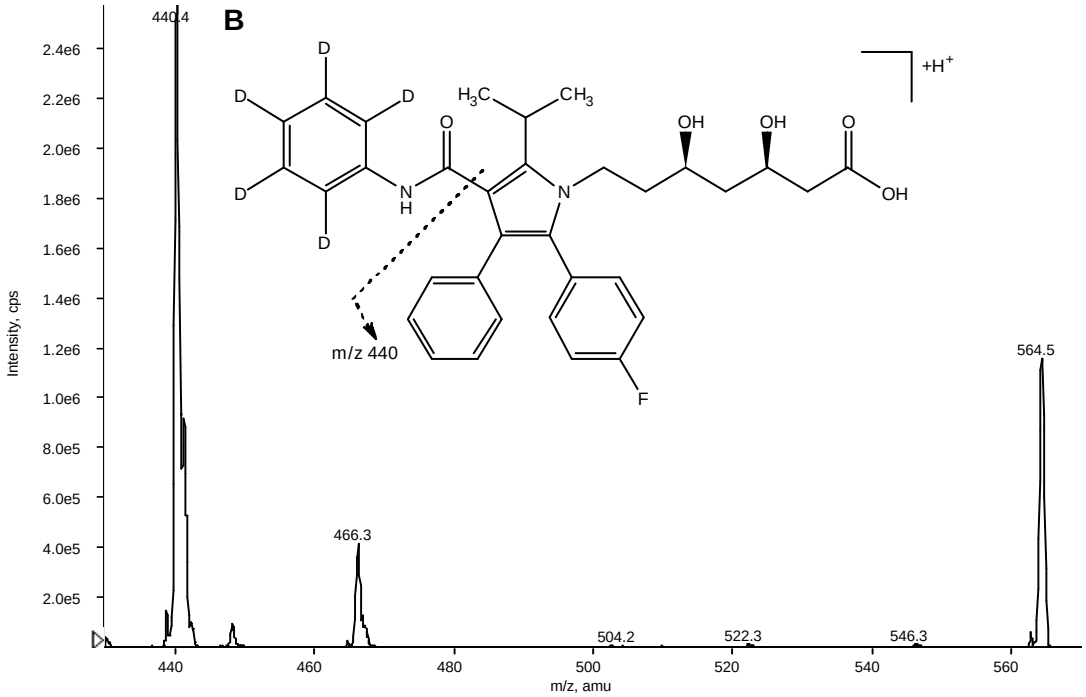
4-Hydroxyatorvastatin: Full-scan (A) and product ion mass spectra (B)



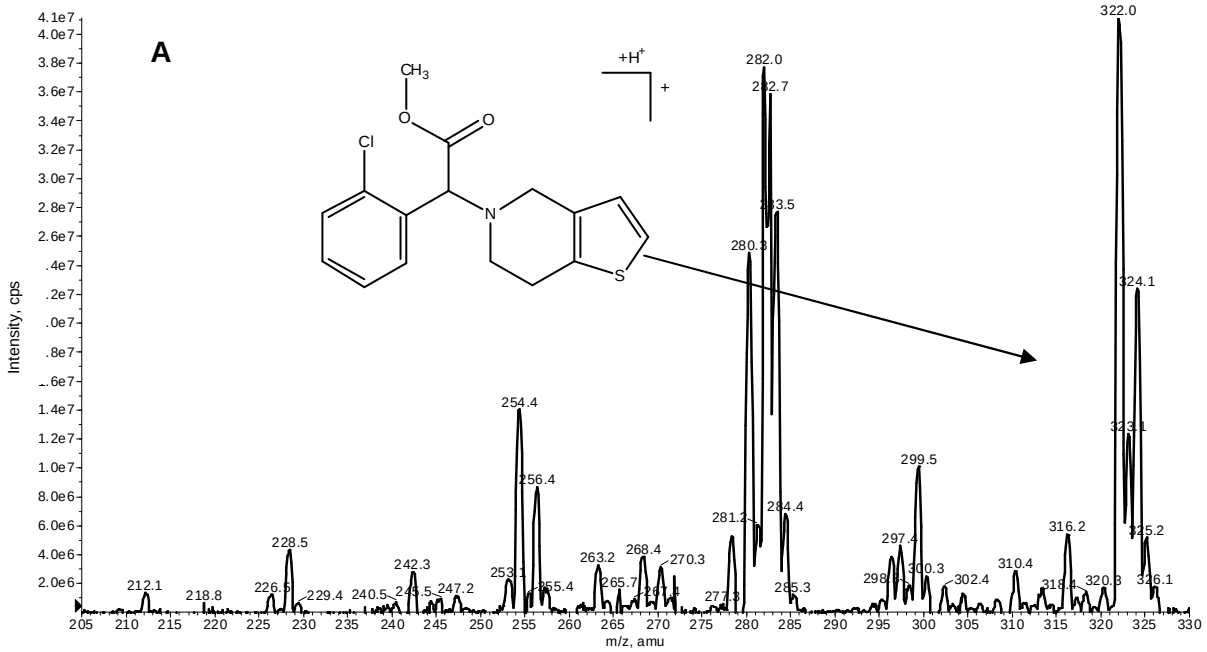


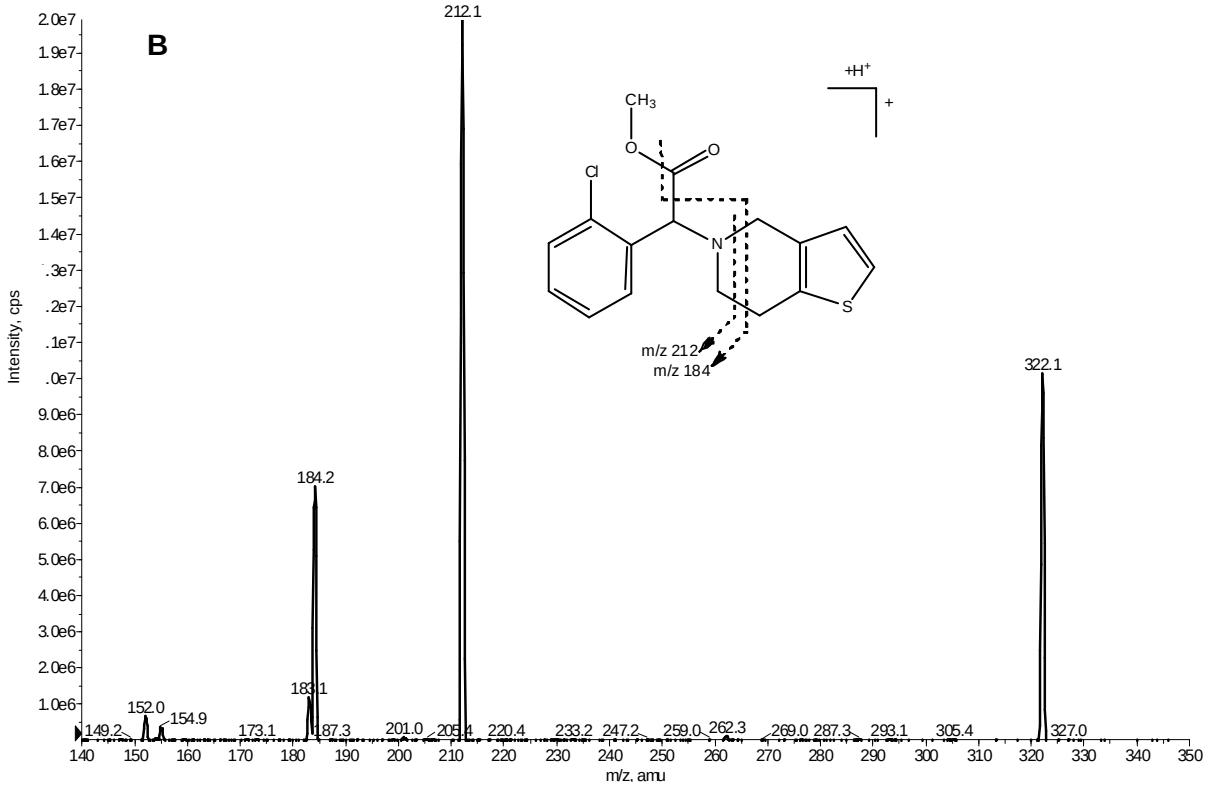
d5-Atorvastatin: Full-scan (A) and product ion mass spectra (B)



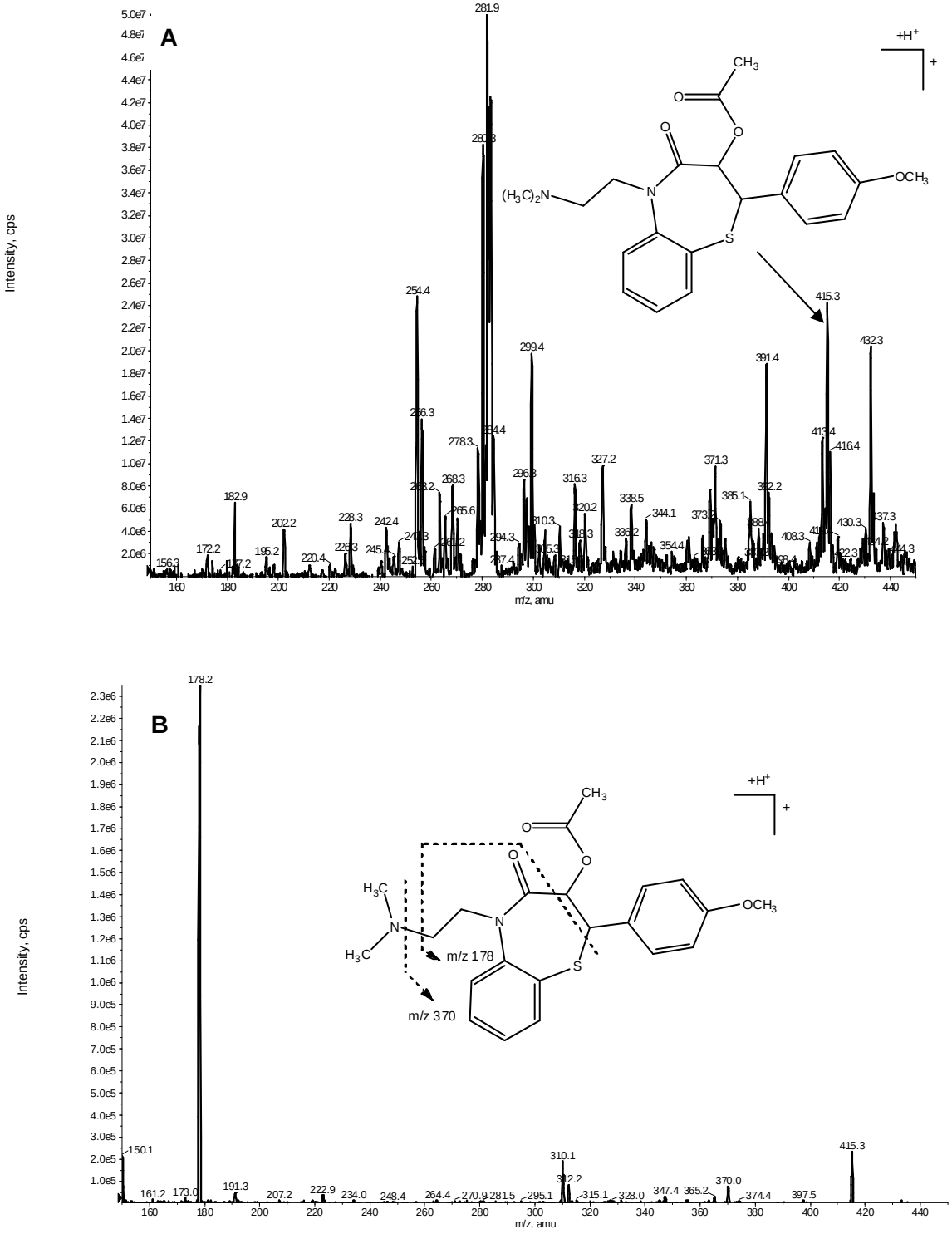


Clopidogrel: Full-scan (A) and product ion mass spectra (B)

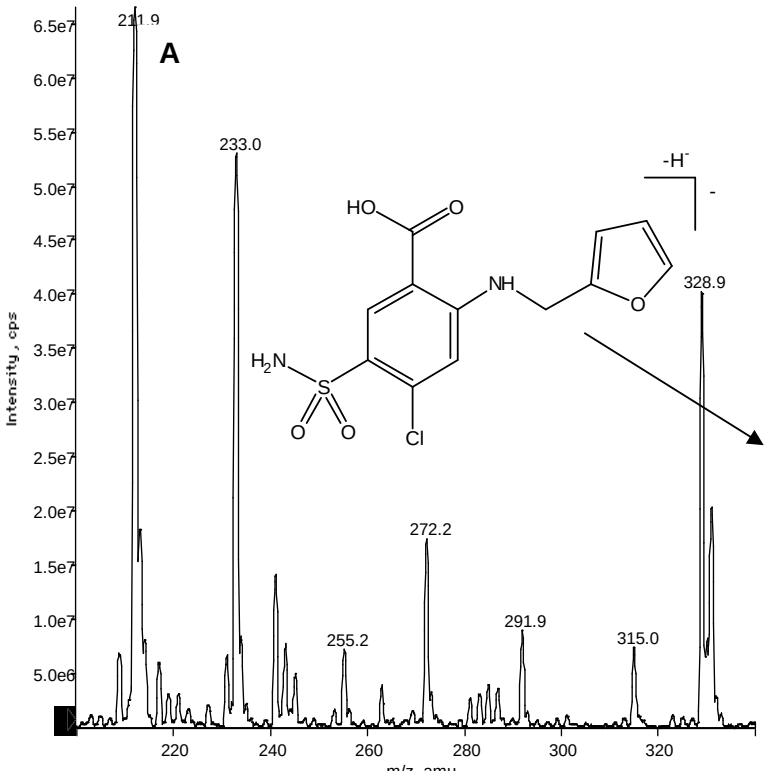


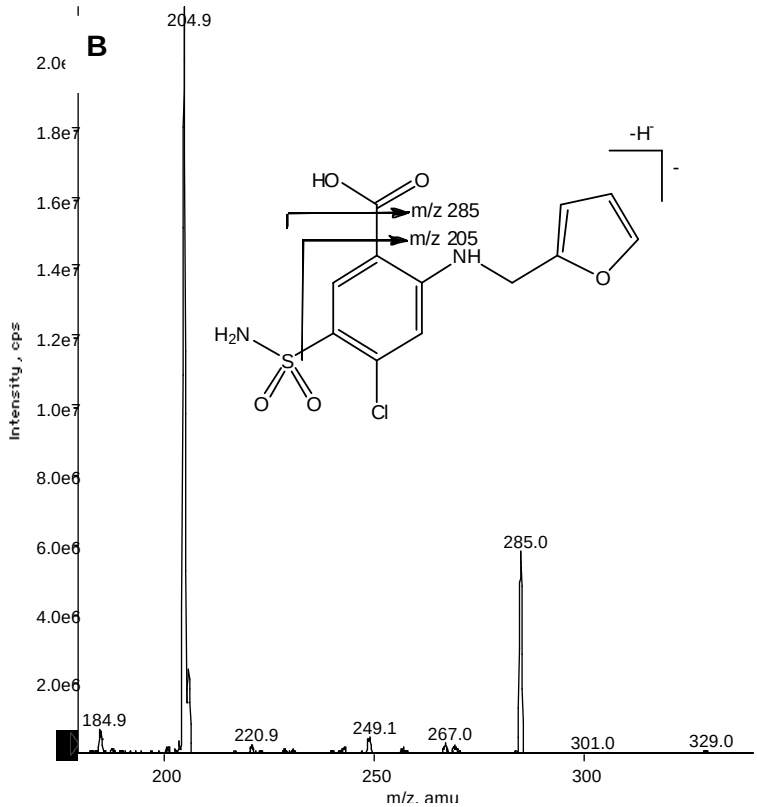


Diltiazem: Full-scan (A) and product ion mass spectra (B)

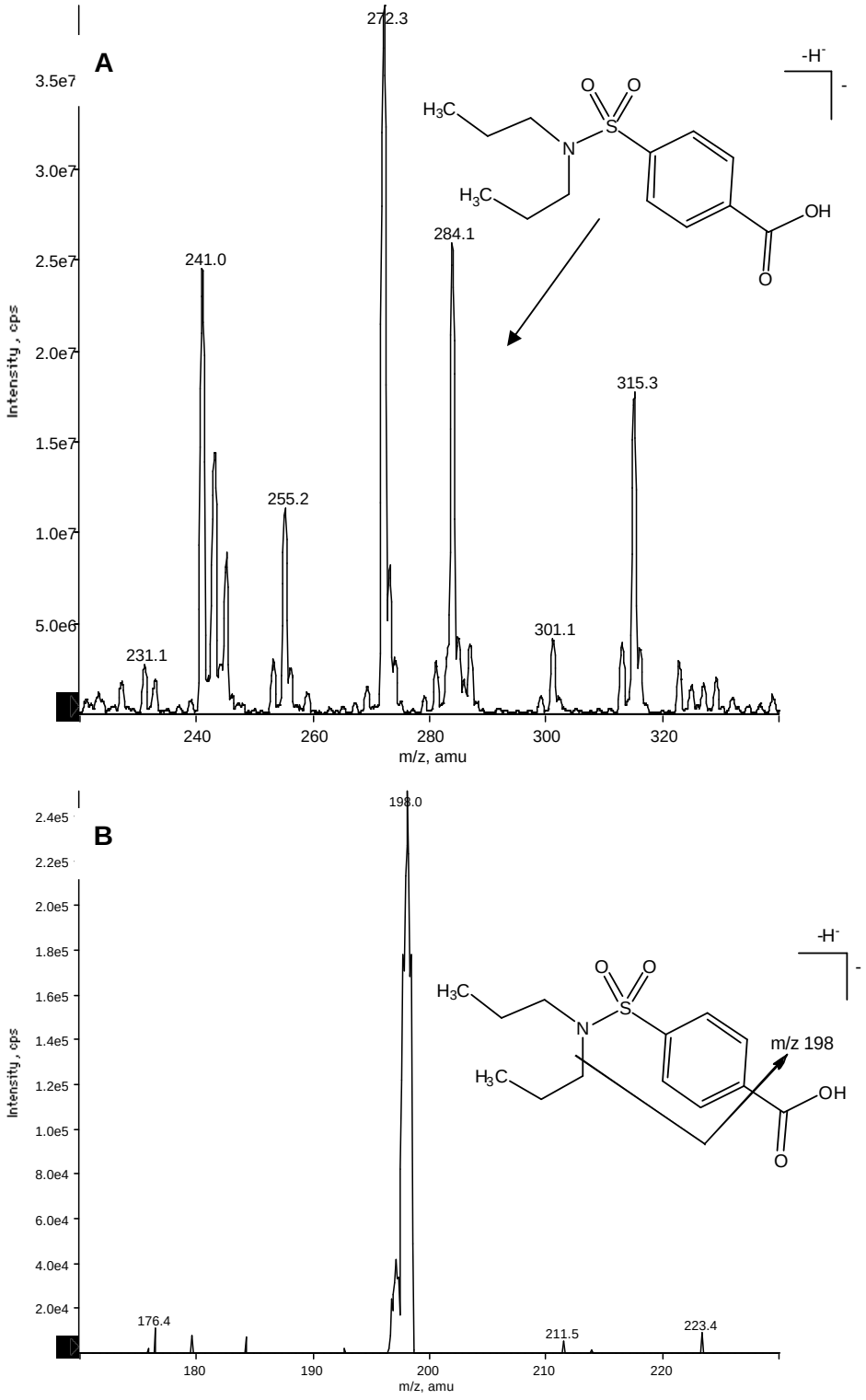


Furosemide: Full-scan (A) and product ion mass spectra (B)

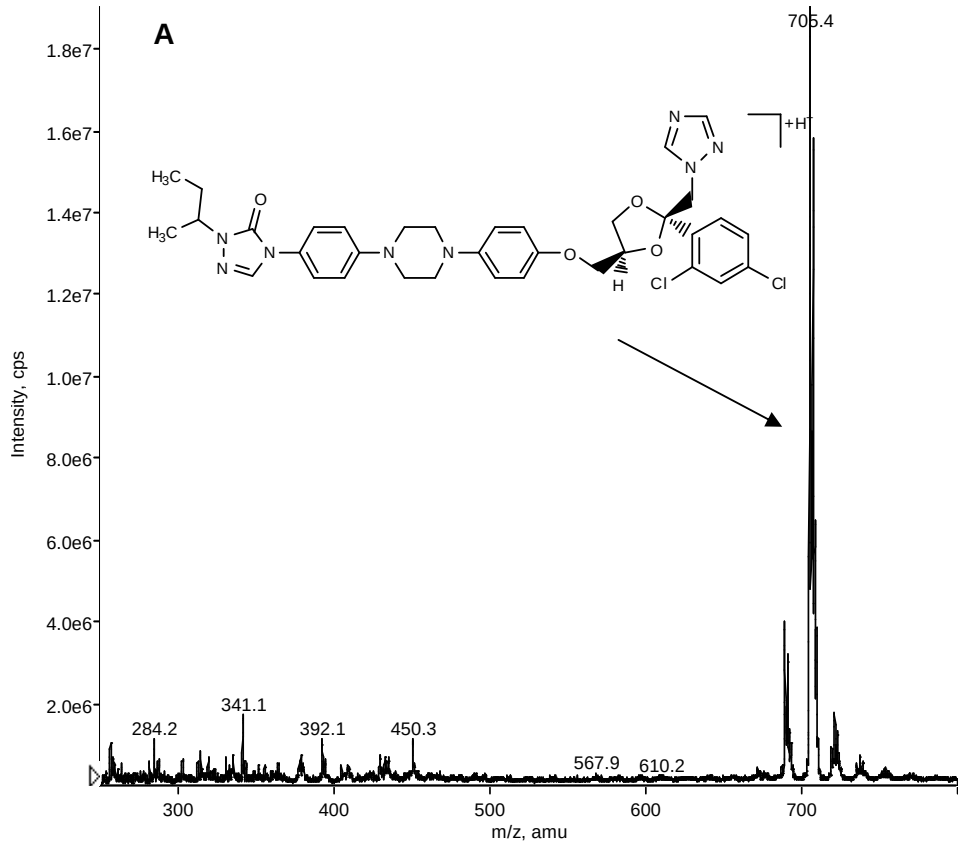




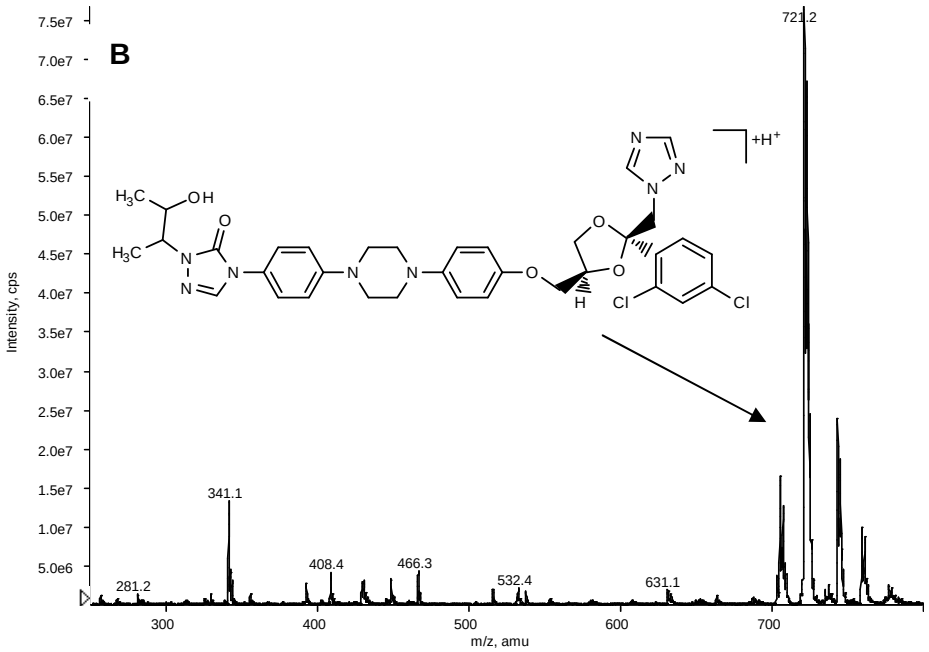
Probenecid: Full-scan (A) and product ion mass spectra (B)



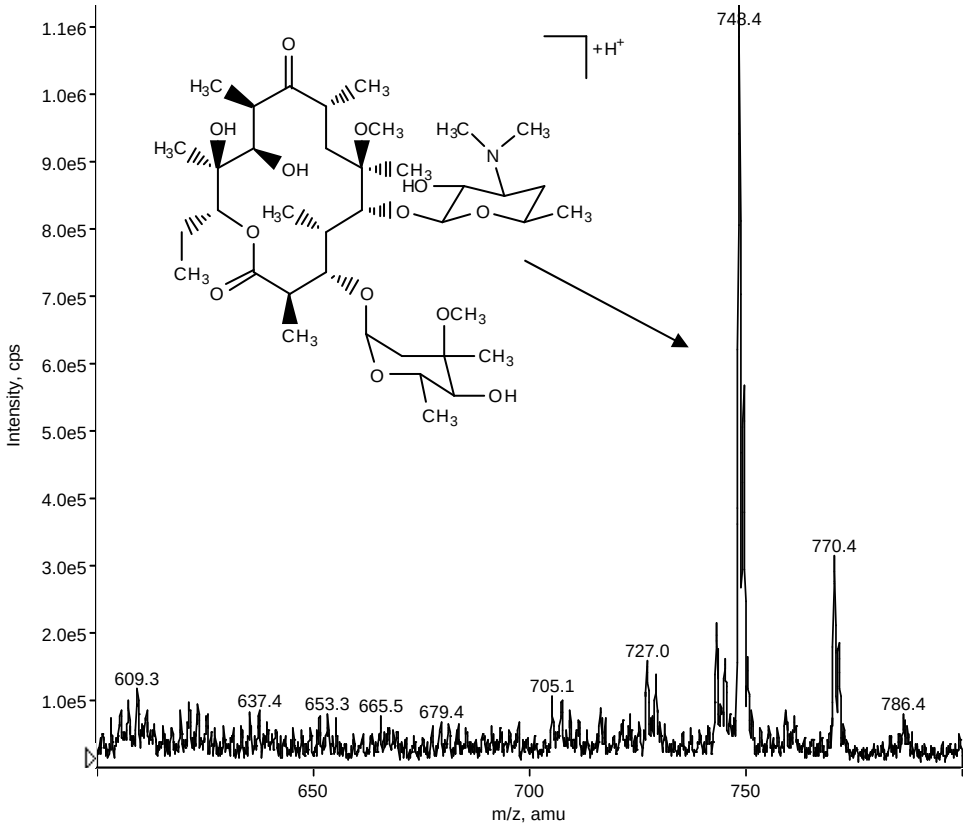
Itraconazole: Full-scan mass spectrum



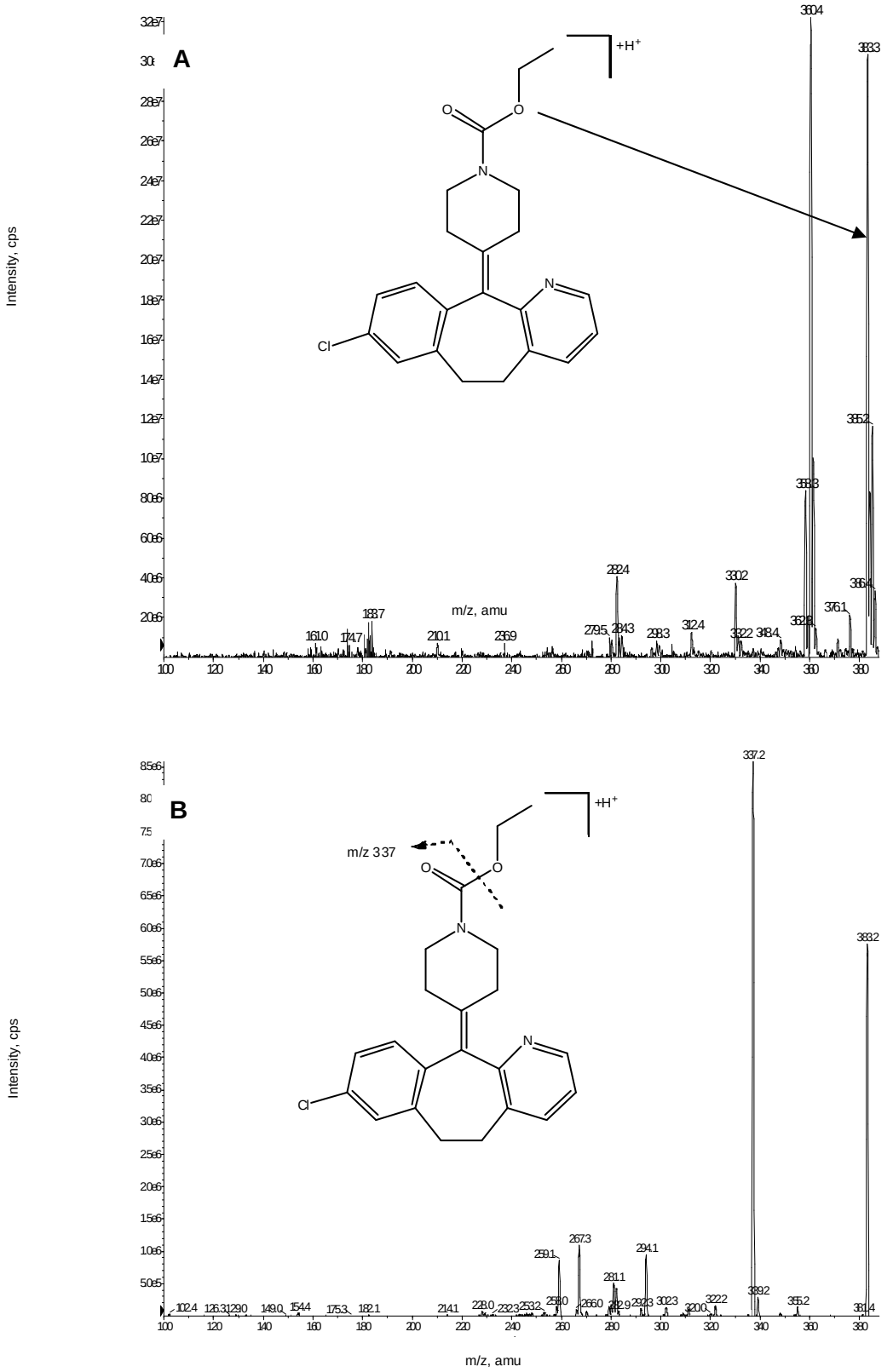
Hydroxyitraconazole: Full-scan mass spectrum



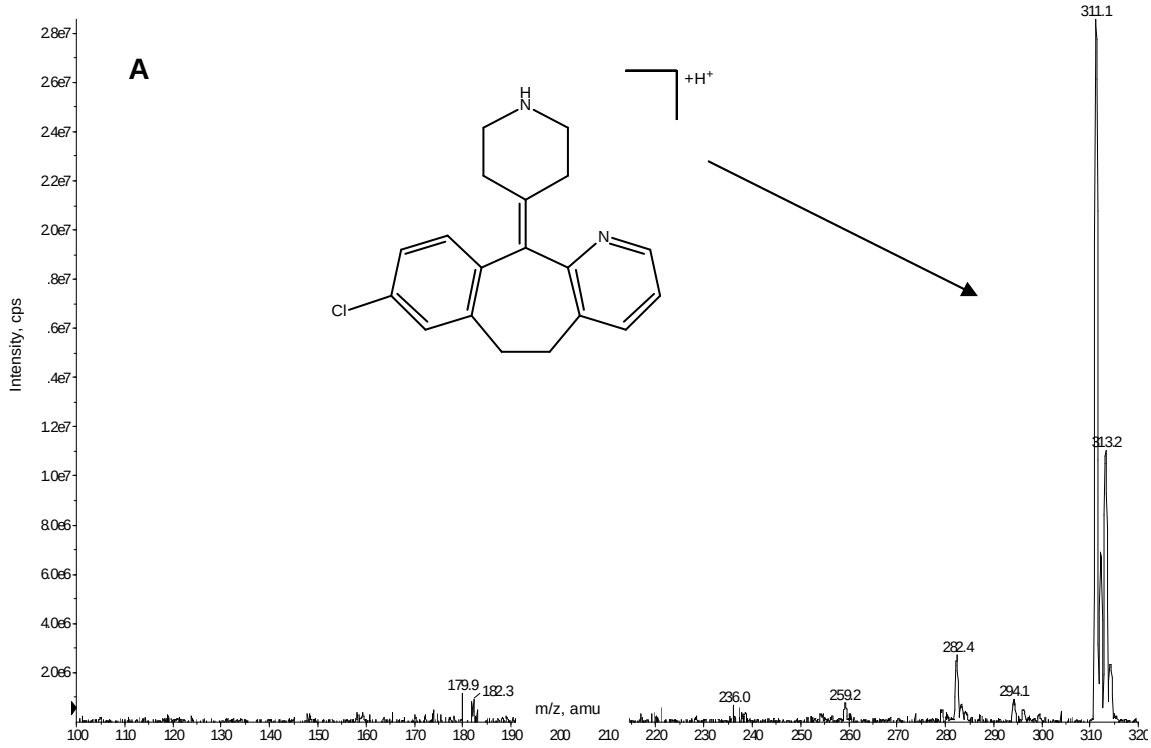
Clarithromycin: Full-scan mass spectrum

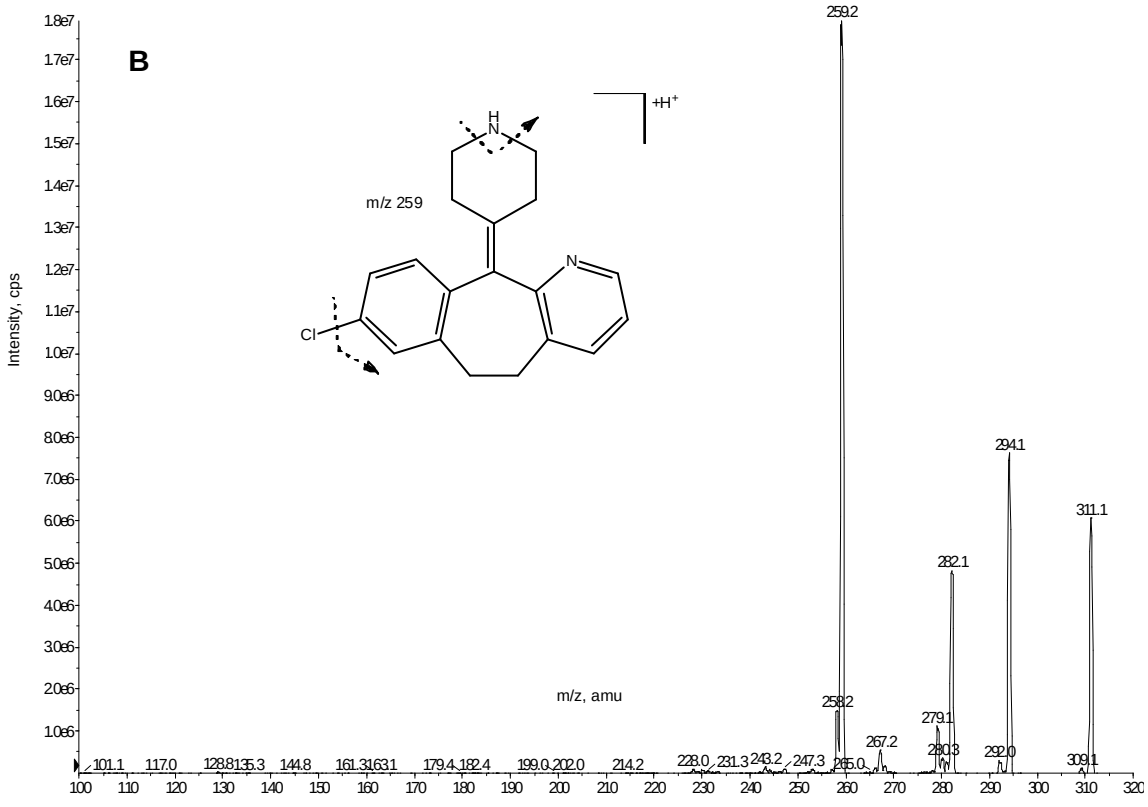


Loratadine: Full-scan (A) and product ion mass spectra (B)

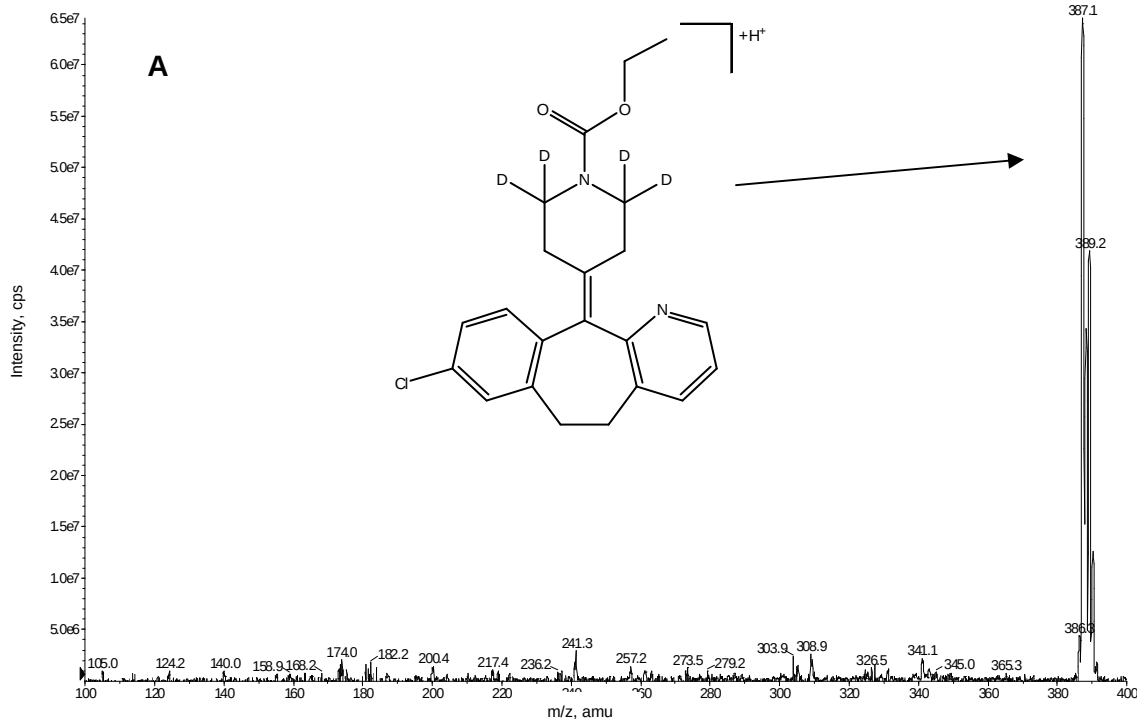


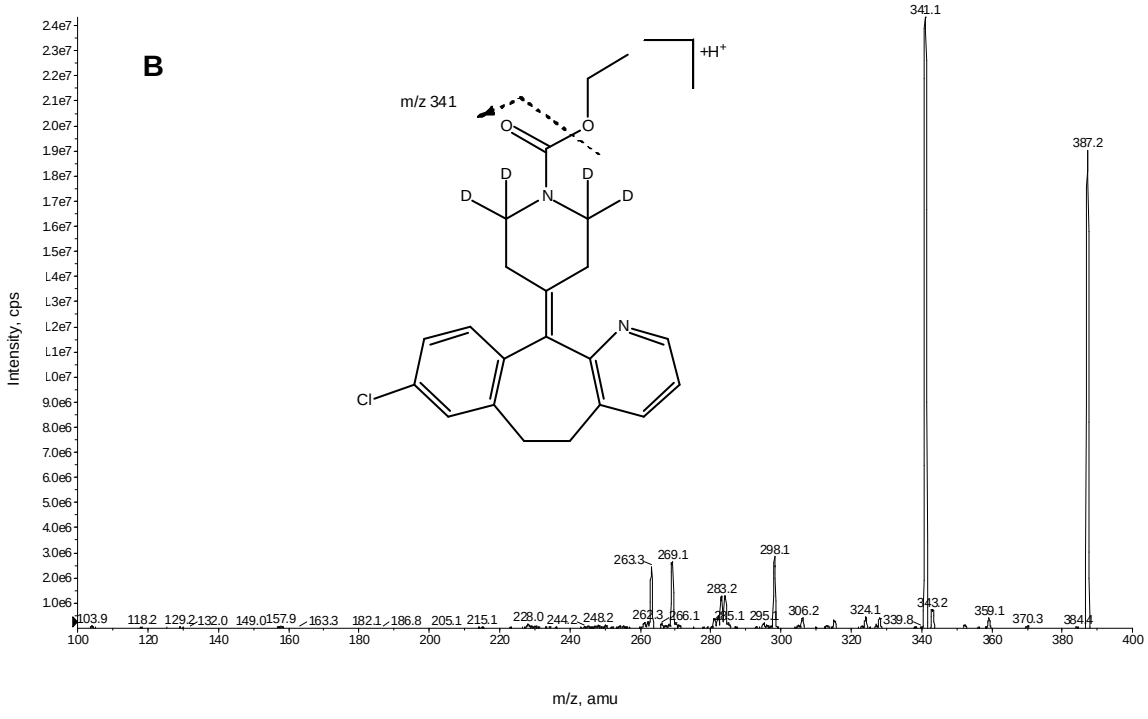
Descarboethoxyloratadine: Full-scan (A) and product ion mass spectra (B)



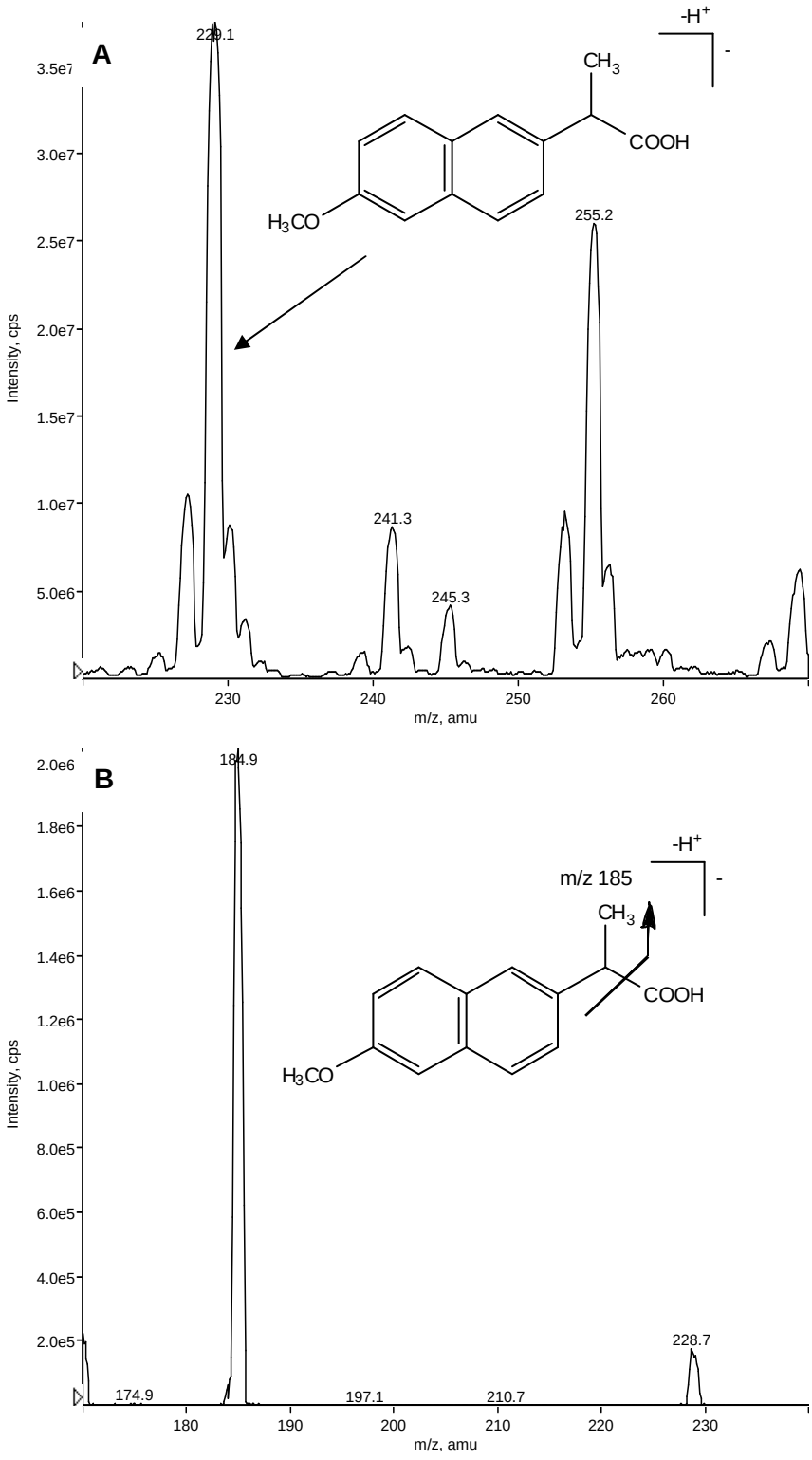


d4-Loratadine: Full-scan (A) and product ion mass spectra (B)

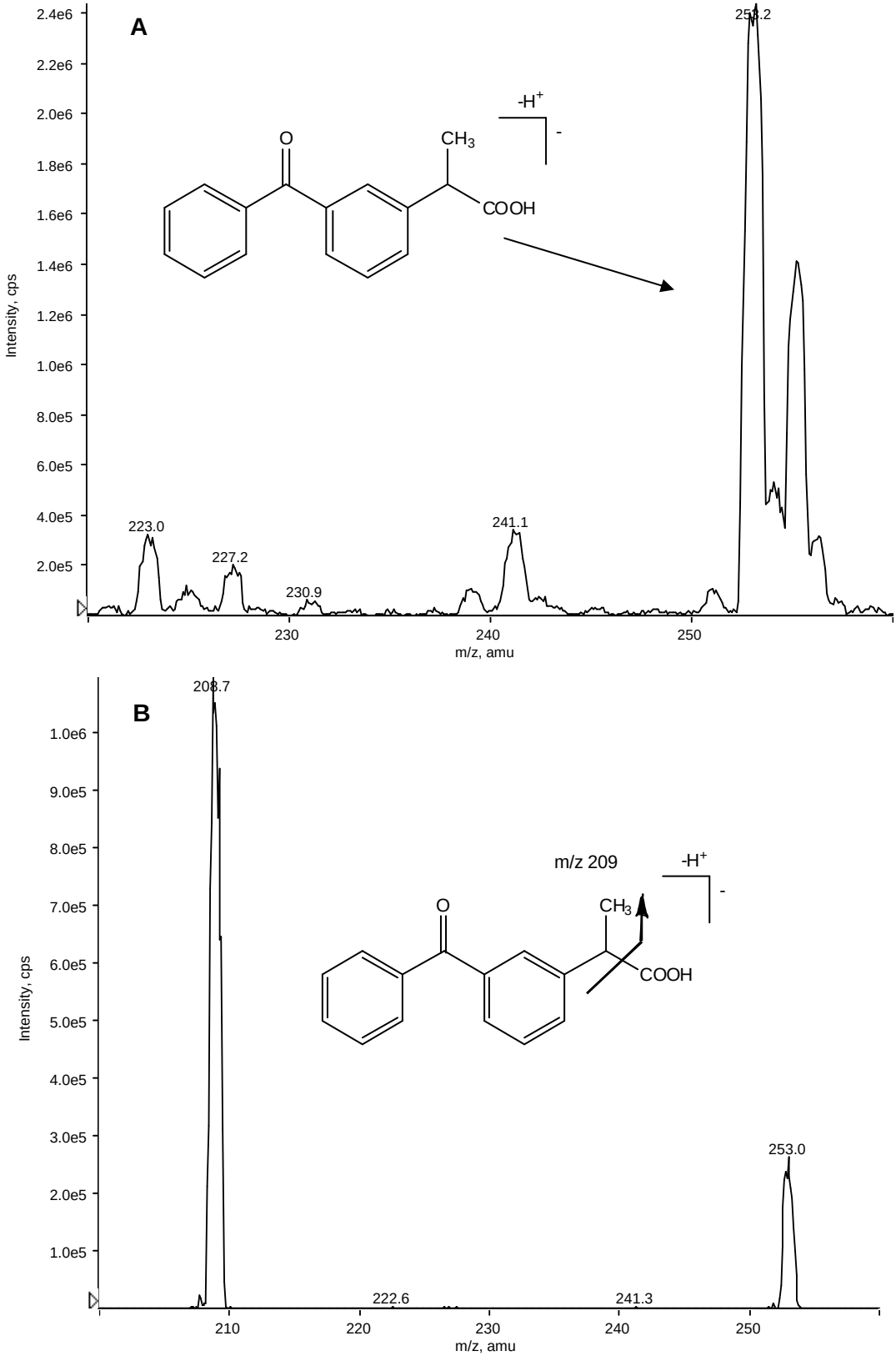




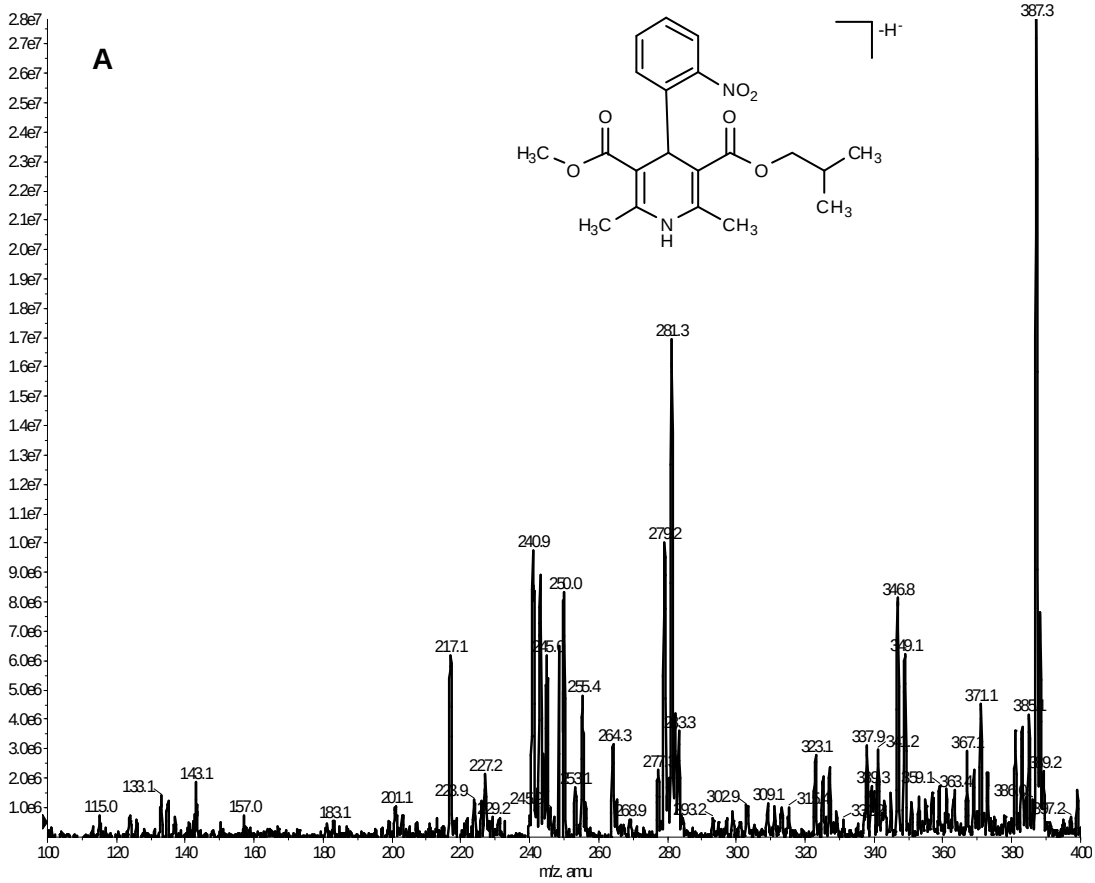
Naproxen: Full-scan (A) and product ion mass spectra (B)

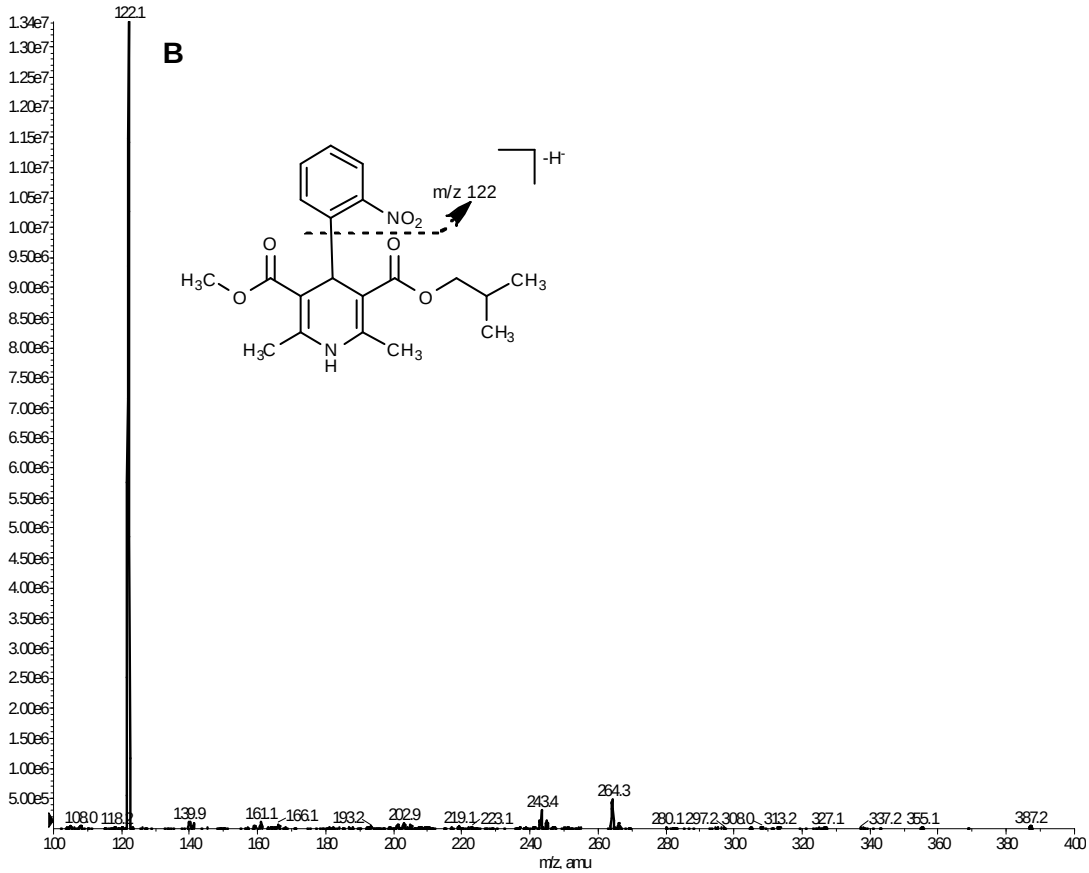


Ketoprofen: Full-scan (A) and product ion mass spectra (B)

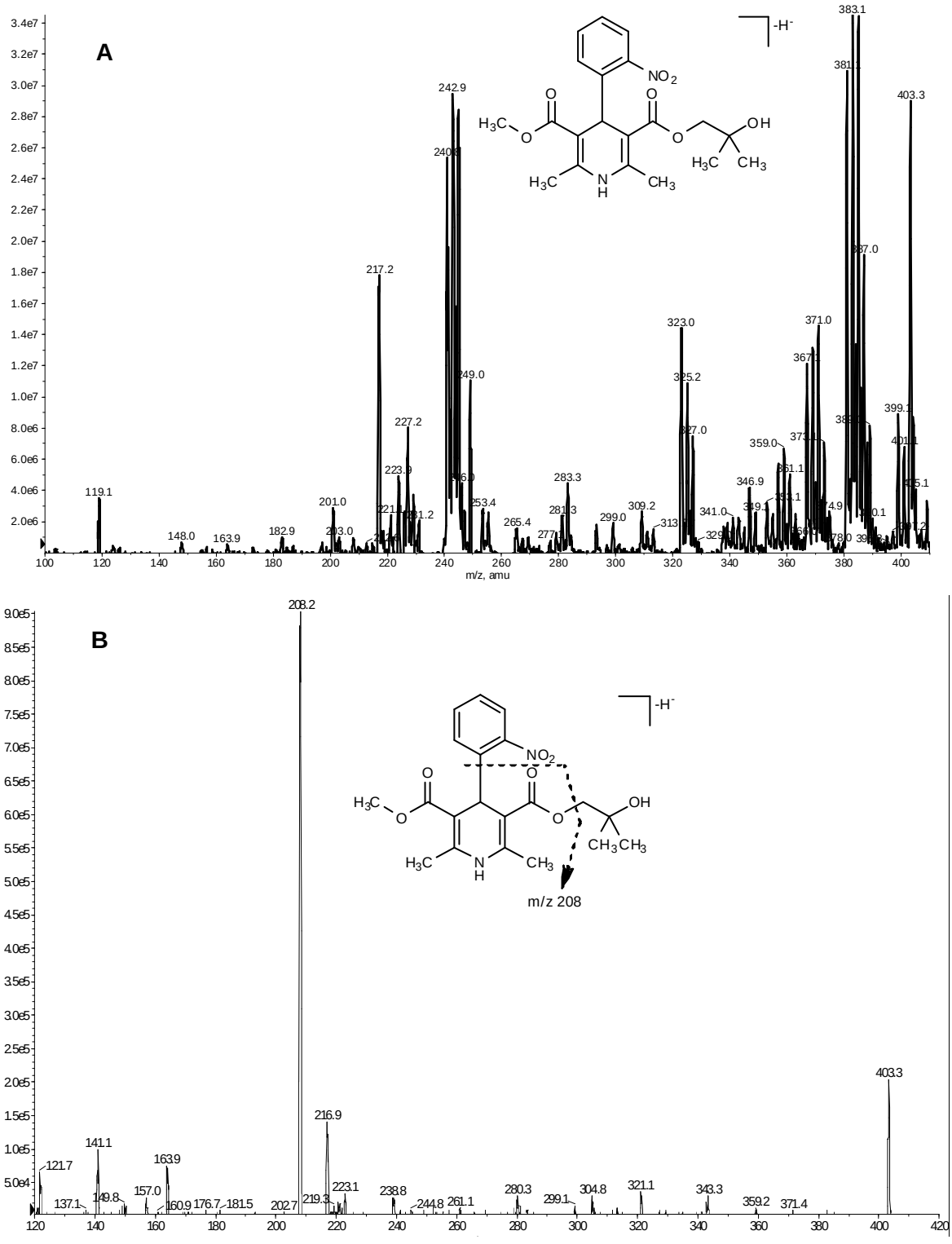


Nisoldipine: Full-scan (A) and product ion mass spectra (B)

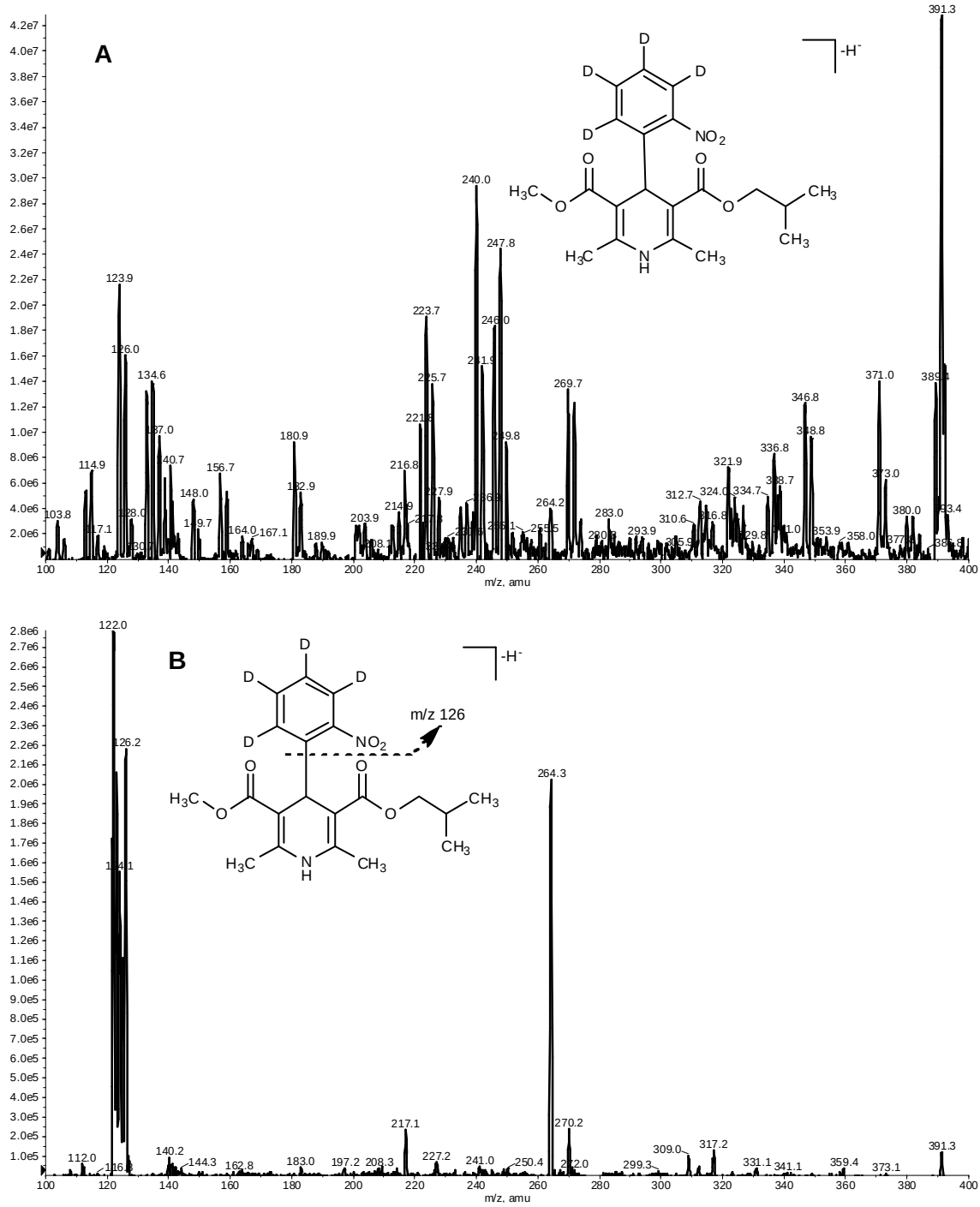




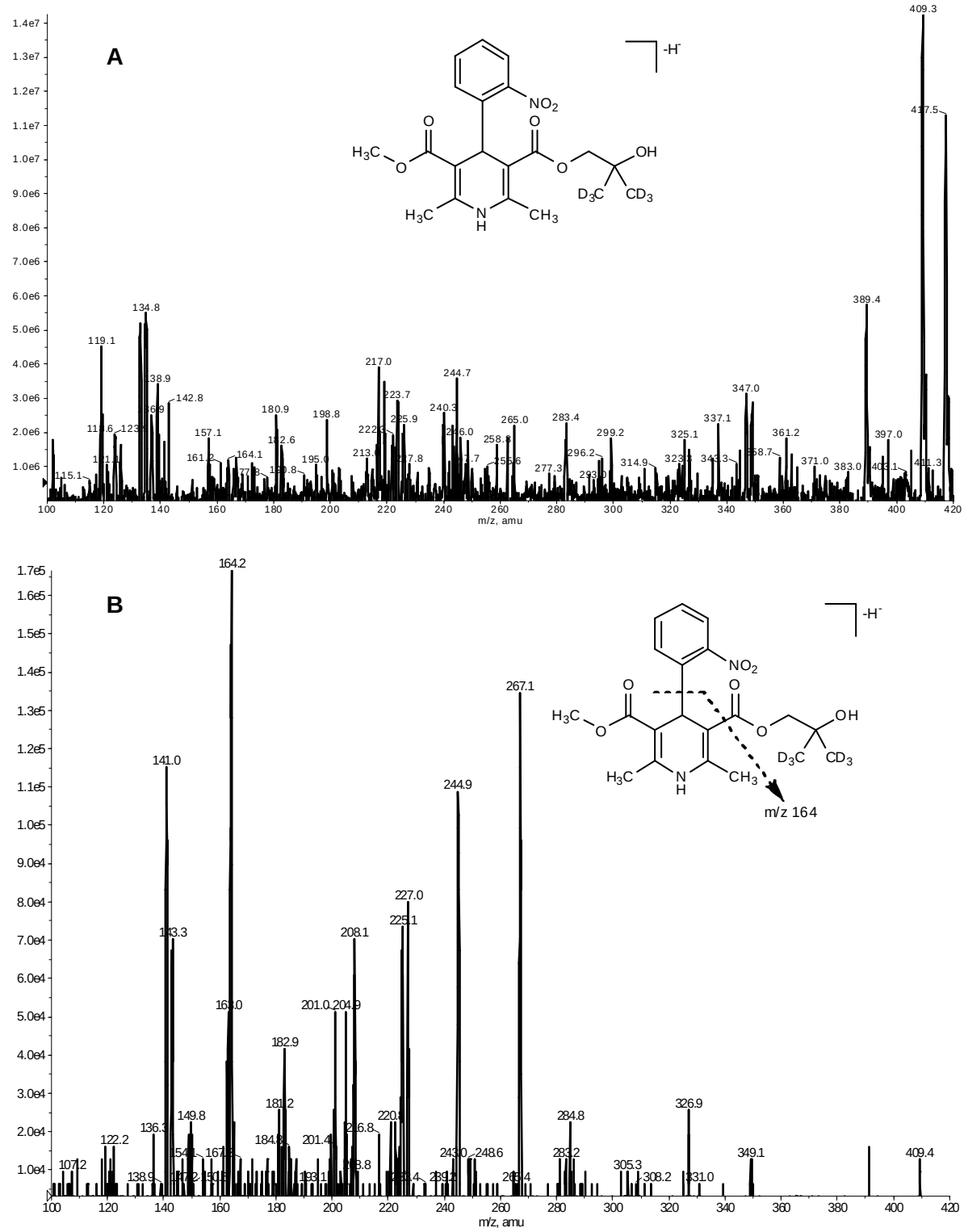
4-Hydroxynisoldipine: Full-scan (A) and product ion mass spectra (B)



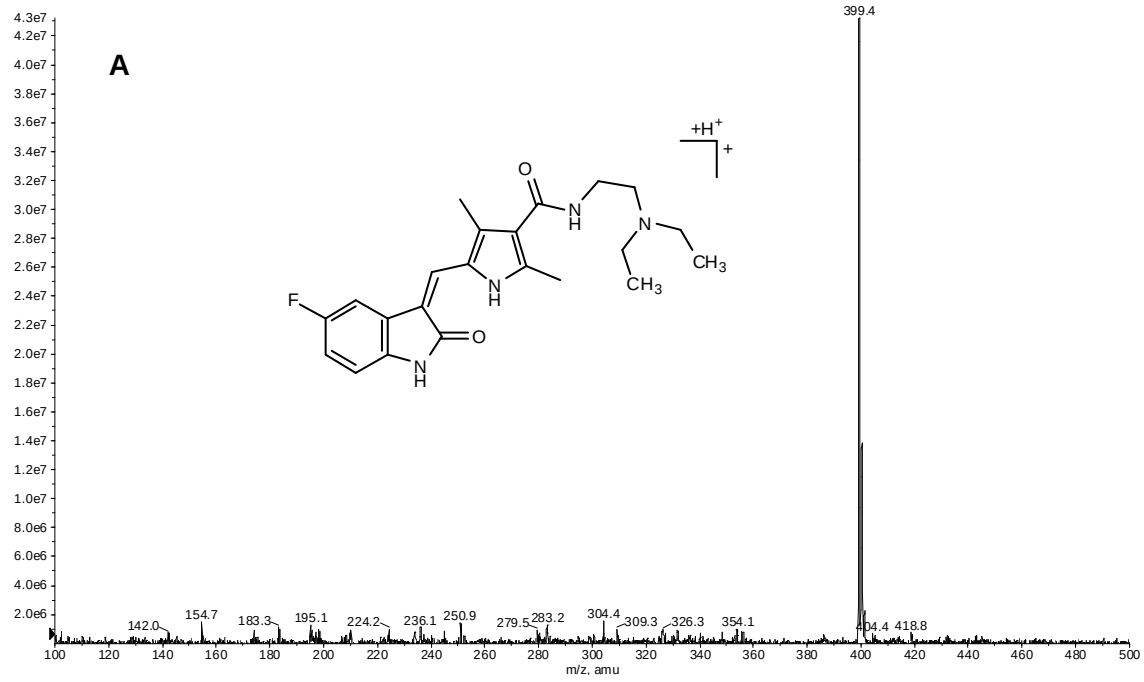
d4-Nisoldipine: Full-scan (A) and product ion mass spectra (B)

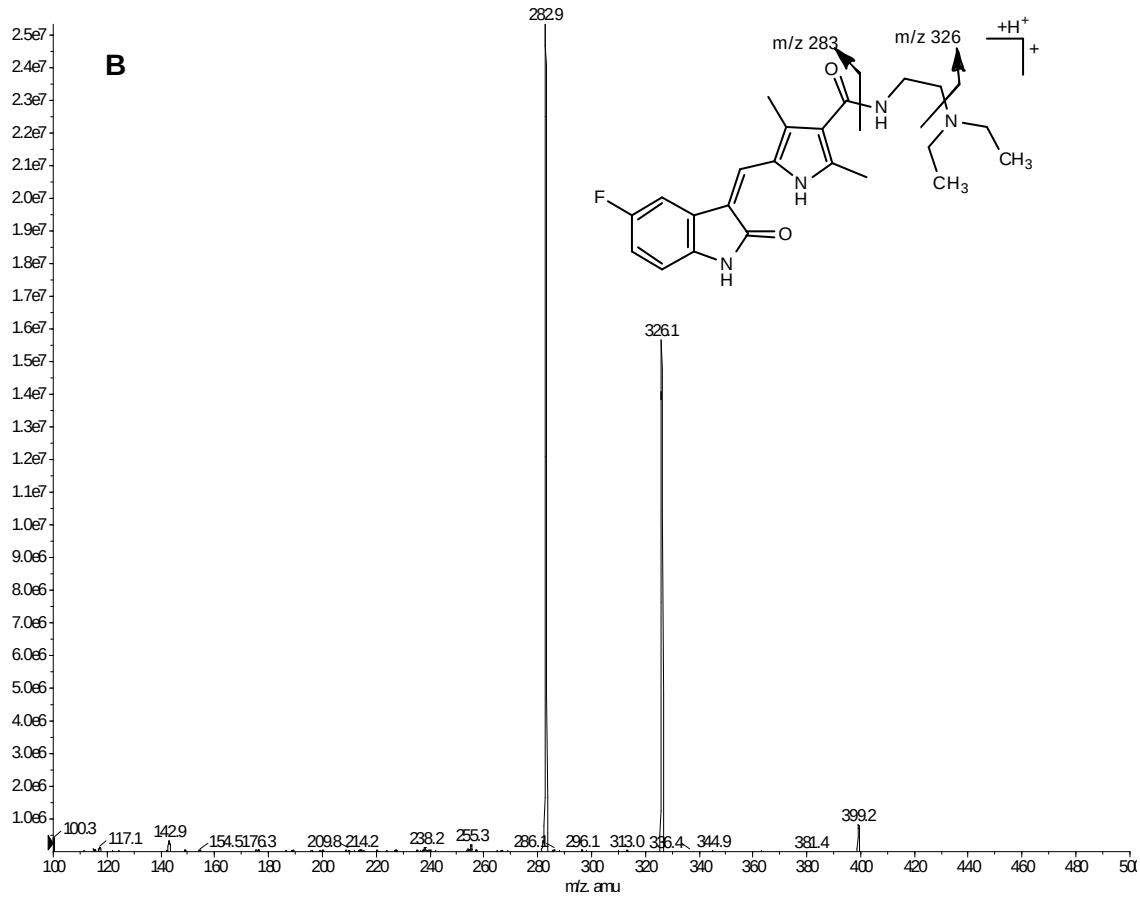


d6-4-Hydroxynisoldipine: Full-scan (A) and product ion mass spectra (B)

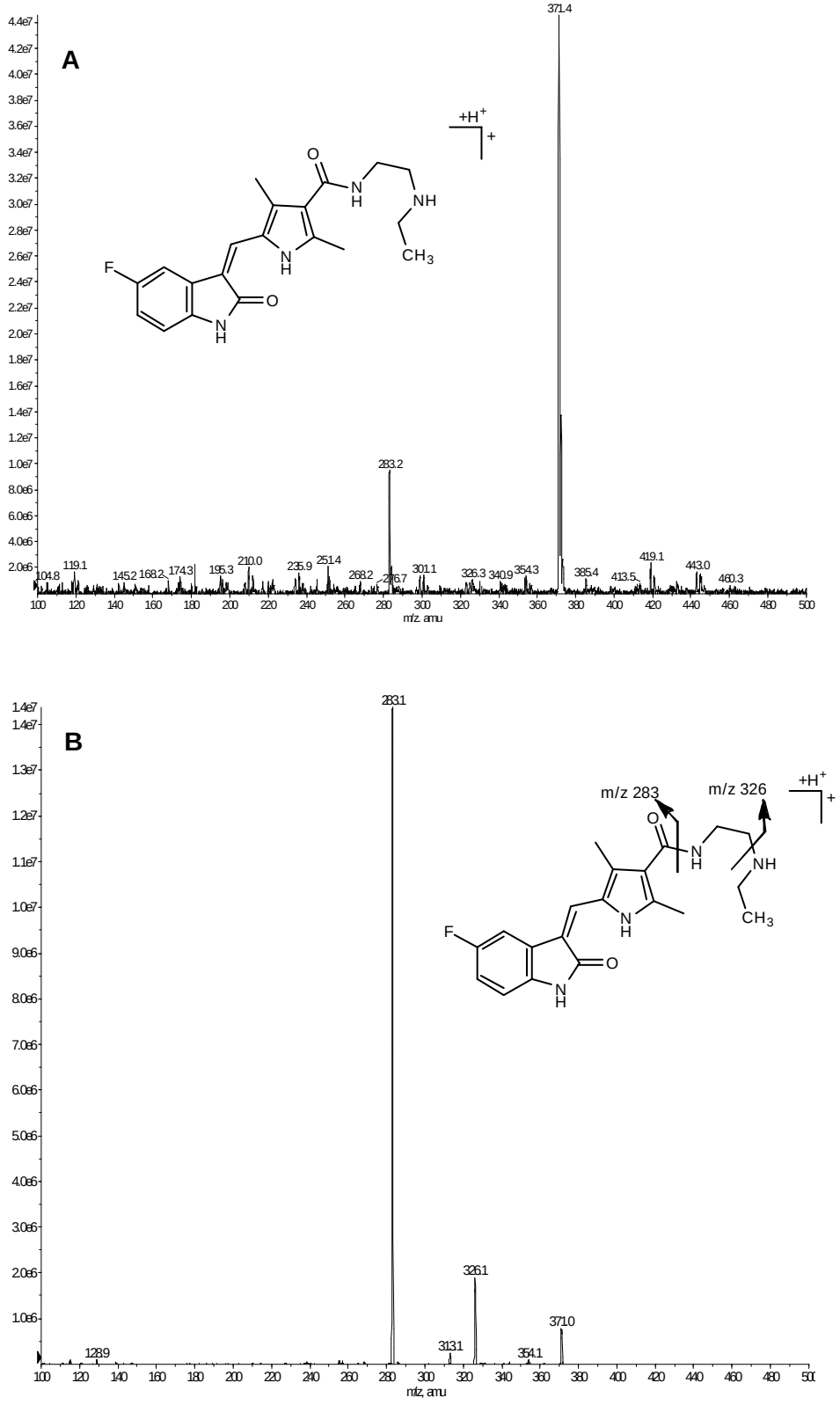


Sunitinib: Full-scan (A) and product ion mass spectra (B)

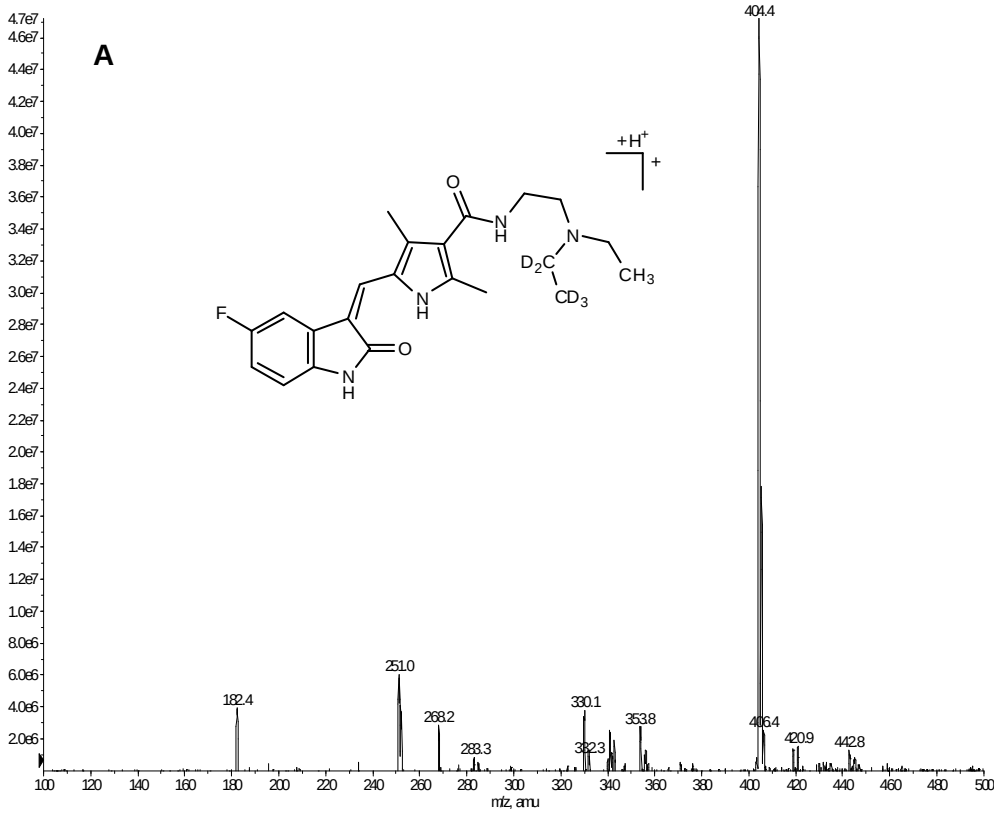


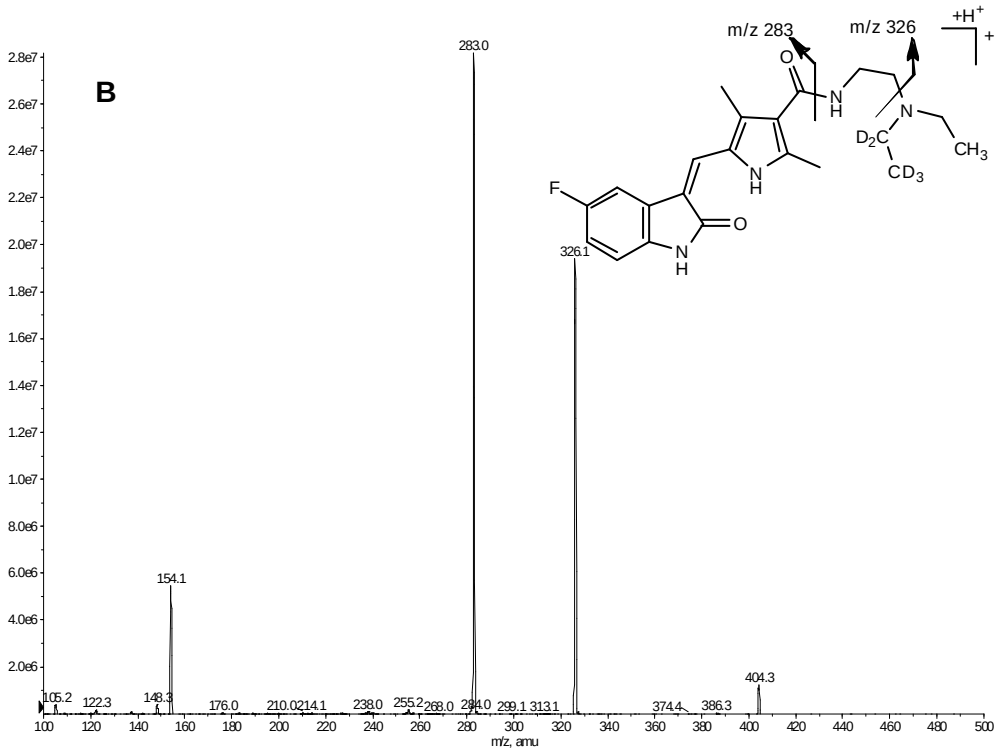


N-Desethylsunitinib: Full-scan (A) and product ion mass spectra (B)



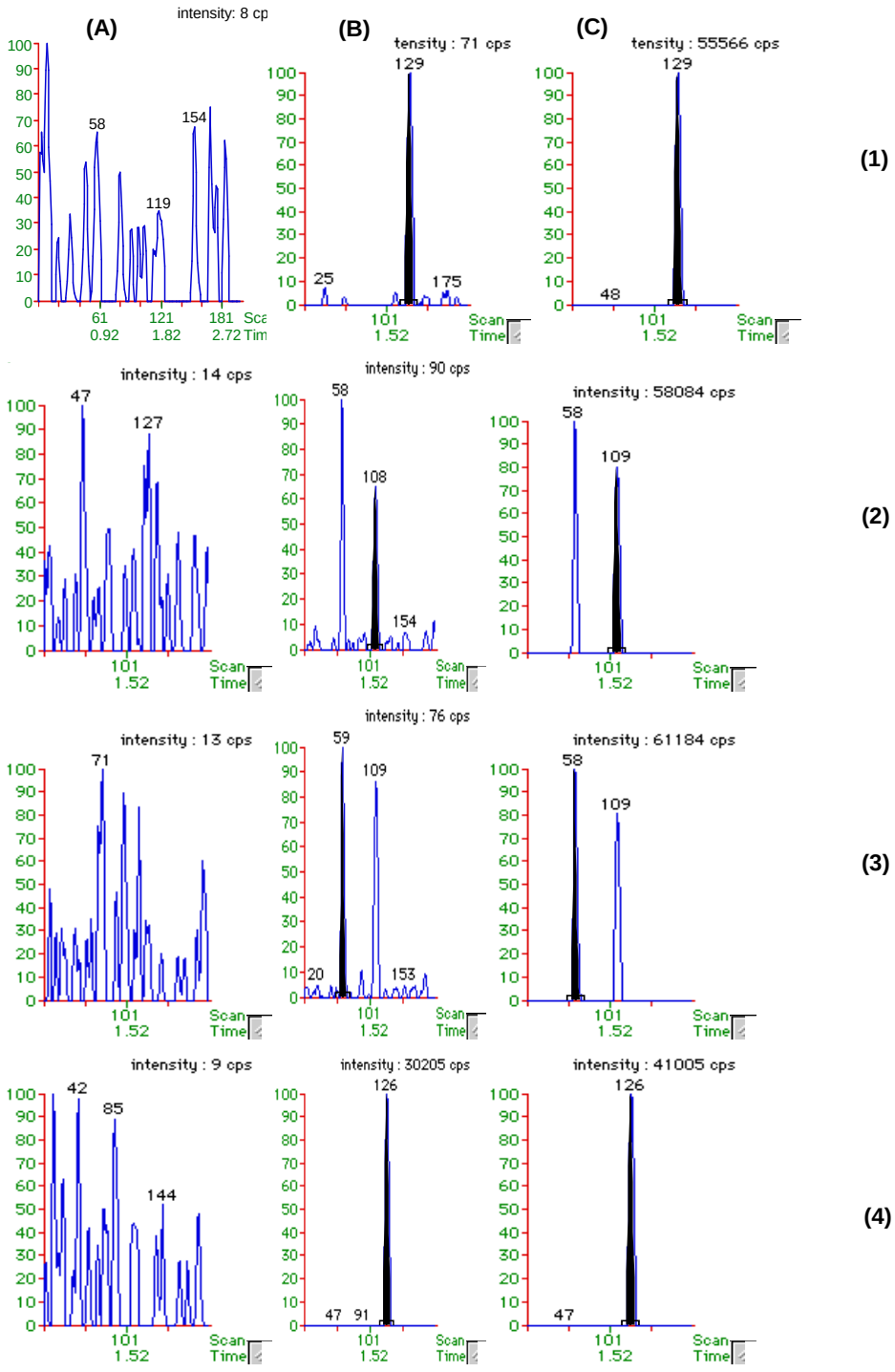
d5-Sunitinib: Full-scan (A) and product ion mass spectra (B)





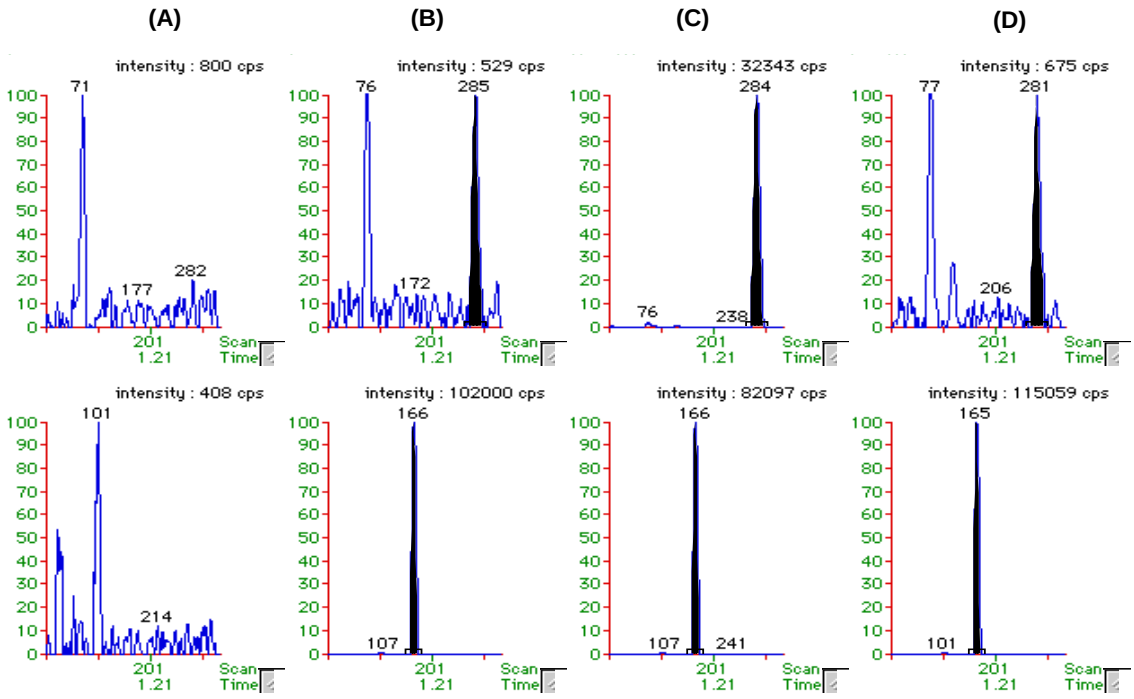
10.2. Chromatograms - Full Validation

Atorvastatin Method API 3000™



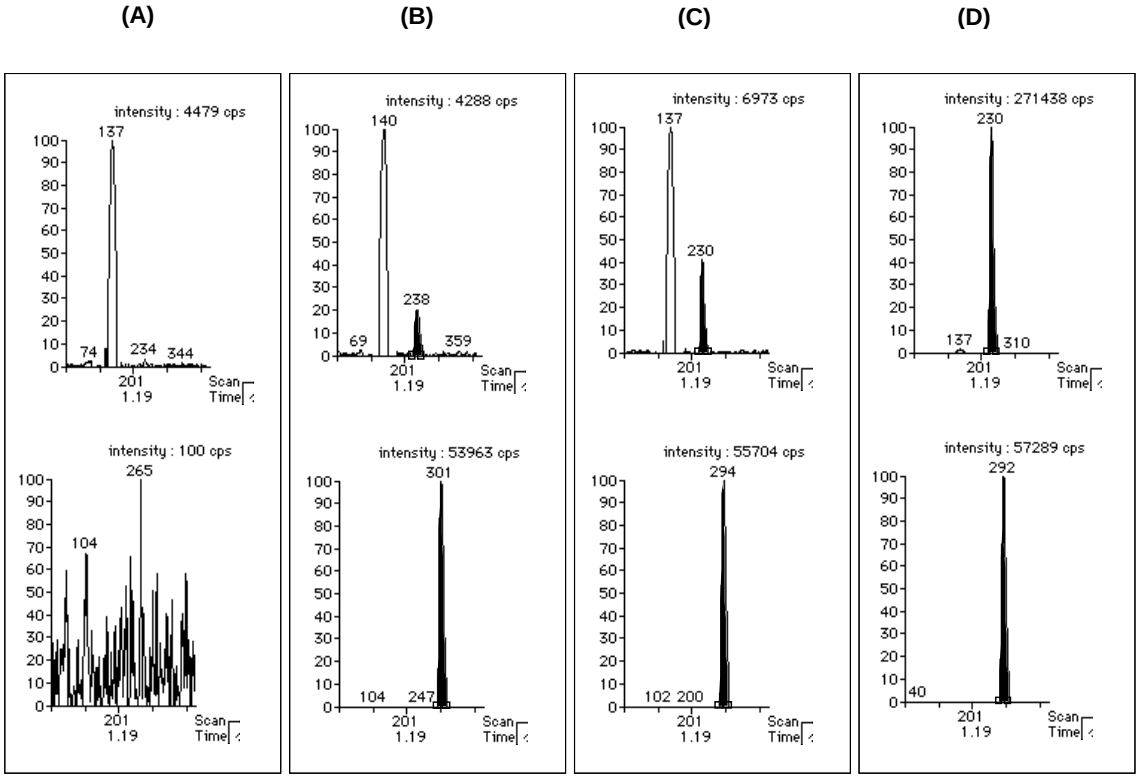
Representative MRM chromatograms for ATO (1), 2-HYD-ATO (2), 4-HYD-ATO (3) and d5-ATO (4) in human plasma: (A) a blank plasma sample; (B) a blank plasma sample spiked with ATO, 2-HYD-ATO, 4-HYD-ATO (at LLOQ= 0.100 ng/mL); (C) a blank plasma sample spiked with ATO, 2-HYD-ATO, 4-HYD-ATO (at L1= 50.0 ng/mL).

Clopidogrel Method API 3000™



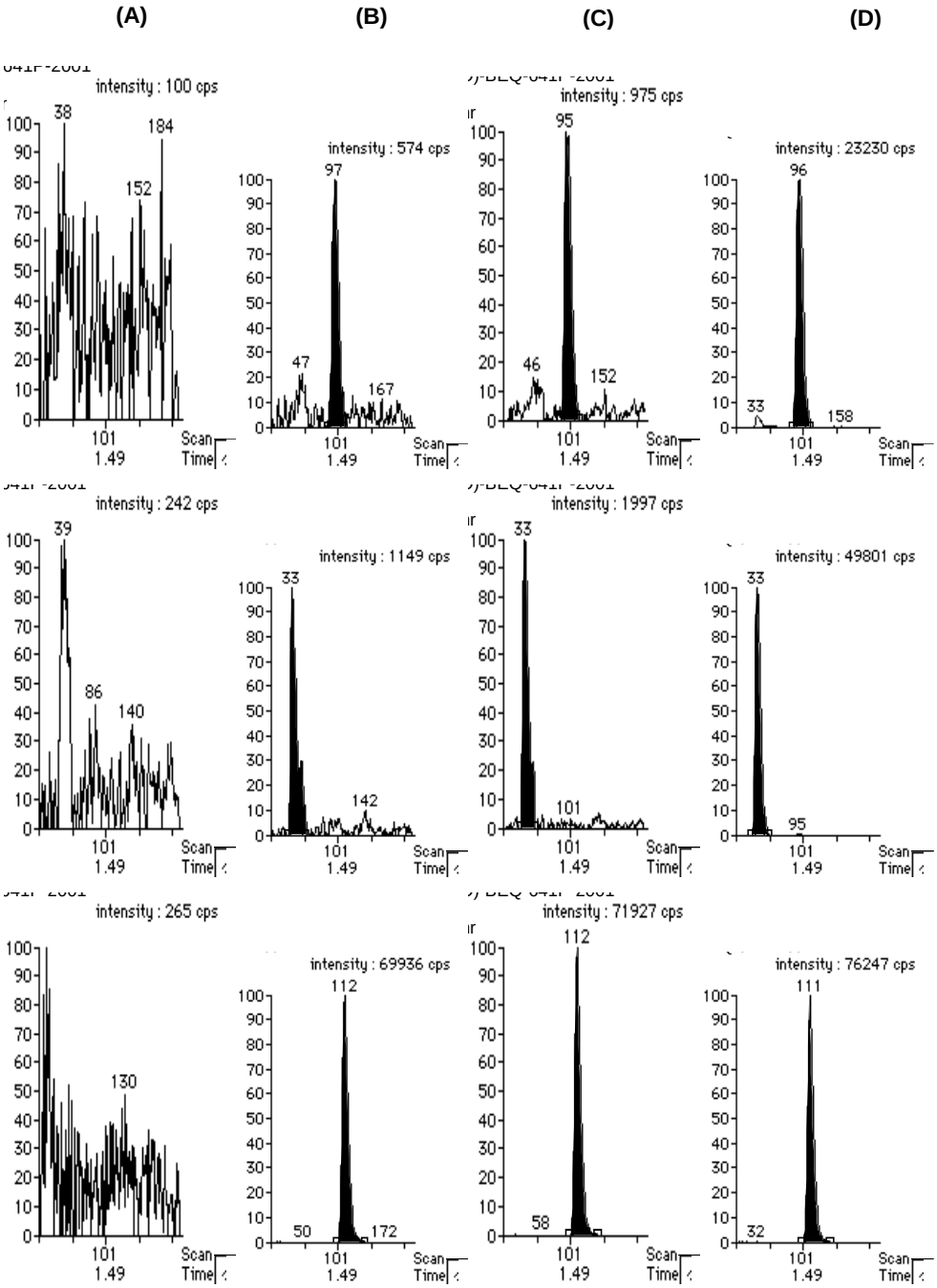
Representative MRM chromatograms for CLP (top) and DIL (IS, bottom) in human plasma: (A) a blank plasma sample; (B) a blank plasma sample spiked with CLP (at LLOQ= 0.0200 ng/mL); (C) a plasma sample from a healthy volunteer 0.5 h after the administration of 75 mg CLP (CLP concentration= 1.604 ng/mL) and (D) a plasma sample from a patient 8.0 h after the administration of 75 mg CLP (CLP concentration= 0.0220 ng/mL).

Furosemide Method API 3000™



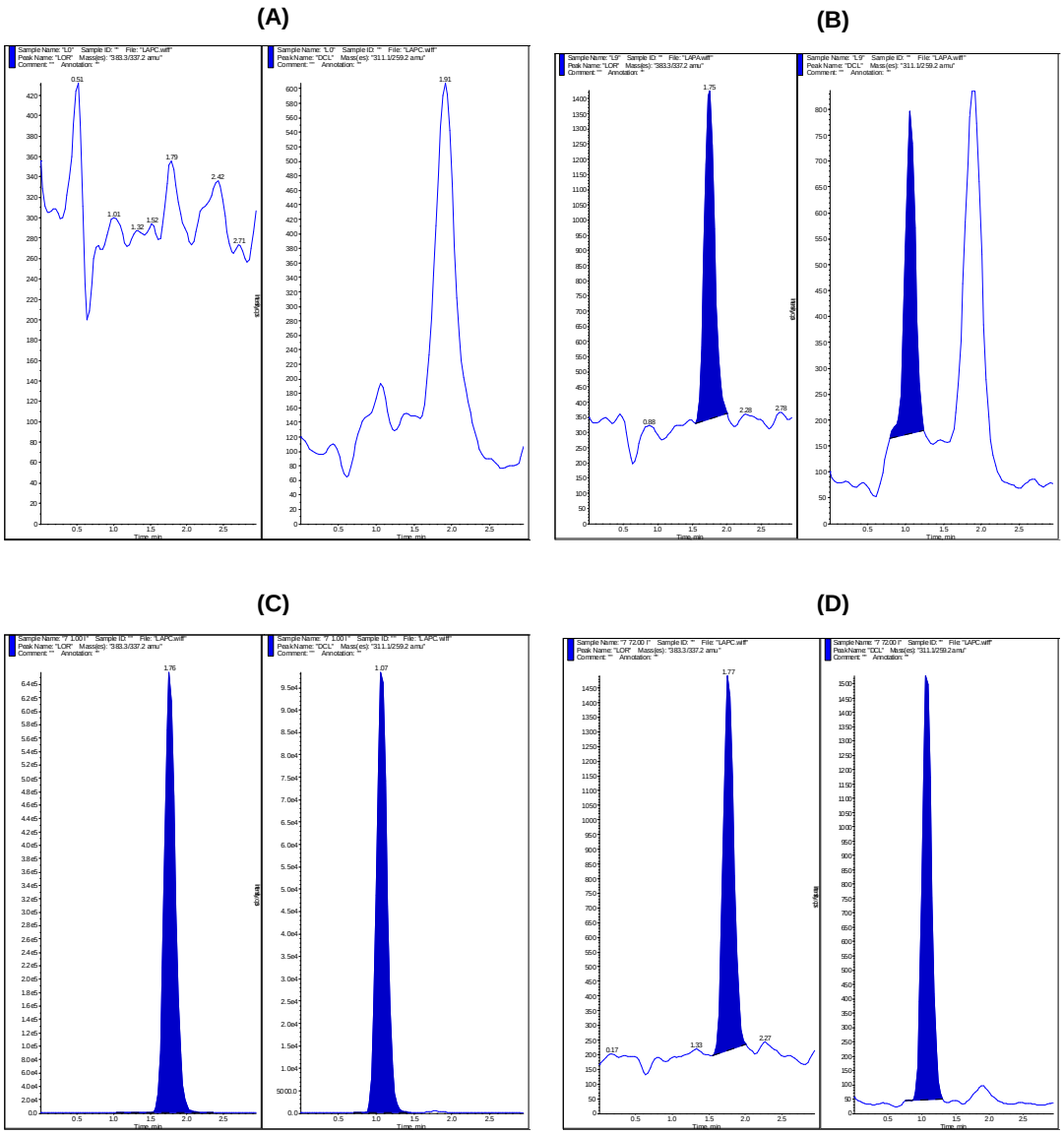
Representative MRM chromatograms for FUR (top) and PRO (IS, bottom) in human plasma: (A) a blank plasma sample; (B) a blank plasma sample spiked with FUR (at LLOQ= 5 ng/mL) and IS; (C) a plasma sample from a patient 12 h after the administration of 40 mg FUR (FUR concentration = 12.4 ng/mL) and (D) a plasma sample from a patient 0.5 h after the administration of 40 mg FUR (FUR concentration = 1156 ng/mL)

Itraconazole Method API 3000™



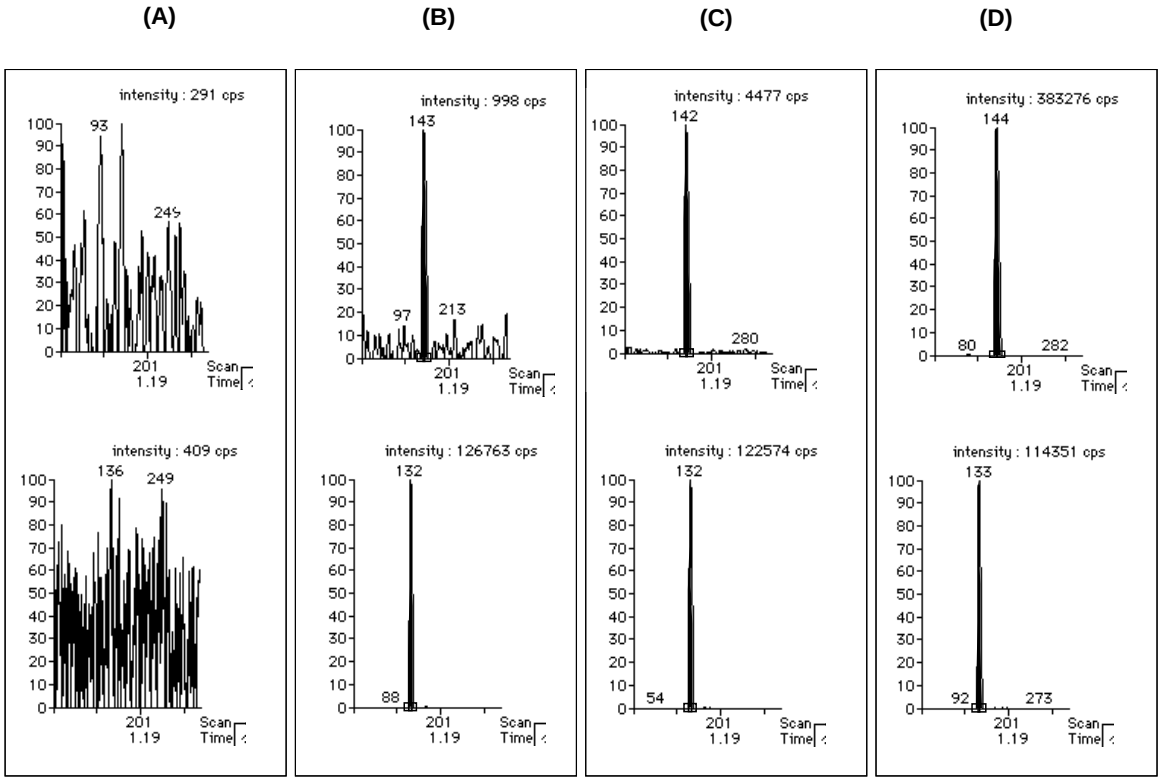
Representative MRM chromatograms for ITR, HYD-ITR and CLA (from top to bottom) in human plasma. (A) a blank plasma sample; (B) a blank plasma sample spiked with ITR and HYD-ITR at the LLOQ (ITR concentration = 3.000 ng/mL, HYD-ITR concentration = 5.000 ng/mL); (C) a plasma sample from a subject 48 h after the administration of 100 mg ITR (ITR concentration = 5.389 ng/mL, HYD-ITR concentration = 8.171 ng/mL) and (D) a plasma sample 4 h after the administration of 100 mg ITR (ITR concentration = 120.82 ng/mL, HYD-ITR concentration = 196.30 ng/mL).

Loratadine Method API 5000™



Representative MRM chromatograms for LOR (left), DCL (right) in human plasma. (A) a blank plasma sample; (B) a blank plasma sample spiked with LOR and DCL at the LLOQ (LOR, DCL concentration = 0.0100 ng/mL); (C) a plasma sample from a healthy volunteer 1.0 h after the administration of 10 mg LOR (LOR concentration = 6.91 ng/mL, DCL concentration = 1.90 ng/mL) and (D) a plasma sample from a healthy volunteer 72 h after the administration of 10 mg LOR (LOR concentration = 0.0129 ng/mL, DCL concentration = 0.0346 ng/mL).

Naproxen Method API 3000™

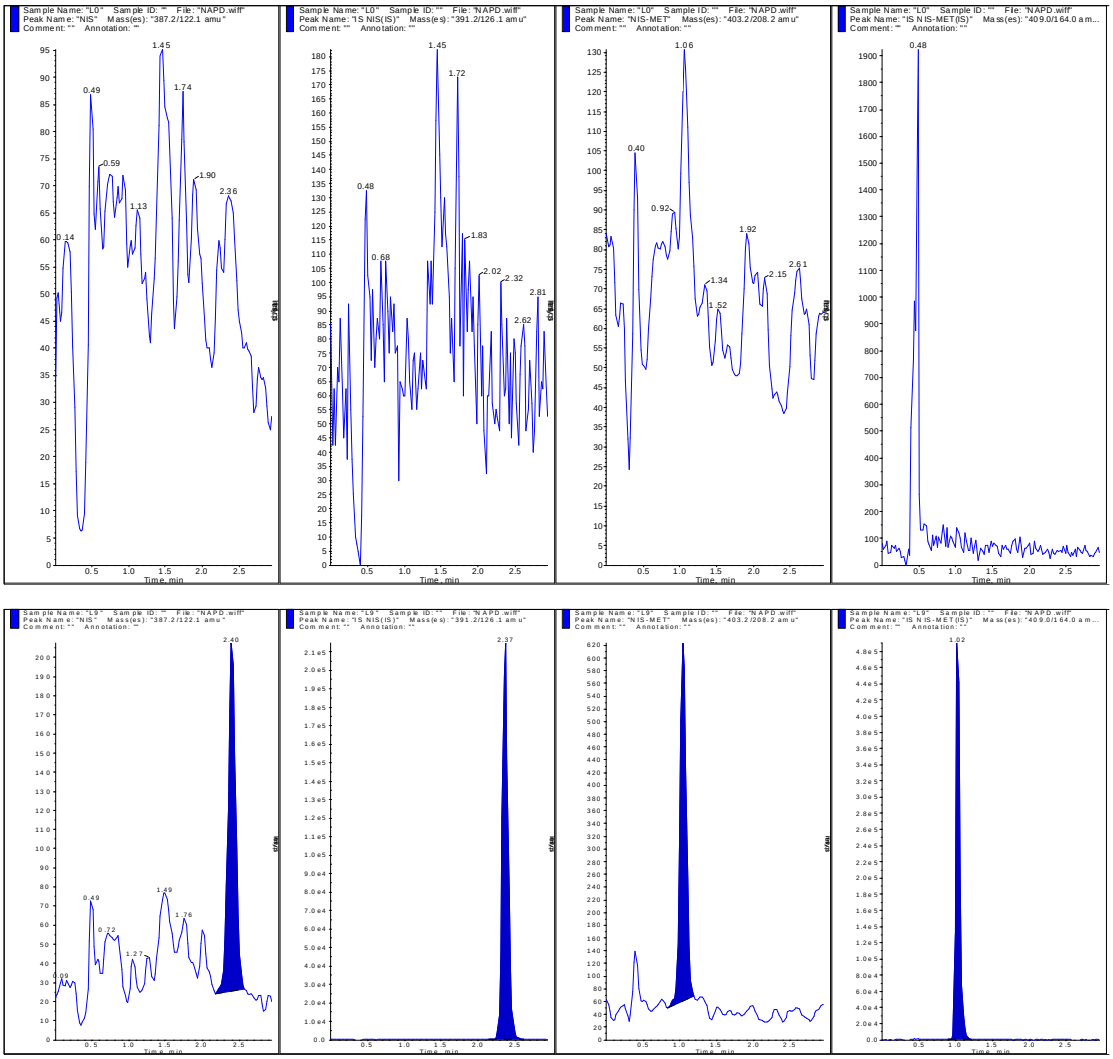


Representative MRM chromatograms for NAP (top) and KEP (IS, bottom) in human plasma. (A) a blank plasma sample; (B) a blank plasma sample spiked with NAP (at LLOQ = 0.100 µg/mL); (C) a plasma sample from a patient 72 h after the administration of 200 mg NAP (NAP concentration= 0.484 µg/mL) and (D) a plasma sample from a patient 0.5 h after the administration of 200 mg NAP (NAP concentration= 44.7 µg/mL).

Nisoldipine Method API 5000™

(A)

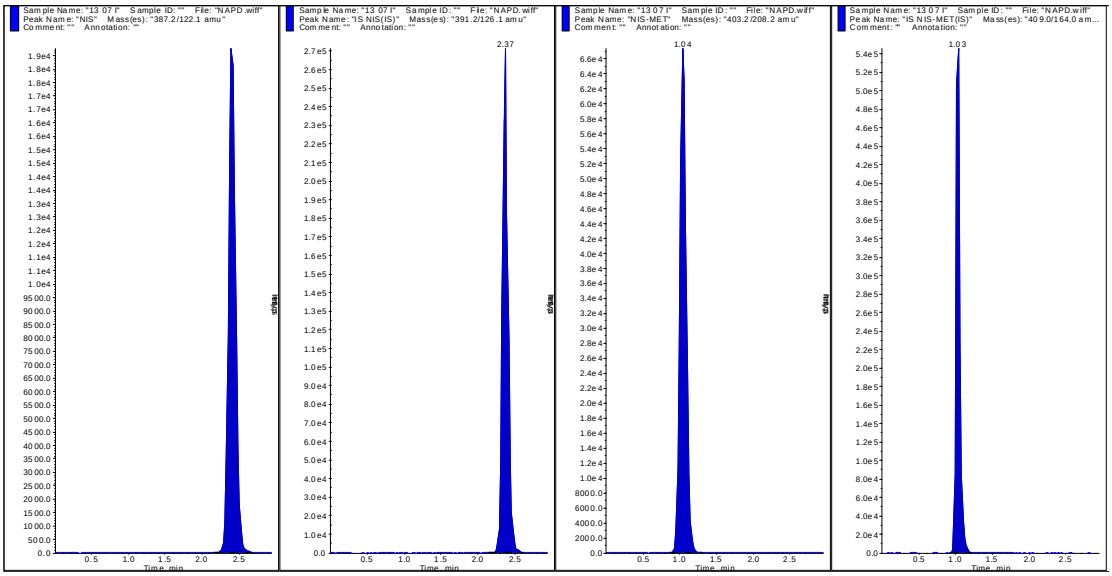
(B)



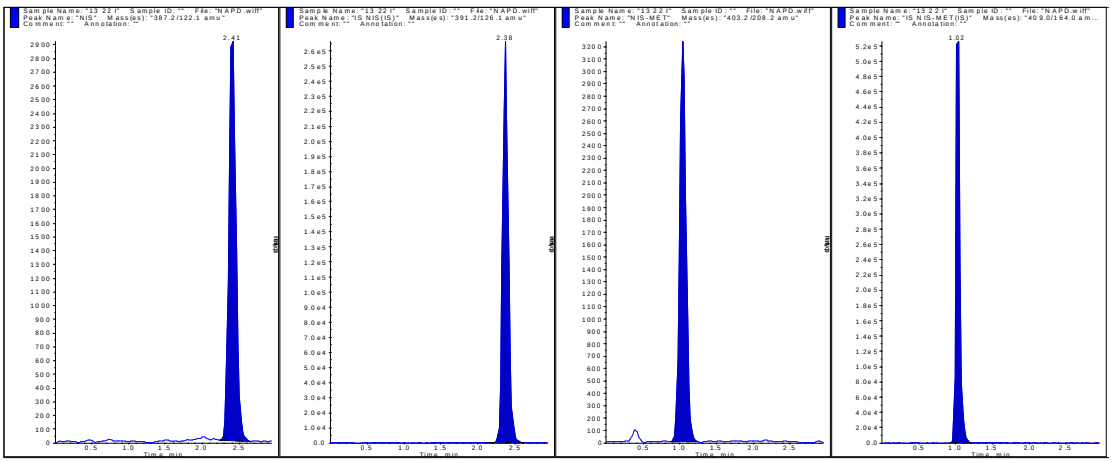
Representative MRM chromatograms for NIS, d4-NIS, 4-HYD-NIS and d6-4-HYD-NIS (left to right) in human plasma. (A) a blank plasma sample and (B) a blank plasma sample spiked with NIS and NIS-MET at the LLOQ (NIS, NIS-MET concentration = 0.005 ng/mL)

Nisoldipine Method API 5000™ continued

(C)



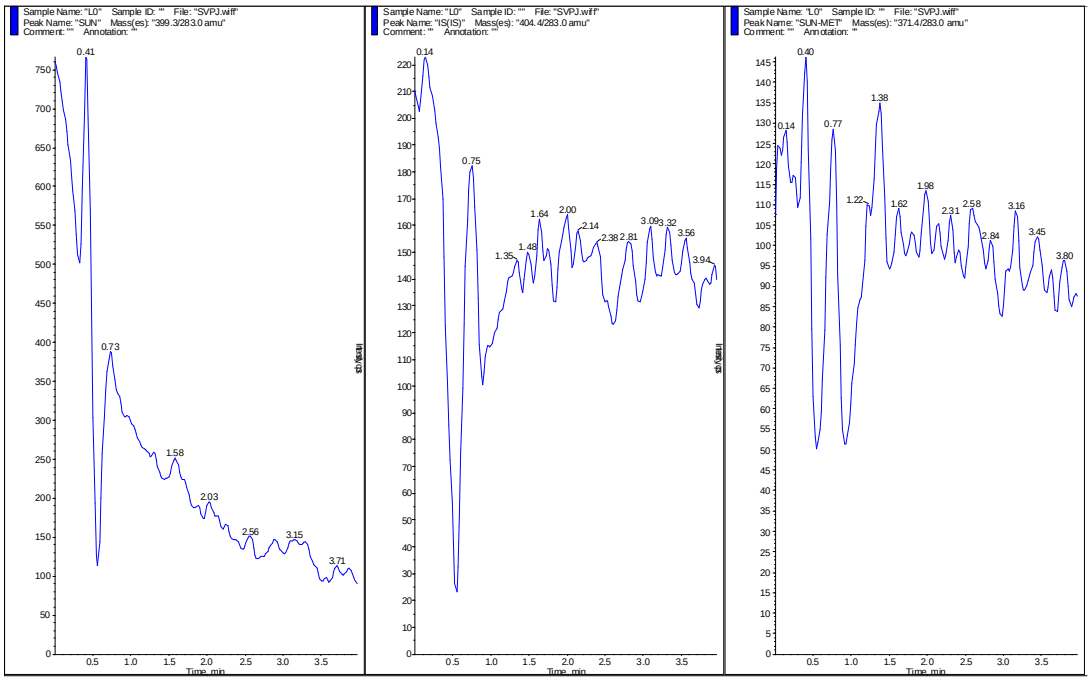
(D)



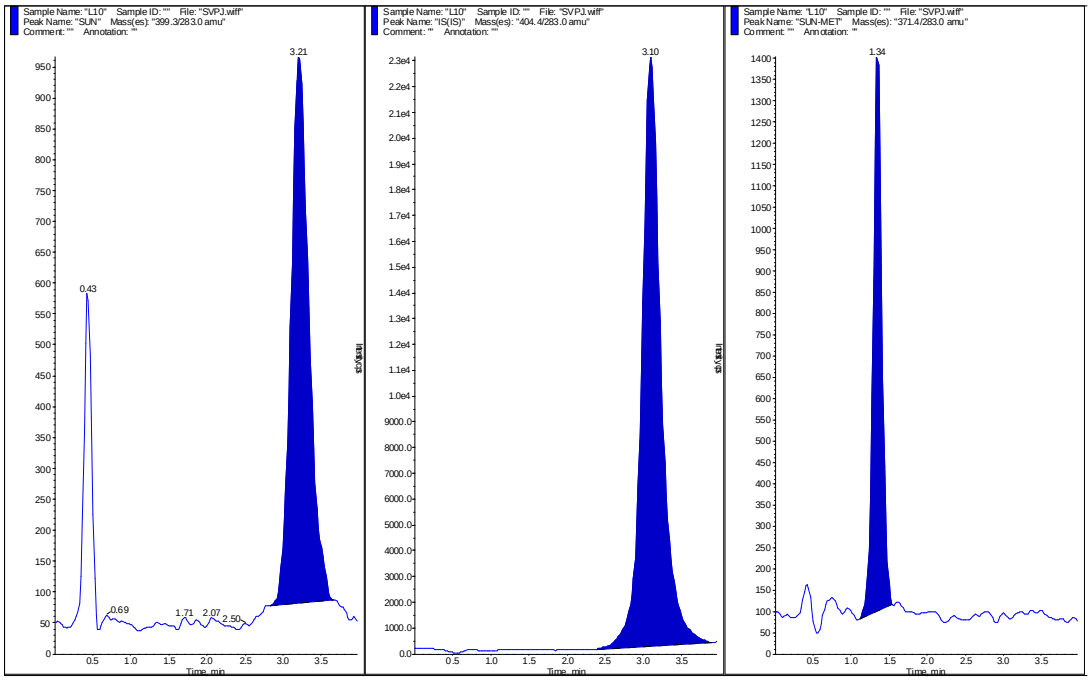
Representative MRM chromatograms for NIS, d4-NIS, 4-HYD-NIS and d6-4-HYD-NIS (left to right) in human plasma. (C) a plasma sample from a healthy volunteer 3.0 h after the administration of 10 mg NIS (NIS concentration = 0.438 ng/mL, 4-HYD-NIS concentration = 0.569 ng/mL) and (D) a plasma sample from a healthy volunteer 48 h after the administration of 10 mg NIS (NIS concentration = 0.0685 ng/mL, 4-HYD-NIS concentration = 0.0277 ng/mL).

Sunitinib Method API 5000™

(A)



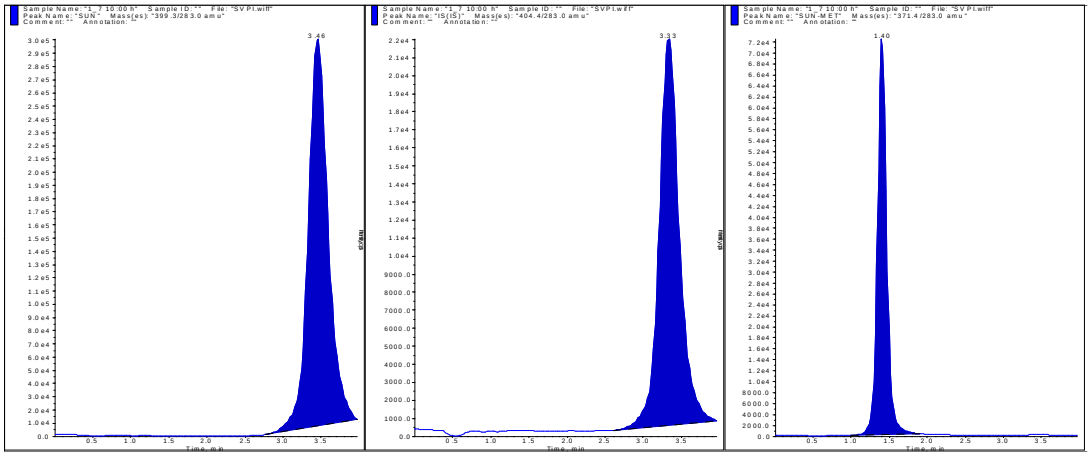
(B)



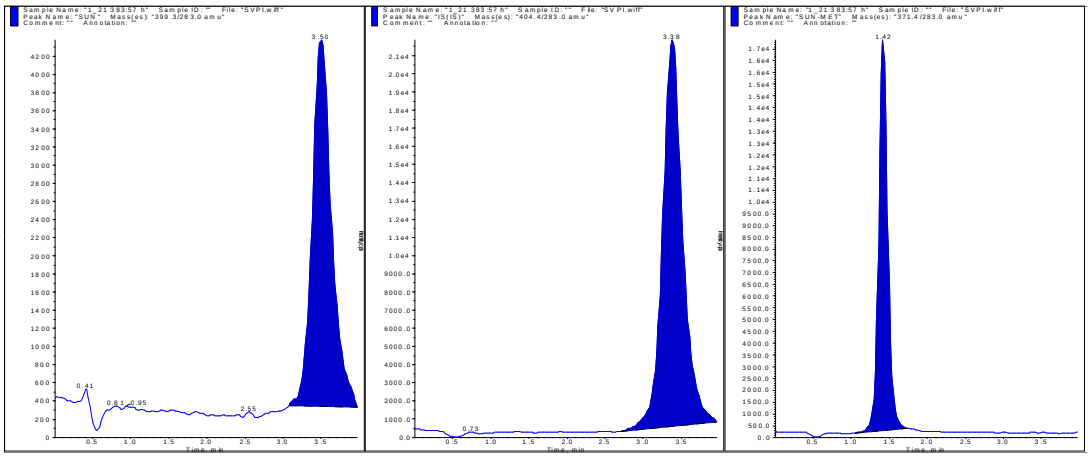
Representative MRM chromatograms for SUN, d5-SUN and DES-SUN (left to right) in human plasma. (A) a blank plasma sample and (B) a blank plasma sample spiked with SUN and DES-SUN at the LLOQ (SUN, DES-SUN concentration = 0.06 ng/mL)

Sunitinib Method API 5000™ continued

(C)



(D)

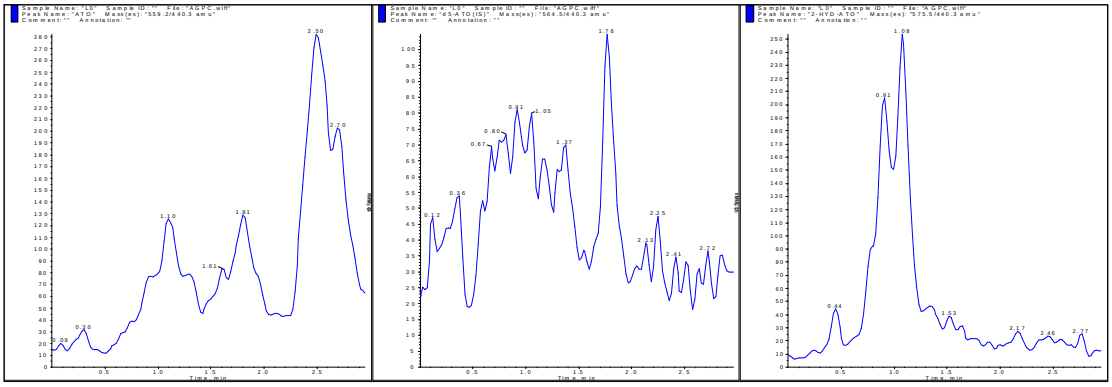


Representative MRM chromatograms for SUN, d5-SUN and SUN-MET (left to right) in human plasma. (C) a plasma sample from a healthy volunteer 10 h after the administration of 50 mg SUN (SUN concentration = 23.9 ng/mL, DES-SUN concentration = 3.60 ng/mL) and (D) a plasma sample from a healthy volunteer 336 h after the last of three daily 50 mg SUN doses (SUN concentration = 0.329 ng/mL, DES-SUN concentration = 0.822 ng/mL).

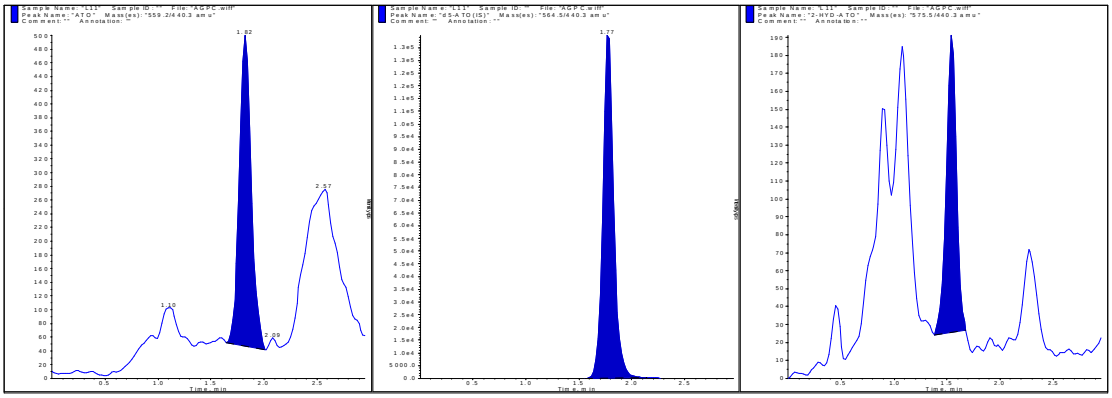
10.3. Chromatograms - Partial Validation

Atorvastatin Method API 5000™

(A)



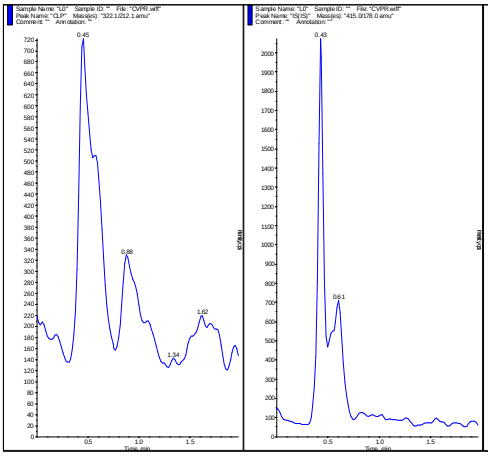
(B)



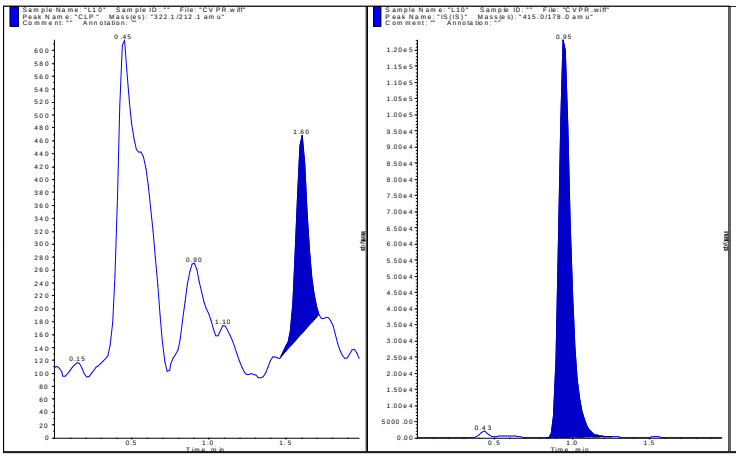
Representative MRM chromatograms for ATO, d5-ATO and 2-HYD-ATO (left to right) in human plasma. (A) a blank plasma sample and (B) a blank plasma sample spiked with ATO and 2-HYD-ATO at the LLOQ (ATO, 2-HYD-ATO concentration = 0.025 ng/mL)

Clopidogrel Method API 5000™

(A)

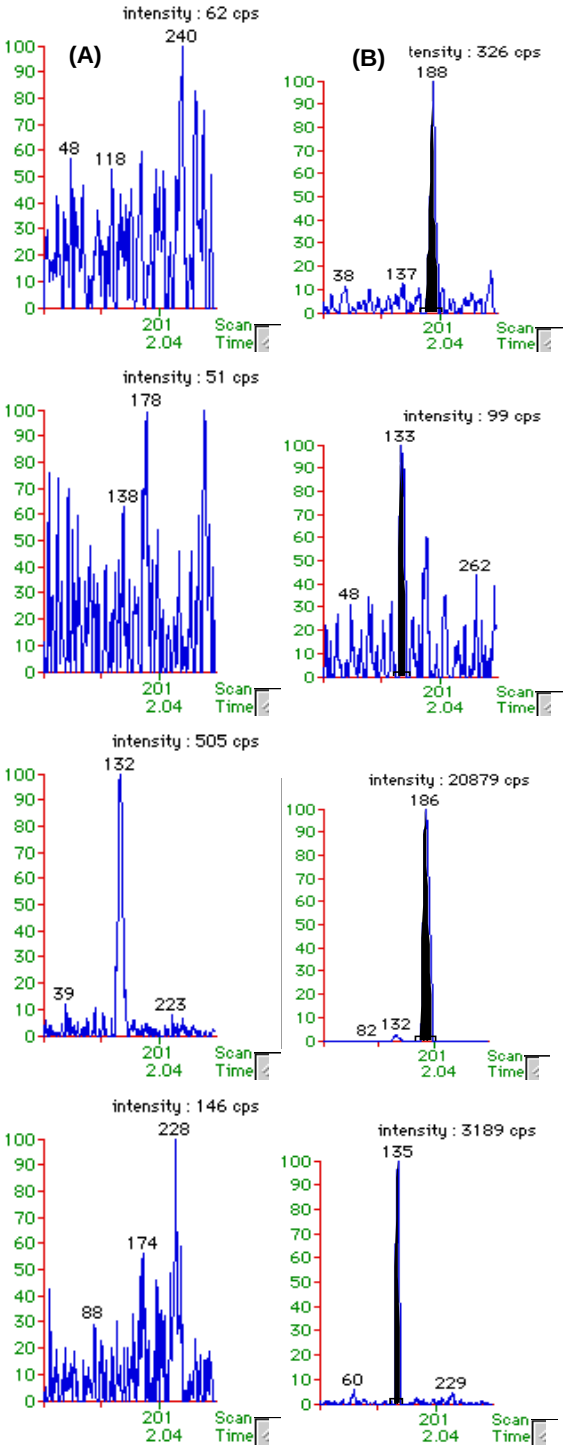


(B)



Representative MRM chromatograms for CLP and DIL (left and right) in human plasma. (A) a blank plasma sample and (B) a blank plasma sample spiked with CLP at the LLOQ (CLP concentration = 0.001 ng/mL)

Loratadine Method API 3000™



Representative MRM chromatograms for LOR, DCL, d4-LOR and d4-DCL (from top to bottom) in human plasma. (A) a blank plasma sample and (B) a blank plasma sample spiked with LOR and DCL at the LLOQ (LOR concentration = 0.100 ng/mL, DCL concentration = 0.150 ng/mL)

10.4. Statistical Evaluation of the Stability Experiments

Atorvastatin

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	49.92	2.0 h	102.90	100.82	105.03	x
		4.0 h	100.09	96.65	103.65	x
	0.2496	2.0 h	103.78	97.69	110.25	x
		4.0 h	104.53	100.82	108.38	x
Post-preparative stability autosampler temperature approximately +4 ℃	49.92	72 h	94.30	92.68	95.95	x
	0.2496	72 h	96.23	93.64	98.89	x
Post-preparative stability approximately -70 ℃	49.92	72 h	93.53	92.59	94.47	x
	0.2496	72 h	99.61	97.42	101.85	x
Freeze-thaw stability	49.92	Once	106.49	104.33	108.69	x
		Twice	94.98	94.04	95.94	x
		Thrice	90.94	88.71	93.22	x
	0.2496	Once	97.04	92.53	101.78	x
		Twice	100.00	97.28	102.79	x
		Thrice	93.12	89.62	96.75	x
Long-term stability at approximately -20 ℃	49.92	8 days	97.55	94.71	100.48	x
		1 month	98.35	95.54	101.24	x
		3 months	101.30	98.47	104.21	x
	0.2496	8 days	94.34	88.28	100.81	x
		1 month	91.54	85.34	98.19	x
		3 months	97.70	91.95	103.80	x
Long-term stability at approximately -70 ℃	49.92	8 days	97.92	95.92	99.97	x
		1 month	98.23	96.54	99.95	x
		3 months	103.15	101.12	105.22	x
	0.2496	8 days	98.42	94.28	102.75	x
		1 month	93.03	90.84	95.27	x
		3 months	100.23	93.66	107.25	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

2-Hydroxyatorvastatin

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	50.11	2.0 h	101.03	96.53	105.74	x
		4.0 h	96.75	93.09	100.56	x
	0.2505	2.0 h	95.60	88.02	103.83	x
		4.0 h	100.93	93.45	109.02	x
Post-preparative stability autosampler temperature approximately +4 ℃	50.11	72 h	99.49	92.68	106.79	x
	0.2505	72 h	102.08	99.06	105.19	x
Post-preparative stability approximately -70 ℃	50.11	72 h	99.82	95.38	104.47	x
						x
	0.2505	72 h	101.06	97.62	104.62	x
						x
Freeze-thaw stability	50.11	Once	105.86	102.65	109.17	x
		Twice	96.90	95.86	97.96	x
		Thrice	96.92	93.24	100.75	x
	0.2505	Once	102.21	98.82	105.72	x
		Twice	102.11	96.29	108.27	x
		Thrice	100.98	94.00	108.49	x
Long-term stability at approximately -20 ℃	50.11	8 days	97.66	94.51	100.92	x
		1 month	105.49	99.69	111.63	x
		3 months	94.19	90.45	98.09	x
	0.2505	8 days	103.43	100.36	106.60	x
		1 month	102.74	95.49	110.54	x
		3 months	93.26	88.74	98.02	x
Long-term stability at approximately -70 ℃	50.11	8 days	98.32	96.73	99.94	x
		1 month	105.34	102.08	108.71	x
		3 months	96.24	91.15	101.61	x
	0.2505	8 days	107.41	102.31	112.76	x
		1 month	91.70	87.44	96.16	x
		3 months	98.10	90.02	106.90	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

4-Hydroxyatorvastatin

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	50.00	2.0 h	103.48	98.78	108.40	x
		4.0 h	99.49	95.36	103.81	x
	0.2500	2.0 h	100.15	97.10	103.29	x
		4.0 h	95.85	89.10	103.12	x
Post-preparative stability autosampler temperature approximately +4 ℃	50.00	72 h	97.30	90.28	104.86	x
	0.2500	72 h	101.70	95.75	108.04	x
Post-preparative stability approximately -70 ℃	50.00	72 h	98.95	93.34	104.90	x
						x
	0.2500	72 h	100.34	94.56	106.48	x
						x
Freeze-thaw stability	50.00	Once	105.83	101.44	110.42	x
		Twice	92.16	90.98	93.35	x
		Thrice	92.27	87.39	97.42	x
	0.2500	Once	92.56	89.47	95.75	x
		Twice	94.70	88.64	101.17	x
		Thrice	99.16	93.58	105.07	x
Long-term stability at approximately -20 ℃	50.00	8 days	95.65	93.87	97.46	x
		1 month	94.31	89.41	99.49	x
		3 months	76.43	73.87	79.07	°
	0.2500	8 days	96.58	90.81	102.72	x
		1 month	98.20	93.14	103.54	x
		3 months	75.27	69.63	81.37	°
Long-term stability at approximately -70 ℃	50.00	8 days	95.32	94.57	96.08	x
		1 month	94.81	90.70	99.11	x
		3 months	76.54	72.85	80.43	°
	0.2500	8 days	97.51	90.50	105.07	x
		1 month	94.29	86.37	102.93	x
		3 months	70.28	63.84	77.38	°

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.
°: Relevant decrease compared to fresh samples.

Clopidogrel

Stability experiment	Clopidogrel concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	10.0	2.0 h	94.58	89.87	99.53	x
		4.0 h	96.76	89.69	104.40	x
	0.0500	2.0 h	107.03	103.63	110.54	x
		4.0 h	104.29	97.77	111.25	x
Post-preparative stability autosampler temperature approximately +4 ℃	10.0	24 h	94.22	88.43	100.39	x
		96 h	106.60	104.19	109.06	x
	0.0500	24 h	96.88	93.87	99.98	x
		96 h	104.13	98.37	110.24	x
Post-preparative stability approximately -70 ℃	10.0	24 h	98.22	90.17	106.99	x
		96 h	103.19	100.67	105.78	x
	0.0500	24 h	100.79	97.37	104.32	x
		96 h	104.67	101.20	108.25	x
Freeze-thaw stability	10.0	Once	93.91	88.42	99.75	x
		Twice	98.85	97.18	100.55	x
		Thrice	94.20	89.39	99.27	x
	0.0500	Once	97.82	90.56	105.66	x
		Twice	107.95	106.19	109.74	x
		Thrice	95.72	88.91	103.06	x
Long-term stability at approximately -20 ℃	10.0	6 days	98.48	93.73	103.47	x
		1 month	98.81	91.31	106.93	x
		5 months	102.74	101.87	103.62	x
	0.0500	6 days	98.57	92.29	105.28	x
		1 month	96.10	89.46	103.23	x
		5 months	98.99	95.02	103.12	x
Long-term stability at approximately -70 ℃	10.0	6 days	97.89	92.52	103.57	x
		1 month	104.76	101.42	108.22	x
		5 months	100.74	98.28	103.26	x
	0.0500	6 days	101.50	95.31	108.10	x
		1 month	99.41	96.93	101.96	x
		5 months	93.29	90.04	96.66	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Furosemide

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	5000	2.0 h	100.30	98.33	102.31	x
		4.0 h	99.78	96.33	103.35	x
	20	2.0 h	102.23	97.25	107.43	x
		4.0 h	103.40	100.38	106.50	x
Post-preparative stability autosampler temperature approximately +4 ℃	5000	24 h	97.65	95.12	100.24	x
		48 h	106.60	102.83	110.49	x
	20	24 h	103.95	100.04	108.00	x
		48 h	94.13	92.46	95.82	x
Post-preparative stability approximately -70 ℃	5000	24 h	96.15	93.54	98.82	x
		48 h	107.11	103.13	111.23	x
	20	24 h	103.92	101.98	105.90	x
		48 h	99.35	94.84	104.04	x
Freeze-thaw stability	5000	Once	98.63	95.18	102.18	x
		Twice	103.33	99.40	107.40	x
		Thrice	95.65	90.86	100.66	x
	20	Once	107.36	104.29	110.51	x
		Twice	101.35	94.02	109.18	x
		Thrice	101.77	96.37	107.43	x
Long-term stability at approximately -20 ℃	5000	4 days	101.66	101.66	107.54	x
		7 days	93.39	93.39	102.61	x
		1 month	92.04	92.04	106.92	x
	20	4 days	97.93	97.93	107.67	x
		7 days	102.43	102.43	108.57	x
		1 month	94.69	94.69	100.71	x
Long-term stability at approximately -70 ℃	5000	4 days	100.85	100.85	107.79	x
		7 days	96.85	96.85	102.43	x
		1 month	97.80	97.80	107.00	x
	20	4 days	100.71	100.71	109.09	x
		7 days	98.62	98.62	108.98	x
		1 month	93.19	93.19	103.01	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Itraconazole

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	1000	2.0 h	100.48	99.61	101.35	x
		4.0 h	104.02	103.13	104.91	x
	10	2.0 h	101.72	97.58	106.02	x
		4.0 h	99.04	92.73	105.71	x
Post-preparative stability autosampler temperature approximately +4 ℃	1000	24 h	98.99	97.81	100.19	x
		48 h	99.49	97.67	101.33	x
		120 h	95.25	93.48	97.04	x
	10	24 h	97.83	95.15	100.57	x
		48 h	96.99	93.69	100.39	x
		120 h	99.76	96.84	102.76	x
Post-preparative stability approximately -70 ℃	1000	24 h	97.60	96.93	98.27	x
		48 h	100.71	99.23	102.21	x
		120 h	94.50	93.82	95.18	x
	10	24 h	96.03	92.81	99.35	x
		48 h	96.71	94.96	98.48	x
		120 h	102.18	98.41	106.07	x
Freeze-thaw stability	1000	Once	104.22	103.22	105.22	x
		Twice	101.55	99.77	103.36	x
		Thrice	99.53	99.10	99.96	x
	10	Once	103.94	98.87	109.25	x
		Twice	101.53	97.19	106.04	x
		Thrice	101.77	99.85	103.71	x
Long-term stability at approximately -20 ℃	1000	3 days	99.91	98.91	100.92	x
		1 month	103.72	101.54	105.94	x
	10	3 days	99.97	97.15	102.86	x
		1 month	96.61	93.48	99.84	x
Long-term stability at approximately -70 ℃	1000	3 days	98.90	96.87	100.95	x
		1 month	103.70	100.62	106.86	x
	10	3 days	104.68	99.31	110.30	x
		1 month	100.60	95.35	106.10	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Hydroxyitraconazole

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	1000	2.0 h	98.49	97.06	99.94	x
		4.0 h	99.62	98.87	100.37	x
	10	2.0 h	99.64	93.81	108.43	x
		4.0 h	100.89	95.48	103.96	x
Post-preparative stability autosampler temperature approximately +4 ℃	1000	24 h	96.68	92.92	100.56	x
		48 h	104.12	102.11	106.17	x
		120 h	97.66	92.62	102.94	x
	10	24 h	100.84	94.91	107.09	x
		48 h	101.50	95.44	107.88	x
		120 h	98.24	92.24	104.56	x
Post-preparative stability approximately -70 ℃	1000	24 h	96.18	92.51	99.97	x
		48 h	104.79	103.35	106.25	x
		120 h	95.31	94.24	96.40	x
	10	24 h	97.57	92.30	103.10	x
		48 h	101.19	95.80	106.84	x
		120 h	95.78	91.75	99.97	x
Freeze-thaw stability	1000	Once	100.10	98.31	101.91	x
		Twice	99.69	98.07	101.33	x
		Thrice	91.40	90.52	92.29	x
	10	Once	99.99	95.79	104.35	x
		Twice	100.66	96.35	105.14	x
		Thrice	98.88	95.22	102.65	x
Long-term stability at approximately -20 ℃	1000	3 days	99.37	98.29	100.47	x
		1 month	97.94	94.25	101.77	x
	10	3 days	99.26	96.27	102.32	x
		1 month	104.02	100.90	107.22	x
Long-term stability at approximately -70 ℃	1000	3 days	98.46	96.99	99.95	x
		1 month	96.98	94.06	99.98	x
	10	3 days	102.43	99.05	105.91	x
		1 month	102.10	99.02	105.26	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Loratadine

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	15.0	2.0 h	95.23	94.18	96.30	x
		4.0 h	95.53	93.70	97.40	x
	0.0250	2.0 h	101.91	99.31	104.58	x
		4.0 h	99.91	96.80	103.11	x
Post-preparative stability autosampler temperature approximately +4 ℃	15.0	24 h	95.06	91.59	98.65	x
		48 h	94.97	92.47	97.53	x
		120 h	96.38	95.55	97.22	x
	0.0250	24 h	97.24	94.77	99.78	x
		48 h	96.23	94.38	98.11	x
		120 h	100.45	98.38	102.57	x
Post-preparative stability approximately -70 ℃	15.0	24 h	95.03	94.45	95.61	x
		48 h	95.32	94.31	96.34	x
		120 h	96.08	95.17	97.00	x
	0.0250	24 h	101.00	98.74	103.31	x
		48 h	96.40	92.12	100.88	x
		120 h	99.14	96.35	102.01	x
Freeze-thaw stability	15.0	Once	96.09	94.93	97.28	x
		Twice	96.44	95.19	97.70	x
		Thrice	96.48	94.50	98.51	x
	0.0250	Once	99.64	97.95	101.36	x
		Twice	99.97	96.45	103.61	x
		Thrice	99.42	96.83	102.08	x
Long-term stability at approximately -20 ℃	15.0	5 days	96.65	94.00	99.37	x
		1 month	100.00	96.24	103.92	x
		3 months	90.32	89.69	90.96	x
	0.0250	5 days	102.44	100.90	104.00	x
		1 month	103.51	98.48	108.79	x
		3 months	91.36	89.35	93.41	x
Long-term stability at approximately -70 ℃	15.0	5 days	97.11	95.64	98.61	x
		1 month	101.08	100.63	101.54	x
		3 months	89.59	88.78	90.40	x
	0.0250	5 days	99.43	95.55	103.46	x
		1 month	106.81	103.84	109.85	x
		3 months	94.12	92.41	95.86	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Descarboethoxyloratadine

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	15.0	2.0 h	98.80	97.39	100.23	x
		4.0 h	99.93	98.13	101.77	x
	0.0250	2.0 h	101.38	96.50	106.50	x
		4.0 h	104.37	101.80	107.00	x
Post-preparative stability autosampler temperature approximately +4 ℃	15.0	24 h	97.36	92.67	102.27	x
		48 h	94.95	92.96	96.98	x
		120 h	95.90	94.56	97.26	x
	0.0250	24 h	97.86	94.78	101.04	x
		48 h	91.54	86.73	96.62	x
		120 h	98.03	91.85	104.63	x
Post-preparative stability approximately -70 ℃	15.0	24 h	98.16	97.22	99.10	x
		48 h	94.22	92.06	96.42	x
		120 h	94.83	93.07	96.63	x
	0.0250	24 h	100.41	97.16	103.76	x
		48 h	91.11	89.01	93.26	x
		120 h	101.98	97.74	106.41	x
Freeze-thaw stability	15.0	Once	98.94	97.63	100.27	x
		Twice	98.54	96.94	100.17	x
		Thrice	98.26	96.17	100.39	x
	0.0250	Once	97.96	95.57	100.41	x
		Twice	101.99	92.99	111.85	x
		Thrice	99.54	94.41	104.94	x
Long-term stability at approximately -20 ℃	15.0	5 days	99.57	97.29	101.90	x
		1 month	102.57	99.15	106.12	x
		3 months	97.33	96.86	97.80	x
	0.0250	5 days	105.09	100.51	109.88	x
		1 month	106.06	100.89	111.51	x
		3 months	97.92	94.64	101.31	x
Long-term stability at approximately -70 ℃	15.0	5 days	98.95	97.21	100.73	x
		1 month	103.94	102.35	105.56	x
		3 months	98.82	98.64	98.99	x
	0.0250	5 days	98.25	94.20	102.48	x
		1 month	106.43	103.98	108.95	x
		3 months	102.83	96.53	109.52	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Naproxen

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	50000	2.0 h	96.06	93.95	98.23	x
		4.0 h	97.11	95.63	98.62	x
	300	2.0 h	103.82	98.96	108.91	x
		4.0 h	102.16	96.57	108.08	x
Post-preparative stability autosampler temperature approximately +4 ℃	50000	24 h	94.62	93.34	95.92	x
		48 h	104.74	103.31	106.20	x
		72 h	107.10	105.61	108.61	x
	300	24 h	95.88	90.40	101.69	x
		48 h	96.47	94.37	98.62	x
		72 h	100.07	97.70	102.49	x
Post-preparative stability approximately -70 ℃	50000	24 h	93.35	92.53	94.17	x
		48 h	105.18	104.21	106.16	x
		72 h	106.66	104.93	108.42	x
	300	24 h	92.65	89.71	95.69	x
		48 h	95.27	92.85	97.74	x
		72 h	100.51	98.34	102.73	x
Freeze-thaw stability	50000	Once	96.28	93.40	99.25	x
		Twice	106.23	105.22	107.25	x
		Thrice	108.65	107.66	109.64	x
	300	Once	98.44	93.83	103.28	x
		Twice	104.60	99.51	109.94	x
		Thrice	106.18	103.56	108.86	x
Long-term stability at approximately -20 ℃	50000	2 days	107.14	104.84	109.48	x
		4 days	98.21	97.09	99.34	x
		3 weeks	107.58	106.12	109.06	x
	300	2 days	100.88	94.53	107.66	x
		4 days	94.68	91.88	97.57	x
		3 weeks	105.25	101.88	108.73	x
Long-term stability at approximately -70 ℃	50000	2 days	107.78	106.70	108.87	x
		4 days	96.69	95.83	97.55	x
		3 weeks	108.41	106.81	110.04	x
	300	2 days	106.97	104.91	109.07	x
		4 days	93.77	90.92	96.71	x
		3 weeks	105.79	104.15	107.45	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Nisoldipine

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	10.0	2.0 h	100.30	99.51	101.09	x
		4.0 h	101.41	97.48	105.50	x
	0.0151	2.0 h	106.12	100.18	112.41	x
		4.0 h	101.44	95.90	107.30	x
Post-preparative stability autosampler temperature approximately +4 ℃	10.0	48 h	100.87	100.16	101.60	x
	0.0151	48 h	103.54	100.30	106.8 9	x
Post-preparative stability approximately -70 ℃	10.0	48 h	99.92	98.74	101.11	x
	0.0151	48 h	101.52	98.47	104.67	x
Freeze-thaw stability	10.0	Once	98.83	97.83	99.84	x
		Twice	99.51	98.42	100.62	x
		Thrice	99.28	98.20	100.38	x
	0.0151	Once	101.50	99.48	103.55	x
		Twice	102.77	98.25	107.49	x
		Thrice	100.36	97.28	103.54	x
Long-term stability at approximately -20 ℃	10.0	2 days	99.17	98.48	99.87	x
		13 days	102.91	98.18	107.87	x
		5 weeks	104.09	103.26	104.93	x
	0.0151	2 days	100.83	99.25	102.43	x
		13 days	103.90	100.30	107.63	x
		5 weeks	103.41	97.77	109.38	x
Long-term stability at approximately -70 ℃	10.0	2 days	99.33	97.94	100.74	x
		13 days	103.82	102.31	105.36	x
		5 weeks	104.00	103.14	104.87	x
	0.0151	2 days	97.59	91.34	104.27	x
		13 days	102.87	97.81	108.19	x
		5 weeks	103.27	100.38	106.25	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

4-Hydroxynisoldipine

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	10.0	2.0 h	94.07	92.41	95.77	x
		4.0 h	95.19	93.57	96.85	x
	0.0150	2.0 h	98.53	93.53	103.81	x
		4.0 h	98.66	95.22	102.22	x
Post-preparative stability autosampler temperature approximately +4 ℃	10.0	48 h	94.96	94.12	95.81	x
	0.0150	48 h	96.47	93.07	99.99	x
Post-preparative stability approximately -70 ℃	10.0	48 h	94.81	93.73	95.90	x
	0.0150	48 h	94.74	91.05	98.57	x
Freeze-thaw stability	10.0	Once	94.44	91.82	97.13	x
		Twice	95.14	93.90	96.39	x
		Thrice	95.58	93.41	97.81	x
	0.0150	Once	96.44	93.74	99.23	x
		Twice	96.53	93.25	99.92	x
		Thrice	96.52	92.98	100.20	x
Long-term stability at approximately -20 ℃	10.0	2 days	95.32	93.45	97.23	x
		13 days	93.31	87.74	99.24	x
		5 weeks	100.43	99.56	101.30	x
	0.0150	2 days	97.62	93.83	101.55	x
		13 days	104.29	102.35	106.26	x
		5 weeks	104.16	99.48	109.05	x
Long-term stability at approximately -70 ℃	10.0	2 days	95.03	93.90	96.18	x
		13 days	95.25	91.24	99.44	x
		5 weeks	98.59	96.99	100.22	x
	0.0150	2 days	96.28	92.65	100.07	x
		13 days	105.86	102.56	109.27	x
		5 weeks	101.59	96.31	107.15	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

Sunitinib

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	100	2.0 h	99.12	98.30	99.94	x
		4.0 h	98.74	98.17	99.31	x
	0.150	2.0 h	99.49	97.15	101.89	x
		4.0 h	102.83	100.24	105.48	x
Post-preparative stability autosampler temperature approximately +4 ℃	100	24 h	92.46	91.82	93.10	x
		48 h	102.36	101.68	103.04	x
	0.150	24 h	98.16	93.44	103.08	x
		48 h	107.93	102.38	113.74	x
Freeze-thaw stability	100	Once	98.88	96.75	101.05	x
		Twice	99.58	97.44	101.76	x
		Thrice	97.52	96.72	98.32	x
	0.150	Once	102.75	100.21	105.35	x
		Twice	100.81	96.05	105.79	x
		Thrice	96.86	92.63	101.25	x
Long-term stability at approximately -20 ℃	100	5 months	98.90	96.11	101.77	x
	0.150	5 months	99.96	96.23	103 .81	x
Long-term stability at approximately -70 ℃	100	5 months	98.77	96.32	101.28	x
	0.150	5 months	98.17	96.46	99. 90	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.

N-Desethylsunitinib

Stability experiment	Concentration (ng/mL)	Time	Point estimator (%)	Lower limit (%)	Upper limit (%)	Sig
Short-term stability	100	2.0 h	101.63	100.37	102.91	x
		4.0 h	101.56	101.05	102.07	x
	0.150	2.0 h	113.20	109.33	117.19	x
		4.0 h	110.07	106.48	113.76	x
Post-preparative stability autosampler temperature approximately +4 ℃	100	24 h	91.95	90.65	93.27	x
		48 h	102.86	102.32	103.40	x
	0.150	24 h	102.49	99.22	105.86	x
		48 h	108.98	105.25	112.83	x
Freeze-thaw stability	100	Once	100.07	96.12	104.16	x
		Twice	100.04	95.83	104.41	x
		Thrice	98.59	96.20	101.04	x
	0.150	Once	101.20	99.15	103.29	x
		Twice	102.95	98.36	107.72	x
		Thrice	98.19	93.28	103.32	x
Long-term stability at approximately -20 ℃	100	5 months	97.58	91.84	103.64	x
	0.150	5 months	97.91	96.67	99.17	x
Long-term stability at approximately -70 ℃	100	5 months	98.91	93.35	104.77	x
	0.150	5 months	98.49	95.28	101.80	x

Sig: Significance (Instability was concluded if both the upper and the lower limit of the confidence interval were greater than -10%).
x: Not a relevant decrease compared to fresh samples.