Development and validation of LC-MS/MS methods to determine PK/PD parameters of anti-infectives

Dissertation

zur Erlangung des naturwissenschaftlichen Doktorgrades
der Julius-Maximilians-Universität Würzburg

vorgelegt von
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Würzburg 2014

Eingereicht bei der Fakultät für Chemie und Pharmazie am

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Doktorurkunde ausgehändigt am

_____

Diese Arbeit wurde am Institut für Biomedizinische und Pharmazeutische Forschung in Nürnberg-Heroldsberg angefertigt.

This thesis has been accomplished at the Institute for Biomedical and Pharmaceutical Research in Nürnberg-Heroldsberg.

Acknowledgement

This thesis was accomplished under the supervision of Professor Dr Fritz Sörgel, Director of the Institute for Biomedical and Pharmaceutical Research (IBMP) in Nürnberg-Heroldsberg and Professor Dr Ulrike Holzgrabe, Chairman of Pharmaceutical Chemistry at the University of Würzburg.

First and foremost, I greatly thank Professor Dr Fritz Sörgel for the assignment of this scientific topic, the provision of the excellent technical equipment, his valuable mentorship and promotion through all the years and during the work of this thesis.
Warmest thanks also go to Professor Dr Ulrike Holzgrabe for the time spent to support this thesis in a very constructive way.

I am very thankful to Dr Martina Kinzig for the introduction and training in LC-MS/MS, expert discussions in the laboratory and strong support during all lab procedures. That included monitoring my work under the international guidelines of FDA and CPMP (now called EMA guidelines) of that time.

Special thanks to the whole IBMP-Team for their outstanding team spirit and collegiality and for their competent advice and practical help during the pharmacokinetic projects. With so many plasma samples it would not have been possible without them. This allowed me to provide in this thesis rich data on drugs in humans helping to improve the quality of life.

The clinical part of this thesis was done by physicians in clinical research centres. I received the plasma samples in the frozen state along with precise information on dose, way of administration and accurate blood sampling. Sincere thanks to the physicians and their medical staff. And not to forget all the healthy volunteers who participated in those studies.

Thank you to my Gymnasium teacher Klaus-Jürgen Heß for his delighting chemistry lessons and his support for the “Jugend forscht”-competition, where we were awarded third in the national competition. That was the beginning of my bioanalytical career. It is very sad to have to accept that he passed away so soon in December of 2012.

Thanks to the “Jugend forscht”-Organization and “Studienstiftung des deutschen Volkes” for supporting me in the program of the promotion of young scientists.

Last, but not least I thank all my family for their stable backing and solid support. I hope I can return this one time.

Meiner Familie

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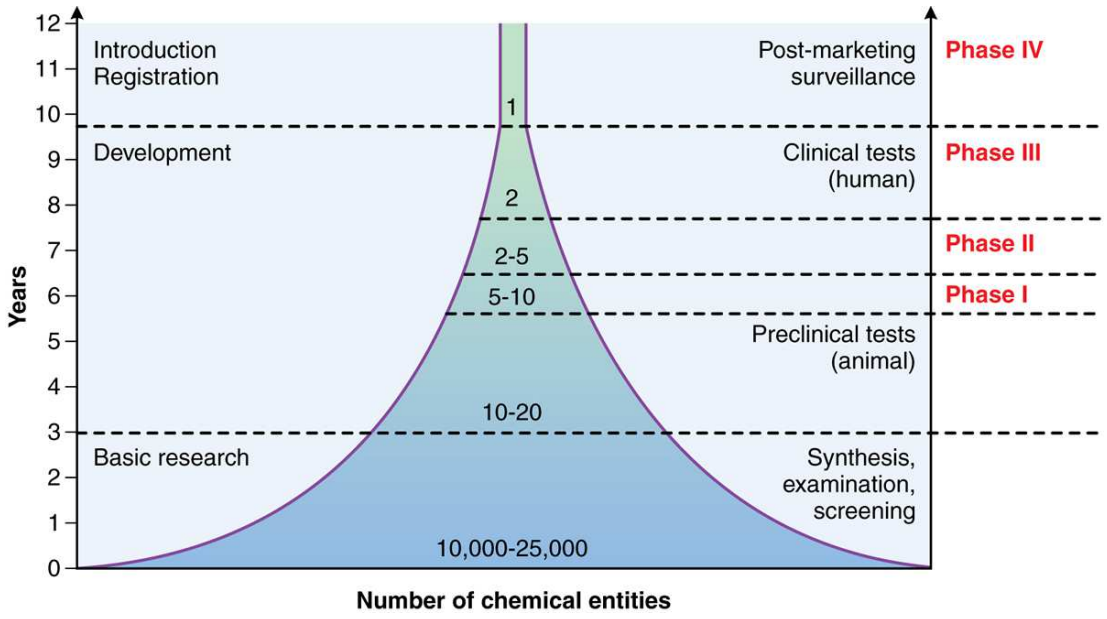
1 INTRODUCTION

1.1 Background on drug development

Drug development includes the long way from screening, synthesis and characterization of thousands of chemical entities towards interaction with a given drug target, pre-clinical tests in animals on efficacy, pharmacology and toxicity, until a variety of clinical trials in human beings. See Figure 1.

Figure 1 *The phases, time lines, and attrition that characterize the invention of new drugs, reprinted by permission from The McGraw-Hill Companies, Inc: Goodman & Gilman's The Pharmacological Basis of Therapeutics by L. Brunton and B. Chabner [1]*



Typically, clinical trials are conducted in four phases: In Phase I (First in Human) the potential new drug is given to a small number of healthy volunteers to assess pharmacokinetics, safety and tolerability. Phase II includes clinical trials in subjects and in a small number of patients to study efficacy and dosing, while during Phase III (Multi-Site) studies hundreds of patients in a large number of clinical sites are evaluated to confirm the efficacy in a large population. Before the first clinical trial can start and before marketing the drug (when the Phase III trials are finished) an application containing all important data of previous investigations must be filed to the appropriate regulatory agency, e.g. Food and Drug Administration (FDA) in USA, European Medicines Agency (EMA) or national institutions, e.g. Federal Institute for

Drugs and Medical Devices (BfArM) in Germany. In the US these applications are called IND (investigational new drug) application and NDA (new drug application). After drug approval, Phase IV (Post-Marketing Surveillance) takes place to record adverse events, compliance and drug-drug-interactions in thousands of patients [1].

A parallel application process has been established, not for studying new chemical entities (NCE), but to compare generic or newly formulated drugs to already marketed drugs. This is called ANDA (abbreviated new drug application) and in general these require only bioequivalence (BE) and bioavailability (BA) trials, which are regulated in detail by the FDA and EMA [2-3].

Recently, a new sort of clinical trials has been established: Phase 0 (Microdosing) trials speed up the drug-development process by enabling decisions on which drug-candidate may be the most promising for further development at an early stage of drug development. Those exploratory IND studies explore pharmacokinetic data in humans by giving a subtherapeutical dose to very few healthy subjects. These in first-in-human studies do not answer safety or efficacy questions [4]. Among other reasons this progress was possible due to especially sensitive and selective bioanalytical methods being able to quantitate biofluid concentrations from a dose that is only one percent of the calculated therapeutic dose.

1.2 Pharmacokinetics and Pharmacodynamics

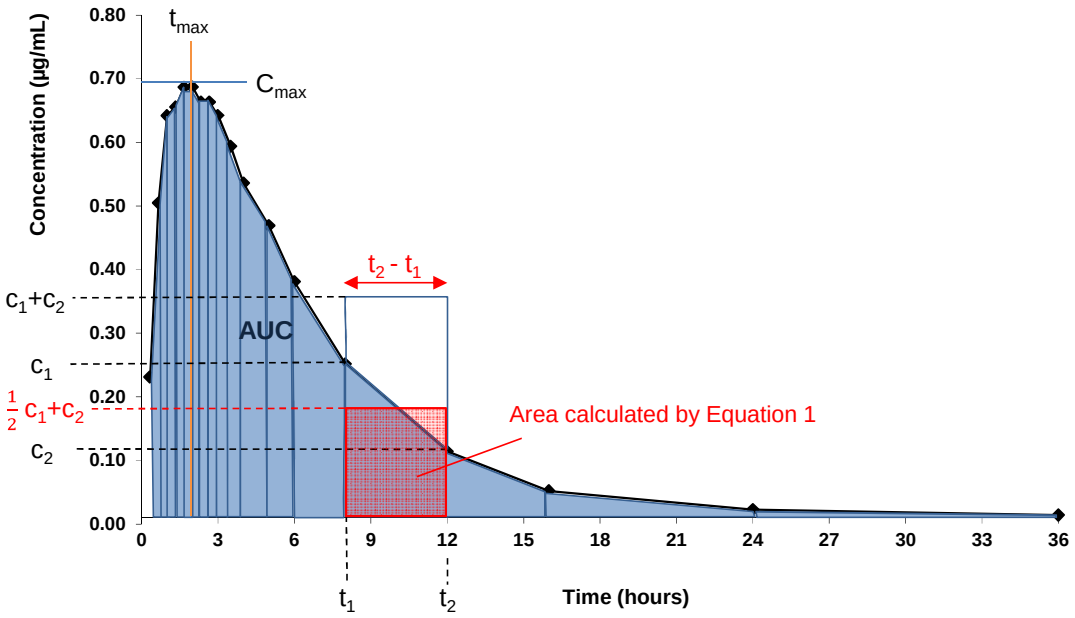
Clinical trials are designed to investigate the pharmacokinetics (PK) and pharmacodynamics (PD) of a potential new drug. While pharmacokinetics are dealing with the absorption, distribution, metabolism and elimination (ADME) of drugs, pharmacodynamics covers the drug's pharmacological and toxic effects.

Pharmacokinetics describe the time course of the drug's and its metabolite's concentration in body fluids and tissues by mathematical equations. As blood is easily available and reflects in many cases the kinetic processes in the organism, plasma or serum concentrations are used as measures of drug levels.

Absorption can be described by three PK parameters: C_{max}, t_{max} and AUC. The maximal plasma concentration (C_{max}, unit e.g. in µg/mL) and the time (t_{max}, unit e.g. in h) when this concentration is reached, can be taken directly from the plasma concentration-time curve. While the amount of absorbed drug is measured by Cmax, the time course of absorption is reflected by the part of the curve before Cmax.

Assuming the same dose, C_{max} is high and t_{max} is short, when drug absorption is quick. When C_{max} is reached, the amount of drug entering the systemic circulation equals the amount of drug being eliminated.

Figure 2 *Determining maximum concentration (C_{max}), time to maximum concentration (t_{max}) and area under the curve (AUC) from the plasma concentration-time curve by the linear trapezoidal rule (data are presented later on page 109)*



The area under the plasma concentration-time curve (AUC, e.g. in µg·h/mL) is a measure for the amount of drug circulating in the organism. The AUC from measured data points starting with the time of administration until the time of the last quantifiable concentration can be easily calculated by the linear trapezoidal rule.

$$AUC_{0\to t} \approx \sum \frac{(t_{i+1} - t_i) \cdot [\, C(t_i) + C(t_{i+1}) \,]}{2} \qquad \textbf{Equation 1}$$

The residual area between the last quantifiable concentration (C_n) until infinity can be extrapolated by assuming a mono-exponential decline of the concentration-time curve. (Determination of k_{el} is discussed later in this chapter.)

$$AUC_{0\to\infty} = \int_0^{\infty} C(t) \cdot dt \quad \approx \sum_{i=1}^{n-1} \frac{(t_{i+1} - t_i) \cdot (C_i + C_{i+1})}{2} + \frac{C_n}{k_{el}} \qquad \textbf{Equation 2}$$

C_{max} and $AUC_{0\to t}$ define the highest and total exposure of a drug in the body as well as the concentration-time curve and therefore are crucial for the decision if two medicinal products are **bioequivalent**. This is the case, if the 90%-confidence

interval for the ratio of the test and reference product is within the acceptance interval of 80-125% [3].

Friedrich Hartmut Dost published 1953 the first text book on pharmacokinetic calculations [5], where he explained that the AUC of a drug, given intravenously (iv) equals the AUC of a drug administered orally (po), if it is absorbed completely into the body. On this basis absolute **bioavailability** is studied:

$$F\,[\%] = \frac{AUC_{po}}{AUC_{iv}} \cdot \frac{Dose_{iv}}{Dose_{po}} \cdot 100 \qquad \textbf{Equation 3}$$

The drug's **Distribution** in the body is expressed as volume of distribution (V, unit e.g. in L), which is a fictive parameter, correlating the dose to the initial concentration (C_0, after bolus injection assuming an immediate distribution over the organism, one compartment):

$$V = \frac{Dose}{C_0} \qquad \left(\text{because} \quad Concentration = \frac{Amount,\ e.g.\ mg}{Volume,\ e.g.\ mL}\right) \qquad \textbf{Equation 4}$$

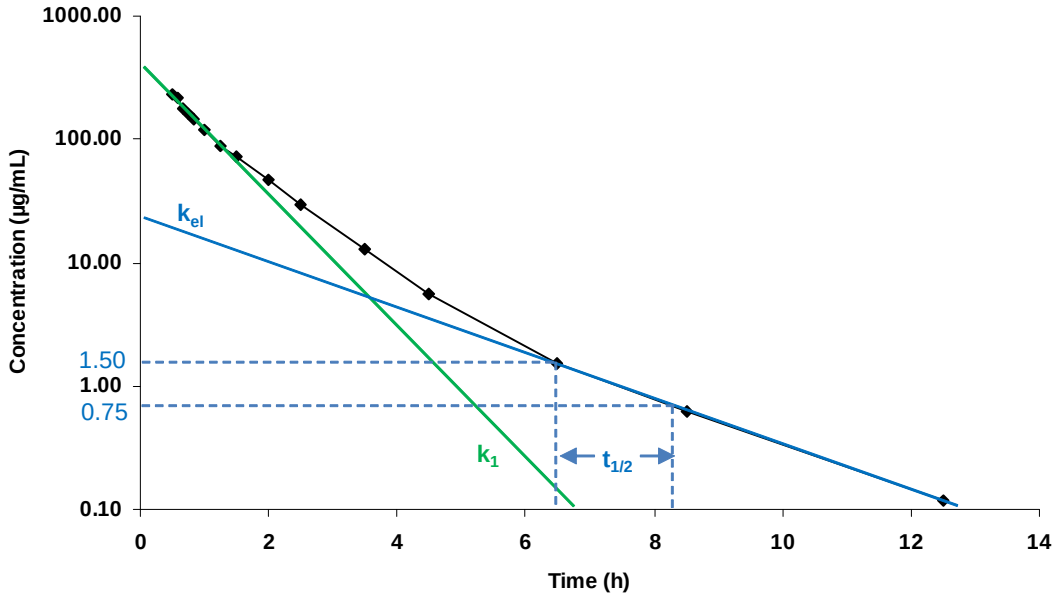
In general, a high volume of distribution indicates a wide distribution of the drug in the body, i. e. organs, tissues, cells, etc. It is influenced by chemical characteristics, and physicochemical properties of the drug. Among those are pKa, solubility, membrane permeability, molecular weight.

The descending part of the plasma concentration-time curve is caused by drug distribution from the blood circulation into body tissues and by drug elimination processes. A sharply falling slope directly after C_{max} indicates either fast distribution, or an early and rapid elimination.

In most cases the drug distributes not immediately and uniformly in the body, but distributes for example faster into heart, liver and lung than into muscle, skin or bone. This is reflected in the log-transformed plasma concentration-time curve after intravenous administration (Figure 3), which descends at first quickly and then slowly. In order to describe such curves mathematically, two (or more) compartment models have been developed, producing operational equations, which are improved by physiologically-based pharmacokinetics (PBPK) [6-8].

A measure for the rate of distribution and elimination is the elimination rate constant (k_1 or k_{el}, unit e.g. 1/h), which is obtained directly from the log-transformed concentration-time by linear regression, see Figure 3:

Figure 3 *Determining the elimination rate constant (k_{el}) from the log-transformed plasma concentration-time curve (which will be presented later on page 161)*



The rate of drug **elimination** can be described by the terminal elimination half-life ($t_{1/2}$, h). Assuming first-order kinetics, $t_{1/2}$ is calculated by the following equation:

$$t_{1/2} = \frac{\ln 2}{k_{el}}$$ **Equation 5**

First-order kinetics can be assumed if absorption, distribution and elimination processes are not saturable. However, many processes exist in an organism [9], that can become saturated, of which the most common saturation is plasma protein binding (PPB) or renal elimination (see below), which affect the linear PK to become non-linear towards dose. PPB may be saturated, when the affinity of a drug to human serum albumin or α_1-glycoprotein and the drug concentration is high, e. g. 10-100 µg/mL plasma [1]. Then, higher free drug concentrations cause V to increase over-proportionally with increasing doses.

A measure for **elimination** is the clearance (CL, mL/min), which defines the volume of blood that is cleared from the drug per unit of time. If elimination processes are not saturated (first-order kinetics), a constant volume of blood is cleared by the organs, and thus, the value of CL is constant over the clinical concentration range.

$$CL_{organ} = \frac{\text{rate of elimination}}{C} = \frac{Q \cdot (C_A - C_V)}{C_A}$$ **Equation 6**

Thus, with known blood flow (Q), venous and arterial drug concentration (C_V and C_A), the clearance of drug eliminating organs (mainly kidney and liver) can be determined and added to yield the total clearance CL_{tot}. As the arterial drug concentration and additional elimination processes in saliva or sweat are difficult to obtain, further equations for clearance have been developed.

Experimentally, CL_{tot} can be determined after a single intravenous dose (complete bioavailability) by the following equation:

$$CL_{tot} = \frac{Dose}{AUC} \qquad \textbf{Equation 7}$$

Accordingly, renal clearance (CL_R) is the volume of blood, which is cleared by the kidneys and can be experimentally determined by the amount of drug excreted in the urine (Ae_∞):

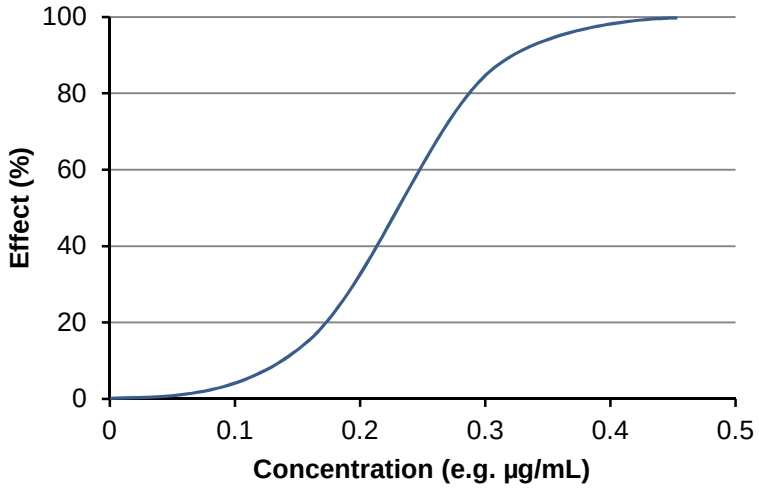
$$CL_R = \frac{Ae_\infty}{AUC} = \frac{Ae_\infty \cdot CL_{tot}}{Dose} \qquad \textbf{Equation 8}$$

Pharmacodynamics is dealing with the effect of drugs on the organism, including the target site, mode of action, resistance mechanisms, potency and efficacy. The mode of action of anti-infectives is for example inhibition of cell wall synthesis (e. g. β-lactams), inhibition of DNA-synthesis (e. g. quinolones), or inhibition of bacterial protein synthesis (e. g. macrolides).

Typically, the effect of antibiotics is evaluated by susceptibility testing of bacterial cultures, which are exposed to different concentrations of the drug. After 18-24 hours of incubation, the minimum inhibitory concentration (MIC) can be assessed [1].

The **PK/PD concept** is originally based on an idea of Eagle et al. [10], who studied in 1950 the effect of the dosing schedule on the efficacy of penicillin. Approximately 50 years later, when systematic studies on various antibiotics were performed, PK/PD became a booming field of research [1, 11-15]. It correlates pharmacokinetics studying concentration-time curves and pharmacodynamics evaluating effect-concentration curves (Figure 4) to effect-concentration-relationships. This makes sense, as the bacteria in a living organism are not subjected to the constant concentration like in a test tube, but to the time course of the concentration curve affecting the efficacy of the antibiotic.

Figure 4 *Typical concentration versus pharmacodynamic effect curve*



Correlating the three main PK parameters to the MIC results in three PK/PD parameters for efficacy:

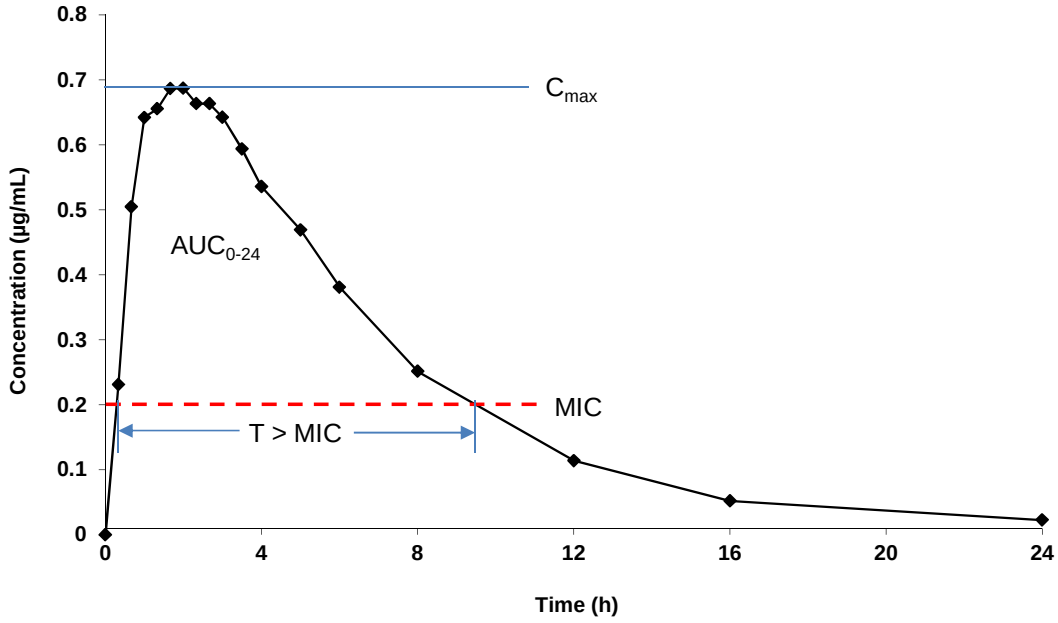
$$\frac{C_{max}}{MIC}; \quad \frac{AUC_{0-24}}{MIC}; \quad T > MIC$$

They can be used for optimising dosing regimens for antibiotics. For example, splitting the single dose every 24 hours into one third of the dose, given every 8 hours, would give the same overall AUC/MIC ratio, but a decreased C_{max}/MIC and in many cases an increased T>MIC.

The endeavour of PK/PD modelling is to find that parameter, which predicts efficacy of a given antibiotic best, giving helpful hints for optimizing treatment and for preventing antimicrobial resistance.

All those calculations and considerations are based on plasma concentration-time curves from measured data points and measured MIC values. Therefore, it is crucial that microbiological and bioanalytical methods, which are intended to generate the data for PK/PD calculations, must fulfil particular requirements, including technical, scientific and also regulatory aspects.

Figure 5 *PK/PD concept for antibiotics (shown on data, which will be presented later on page 109)*



1.3 LC-MS/MS – techniques and progress with time

Mass spectrometry (MS) was born in 1897, when Sir J. J. Thomson discovered the electron, for what he was awarded the Nobel Prize in Physics in 1906. At the beginning of the 20th century he constructed the first mass spectrometer, sorting ions according to their trajectory in the electromagnetic field. Thomson's scholar F. W. Aston (1922 Nobel Prize in Chemistry) and A. J. Dempster improved resolution and developed the first ionisation technique EI (electron ionisation). Chemists sought high resolution mass analyzers, being able to detect elements and small molecules, which led to the development of time-of-flight and quadrupole mass spectrometers. Those could be coupled to gas chromatography (GC) and later in the 1950s to liquid chromatography (LC) by W. Paul, who was awarded the Nobel Prize in Physics in 1989 for his evolutions in ion-trap MS.

Despite high improvements in resolution and quantitative analysis by coupling to liquid chromatography were achieved, in the 1980s still no large molecules could be analyzed as these were fragmented already during ionisation or evaporation. It was only when J. B. Fenn applied the soft ionisation technique ESI (electrospray ionisation) to peptides and F. Hillenkamp, M. Karas and K. Tanaka developed the MALDI technique (matrix assisted laser desorption ionisation), biological

experiments with biological macromolecules became possible. J. B. Fenn and K. Tanaka were awarded with part of the Nobel Prize in Chemistry in 2002 for this work [16].

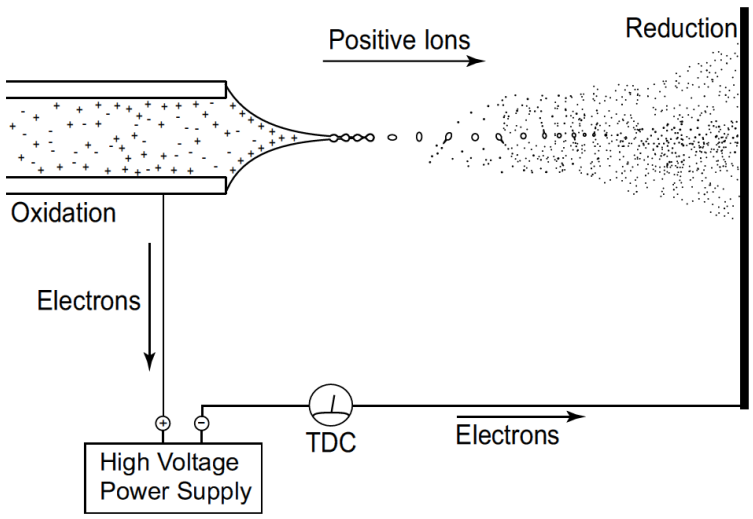
Here, triple-quadrupole mass spectrometers with soft ionisation techniques coupled to liquid chromatography were applied to quantification of pharmaceutical molecules in complex biological matrices.

1.3.1 Ionisation Sources

The ionisation source plays an important role for mass spectrometry as it is responsible for generating detectable ions from the sample solution. A number of ionisation sources for solid and liquid samples are available. The most common techniques for transferring compounds from liquids into the gas phase are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). As they are soft ionisation techniques they are widely used for the analysis of pharmaceuticals.

Electrospray ionisation (ESI) is the most common ionisation technique for (bio)-pharmaceuticals. It was invented by M. Dole [17] and first used for bio-molecules by J. B. Fenn. The analyte solution from the LC is sprayed (vaporised) through a capillary with a voltage of ±2-5 kV with the goal to produce gas phase ions to enter the mass analyzer. ESI requires already in solution protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecular ions.

Figure 6 *Mechanism of electrospray ionisation (ESI), reprinted by permission from John Wiley and Sons: Mass spectrometry reviews [18]*

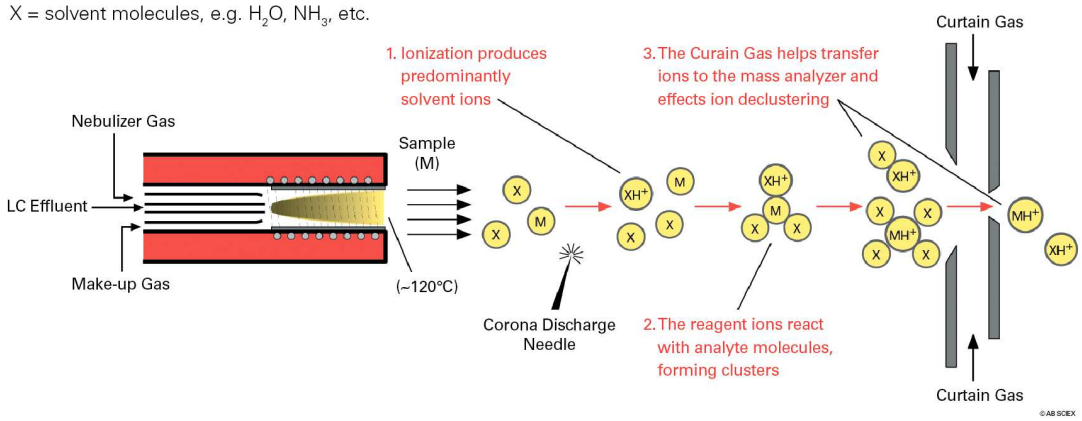


By evaporation of solvent molecules, the droplets shrink until the charge repulsion is high enough for Coulomb explosion. These smaller droplets run through the same process until smallest charged droplets are formed. Still, research is ongoing how the gaseous phase ions are formed from these last highly charged droplets. Two different mechanisms have been postulated: the ion evaporation model (IEM) first linked to the names of Iribarne and Thomson [19-21], and the charged residue model (CRM) first postulated by Dole et al. [17, 22]. Kebarle et al. [18, 23] summarized the mechanisms and concluded, that IEM is supposed for small ions, while the CRM is more plausible for large macromolecules.

As the analytes need to be charged already in solution, this technique is suitable for moderate polar to polar compounds. ESI is able to produce multiply charged ions, useful for the analysis of peptides and proteins. Moreover, the technique allows the use of very low flow rates, enabling the analysis of samples with less sample volume (e. g. proteins). Here, a TurboIonSpray® interface was used, which facilitates desolvation by a heated inert gas.

Non-polar compounds often show poor sensitivity after ESI and are much more accessible using APCI.

Figure 7 *Mechanism of atmospheric pressure chemical ionisation (APCI), reprinted by permission from AB Sciex Pte. Ltd.*



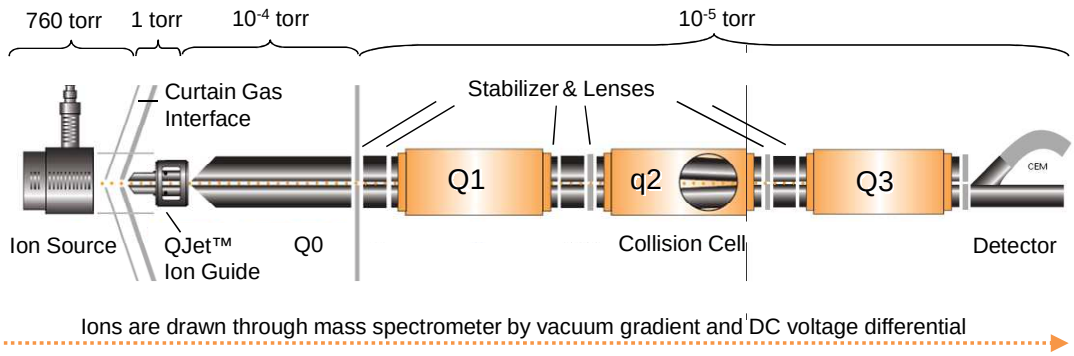
Atmospheric pressure chemical ionisation (APCI) is slightly different from ESI. The compounds need not to be charged in solution, which is vaporized in a quartz tube (temperatures between 300 and 500 °C), since they are ionized by primary ions from solvent molecules or from air. Primary ions are generated on a corona discharge needle with a current of ±3-5 µA and can be $CH_3OH_2^+$, H_3O^+, OH^-, CH_3O^-, CH_3CNH^+ from solvent molecules or e. g. O_2^+, O_2^- from air. Ionisation of the analyte takes place via charge transfer or proton transfer from the primary ion onto the

analyte. However, also adducts may be formed of the analyte with NH_4^+ or Na^+ from LC modifiers, which are often not reproducible enough for a sound quantification of pharmaceuticals in plasma samples from a high number of individuals.

1.3.2 Triple quadrupole technology

Molecular ions, produced in the ion source at atmospheric pressure (760 torr, 1013 mbar), enter the first region of the mass spectrometer via a small orifice; here through a so-called “curtain gas” (inert gas), which assists declustering from solvent molecules. This part of the mass spectrometer is evacuated by a first turbo pump and a backing pump to ensure a high vacuum and avoid collisions with other molecules. In this region, the molecular beam is focused and led through a first quadrupole, the Q0, see Figure 8. Focussing in this first region is achieved in different ways in different mass spectrometers, see chapter 1.3.3. The actual mass analysis takes place in the high vacuum region (10^{-5} torr), which is evacuated by a second turbo pump and backing pump. Additional stabilizers and lenses are involved to keep the ions on their path towards the detector, which is in this case a channel electron multiplier. Two main principles accelerate the ions into and through the mass spectrometer: the vacuum gradient and the DC voltage differential, see Figure 8.

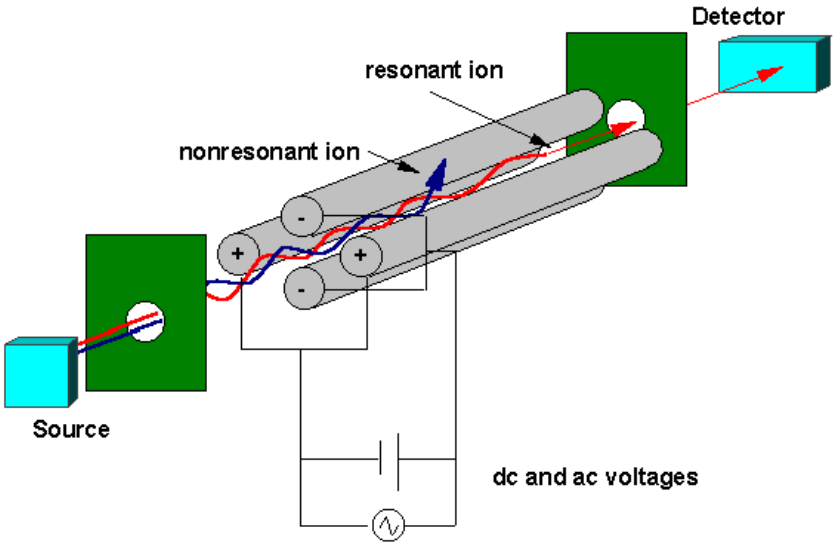
Figure 8 *Schematic ion path of the triple quadrupole mass spectrometer API 5000™ LC-MS/MS system, adapted from AB Sciex Pte. Ltd.*



Quadrupole mass spectrometers are also called mass filters, as they have the ability to selectively filter ions with a given mass-to-charge ratio (m/z) by a combination of direct current (DC) and alternating current (AC), applied to each quadrupole. A single quadrupole consists of four parallel metal rods (Figure 9), of which the two opposite ones are equally charged. The potential of opposite rods is ±(U + Vcos(wt)), where

U is a DC voltage and Vcos(wt) is an AC voltage with V being the amplitude and w the frequency.

Figure 9 *Schematic of a quadrupole mass filter, reprinted by permission from B.M. Tissue [24]*



If only AC is applied to the rod pairs, all ions move on spiral-shaped flight paths towards the detector. If additionally a certain DC is applied to the rods, all ions (except the ones with the appropriate m/z) are dropped from their trajectory and filtered by collision with the rods. By scanning a quadrupole, the DC/AC ratio is elevated constantly to allow each m/z ratio to reach the detector for a certain time. Dependent on which currents are applied to which quadrupoles, different scan modes can be introduced:, see Table 1.

For known compounds, as is normally the case during bioanalytical measurements of clinical trial samples, the Q1 spectrum is recorded for confirming and optimizing the intensity of the drug's molecular ion in the spectrum. The product ion spectrum is recorded for selecting and optimizing a characteristic fragment ion of the drug. These fragment ions are most often build within the Q2 by collision-activated dissociation (CAD) with an inert gas (here: nitrogen), but may also be produced in the first region of the mass analyzer by collision-induced dissociation (CID) with gas molecules from the curtain gas or due to the lower vacuum.

Table 1 *Types of spectra with a triple-quadrupole mass spectrometer according to different application modes of the three quadrupoles*

Type of spectrum	Q1	Q2 / Collision gas	Q3
Q1 spectrum	Scanning	AC only / no	AC only
Q3 spectrum	AC only	AC only / a little	Scanning
Product ion spectrum	Filtering	AC only / yes	Scanning
Precurser ion spectrum	Scanning	AC only / yes	Filtering
Multiple reaction monitoring	Filtering	AC only / yes	Filtering
Neutral loss scan	Scanning	AC only / yes	Scanning
	(in sync with Q3)		(in sync with Q1)

The exact masses of the precursor ion from the Q1 and normally one characteristic fragment from the product ion spectrum are then used to set the triple quadrupole to the MRM (multiple reaction monitoring) mode, which is used for quantitative analysis: The first quadrupole (Q1) selects the precursor ion, which is then fragmented in the second quadrupole (Q2). In the third quadrupole (Q3) a specific fragment, which is characteristic for the pharmaceutical compound, is selected. For that reason the triple quadrupole is highly selective as not only the molecular ion but also one (or more) of its characteristic fragments is monitored. This plays an important role for quantification of compounds in complex matrices, such as human plasma or urine.

The selectivity does not only affect the analyte of interest but also the background ions. A decrease of background ions is observed which results in an overall increase of the signal-to-noise ratio of the analyte, resulting in an extended sensitivity. These two characteristics – sensitivity and selectivity – make the triple quadrupole mass spectrometer superior to UV or fluorescence detection techniques and the ideal instrument for bioanalytical measurements in samples from clinical trials.

1.3.3 Progress in the construction of mass spectrometers

During the past decade a fast improvement of mass analyzers occurred, making it possible to selectively quantify smaller and smaller amounts of analytes in complex matrices, like human plasma. The most obvious alternation is the ionisation source: Whereas the API III and API 3000™ were equipped with the horizontal spraying

TurboIonSpray® (TISP) source, the API 5000™ is now equipped with the vertical spraying TurboV™ (TVSP) source, allowing higher flow rates and thus better separation of ions from non-charged particles.

But the major improvements are located within the ion path. The orifice diameter, for example, grows from 0.1 mm [25] over 0.25 mm [26] to 0.62 mm [27] allowing a larger number of molecular ions to enter the mass spectrometer, but making improved declustering and ion focusing necessary. This can be achieved by a skimmer, which is a funnel-shaped component directly behind the orifice, functioning by gas dynamics. It was later replaced by a more complex component, the QJet™ ion guide, which uses a combination of gas dynamics and radio frequency fields [27].

All those technical alterations were done to improve system performance and facilitate quantification methods. In order to keep those methods to the state of the art, they have to be transferred regularly to the newer instruments.

1.4 LC-MS/MS method development

Bioanalytical LC-MS/MS method development comprises three main parts: At first, MS/MS spectra are recorded and mass spectrometry settings are optimized, to find a specific fragment ion and the most appropriate system parameters. Second, a fast liquid chromatography method, suitable for MS/MS detection, is developed to separate the analytes from matrix components. Third, a cost-effective and selective sample preparation technique is selected and optimized, supporting the analysis by LC-MS/MS. These steps are described in the following chapters and are complemented by the selection of a suitable internal standard and setting the calibration range.

1.4.1 MS/MS spectra and system optimization

To evaluate the most intensive signals, different ionisation sources and both, positive and negative scan mode should be tested. The ionisation can be evaluated best by infusing an analyte solution directly into the ion-source with the temperature set to zero. In most cases, heating the nitrogen stream in the TISP or TVSP source step by step to a maximum temperature of 350 °C (API 3000™) or 600 °C (API 5000™), is useful to optimize desolvation of the analyte ions and to observe analyte instabilities in the heat. Doing so, an appropriate equilibration time should be kept after each

step. APCI cannot be used without heat as the complete solution needs to be vaporised within the quartz tube. Therefore, optimizing the temperature should be done in relation to the solvent flow.

With each ionisation source, a precursor ion spectrum (Q1) and a product ion spectrum of the protonated or deprotonated molecular ion should be recorded in the positive and in the negative scan mode. Using the most promising ionisation source and polarity, the mass spectrometer settings have to be optimized for the precursor ion. Analyzing pharmaceuticals with low molecular masses, in most cases the mass-to-charge ratio (m/z) of the precursor ion is equal to the exact mass plus or minus one (depending on the polarity of the scan mode). The aim is to intensify the m/z signal of $[M+H]^+$ or $[M-H]^-$ by scanning the Q1 (without collision gas) and incrementing the gas settings and the electronic settings, i. e. the declustering potential (DP) or the orifice voltage (OR).

Using the optimized settings for the precursor ion, the electronic settings for the product ion is optimized. The most important parameters for fragmentation are collision energy and collision gas density. In most cases, the most abundant product ion can be used for the mass transition, but two more factors have to be considered for selecting the fragment:

Analyzing small amounts of an analyte in complex matrices like plasma samples, not only the intensity of a fragment signal is crucial, but also the specificity of its underlying fragmentation reaction. Another molecule which has co-incidentally the same molecular mass may also loose water or carbon dioxide, but will most likely not loose the same mass if divided by an intra-molecular reaction into large fragments.

The second case considers analyzing a metabolite. One should be aware that a second metabolite in the plasma sample may have the same molecular mass and probably has some fragment ions in common. This can be the case if, for example, a hydroxyl group is connected to another carbon atom. In such a case, not only the selection of a useful fragment ion is essential, but also the sensible separation via chromatography.

At last, a Q1 spectrum and a product ion spectrum of the analyte with all optimized parameters are recorded to select the exact m/z values of the precursor ion and the desired fragment ion for the mass transition in the MRM mode.

1.4.2 Liquid chromatography for combined use with mass spectrometry

In contrast to conventional HPLC (high performance liquid chromatography) and due to the ionisation techniques for mass spectrometric detection, only volatile solvents and buffers may be used for LC with MS or MS/MS detection. These are acetonitrile, methanol, water, 2-propanol, acetic acid and formic acid (0.1-0.5 % v), ammonia, ammonium acetate and ammonium formate (2-10 mM in positive ion mode, 2-50 mM in negative ion mode). The use of TFA (trifluoroacetic acid) or TEA (triethylamine) as ion-pairing reagents in the mobile phase to improve peak shape and resolution should be carefully balanced against sensitivity issues as they may suppress the signal intensity of the analyte by preventing efficient desolvation and ionisation in the MS ion source.

In LC with MS/MS detection, the mobile phase composition is pre-determined by the ionisation process to a certain extend. Though, depending on which ionisation technique serves best for the analyte (e. g. polar compounds for ESI), the mobile phase is pre-determined to a certain extend. This means that for ESI ionisation, the compound should be already ionized within the mobile phase, which is achieved by setting the pH at least two units above or below the compound's pKa. In order to avoid ion suppression at the same time, the mobile phase should have a rather small ion strength, typically 2-10 mM. Additionally, for ESI acetonitrile should be preferred versus methanol, as it assists desolvation in the gaseous phase slightly better. However, if APCI showed better intensity during pre-tests, the mobile phase should consist of the protic solvent methanol and slightly higher ion strength by ammonium acetate or formate.

Consequently, column selection permits more degrees of freedom, as a variety of reversed-phase columns had been developed in the past years. The most common columns are based on silica modified by C18 or C8 chains, which are preferably used for hydrophobic compounds. Starting with a certain water (or buffer) content in the mobile phase an increase of the organic content will increase elution of the analytes. Especially silica columns with steric large C18 chains, still contain not-modified silanol groups. Depending on the concrete application, remaining silanol groups can assist or trouble chromatography. Therefore, columns with TMS (trimethylsilane) "endcapping" are a good alternative. The carbon content of C18 and C8 columns gives a good information on how many (steric large) C18 or C8 chains are present on a given column packing. For more polar or aromatic compounds, phases modified with $-NH_2$, -CN or $-C_6H_5$ are recommendable.

As modified silica phases are not stable in basic conditions over pH 8 and only for short time in acidic conditions below pH 2, column packings based on polymers have been developed, which allow for instance retention of hydrophobic and basic compounds under basic pH conditions.

Due to the MRM mode in MS/MS detection, peaks need not necessarily be separated from each other; but from matrix components avoiding matrix effects within the ionisation source. Nevertheless, as metabolites often differ only in the position of a hydroxyl group, the m/z of the fragments may be equal and therefore must be clearly separated in the LC. Caution has to be taken, if fragments with the same m/z of pharmaceutical and metabolites had to be chosen for MRM transitions.

Therefore, in most cases, it is possible to use short columns with small inner diameters to produce short run times by retaining the analytes for one or two minutes on the at most specific column and in this way creating high-throughput methods.

1.4.3 Sample preparation techniques

Before samples can be analyzed with LC-MS/MS, matrix components need to be removed to prevent columns from clogging and to ensure a low background during the ionisation process. In general, there are three common sample preparation procedures for LC-MS/MS: protein precipitation (PPT), liquid-liquid extraction (LLE) and solid phase extraction (SPE).

PPT is the method of choice to achieve sample extracts from human plasma, because ideally, only one or two pipetting steps are necessary per sample and only small amounts or low-cost chemicals are needed. Typically, sample and precipitation reagent (e. g. acetonitrile) are mixed at a ratio of 1:2 or 1:3 (v/v). Addition of acids (formic acid, acetic acid, trifluoroacetic acid) to the precipitation reagent may result in a more efficient precipitation process and needs to be investigated during method development. After centrifugation, the supernatant can additionally be diluted with buffer to reduce the organic content in the sample, which may result in sharper chromatography. Instable drugs can be stabilised before precipitation by adding a buffer with the corresponding pH-value or special stabilising agents. Polson et al. [28] evaluate various protein precipitation methods and conclude that the overall ion strength should be as low as possible for ESI ionisation, including the sample extract as well as the mobile phase. However, matrix effects can be compensated by the internal standard, which is tested during method development and validation.

Low-dosed or rapid-metabolized drugs often require concentration of the sample, which can be achieved by liquid-liquid extraction (LLE) or solid phase extraction (SPE). According to Bonfiglio et al. [29], liquid-liquid extracts demonstrate the least matrix effect in ESI ionisation. During LLE, an excess of organic solvent is added to a certain amount of biological sample and shaken virtuously. Typically, the organic layer is then evaporated to dryness and reconstituted in a water containing solvent. According to the physical and chemical properties of the analyte, an appropriate extraction solvent needs to be selected. Moreover, the extraction efficiency strongly depends on the pH value, which needs to be investigated during method development. The major drawback of this technique is lipids, which might be extracted by certain organic solvents from the biological sample together with the analyte and can disturb the chromatography. Additionally, a large amount of costly organic solvents is needed and automation of LLE methods is rather not trivial.

The third common sample preparation technique is SPE, where a stationary phase is used for sample purification and enrichment. Like HPLC columns, a variety of stationary phases are available for analytes with different physical and chemical properties. After column conditioning each sample is loaded on its own SPE column, where the analyte molecules bind to the stationary phase. Then, matrix components are removed from the sample by pre-defined washing steps. Finally, the purified analyte is eluted with an appropriate solvent and evaporated to dryness. The re-dissolved extract is then injected into the LC-MS/MS system. This technique evolves most expenses for material and time, but is sometimes unavoidable when very small amounts of analyte have to be detected with high precision. Then, analytes are concentrated by using a relatively large plasma sample volume and re-dissolving the dried analyte in a much smaller volume.

Optimising these techniques with regard to high-throughput has been an emerging field in past years, which was reviewed previously [30-31]. The most important innovation is the use of 96-well plates, available for all three sample preparation techniques, where only the first step involves pipetting single samples, while all further steps are carried out with 8-channel or 12-channel pipettes, or with robots, which are able to transfer up to 96 samples at the same time, which is also used here, see chapter 5.1.3.

1.4.4 Selecting the Internal Standard

The internal standard (IS) plays an important role in bioanalytical methods for high sample numbers from many different individuals. When the IS is added to each sample as first step of sample preparation, the IS response in each sample can correct for imprecision (variation) and inaccuracy (correctness) of the analyte response deriving for example from sample handling during the work-up procedure or from different endogenous plasma matrix components in calibration samples and in unknown samples from clinical trials. The latter is known as matrix effect, predominantly occurring in bioanalytical methods using LC-MS/MS.

Stable isotopically labelled compounds show virtually similar behaviour to the analyte and are, therefore, preferred as internal standards. However, limited availability or high costs for synthesis (especially when new chemical entities have been developed for first pharmaceutical use in man) force the analyst to use structural analogues for internal standardization of sample preparation and analysis.

For practical purposes, the IS response (i.e. peak area or peak height) should be high enough to give stable signals and well integrable peaks, but it should not be too high to avoid analyte signals in blank samples, e.g. caused by isotopic impurity of stable isotopically labelled compounds.

1.4.5 Setting the calibration range

Ideally, for the determination of a complete pharmacokinetic profile, the lower limit of quantification (LLOQ) needs to be zero, because at the end the drug should leave the organism completely. However, the goal and the design of the clinical trial may not foresee sampling until plasma concentrations are zero and in addition, extraordinary sensitive assays often involve cost intensive equipment and time consuming sample preparation techniques.

For bioequivalence studies minimal requirements for the LLOQ have been defined by the EMA [3]: “The lower limit of quantification should be 1/20 of C_{max} or lower, as pre-dose concentrations should be detectable at 5 % of C_{max} or lower.” The reason behind is assuring an un-biased C_{max} from study periods one and two, which must be separated by a sufficient long wash-out period. This rule is also rational for other than BE studies, but one should be aware of the fact that C_{max} is not a fixed value, but underlies a certain inter-subject variation. Therefore, for the estimation of the expected C_{max} it is crucial not only to consider drug, dose, formulation (e. g. extended

release), study design (fed or fasted), but also the study population, i. e. healthy, elderly, pediatric, renal or hepatic impaired patients. The last sampling time and half-life of the drug also play an important role for estimating the concentration in the last plasma sample. Often, also metabolites should be included in those pharmacokinetic considerations.

If a bioanalytical assay is very sensitive per se, which means that the simplest and cheapest techniques already allow a very sensitive method, then it is advisable to validate the lowest possible LLOQ, even if higher concentrations are expected. The reason is: Since drug development implies various clinical trials with different dosages or formulations, the calibration range may have to be adjusted later on. Then, it is much easier to add a partial validation to elevate the concentration range than screwing more sensitivity out of a method without changing equipment (which would require a complete new validation).

In some cases, the needed concentration range is wider than the linearity range of the assay, in particular of the detector. Then, the calibration range is set to the required LLOQ and the highest linearly detectable concentration is set as the ULOQ (upper limit of quantification). Samples with concentrations exceeding the calibration range can be diluted before analysis, which then has to be proven in an appropriate validation experiment (see chapter 2.5.11 Dilution integrity). Nevertheless, it is not advisable to pre-dilute a large number of the study samples.

1.5 Why do we have to validate?

The data obtained from measurement of samples from clinical trials is used for the decision, if a new chemical entity can be applied to ill human beings. This decision is basically an ethical question, which is therefore secured against errors and manipulation.

In 1995 and 1996 the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use within the EU, Japan and the USA) published the first, but rather general guidance for industry to harmonize the procedure for validation of analytical procedures to ensure valid interpretation of clinical trial data. The ICH guideline was renewed in 2005 [32] and is still the basis for bioanalytical work.

However, in 2001 the first concrete guideline on BMV was implemented by the FDA [33], addressing accuracy, precision and selectivity like ICH, and adding acceptance criteria for analytical runs. In 2012, the first guideline on BMV by the EMA became effective [34], including experiments that have been grated as additionally important since 2001, like ISR (incurred sample re-analysis) [35-42], or should be determined in a different way, like ME (Matrix Effect) [43-46].

These new concepts target basically the same idea: Calibration standards are normally prepared in a pool of blank matrix (e. g. plasma or urine) of healthy donors, while the samples with unknown concentrations come in an extreme case from thousands of ill patients (i. e. in Phase IV studies) with highly varying composition of plasma components. Analyzing part of those samples a second time as ISR gives a good idea on assay reproducibility, accuracy and precision; in some cases even a better idea than validation experiments with spiked blank matrix pools.

The second new concept is focused on the ME which is unwanted when co-eluting matrix components from single unknown samples enhance or suppress the ionisation of the analyte in the ion source of the MS, which causes an over- or under-estimation of the signal compared to the calibration standards. In principle, some sentences addressing this issue are already included in the FDA guideline on BMV [33], but conduct and expressiveness of ME assessment differed a lot between laboratories [45], which was 2012 brought to a consensus in the EMA guideline on BMV [34].

For the sake of completeness: The third important new concept introduced and referenced by the new EMA guideline is GCLP (Good Clinical and Laboratory Practice), which is explained in a new EMA Reflection paper [47]. It emphasizes the ethical responsibility also of bioanalyticists who should act with due diligence, for example regarding written informed consent by the subjects having donated the samples. This is regarded as a step towards applying the principles of GLP (Good Laboratory Practice), which were originally intended for non-clinical studies, also to clinical studies to support the international acceptance of laboratory data within the OECD (Organisation for Economic Cooperation and Development).

Current developments include release of the Guideline on Bioanalytical Method Validation in Pharmaceutical Development [48] by the Japanese Ministry of Health, Labour and Welfare (MHLW) in September 2013, which is a well conceived document concording with the main ideas of the EMA guideline. In the same month the FDA published their second draft Guidance for Industry on Bioanalytical Method

Validation, which is differing in several topics from previous guidelines by FDA and EMA as was recently commented by different organisations like the American Association of Pharmaceutical Scientists (AAPS), the European Bioanalysis Forum (EBF), and the Global Bioanalysis Consortium (GBC). The release of the final document is planned for March 2014.

These developments took place after the experimental part of this thesis was completed.

1.6 Aims of this thesis

The aim was to develop and validate fast, highly sensitive, precise, and accurate LC-MS/MS methods, complying with regulatory guidelines, for the analysis of human plasma and urine from clinical trials and to use the concentration data for the calculation of pharmacokinetic parameters in the context of pharmacokinetic-pharmacodynamic principles.

Several therapeutically important drugs were investigated.

a) Erythromycin ethylsuccinate has been marketed for many years, yet it's pharmacokinetics were not well described since appropriate methods did not become available. In particular, the extraction process was in many cases not suitable to determine the prodrug erythromycin ethylsuccinate from the active base erythromycin A. For the simultaneous quantification of erythromycin ethylsuccinate and erythromycin A in human plasma only sparse data on analyte stability and methods had been previously published, which used liquid-liquid extraction (not under cooled conditions). These methods were not suitable for the application in regulated high-throughput analysis of samples from a clinical trial.

b) Another macrolide widely used in Germany is roxithromycin. However, it has never been approved for use in the United States. Since pharmacokinetic data on roxithromycin are characterized by a high variability (different expression of CYP3A4 in liver and intestine and saturable protein binding at higher concentrations), a pharmacokinetic study until 120 hours after administration was desirable, leading to the need for an LC-MS/MS method, which is much more sensitive than previous methods. Only three LC-MS/MS methods for the determination of roxithromycin concentrations in human plasma were previously reported, which were characterized

by too high LLOQs [49-50], too laborious sample preparation procedures [49], or too long analysis times [49, 51].

c) Clarithromycin is also widely used and has worldwide importance. Clarithromycin is metabolized to a hydroxylated metabolite, which exhibits at least one half of the parent compound's antimicrobial activity. Therefore, it is crucial to analyze both compounds in samples from a clinical trial studying clarithromycin. Several LC-MS/MS methods have been reported for the analysis of clarithromycin in human plasma, but only four of them determined both, clarithromycin and 14-hydroxy clarithromycin. Two of them estimated 14-hydroxy clarithromycin only semi-quantitatively [52-53]. Since the reported LLOQs of the remaining two methods [54-55] were too high for the quantification of all concentrations until 36 hours (including inter-subject variability) after the administration of 250 mg clarithromycin, a new method needed to be developed and validated.

The ruggedness of these macrolide methods should be tested by comparing the selectivity, sensitivity, linearity range, matrix effects, accuracy and precision data in validation experiments on three LC-MS/MS systems, differing from each other in the way of construction of the ionisation source and the ion focussing region of the ion path.

d) Many β-lactams have been introduced since the early days of penicillin G [56]. Flucloxacillin was considered an important step forward, however, its pharmacokinetics are not fully understood. For the determination of flucloxacillin only two LC-MS/MS methods had been previously published: One was designed to analyze commercially available drug products [57] and the second one was characterized by a too high LLOQ [58], since it was applied to a PK study of intravenous flucloxacillin doses (1.5 to 5 g). Therefore, a highly sensitive method should be developed and validated for the regulated quantification of plasma concentrations from a 250 mg oral dose of flucloxacillin.

e) Piperacillin can be considered a real breakthrough into broad spectrum antibiotics and will continue to be a substantial agent in antimicrobial therapy. Only four LC-MS/MS methods for determining piperacillin in human plasma or serum have been reported to day [59-62], but none of them was validated for analysis of urine samples. These methods are characterised by relatively long analysis times per sample (5.5 to 8.0 minutes), expensive and laborious sample preparation techniques or reported issues with poor precision [59] since they were not designed specifically for piperacillin, but for detecting a large number of β-lactams. The aim was to

develop and validate a new LC-MS/MS method for the fast and cost-effective determination of piperacillin concentrations in human plasma and urine from a clinical trial studying a 4 g intravenous dose of piperacillin.

f) Moxifloxacin is the latest fluoroquinolone that was not only successfully introduced into the market, but also received great acceptance by physicians. Yet, recently adverse events [63-64] have been reported that make any further investigation into moxifloxacin questionable. Only two LC-MS/MS methods for the analysis of moxifloxacin in human plasma samples had been reported [65-66]. However, both were not useful for the intended application since they were characterised by either a very high sample volume of 1 mL plasma or by less sensitivity (0.050 µg/mL). Therefore, the aim was to develop and validate an ultra-fast and highly precise method using protein-precipitation for the determination of moxifloxacin in human plasma samples from a clinical trial studying a 400 mg oral dose of moxifloxacin.

Summary of aims of this thesis

All methods were fully characterized by using parameters like accuracy, precision, sensitivity, selectivity, run time, linearity, extraction recovery and matrix effects. These parameters were discussed in detail and compared to methods from the literature. Moreover, the method parameters of the macrolides were compared on three different mass spectrometers and discussed with respect to technical construction.

Acquired stability data of the examined anti-infectives and their metabolites were explained by chemical reaction mechanisms and were set into the context of stability data from the literature as well.

The purpose of the methods was to analyse samples from clinical trials, which in turn are designed to obtain pharmacokinetic data on the investigated drugs. The gained parameters like AUC, C_{max}, t_{max} and $t_{1/2}$ were compared to data from previously reported comparable trials. The obtained plasma concentration-time curves were correlated graphically with MIC values of popular microorganisms which might be a starting point for further PK/PD investigations.

2 MATERIAL AND METHODS

2.1 Instrumentation and software

Mass spectrometers and software products

API III Plus	RAD 1.6, MacQuan 1.5
API 3000™	SampleControl 1.4, LC2Tune 1.4, MacQuan 1.6
API 5000™	Analyst 1.4.2

all from AB SCIEX (Concorde, Ontario, Canada),
supplied by Applied Biosystems GmbH (Darmstadt, Germany)

Calculation Software

Microsoft Excel 2000	Microsoft Corporation, Redmond, WA, USA
WinNonlin™ Professional 2.0	Pharsight Corporation, Palo Alto, USA

HPLC pumps

L-6000 Lichrograph HPLC pump	Merck KGaA, Darmstadt, Germany
L-6200A intelligent HPLC pump	Merck KGaA, Darmstadt, Germany
HPLC pump 1200 Series	Agilent Technologies, Waldbronn, Germany
Harvard apparatus syringe pump 11	Harvard Apparatus Inc., Massachusetts, USA

Autosamplers

L-7250 LaChrom autosampler	Merck KGaA, Darmstadt, Germany
CTC CombiPal autosampler	CTC Analytics, Zwingen, Switzerland
CTC HTS Pal autosampler	CTC Analytics, Zwingen, Switzerland

Automated 96-Channel Pipettor

Multimek™	Beckmann Coulter GmbH, Krefeld, Germany

Chromatography columns

Aqua C18, 5µm (50x4.6 mm)	Phenomenex Ltd., Aschaffenburg, *
Betasil C18, 5µm (50x4.6 mm)	Thermo Electron Corporation, Dreieich, *
Nucleosil 100 NH_2, 5µm (40x4.6 mm)	Alltech Grom GmbH, Rottenburg-Hailfingen, *
Spherisorb Phenyl, 5µm (60x4.6 mm)	Alltech Grom GmbH, Rottenburg-Hailfingen, *
Symmetry C8, 5µm (50x4.6 mm)	Waters GmbH, Eschborn, *
YMC ODS AM, 3µm (50x4.6 mm)	YMC Europe GmbH, Dinslaken, *
YMC Pack CN, 3µm (50x4.0 mm)	YMC Europe GmbH, Dinslaken, *
	*Germany

Pipettes

Eppendorf Reference® 10-100µL	Eppendorf, Hamburg, Germany
Eppendorf Reference® 100-1000µL	Eppendorf, Hamburg, Germany
Eppendorf Reference® 500-2500µL	Eppendorf, Hamburg, Germany
Eppendorf Reference Multipette®	Eppendorf, Hamburg, Germany

Centrifuges

Allegra™ X-12R Centrifuge	Beckmann Coulter GmbH, Krefeld, Germany
Biofuge primo R	Heraeus Sepatech, Osterode, Germany

Laboratory mixer

Heidolph REAX 2000 Vortex Mixer	Heidolph-Elektro GmbH, Kelheim, Germany
Multitube Vortex Mixer	VWR Scientific, South Plainfield, NJ, USA

Analytical balance

Mettler AT261 Delta Range® FACT	Mettler-Toledo GmbH, Gießen, Germany

pH-Meter

WTW pH 521	WTW GmbH, Weilheim, Germany

Ultrasonic bath

Sonorex Super RK 255	Bandelin electronic, Berlin, Germany

Consumables

Pipette tips for Eppendorf pipettes	VWR International GmbH, Ismaning, Germany
Disposable nitril gloves, powder free	VWR International GmbH, Ismaning, Germany
Microtubes, PP, 0.5 mL, snap-on lid	VWR International GmbH, Ismaning, Germany
Microtubes, PP, 2 mL, screw cap	VWR International GmbH, Ismaning, Germany
Microplates, 96 Deep-Well	Porvair Sciences Ltd, Shepperton, UK

2.2 Reference compounds, chemicals and biological specimens

Reference compound

Clarithromycin (CLA)	Hexal AG, Holzkirchen, *
14-Hydroxy clarithromycin (CLA-MET)	Hexal AG, Holzkirchen, *
Erythromycin (ERY A)	Sigma Aldrich Chemie GmbH, Schnelldorf,*
Erythromycin ethylsuccinate (EES)	Sigma Aldrich Chemie GmbH, Schnelldorf,*
Flucloxacillin (FLU)	GlaxoSmithKline, West Sussex, UK
Gatifloxacin (GAT)	Grünenthal GmbH, Aachen, *
Moxifloxacin hydrochloride (MOX)	Hexal AG, Holzkirchen, *
Oleandomycin (OLE)	Sigma-Aldrich Chemie GmbH, Schnelldorf,*
Piperacillin (PIP)	Wyeth Pharma GmbH, Münster, *
Roxithromycin (ROX)	Hexal AG, Holzkirchen, *
Sulbactam sodium (SUL)	Pfizer GmbH, Karlsruhe, *
Tolbutamide (TOL)	Sigma-Aldrich Chemie GmbH, Schnelldorf,*

*Germany

Chemicals and solvents

Acetic acid, 98% p. a.	VWR International GmbH, Darmstadt, *
Acetonitrile, HPLC-grade	Fisher Chemical, Fisher Scientific GmbH, *
Ammonia solution, 25% p. a.	VWR International GmbH, Darmstadt, *
Ammonium acetate, p. a.	VWR International GmbH, Darmstadt, *
Ammonium formate, p. a.	VWR International GmbH, Darmstadt, *
Formic acid, 98% p. a.	VWR International GmbH, Darmstadt, *
Methanol, HPLC-grade	VWR International GmbH, Darmstadt, *
	*Germany

Ultra pure water

Milli-Q purification system	Millipore Corporation, Bedford, MA, USA

Drug-free biological matrix

Drug-free human EDTA plasma	pooled plasma from healthy volunteers
Drug-free human heparinate plasma	pooled plasma from healthy volunteers
Drug-free human urine	pooled urine from healthy volunteers

Haemolysed plasma was prepared by adding 1 % of frozen and re-thawed drug-free whole blood to drug-free human plasma containing ammonium heparinate or EDTA as anticoagulant.

2.3 Stock solutions

Before weighing, reference compounds are allowed to equilibrate to room temperature. The amount to be weighed is calculated with respect to purity, salt form (conversion factor) and desired concentration, see Table 2. Then, the substance is accurately weighed, dissolved in the appropriate solvent and diluted to the planned volume. The stock solutions were aliquoted into polypropylene tubes and stored at approximately -70 °C.

Table 2 *Stock solutions of analytes and internal standards (IS)*

Analyte	Purity [%]	Content [%]	Solvent	Concentration [mg/mL]
Clarithromycin (CLA)	98.1	98.1	Acetonitrile	0.40
14-Hydroxy Clarithromycin (CLA-MET)	100.0	100.0	Acetonitrile	0.40
Clarithromycin-d_3 (IS)	98.0	98.0	Acetonitrile	0.40
Erythromycin A (ERY A)	94.0	88.2	Acetonitrile	0.20 and 0.16
Erythromycin Ethylsuccinate (EES)	97.4	97.4	Acetonitrile	0.50 and 0.40
Flucloxacillin (FLU)	91.6	91.6	Ultra-pure water	1.00
Gatifloxacin (GAT, IS)	92.5	92.5	Ultra-pure water	0.20
Moxifloxacin (MOX)	96.8	88.7	Ultra-pure water	0.50
Oleandomycin (OLE, IS)	100.0	100.0	Acetonitrile	0.10
Piperacillin (PIP)	96.0	96.0	Methanol	1.00 and 12.50
Roxithromycin (ROX)	97.6	97.6	Acetonitrile	0.40 and 1.00
Sulbactam (SUL, IS)	92.4	84.4	Ultra-pure water	0.10
Tolbutamide (TOL, IS)	100.0	100.0	Acetonitrile	2.00

Content: Percentage of analyte in the reference substance with respect to purity and salt form

2.4 Calibration standards and spiked quality control samples

On each validation day and before study sample analysis, two stock solutions (stock solutions I and II) are precisely prepared and used for the preparation of the calibration standards (stock solution I) and the spiked quality control (SQC) samples (stock solution II), respectively.

Calibration standards as well as SQC samples in human plasma were prepared by serial dilution, adding the appropriate amount of stock solution (I or II) or the higher concentrated calibration standard or the higher concentrated SQC to drug-free matrix (e. g. human plasma or urine), respectively.

For method validation and for calibration of pharmacokinetic measurements a calibration curve (including two blank samples with and without IS, which were not used for calculation of linear regression) was measured with each analytical run. SQC samples were prepared for method validation and to control the precision and accuracy of the assay during the measurement of study samples.

Table 3 *Calibration standards and SQC samples for validation*

Analyte (Matrix)	Calibration standard concentrations [µg/mL] SQC concentrations [µg/mL]
CLA (P)*	3.97, 2.98, 1.98, 0.982, 0.487, 0.193, 0.0962, 0.0480, 0.0191, 0.0144, 0.00958 2.96, 0.980, 0.0949, 0.0241
CLA (E)**	4.00, 3.00, 2.00, 0.500, 0.200, 0.100, 0.0500, 0.0200, 0.0150, 0.0100 4.00, 0.500, 0.0200, 0.0100
CLA (P)**	4.00, 3.00, 2.10, 0.400, 0.100, 0.0250, 0.0125, 0.00313, 0.00156, 0.000781 4.00, 1.60, 0.100, 0.00313, 0.00156
CLA (P)***	4.00, 3.00, 2.10, 0.400, 0.100, 0.0250, 0.0125, 0.00313, 0.00156, 0.000781 4.00, 1.60, 0.100, 0.00313, 0.00156
CLA-MET (P)*	3.96, 2.97, 1.97, 0.980, 0.486, 0.193, 0.0959, 0.0479, 0.0191, 0.0143, 0.00955 2.97, 0.98, 0.0951, 0.0242
CLA-MET (E)**	4.00, 3.00, 2.00, 0.500, 0.200, 0.100, 0.0500, 0.0200, 0.0150, 0.0100 4.00, 0.500, 0.0200, 0.0100
CLA-MET (P)**	4.00, 3.00, 2.10, 0.400, 0.100, 0.0250, 0.0125, 0.00625, 0.00313, 0.00156 4.00, 1.60, 0.100, 0.00625, 0.00313
CLA-MET (P)***	4.00, 3.00, 2.10, 0.0125, 0.00625, 0.00313, 0.00156, 0.000781, 0.000391 4.00, 0.100, 0.00625, 0.000781, 0.000391
EES (P)*	5.00, 4.00, 2.00, 1.00, 0.200, 0.100, 0.050, 0.020, 0.0150, 0.0100 4.00, 0.400, 0.100, 0.0200, 0.0100
EES (P)**	4.00, 3.00, 2.10, 0.420, 0.0700, 0.0125, 0.00250, 0.00125 4.00, 0.420, 0.0125, 0.00625, 0.00250
EES (P)***	4.00, 3.00, 2.10, 0.0125, 0.00250, 0.00125, 0.000625, 0.000313, 0.000156 4.00, 0.420, 0.0125, 0.000313, 0.000156
ERY A (P)*	2.00, 1.60, 0.800, 0.400, 0.0800, 0.0400, 0.0200, 0.00800, 0.00600, 0.00400 1.60, 0.160, 0.0400, 0.00800, 0.00400
ERY A (P)**	2.10, 1.60, 0.400, 0.100, 0.0125, 0.00313, 0.00156, 0.000781 2.10, 0.400, 0.0125, 0.00156
ERY A (P)***	2.10 1.60, 0.400, 0.0125, 0.00313, 0.000781, 0.000391, 0.0000977 2.10, 0.400, 0.0125, 0.000391, 0.000195
FLU (P)**	15.0, 7.50, 3.75, 1.88, 0.750, 0.375, 0.150, 0.0750, 0.0400, 0.0100 15.0, 7.50, 0.375, 0.0375, 0.0100
MOX (P)**	5.00, 3.75, 1.00, 0.250, 0.0500, 0.0250, 0.0100, 0.00800, 0.00500 5.00, 1.00, 0.125, 0.0125, 0.00500
PIP (P)**	125, 100, 12.5, 2.50, 1.25, 0.250, 0.125, 0.0500 125, 12.5, 1.25, 0.125, 0.0500
PIP (U)**	1250, 1000, 350, 35.0, 17.5, 3.50, 2.00, 1.25 1250, 350, 35.0, 3.50, 1.25
ROX (P)*	3.95, 2.97, 1.99, 0.984, 0.485, 0.189, 0.0938, 0.0466, 0.0182, 0.0137, 0.00921 2.79, 0.908, 0.0873, 0.0211
ROX (P)**	4.00, 3.00, 2.10, 1.60, 1.20, 0.404, 0.101, 0.0253, 0.0126, 0.00632, 0.00316 4.00, 0.101, 0.00632, 0.00316
ROX (P)***	1.61, 1.21, 0.404, 0.101, 0.0253, 0.00632, 0.00158, 0.000790, 0.000395 1.61, 1.21, 0.101, 0.000790, 0.000395

(P): human ammonium heparin plasma, (E): human EDTA plasma, (U): human urine

*: API III Plus, **: API 3000™, ***: API 5000™

2.5 Validation experiments – procedure and acceptance criteria

The realisation and acceptance criteria of the following validation experiments were performed as recommended by the guidelines on bioanalytical method validation introduced above (FDA, EMA [33, 67]).

2.5.1 Specificity

The specificity of an analytical method is the ability not to detect potential interfering compounds from the biological matrix. The specificity is determined by analyzing drug-free human plasma or urine from at least six different individuals (males and females) with addition of IS and without IS.

The specificity experiment is successful, if no co-eluting peaks with areas of more than 20 % of the analyte peak area at the lowest level of quantification (LLOQ) occurred (FDA, EMA [33, 67]).

2.5.2 Selectivity

The selectivity of an analytical method is the capability to differentiate the analyte from potential interfering compounds in the biological matrix. The influence of at least six different batches of biological matrix on the determination of the analytes was determined. Each batch of matrix was spiked with two different concentrations, which were prepared thrice. For each concentration level, the mean value, standard deviation and precision had to be within 15 %.

2.5.3 Linearity and sensitivity

For the determination of linearity and sensitivity, a calibration curve of at least eight concentration levels was freshly prepared in biological matrix on each validation day and analyzed in at least five validation runs (at least three runs are requested by FDA and EMA) together with two blanks (with/without IS, not used for linear regression).

Each calibration curve was evaluated by weighed linear regression and accepted, if it contained maximal two outliers (back-calculated concentration outside 15 %-limit,

or 20 %-limit at LLOQ) not being adjacent. The slopes, intercepts and the correlation coefficients of the corresponding individual curves are calculated.

During study sample analysis the same specifications were applied, except for the LLOQ, which had to be within 15 %.

The sensitivity of a method is defined by its LLOQ (lower limit of quantification), which is the lowest concentration that can be measured with acceptable accuracy and precision within 20 % and a signal-to-noise ratio greater or equal to 5. The ULOQ (upper limit of quantification) is the calibration standard, but not necessarily the highest quantifiable concentration (see pre-dilution of study samples in chapter 2.5.11 Dilution integrity).

The signal-to-noise ratio at the LLOQ is determined on API III Plus and API 3000™ by the peak signal divided by the upper noise level. The signal-to-noise on API 5000™ is calculated with the AB SCIEX script using 3 STD of the noise.

2.5.4 Precision and accuracy

For the evaluation of the within-run and the between-run precision and accuracy of the assay, five replicates of each SQC concentration level were prepared and analyzed on each validation day. The concentrations were calculated by the corresponding calibration curve. Mean, standard deviation (SD), accuracy and precision are calculated for each concentration level:

$$\text{Accuracy } (\%) = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \cdot 100 \qquad \textbf{Equation 9}$$

$$\text{Precision } (\%) = \frac{\text{SD of measured concentrations}}{\text{Mean of measured concentrations}} \cdot 100 \qquad \textbf{Equation 10}$$

2.5.5 Stability experiments

The stability experiments reflect the drug stability in the study samples during collection, handling, preparation, measurement and storage. Therefore, the stability of all analytes in the given matrix was evaluated by SQC samples with known nominal concentrations at the ULOQ and near or at the LLOQ. Five replicates at each concentration level were evaluated, although only three are requested in the guidelines. The tested conditions are summarized in Table 4.

Table 4 *Conditions of stability experiments*

Experiment	Temperature	Time	Experiment reflects…
Short-term	RT or 4 °C	2 and 4 hours	sample handling and preparation
Long-term	-20 °C and -70 °C	days to months	storage conditions
Freeze/Thaw	-70 °C to RT	FT 1: >24 hours, FT 2, 3: 12 hours	freezing and thawing of samples
Post-preparative (autosampler)	4 °C and -70 °C	24 up to 120 hours	storage in the autosampler or freezer between sample preparation and measurement
Stock solution	RT or 4 °C	6 hours	stability of stock solutions
Light stability	-	reasonable time intervals	stability in daylight or artificial light

RT: room temperature, FT 1: freeze and thaw cycle 1

Directly after application of the storage conditions, the stored SQC samples and freshly prepared calibration standards and SQC samples are prepared and analyzed together in the same validation run.

While short-term, long-term and freeze/thaw conditions are subjected directly to the SQC samples in matrix, the post-preparative stability starts after the sample work-up procedure, when matrix components, including enzymes have been removed.

The stability of stock solutions is evaluated by preparing two stock solutions of the same concentration and storing them for 6 hours at the intended temperature, which depends on the expected analyte stability and the intended handling of the stock solutions. After 6 hours two freshly prepared stock solutions and the stored ones are diluted five times to a concentration within the calibration range and analyzed alternately.

To determine the stability of an analyte towards light, human plasma and human whole blood is spiked to a high concentration and aliquoted to 75 SQC samples, each. 25 of those aliquots in each matrix, are stored in the dark, 25 in neon light and 25 in daylight (window sill). After 0, 1, 2, 4 and 6 hours, 5 aliquots each are transferred into a freezer at -70 °C. At the end, all samples are thawn and analysed.

Statistical evaluation of the stability experiments is performed by calculating 95 % confidence intervals for the ratios between the concentrations measured after applying the respective test conditions and the corresponding control samples (to

allow for any contribution of assay imprecision). Instability is concluded if both the upper and lower limit of the confidence interval are greater than 10 %.

2.5.6 Recovery

For the determination of the absolute extraction recovery of analyte and IS, five concentration levels in blank matrix, and the same concentration levels in *processed* blank matrix were prepared by serial dilution. Five replicates of each concentration level were analyzed. The recovery was evaluated by the equation:

$$\text{Recovery } (\%) = \frac{\text{normalized mean peak area in matrix}}{\text{normalized mean peak area in processed matrix}} \cdot 100 \qquad \textbf{Equation 11}$$

Normalization is performed by dividing the mean peak area (n=5) by the corresponding theoretical concentration. The recovery may be below 100 %, but the extent should be “consistent, precise and reproducible” (FDA [33]). Recovery is not mentioned in the EMA guideline on BMV [34].

2.5.7 Matrix Effect

Matrix components may enhance or suppress the signal in LC-MS/MS assays (see chapter 1.3.1 Ionisation Sources). There are two approaches how to determine the matrix effect.

The **Matrix Factor (MF)** is determined by spiking the analyte and the IS into six different lots of processed blank matrix (to exclude extraction recovery) and at the same concentration into pure solution (e. g. mobile phase) and injecting three replicates each directly into the system. The MF for each lot of matrix and each analyte is then calculated by the following equation:

$$\text{MF} = \frac{\text{peak area in processed matrix}}{\text{peak area in solution}} \qquad \textbf{Equation 12}$$

A MF greater or smaller than 1 suggests ion enhancement or ion suppression due to matrix components and should be correctable by the MF of the internal standard. Therefore, the precision of the IS-normalized MF, calculated by dividing the analyte-MF by the IS-MF, should not be greater than 15%.

Post-column infusion can be used to visualize the matrix effect by infusing a solution with a low or medium analyte concentration via a T connector behind the

analytical column by means of a syringe pump. The injection of different lots of processed blank matrix samples onto the chromatographic system can increase or decrease the signal, which reflects enhancement or suppression of the analyte's ionisation at the corresponding time point.

2.5.8 Haemolysed plasma

During venous blood collection into vacutainers or monovettes, the vacuum may cause some blood cells to lyse and release haemoglobin, which is visible by the orange to red colour of the plasma after centrifugation. Analytes may bind to such plasma components, resulting in a different behaviour during extraction and analysis than in non-haemolysed human plasma. As calibration standards and SQC samples are prepared in a pool of normal human plasma, the measured concentration in haemolysed plasma may be underestimated. Therefore, the influence of haemolysed plasma has to be evaluated during method validation.

Haemolysed human plasma can be produced by freezing whole blood and re-thawing it, which causes lysis of blood cells. The orange to red colour of haemolysed clinical trial samples can be produced by spiking 1% of lysed blood into normal human plasma.

The influence of haemolysed plasma on the determination of pharmaceuticals was determined by spiking haemolysed human plasma at two concentration levels and analyzing five replicates together with a calibration curve in normal human plasma. No influence was concluded, if the accuracy and precision at each concentration level did not exceed 15 %.

2.5.9 Hyperlipidaemic plasma

Especially in clinical trials evaluating a food-effect, very different plasma samples regarding the content of lipids have to be analyzed in the same analytical run to assure comparability of study periods. Therefore, hyperlipidaemic and non-hyperlipidaemic samples have to be quantified using calibration standards in normal human plasma. This kind of matrix difference may affect the resulting concentration data at most, if the samples are prepared using a liquid-liquid extraction method, where also lipids can be extracted from the plasma by the organic solvent.

The influence of hyperlipidaemic plasma is evaluated similarly to the experiment for haemolysed plasma. Five replicates per concentration level in hyperlipidaemic plasma are prepared and analyzed together with calibration standards in normal human plasma. No influence is concluded, if the accuracy and precision at each concentration level does not exceed 15 %.

2.5.10 Anticoagulant

The influence of the anticoagulant on the assay is determined by preparing SQC samples in plasma containing one anticoagulant (e. g. EDTA) and calibration standards in plasma with another anticoagulant (e. g. ammonium heparin). If the anticoagulant has no enhancing or suppressing effect on the sample analysis, the measured concentrations are within the 15-% accuracy and precision of the nominal SQC concentration.

2.5.11 Dilution integrity

Despite the proper planning of the calibration range based on literature data, the concentrations of some study samples may exceed the validated calibration range. In order to allow for a valid concentration value, those samples can be diluted with drug-free human plasma (or urine) prior to the sample preparation procedure and the re-analysis. This pre-defined dilution step must also be validated, whereby in most cases, a dilution by factor 5 is sufficient.

The influence of sample dilution on the assay is determined by spiking the analyte into the respective blank matrix at a concentration that exceeds the calibration range to simulate a high study sample. This high SQC is first diluted by the intended factor with drug-free matrix (at least five determinations per dilution factor) and then processed and analyzed as all other samples. This experiment is also stipulated in the bioanalytical method guidelines [33, 67] by the EMA and FDA, where accuracy and precision should be within the set criteria, i.e. within ±15 %.

2.5.12 Incurred sample re-analysis (ISR)

To check the accuracy and precision not only in spiked matrix samples, but in real clinical trial samples, a defined percentage of study samples is re-analyzed as

incurred samples. For the best assessment, values around C_{max} and during the elimination phase are selected. Depending on the study sample number (below or exceeding 1000), 10 % or 5 % should be re-analyzed as incurred samples, but less than 20 samples for ISR is not recommended. For 67 % of the incurred samples, the accuracy should be within 20 % of the mean of the original result and the ISR result. The ISR concept developed through many publications, but the above procedure and acceptance criteria is currently only included in the guidance by the EMA [34].

2.6 Study sample collection and sample handling

All study samples analyzed here, were from clinical trials with healthy volunteers, who have signed informed consent forms in accordance with the “Declaration of Helsinki” and its amendments. Shortly after collecting the human blood samples in monovettes containing the respective anticoagulant, the samples are centrifuged for 10 minutes at approximately 1500 g in a cooled centrifuge (approx. 4 °C). Each plasma sample is immediately transferred into at least two storage tubes (aliquots) and frozen at the recommended temperature until analysis.

The shipment of human plasma samples from the study site to the bioanalytical laboratory is done in dry-ice containing Styrofoam containers, attended with temperature loggers to ensure analyte stability. Only after the first aliquot arrived in good conditions at the bioanalytical laboratory, the second aliquot is shipped and subsequently stored in another freezer.

On the day of analysis, the human plasma samples are thawed in a cold water bath and mixed thoroughly. After centrifuging the tubes for 5 minutes at approximately 4 °C and 1500 g, an aliquot of the plasma sample is taken and prepared for analysis according to the validated sample preparation procedure for the respective assay.

2.7 Pharmacokinetic analysis

All pharmacokinetic parameters were determined from actual sample collection times and assayed plasma concentrations at these times. Concentration values below the lower limit of quantification were set to zero. Pharmacokinetic parameters were calculated by using the formulas described in chapter 1.2 Pharmacokinetics and Pharmacodynamics.

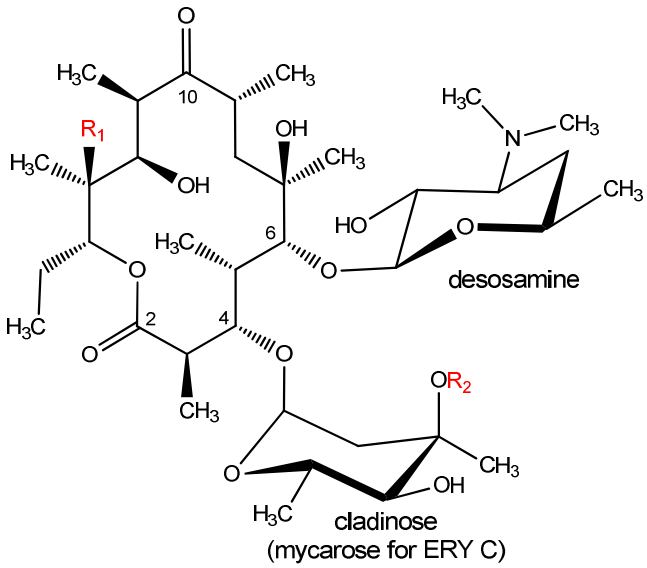
3 MACROLIDES

3.1 Erythromycin Ethylsuccinate and Erythromycin A

3.1.1 Chemical and pharmaceutical properties

Being of bacterial origin erythromycin is a mixture of structurally very similar components, chemically corresponding to a 14-membered lactone ring, which is substituted by two sugar groups at C(4) and C(6), (for atom membering, see Scheme 1). The main component, erythromycin A, is composed of this 14-membered ring containing ten chiral carbon atoms, two of them being attached to cladinose C(4) and desosamine C(6). Erythromycin A, $C_{37}H_{67}NO_{13}$ (exact mass 733.5 amu, mol. wt. 733.9 g/mol) is a water-insoluble weak base with a logP of 3.06 [68] and a pK_a of 8.8 [69].

Scheme 1 *Chemical structures of erythromycin A (ERY A), B and C*



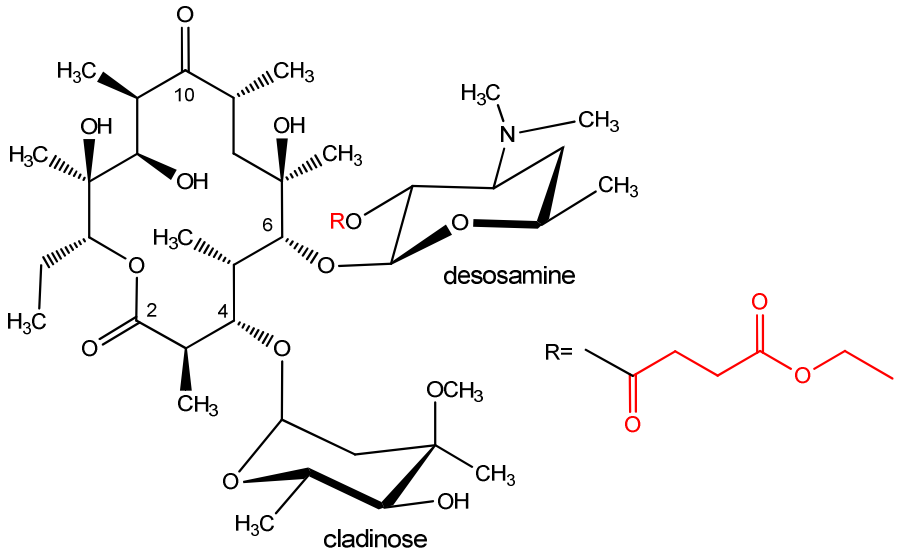
ERY	R1 / R2	Formula	Mol. weight	Exact mass	pK_a	logP
A	-OH / -CH_3	$C_{37}H_{67}NO_{13}$	733.9 g/mol	733.5 amu	8.8	3.06
B	-H / -CH_3	$C_{37}H_{67}NO_{12}$	717.9 g/mol	717.5 amu	-	-
C	-OH / -H	$C_{36}H_{65}NO_{13}$	719.9 g/mol	719.5 amu	-	-

IUPAC Name of ERY A: (3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-6-(((2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-14-ethyl-7,12,13-trihydroxy-4-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,7,9,11,13-hexamethyloxacyclotetradecane-2,10-dione

Erythromycin is the first broad-spectrum macrolide antibiotic that has been used for the treatment of infections of the respiratory tract and other infections. It serves as an important alternative to penicillins.

The antibacterial activity of macrolides results from a blocking of bacterial protein synthesis in the ribosomes, more precisely, by interacting with the 23S ribosomal RNA (rRNA) of the 50S ribosomal subunit. This prevents the formation of new ribosomes and subsequently the translation of messenger RNA in active ribosomes [70]. This target site may be methylated by bacterial methyltransferase, the major mechanism of macrolide resistance [71].

Scheme 2 *Chemical structure of erythromycin ethylsuccinate (EES)*



Formula	Mol. weight	Exact mass	pKa	logP
$C_{43}H_{75}NO_{16}$	862.1 g/mol	861.5 amu	7.1	2.1

IUPAC Name: (2S,3R,4S,6R)-4-(dimethylamino)-2-(((3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-14-ethyl-7,12,13-trihydroxy-4-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3,5,7,9,11,13-hexamethyl-2,10-dioxooxacyclotetradecan-6-yl)oxy)-6-methyltetrahydro-2H-pyran-3-yl ethyl succinate

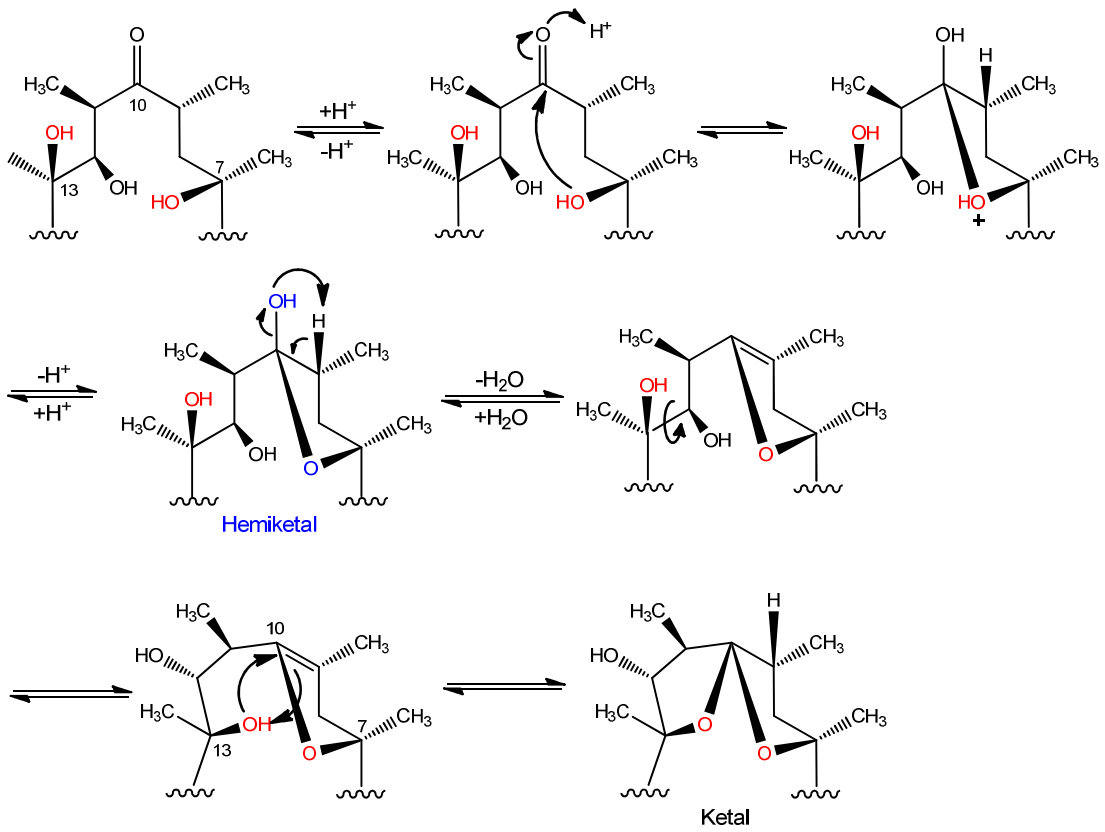
Since erythromycin has been discovered, many structural modifications and formulations have been developed to improve its bitter taste, its acid stability in the gastric environment and, by implication, its bioavailability too. One of those erythromycin derivatives is erythromycin ethylsuccinate (pKa 7.1 [72], logP 2.1 [73]), see Scheme 2. It only differs from erythromycin A by esterification of the desosamine-hydroxyl group. Erythromycin ethylsuccinate serves as prodrug for

erythromycin A as the desosamine-hydroxyl group is essential for its pharmacological activity [74].

Erythromycin ethylsuccinate was developed as a paediatric suspension because of its poor water-solubility (devoid of the bitter taste of erythromycin), but it is also available as enteric-coated or film-coated tablet or suspension for adults.

Erythromycin base is incompletely but reasonably absorbed from the upper small intestine, but inactivated by gastric acid (see Scheme 3), especially in combination with food [1]. The absorption of erythromycin ethylsuccinate is rapid (half an hour after drug administration [75]) and less altered in the presence of food [76].

Scheme 3 *Acid-catalyzed intramolecular ketal formation of erythromycin*



Data from studies using microbiological assays revealed peak concentrations of erythromycin ethylsuccinate of 1.5 µg/mL (0.5 µg/mL of base), at 1 to 2 hours after administration of a 500-mg dose, indicating that concentrations of the free base comprise 20 to 35 % of the total concentration and that the concentration of the microbiologically active erythromycin base in serum is similar for various preparations [1]. For a more detailed view on the pharmacokinetics of the prodrug erythromycin ethylsuccinate and its active metabolite erythromycin base in human

plasma, more specific methods are needed that are able to distinguish between erythromycin ethylsuccinate and erythromycin base.

Erythromycin distributes readily into body tissues and fluids, achieving antibacterial concentrations, except for brain and CSF. The latter may derive from the rather large molecular size of erythromycin, even since the molecule would be lipophilic enough (logP 2.1) to enter the blood brain-barrier. The volume of distribution of erythromycin base is 0.78±0.44 L/kg and it is bound to plasma proteins by 84±3 % [1].

Metabolism of erythromycin occurs by CYP3A4 in the liver, mainly producing the *N*-demethylated analogue [69]. Erythromycin inhibits CYP3A4, not only affecting its own metabolism, but also the efficacy and tolerability of many other drugs, including terfenadine, felodipine, midazolam or carbamazepine. There is still uncertainty about the mechanism of this non-competitive inhibition since it seems to be difficult to distinguish between either a slowly reversible [77] or a mechanism-based irreversible (MBI) [78] mechanism. Data from a recently published study support the mechanism-based inactivation of CYP3A4 by macrolides. Studying the inhibition kinetics of genetic CYP3A4 variants, the equations involving an enzyme-inhibitor intermediate complex resulted in inhibition constants being quite likely attributable only by MBI [79]. This interaction may cause beneficial (dose reduction of cyclosporines) or mostly adverse drug response, like QT interval prolongation, hypotension, excessive sedation or ataxia [77]. However, serious side effects have been observed rarely. The most abundant side effects of erythromycin are dose-related and either gastrointestinal, including nausea, vomiting, and diarrhoea, or hepatic .

3.1.2 Current state of published analytical methods

Several methods are published determining erythromycin or its derivatives in human plasma [80-85]. However, only few methods can be found that quantify erythromycin ethylsuccinate in matrices like pharmaceutical formulations [86-89], urine and milk [90] and serum or plasma [91-95]. The methods for erythromycin ethylsuccinate in human plasma or serum use liquid chromatography with electrochemical detection (LC/ED) [94], or with derivatisation and spectrofluorometric detection (LC/FD) [95]. Others reported (FAB-MS) methods [92-93], one of them being coupled to an LC system [92]. Previously reported LC/ED, LC/FD and FAB-MS methods present low sensitivity, as well as expensive or time-consuming sample preparation procedures using high sample volumes and long run times.

For a specific and fast determination, LC/MS and LC-MS/MS are especially useful. In particular, MS/MS triple quadrupole mass spectrometry used in the multiple-reaction-monitoring mode (MRM) provides surpassing speed, sensitivity and high selectivity in quantitative analysis.

Several LC-MS/MS methods have been reported which determine erythromycin in a series of matrices like fish [96-97], commercial preparations [98-100], aqueous solution [101] and human plasma [102]. However, only one LC-MS/MS method has been published for erythromycin ethylsuccinate in human plasma [91]. They used a cost-ineffective and time-consuming extraction method combined with a comparably long analysis run time. With regard to the high sample amount in large pharmacokinetic studies, this is considered as inappropriate.

3.1.3 LC-MS/MS method

The original LC-MS/MS method was provided by Professor Dr Fritz Sörgel and is discussed in detail in the following chapter. As EES and ERY A are non-polar compounds, APCI is the most promising ionisation technique. It was used in the positive ion mode, as both analytes are weak bases and can be easily protonated. The positive ion Q1 spectra of EES and ERY A contained intensive peaks correspondent to the protonated molecules $[M+H]^+$ with m/z 862.7 (EES) and m/z 734.6 (ERY A). No ERY A signals could be observed in the Q1 spectrum of EES, indicating that no in-source fragmentation of the EES ester bond occurred, see Figure 10.

The protonated molecules of EES (m/z 862.7) and ERY A (m/z 734.6) are fragmented by collision-activated dissociation resulting in the MS/MS product ion mass spectra depicted in Figure 11 and Figure 12. The system parameters are optimized for the most intensive fragment signals, which are m/z 286.1 (EES) and m/z 158.0 (ERY A), corresponding to the protonated (and esterified) desosamine.

Figure 10 *Q1 mass spectrum of erythromycin ethylsuccinate (EES)*

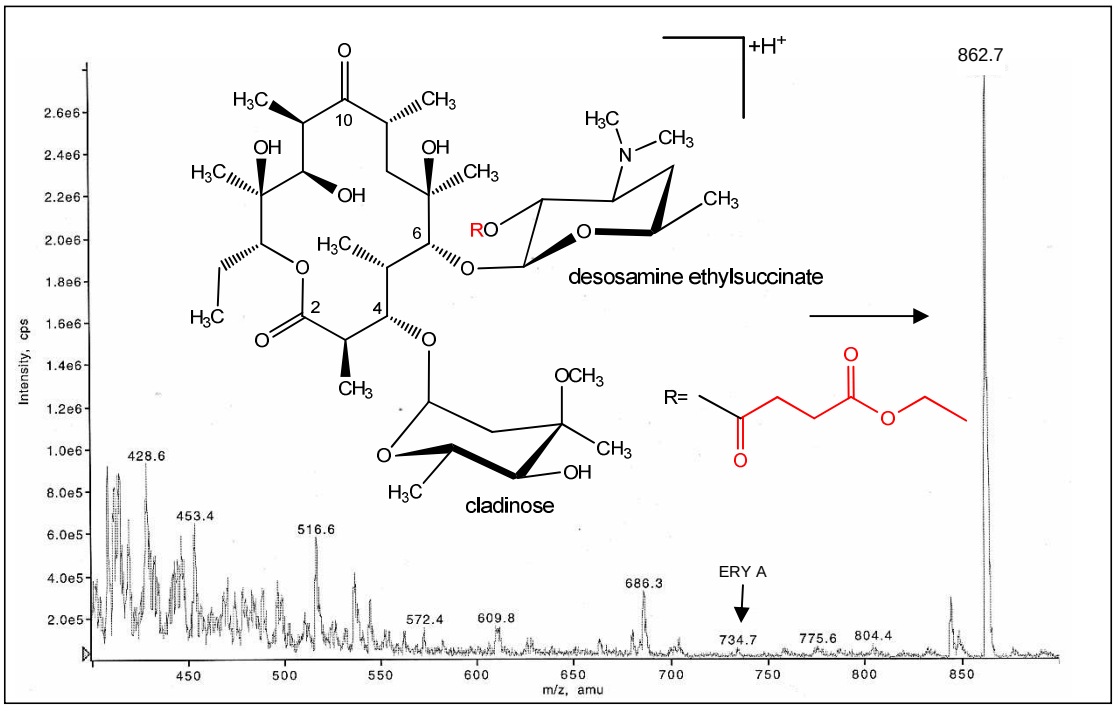


Figure 11 *Product ion spectrum and fragmentation scheme of EES*

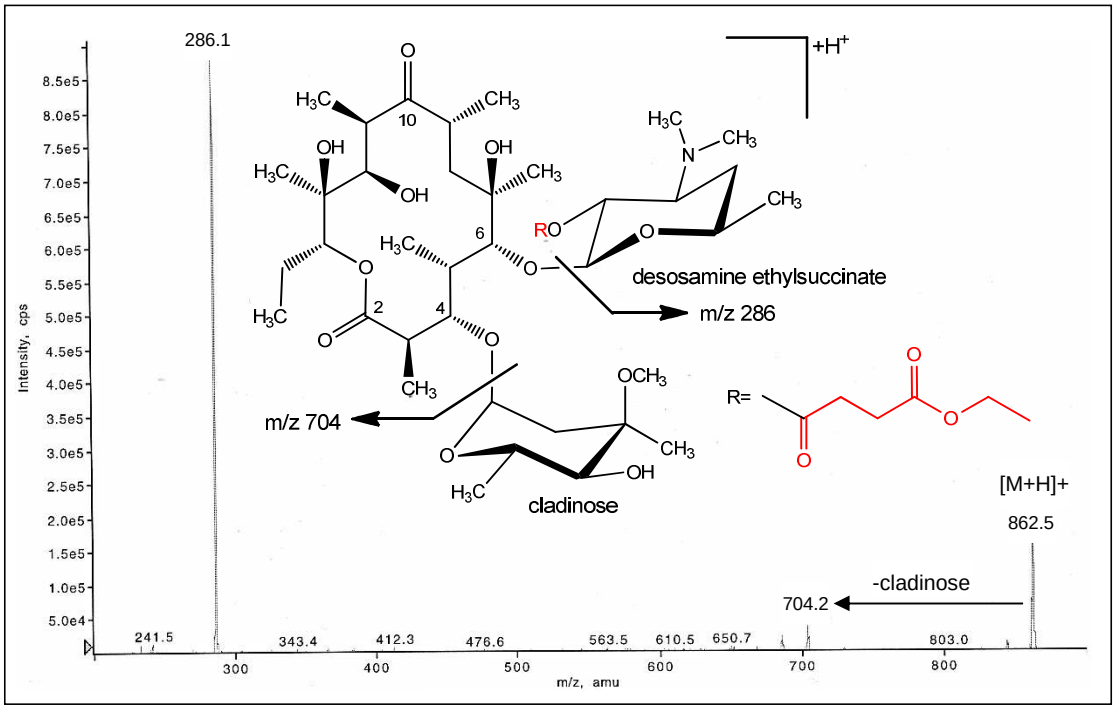
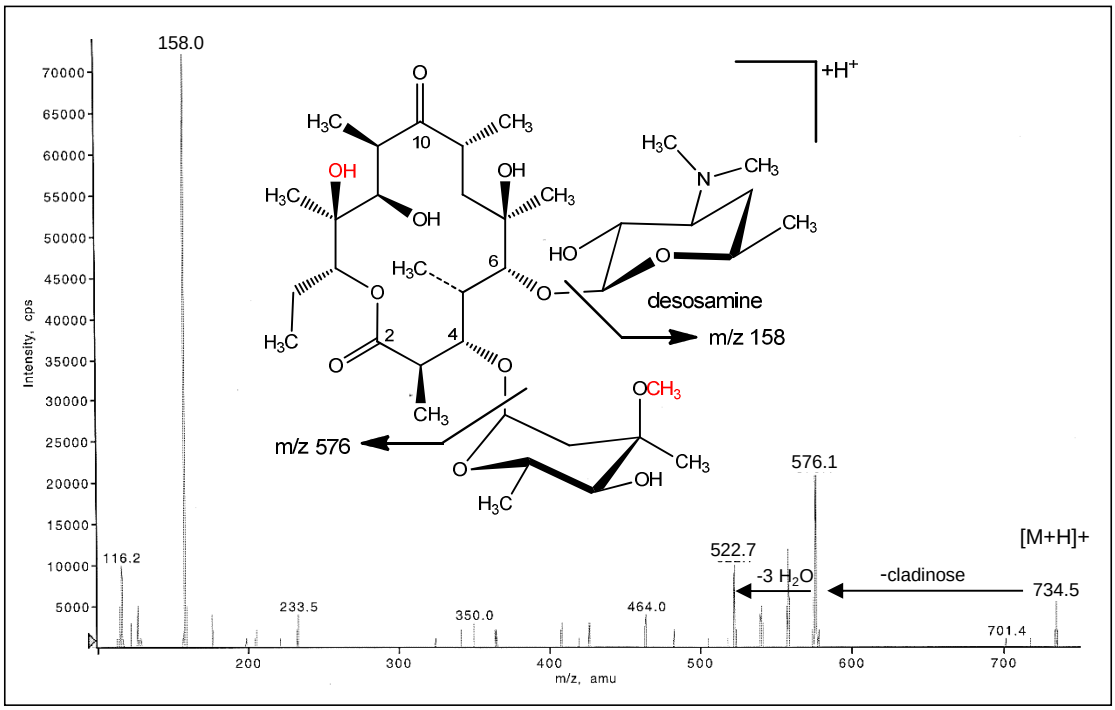


Figure 12 *Product ion spectrum and fragmentation scheme of erythromycin A*

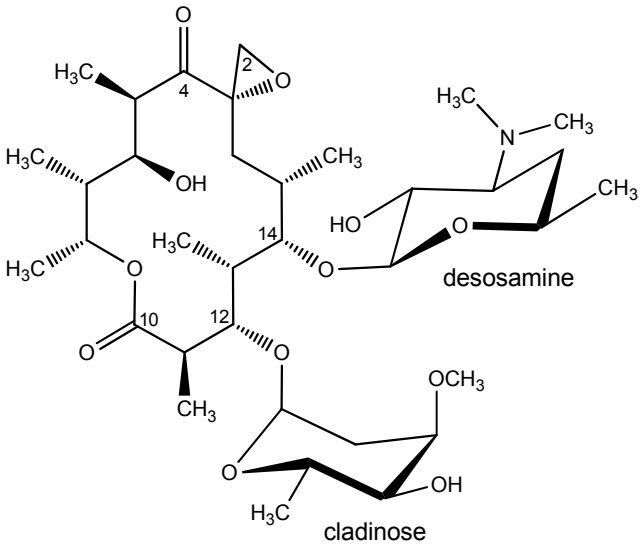


Oleandomycin was used as internal standard, as it is chemically analogous to the analytes (pKa 8.84, logP 1.69 [68]) and pharmaceutically not applied anymore, displayed in Scheme 4.

The spectra of the internal standard oleandomycin (OLE) represented the protonated molecule $[M+H]^+$ with m/z 687.9 and the most abundant fragment ion with m/z 158.3. Thus, the mass spectrometric experiments were performed using an APCI interface operating in the positive ion mode by means of MRM of the following transitions: m/z 863 → m/z 286 (EES), m/z 735 → m/z 158 (ERY A), and m/z 688 → m/z 158 (OLE).

System parameters were optimized for each system during spectra acquisition. Settings summarized in Table 5 were chosen to obtain the highest analytical sensitivity for EES and ERY A.

Scheme 4 *Chemical structure of oleandomycin (OLE)*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{35}H_{61}NO_{12}$	687.9 g/mol	687.4 amu	8.84	1.69

IUPAC Name: (3S,5R,6S,7R,8R,11R,12S,13R,14S,15S)-14-(((2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-6-hydroxy-12-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-5,7,8,11,13,15-hexamethyl-1,9-dioxaspiro[2.13]hexadecane-4,10-dione

Table 5 *Optimized MS/MS parameters for EES, ERY A and OLE*

	API III Plus	API 3000™	API 5000™
Probe temperature	500 °C	500 °C	350 °C
Discharge needle current	3 µA	3 µA	5 µA
Orifice voltage / Declustering potential	40 V (OR)	46 V (OR)	50 V (DP)
Collision energy	40 eV	38 eV	40 eV
Nebulizer gas (APCI)	80 psi	80 psi	50 psi (GS1)
Auxiliary gas (APCI)	2 L/min	2 L/min	50 psi (GS2)
Nebulizer, auxiliary and curtain gas	High purity nitrogen	High purity nitrogen	High purity nitrogen
Collision gas	High purity argon	High purity nitrogen	High purity nitrogen
Collision gas thickness	$240 \cdot 10^{13}$ atoms/cm^2	4 ($2.4 \cdot 10^{-5}$ torr)	5 ($1.7 \cdot 10^{-5}$ torr)

As the ethylsuccinate ester of erythromycin is unstable in methanol but stable in acetonitrile [103], chromatographic separations were done without methanol in the

mobile phase. The ionisation processes in the APCI source can be supported by a buffer with low ion-strength in the mobile phase, which was done with ammonium acetate (5 mM). The stability of erythromycin in acidic solution has been evaluated by Nakagawa et al. [104]. They found that erythromycin degraded more than 20 % within one hour at pH 4, but was stable at pH 5. Cachet et al. [105] stated that stability is critical during sample preparation and observed no degradation during measurement, even with a mobile phase of pH 3. Therefore, the plasma samples were buffered with ammonium acetate buffer (50 mM, pH 4.8) immediately before precipitation. For the mobile phase, an ammonium acetate buffer (5 mM, pH 4.0) was mixed with acetonitrile (55/45, v/v), which results in an experimental pH of the mobile phase of approximately pH 5.

This mobile phase composition was delivered at a flow rate of 1.2 mL/min to a CN reversed phase column, resulting in a baseline separation of EES and ERY A within a run time of approximately 3.0 minutes (1.0 minute (ERY A, OLE) and 1.5 minutes (EES)).

As previously discussed, EES reaches peak serum concentrations of 1.5 µg/mL following administration of a 500-mg dose, whereby ERY A reaches peak serum concentrations of 0.5 µg/mL [1]. In order to detect 5 % of C_{max}, the LLOQs of EES and ERY A should be lower than 75 ng/mL, and 25 ng/mL, respectively. In order to be prepared for other dosing regimens, the following calibration ranges were defined: 10.0 to 5000 ng/mL for EES and 4.00 to 2000 ng/mL for ERY A.

The calibration standards and the SQC samples were prepared in drug-free human plasma containing ammonium heparinate as anticoagulant because the study samples were planned to be collected using the same anticoagulant. To control probable conversion of EES to ERY A in human plasma, SQC samples containing EES+ERY A and SQC samples containing either EES or ERY A were prepared.

To prevent erythromycin from intramolecular ketal formation (Scheme 3), all sample handling as well as the sample preparation procedure was done in an ice-water bath. After thawing the human plasma samples, calibration standards and SQC samples in a cold water bath, the samples are mixed thoroughly and centrifuged for 5 minutes at approximately 4 °C and 2380 g to separate eventually present solid components.

As the most cost-effective and fastest sample preparation technique, protein precipitation is preferred: An aliquot of 100 µL of human plasma is stabilized by addition of 50 µL of ammonium acetate buffer (50 mM, pH 4.8) and subsequently deproteinised by adding 250 µL of acetonitrile containing the internal standard (IS)

oleandomycin, see Scheme 4. The IS working solution at a concentration of 500 ng/mL resulted in good peak area ratios (EES/IS and ERY A/IS) on API III Plus. For the more sensitive systems API 3000™ and API 5000™, the IS working solution was further diluted by factor ten.

The precipitated sample, containing the IS, is mixed thoroughly to facilitate deproteinisation. The precipitate is separated by centrifugation for 5 minutes at 3600 rpm (2380 g) at approximately 4 °C. Lowering the ion strength and raising the water content of the sample gives more constant and sharper peaks. Therefore, an aliquot of 100 µL of the supernatant is diluted with 100 µL of ammonium acetate buffer (5 mM, pH 4.8) and after mixing, 30 µL of each sample is injected onto the LC-MS/MS system. In Table 6 the optimized sample preparation procedure and LC-MS/MS conditions are summarized.

Table 6 *Summary of the assay for EES and ERY A in human plasma*

Characteristic	***Details***
Sample preparation	100 µL plasma + 50 µL NH_4CH_3COO (50 mM, pH 4.8) + 250 µL CH_3CN /IS 100 µL supernatant + 100 µL NH_4CH_3COO (5 mM, pH 4.8)
Internal standard	Oleandomycin (OLE)
Column	YMC-Pack Cyano, 3 µm (50 x 4.0 mm)
Mobile phase	45 % CH_3CN 55 % NH_4CH_3COO (5 mM, pH 4.0)
Flow rate	1.2 mL/min
Run time	3.0 min
Ionisation	APCI – atmospheric pressure chemical ionisation
Polarity	Positive ion mode
Mass transitions	m/z 863 → m/z 286 (EES) m/z 734 → m/z 158 (ERY A) m/z 688 → m/z 158 (OLE)

CH_3CN /IS: acetonitrile containing the internal standard oleandomycin (500 ng/mL)

3.1.4 Validation

This method was validated according to the FDA guideline on BMV [33], as it was intended for measurement of concentration data for pharmacokinetic studies. The data were obtained from a previous validation and the results are explained in this chapter.

The specificity was determined in drug-free human plasma from six individuals. No co-eluting peaks from matrix components interfered with the peaks of erythromycin ethylsuccinate, erythromycin A and the IS.

For the determination of linearity and sensitivity (LLOQ) of the assay a calibration curve for each analyte, including two blank samples which were not used for linear regression, was prepared and analyzed on each of the five validation days. The precision of the calibration standards is summarized in Table 7.

Table 7 *Inter-day precision and accuracy of the calibration standards containing erythromycin ethylsuccinate or erythromycin A in human plasma*

	EES	ERY A
Precision (range)	1.4 to 12.3 %	1.2 to 9.2 %
Accuracy (range)	-4.4 to 6.1 %	-3.8 to 5.0 %
Precision (LLOQ)	12.3 %	9.2 %
Accuracy (LLOQ)	5.4 %	1.7 %

The calibration curves are linear over the whole concentration range, the correlation coefficient of all curves being better than 0.999. The mean linear regression equations of the calibration curves generated during the validation are:

EES: $y = 0.0009\ (\pm 0.0005) + 0.0006\ (\pm 0.0000)\ x,\ r^2 > 0.999$

ERY A: $y = 0.0008\ (\pm 0.0004) + 0.0008\ (\pm 0.0000)\ x,\ r^2 > 0.999$

y: ratio of analyte peak area and IS peak area, *x*: plasma concentration of analyte

Both, the slope and the intercept show very good consistency during all five days, as does the precision and accuracy of the calibration standards, especially the lower limit of quantification.

The intra-day and the inter-day precision and accuracy of the assay was evaluated preparing three types of SQC samples. These types include SQCs in human plasma containing EES or ERY A solely, or containing both, EES and ERY A together in one sample. In this way, a probable conversion of EES to ERY A could be observed. Each SQC type was prepared at four concentration levels and analyzed five times on five consecutive validation days. The concentrations of the SQCs were calculated by the corresponding calibration curve. Precision and accuracy data are summarized in Table 8 and Table 9.

Table 8 *Intra- and inter-day precision and accuracy for the analysis of spiked quality control samples containing EES or ERY A in human plasma*

Nominal concentration [ng/mL]	Analyte	Intra-day (N = 5)		Inter-day (N = 25)	
		precision [%]	accuracy [%]	precision [%]	accuracy [%]
4000	EES	3.5	107.0	3.5	105.3
400.0	EES	5.3	100.2	4.6	103.8
100.0	EES	4.8	106.7	4.5	105.6
20.00	EES	6.6	102.9	6.4	99.0
10.00	EES	7.0	101.0	8.4	98.9
1600	ERY A	3.3	105.2	4.6	99.6
160.0	ERY A	3.9	102.4	3.9	100.4
40.00	ERY A	5.6	102.4	5.6	97.8
8.000	ERY A	7.2	105.0	7.4	100.8
4.000	ERY A	7.4	101.1	7.6	101.7

Table 9 *Intra- and inter-day precision and accuracy for the analysis of spiked quality control samples containing EES and ERY A in human plasma*

Nominal concentration [ng/mL]	Analyte	Intra-day (N = 5)		Inter-day (N = 25)	
		precision [%]	accuracy [%]	precision [%]	accuracy [%]
4000	EES	3.0	96.5	5.5	98.5
400.0	EES	1.7	95.7	6.1	97.7
100.0	EES	4.7	98.2	5.8	98.4
20.00	EES	8.0	97.8	7.1	97.5
10.00	EES	7.3	98.6	8.6	98.0
1600	ERY A	3.8	106.3	3.2	104.0
160.0	ERY A	1.3	103.6	3.8	101.8
40.00	ERY A	2.8	100.9	5.1	100.6
8.000	ERY A	6.6	102.2	8.9	100.9
4.000	ERY A	5.2	107.6	4.4	101.0

Erythromycin ethylsuccinate and erythromycin A can be measured with high precision and accuracy in human plasma, when analyzed alone in each sample. As the human plasma samples from the erythromycin ethylsuccinate study will contain both analytes, it should be tested if both analytes can be determined reliably in a

simultaneous analysis. Table 9 summarizes the results of the validation samples containing both analytes at the same time.

The small negative deviation from 100 % of EES accuracies combined with the small positive deviation of ERY A accuracies in Table 9 may indicate a slight conversion from EES to ERY A, but these values are statistically not significant. This is also supported by the data in Table 8, confirming that simultaneous determination of EES and ERY A is possible with very high accuracy and precision.

The mean (±SD) absolute extraction recovery of EES and ERY A was determined to be 99.5 ± 5.0 % (EES) and 95.8 ± 1.6 % (ERY A) over the whole concentration range. The mean absolute extraction recovery of the IS oleandomycin at the working concentration is 91.3 ± 1.2 %. The simple protein precipitation introduced to extract the analytes from plasma shows satisfactory recovery for both analytes. The precision of all analytes was ≤5.1 %.

No influence of haemolysed plasma on the precision and accuracy of EES and ERY A in five replicates at two concentration levels, each, was observed. The mean concentration values of EES and ERY A in haemolysed human plasma were within ±7 % of the nominal concentrations. The precision was better than 4 %, see Table 10. Therefore, haemolysed human plasma samples can be analysed with the same precision and accuracy as normal human plasma samples.

Table 10 *Accuracy and precision of EES and ERY A in haemolysed plasma*

Concentration [ng/mL]	Analyte	Accuracy [%]	Precision [%]
4000	EES	101.9	2.0
100.0	EES	102.9	3.9
1600	ERY A	93.3	1.5
40.00	ERY A	94.2	3.5

Stability data for EES and ERY A in human plasma are presented in Table 11 and Table 12. The stability samples for EES and ERY A were prepared separately in human plasma: five replicates of EES (4000 ng/mL and 100 ng/mL) and ERY A (1600 ng/mL and 40 ng/mL), respectively.

The short-term stability data indicate that no ketal formation occurred in plasma samples at 4 °C within 1.5 hours. Therefore, plasma samples containing EES and ERY A can be prepared for analysis in an ice-water bath within 90 minutes, which is

enough time for precipitation of 100 samples during three freeze-thaw cycles. The results of the stability experiments indicate that EES and ERY A are stable in human plasma for at least 1.5 hours at 4 °C, and for at least 2 months at -20 °C and -70 °C.

Table 11 *Statistical evaluation of the stability experiments for EES*

Stability experiment	Time	Concentration [ng/mL]	Point estimator [%]	Lower Limit [%]	Upper Limit [%]
Short-term stability (approximately 4 °C)	30 min	4000	104.0	101.2	106.8
	60 min		101.8	99.9	103.7
	90 min		102.7	101.8	103.6
	30 min	100.0	101.4	98.5	104.3
	60 min		103.7	98.3	109.3
	90 min		101.6	96.7	106.9
Post-preparative stability (autosampler, approx. 4 °C)	24 hours	4000	101.6	97.8	105.6
	24 hours	100.0	101.3	98.6	104.2
Post-preparative stability (approximately -70 °C)	24 hours	4000	102.8	102.2	103.4
	24 hours	100.0	98.8	88.8	109.8
Freeze-thaw stability (approximately -70 °C)	Once	4000	102.3	97.1	107.7
	Twice		101.1	98.3	103.9
	Thrice		102.3	99.8	104.8
	Once	100.0	102.9	100.6	105.2
	Twice		96.4	89.4	104.0
	Thrice		103.0	102.1	103.9
Long-term stability (approximately -20 °C)	11 days	4000	98.6	96.2	101.0
	2 months		98.2	93.7	102.9
	11 days	100.0	98.9	93.3	104.7
	2 months		105.0	103.0	107.0
Long-term stability (approximately -70 °C)	11 days	4000	101.6	97.6	105.8
	2 months		101.8	97.4	106.4
	11 days	100.0	97.8	94.0	101.8
	2 months		101.4	97.7	105.3

For the stability in prepared human plasma and during two freeze-thaw cycles, the lower limits of the confidence intervals are 88.8 and 89.4 % respectively. This indicates that the data in these two experiments was little less precise as the data of the other experiments. This is not critical since the precision is generally rated acceptable within 85-115%. In addition, the point estimators, reflecting the accuracy of the stability data, are very close to 100% (98.8 and 96.4 %). Therefore, both

analytes are stable in prepared human plasma for at least 24 hours and during three freeze-thaw cycles, each longer than 12 hours.

The hydrolysis of erythromycin ethylsuccinate in human plasma has also been studied by Croteau et al. [94] who found half-lives of 1 hour at 37 °C and 4 hours at 4 °C, whereas Tsuji et al. [95] found approximately 10 % hydrolysis of EES during 13 days at -20 °C, but no further decrease after 36 days. Others reported a combined experiment of 1 week at -20 °C followed by three freeze-thaw cycles to 37 °C, which affected the stability to a certain extent [91].

Table 12 *Statistical evaluation of the stability experiments for ERY A.*

Stability experiment	**Time**	**Concentration [ng/mL]**	**Point estimator [%]**	**Lower Limit [%]**	**Upper Limit [%]**
Short-term stability (approximately 4 °C)	30 min	1600	98.6	95.5	101.7
	60 min		98.9	97.0	100.8
	90 min		99.4	96.8	102.0
	30 min	40.00	102.9	97.4	108.8
	60 min		100.5	96.1	105.1
	90 min		96.7	92.5	101.1
Post-preparative stability (autosampler, approx. 4 °C)	24 hours	1600	95.6	95.1	96.1
	24 hours	40.00	97.2	91.5	103.1
Post-preparative stability (approximately -70 °C)	24 hours	1600	96.3	94.5	98.1
	24 hours	40.00	98.6	93.2	104.2
Freeze-thaw stability (approximately -70 °C)	Once	1600	98.5	97.5	99.7
	Twice		95.8	94.1	97.7
	Thrice		102.0	100.1	103.9
	Once	40.00	99.8	93.1	106.9
	Twice		102.0	96.2	108.2
	Thrice		97.6	93.8	101.6
Long-term stability (approximately -20 °C)	11 days	1600	103.9	101.6	106.2
	2 months		100.1	97.7	102.5
	11 days	40.00	98.9	97.2	100.6
	2 months		99.1	94.0	104.4
Long-term stability (approximately -70 °C)	11 days	1600	100.5	98.1	102.9
	2 months		98.3	96.2	100.4
	11 days	40.00	100.9	97.4	104.6
	2 months		102.9	94.7	111.7

3.1.5 Method comparison on three different LC-MS/MS systems

During method transfer all tuning parameters were optimized, assuring the real optimum for the erythromycin method on all instruments and therefore producing absolute comparability of the validation experiments. For method comparison experiments were chosen that are influenced by either the ionisation process or the alternated ion path on the quantification of EES and ERY A. These experiments were performed by using the same analytical column, mobile phase and sample preparation technique to assure absolute comparability on API 3000™ and API 5000™. Details to the experiments in principle are detailed in chapter 2.5.

Data from system-dependent validation experiments are compared to previously obtained data from API III Plus, see Table 13 and Table 14.

Table 13 *Validation data of EES on three different LC-MS/MS systems*

Experiment	**Unit**	**API III Plus**	**API 3000™**	**API 5000™**
Selectivity in plasma	[-]	no interference in 6 individuals	no interference in 20 individuals	no interference in 20 individuals
Linearity range Order of magnitude	[ng/mL]	10 to 5000 $5.0 \cdot 10^2$	1.25 – 4002 $3.2 \cdot 10^3$	0.156 – 4002 $2.6 \cdot 10^4$
Correlation Coefficient	[-]	>0.999	>0.997	>0.999
LLOQ **Gain for newer API**	[ng/mL]	10 -	1.25 **8**	0.156 **8**
Precision of LLOQ Accuracy of LLOQ	[%] [%]	12.3 105.4	2.7 101.3	4.1 101.7
Highest SQC Lowest SQC	[ng/mL] [ng/mL]	4000 10	4002 2.50	4002 0.156
Precision of SQCs Accuracy of SQCs	[%] [%]	3.5 to 8.4 98.9 to 105.6	6.8 to 8.8 95.7 to 105.2	3.9 to 12.4 96.9 to 104.5
Matrix Effect EES EES/IS	 MF, Prec [%] MF, Prec [%]	 - -	 0.84, 10.8 % 0.92, 1.7 %	 0.84, 8.7 % 0.88, 1.8 %

SQC: spiked quality control, LLOQ: lower limit of quantification, EES: erythromycin ethylsuccinate, IS: internal standard, MF: matrix factor, Prec: precision in plasma from 6 individuals

Table 14 *Validation data of ERY A on three different LC-MS/MS systems*

Experiment	**Unit**	**API III Plus**	**API 3000™**	**API 5000™**
Selectivity in plasma	[-]	no interference in 6 individuals	no interference in 20 individuals	no interference in 20 individuals
Linearity range Order of magnitude	[ng/mL]	4.00 - 2000 $5.0 \cdot 10^2$	0.781 – 2100 $2.7 \cdot 10^3$	0.0977 – 2100 $2.1 \cdot 10^4$
Correlation Coefficient	[-]	>0.999	>0.999	>0.998
LLOQ **Gain for newer API**	[ng/mL]	4.00 -	0.781 **5**	0.0977 **8**
Precision of LLOQ Accuracy of LLOQ	[%] [%]	9.2 101.7	1.2 96.4	6.9 106.5
Highest SQC Lowest SQC	[ng/mL] [ng/mL]	1600 4	2100 1.56	2100 0.195
Precision of SQCs Accuracy of SQCs	[%] [%]	3.9 to 7.6 97.8 to 101.7	2.2 to 10.0 97.6 to 101.0	1.6 to 9.7 100.8 to 104.2
Matrix Effect ERY A ERY A/IS	 MF, Prec [%] MF, Prec [%]	 - -	 1.05, 2.0 % 1.02, 1.9 %	 0.98, 3.0 % 1.03, 1.0 %

SQC: spiked quality control, LLOQ: lower limit of quantification, ERY A: erythromycin A, IS: internal standard, MF: matrix factor, Prec: precision in plasma from 6 individuals

This high sensitivity on API 5000™ derives from the large orifice, which enables a relatively high number of molecular ions to enter the mass spectrometer, whereas the vertical spraying ion source enables a relatively high number of analyte ions among relatively few interfering un-charged molecules to enter the mass spectrometer.

The linear regression of the peak area ratios versus concentrations were fitted over the mentioned concentration ranges. The mean linear regression equations of the calibration curves were:

API III Plus, EES: $y = 0.0009\ (\pm 0.0005) + 0.0006\ (\pm 0.0000) \cdot x \quad (r^2 \geq 0.999)$

API 3000™, EES: $y = 0.0001\ (\pm 0.0020) + 0.0068\ (\pm 0.0016) \cdot x \quad (r^2 \geq 0.997)$

API 5000™, EES: $y = 0.0000\ (\pm 0.0001) + 0.0127\ (\pm 0.0125) \cdot x \quad (r^2 \geq 0.999)$

API III Plus, ERY A: $y = 0.0008\ (\pm 0.0004) + 0.0008\ (\pm 0.0000) \cdot x \quad (r^2 \geq 0.999)$

API 3000™, ERY A: $y = -0.0001\ (\pm 0.0006) + 0.0084\ (\pm 0.0012) \cdot x \quad (r^2 \geq 0.999)$

API 5000™, ERY A: $y = 0.0010\ (\pm 0.0003) + 0.0130\ (\pm 0.0003) \cdot x \quad (r^2 \geq 0.998)$

y: ratio of the EES or ERY A peak area to the IS peak area

x: plasma concentration of EES or ERY A

Excellent linearity and correlation coefficients were obtained for all linearity ranges.

The overall precision and accuracy of the back-calculated calibration standards in human plasma on API III Plus ranged from 1.2 to 12.3 % and from 95.6 to 106.1 %, on API 3000™ it ranged from 0.3 to 8.2 %, and from 95.4 to 104.2 % for both analytes. On API 5000™ the precision and accuracy of the calibration standards ranged from 0.8 to 8.2 % and from 90.4 to 106.5 %, for both analytes. Therefore no remarkable difference in precision or accuracy of the calibration standards was observed on all three mass spectrometers.

Increasing sensitivity was achieved while transferring the method from one system to the next. This can be seen by the LLOQ, which was improved two times by a factor of 8 for EES and by the factors 5 and 8 for ERY A. The precision and accuracy obtained at the LLOQs ranged from 1.2 to 12.3 % and from 96.4 to 106.5 %, respectively, for both analytes on all three instruments, still showing a high precision at the lowest limits of quantification. In addition, the signal-to-noise ratio was greater than 5 for both analytes on all systems, except for EES at API 5000™ where it was greater than 10.

Due to improved sensitivity of the newer mass analyzers, interferences in the extended lower calibration range may occur. Therefore, the selectivity of the method was tested by preparing drug-free human plasma from twenty (ten males and ten females) individuals with and without IS. With none of the tested mass analyzers interference between matrix components and EES or ERY A, or the IS (oleandomycin) occurred in the observed concentration ranges. Even with the most sensitive instrument, the API 5000™, samples were free from interferences down to 0.0488 ng/mL.

For the evaluation of the intra-day and inter-day precision and accuracy on the API 3000™ and API 5000™, SQC samples containing EES or ERY A in human plasma were analyzed on three validation days, respectively. Sample preparation and calculations were performed as described above in the validation procedure. The overall inter-day precision of the SQC samples containing EES or ERY A in human plasma fulfilled the pre-defined acceptance criteria very well. All precision data were below 15 % and accuracy was well within 85-115 % deviation from nominal concentration.

As mass analyzers with quite different ionisation sources are compared, the test for matrix effect is important. The matrix effect was investigated quantitatively as matrix

factor (MF) and qualitatively with post column infusion (for details on experimental set-up see chapter 2.5.7).

All matrix factors (MF, mean ± SD) of ERY A were close to 1.0 on API 3000™ and on API 5000™ indicating the absence of a matrix effect of ERY A in both analytical systems. For EES, the MF calculated only by peak areas were slightly lower than the MF corrected by the internal standard oleandomycin. This shows that slight ion suppression of EES in both ionisation sources of API 3000™ and API 5000™ occurs. However, as the IS oleandomycin undergoes slight suppression to the same extent, the overall matrix factor is compensated. The MF does not need to be 1.0 (see EMA [34]), but has to be constant for all analyzed matrices, assuring no difference for calibration standards and different subjects' samples. That is the case in this experiment, proven by the small standard deviations.

Via post column infusion no increase or decrease of the signal was observed at and near the retention times of EES, ERY A and OLE, respectively, and therefore, a matrix effect was excluded for both instruments.

3.1.6 Comparison of the LC-MS/MS method with procedures reported in the literature

Table 15 shows LLOQ, run time, sample volume, sample preparation and quality control data for several previously reported analytical methods determining erythromycin ethylsuccinate in human plasma [91-95] in comparison to the present LC-MS/MS method.

All previously reported methods are based on extraction procedures requiring high sample volumes (up to 3.0 mL), expensive organic solvents and much time for sample preparation procedures. Such high sample volumes should be avoided for two reasons: First, blood volume taken from volunteers or patients should be kept low for ethical reasons and 3 mL of plasma would require approximately 6 mL of blood for one single aliquot and one single timepoint. Second, re-analysis of samples due to analytical or pharmacokinetic reasons is hardly possible when sample volume is enough for only one analysis.

This new method needs 0.100 mL sample volume only, and is a fast and low-cost precipitation method featured by excellent recovery data (>95% for ERY and >99% for EES) with high precision (CV ≤ 5%). Comparison with previously published data reveals that all LLE methods are characterized by lower recoveries. They did not

control pH or used weak alkaline pH conditions, though two pH steps above the pKa of 8.8 would be needed to achieve fully neutral ERY A and EES molecules for complete extraction into organic solvent. This was overcome by one older method [95], where a higher recovery is produced by a very laborious triple LLE.

Furthermore, the method saves analyzing time, with 3 minutes being more than twice as fast as the fastest previously reported method [91]. Other reported methods need up to 40 minutes per sample.

Only one previous method reported a similar number of validation experiments [91], but no stability assessment during plasma sample handling and their sample preparation process, which used LLE and evaporation at 50°C. The developed method used cooled PPT and was tested by extensive validation experiments, like different stability investigations, the influence of hemolyzed plasma and matrix effects.

The most important advance of the new method is the enhanced sensitivity down to 0.156 ng/ml for erythromycin ethylsuccinate and down to 0.098 ng/mL for erythromycin base. This in an improvement by a factor of 3 (EES) and by a factor of 10 (ERY A).

With the new LC-MS/MS method erythromycin ethylsuccinate and erythromycin base can be measured with high sensitivity using simple protein precipitation sample work up. With only 3 minutes runtime about 400 human plasma samples can be measured per LC-MS/MS system per day.

Table 15 *Analytical methods for the determination of erythromycin ethylsuccinate and erythromycin A in human plasma; grouped by analytical technique and descending years of publication*

Method		Linearity Range [ng/mL]	Approx. run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of SQCs [%]	Recovery (mean ± SD) [%]	Sample Vol. [mL]	Sample prep.	Internal standard	Additional VAL exp.	Application
LC-MS/MS (current method)	EES ERY	10-5000 4.0-2000	3.0	12.3 / 105.4 9.2 / 101.7	3.5 to 8.4 / 98.9 to 105.6 3.9 to 7.6 / 97.8 to 101.7	99.5±5.0 (EES) 95.8±1.6 (ERY) 91.3± 1.2 (IS)	0.100	PPT	oleandomycin	1, 2a, 2b, 2c, 2d, 3	500-mg PK
API 3000™ (current method)	EES ERY	1.25-4002 0.781-2100		2.7 / 101.3 1.2 / 96.4	6.8 to 8.8 / 95.7 to 105.2 2.2 to 10.0 / 97.6 to 101.0					4, 5	
API 5000™ (current method)	EES ERY	0.156-4002 0.098-2100		4.1 / 101.7 6.9 / 106.5	3.9 to 12.4 / 96.9 to 104.5 1.6 to 9.7 / 100.8 to 104.2					4, 5	
LC-MS/MS [91] Gu 2006	EES ERY	0.5-5000	6.5	17.4 / 96.0 16.1 / 96.0	0.8 to 2.1 / 94.0 to 97.3 1.5 to 5.9 / 100.9 to 102.4	56±3 to 59±4 77±7 to 82±3	0.200	LLE	diazepam	1, 2b, 2c, 2d, 4	500-mg PK
LC-(FAB)-MS [92] Kokkonen 1991	EES	105-10500	13	36 / 100	12.0 / 88.6	77±4 (5250 ng/mL)	1.0	LLE	$[^2H_5]$-EES	n.r.	n.r.
FAB-MS [93] Ottoila 1987	EES ERY	50-5000 100-5000	n.r.	6.3 / n.r. 5.5 / n.r.	n.r.	86±4 (1000 ng/mL) 90±1 (1000 ng/mL)	1.0	LLE	$[^2H_5]$-EES $[^2H_3]$-ERY	n.r.	PK (no data)
LC/ED [94] Croteau 1987	EES ERY	250-3000 250-10000	40	n.r.	3.2 / n.r. 10.3 / n.r.	55.8 74.0 (ERY), 68 (ROX)	2.0	LLE	roxithromycin	2a	600mg-PK (graphical)
LC/FD [95] Tsuji 1978	EES ERY	10-n.r.	24	n.r.	n.r.	96.2±4.5 (600ng/mL) 102.2±6.0 (600ng/mL)	1-3	LLE (3x)	n.r.	2b	200-mg PK (no data)

Prec: Precision, Acc: Accuracy, SD: standard deviation, ERY: erythromycin A, EES: erythromycin ethylsuccinate, PPT: protein precipitation, LLE: liquid-liquid extraction, n.r.: not reported; 1: Specificity, 2: Stability (a) short-term, (b) long-term, (c) post-preparative, (d) freeze-thaw, 3: haemolysed plasma, 4: Matrix Effect, 5: Signal-to-noise

3.1.7 Application to a pharmacokinetic study

3.1.7.1 Study design and sample handling

The LC-MS/MS method was used to investigate the pharmacokinetics of 587.25 mg erythromycin ethylsuccinate, equivalent to 500 mg erythromycin base in suspension, given as a single oral dose to 10 healthy male and female volunteers under fasting conditions. Blood collection was performed immediately before administration and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00, 16.00, and 24.00 hours after oral administration of study drug.

The samples were collected into ammonium heparinate monovettes and cooled in an ice-water bath to assure the stability of erythromycin ethylsuccinate. This procedure surpasses serum collection, where the blood has to clot for several minutes at room temperature. The blood samples were cooled to approximately 4 °C during centrifugation for 10 minutes at 1500 g. The plasma was subsequently transferred into polypropylene tubes and immediately frozen at approximately -70 °C until analysis.

3.1.7.2 Determination of plasma concentrations

The concentrations of erythromycin ethylsuccinate and its active metabolite erythromycin A were determined using the validated LC-MS/MS method as summarized in Table 6.

The human plasma samples were analyzed for erythromycin ethylsuccinate and erythromycin A together with injection standards, calibration standards, study samples and SQC samples was measured to assure quality during measurement.

Figure 13 depicts typical MRM-chromatograms of EES, ERY A and the IS (oleandomycin) in human plasma samples:

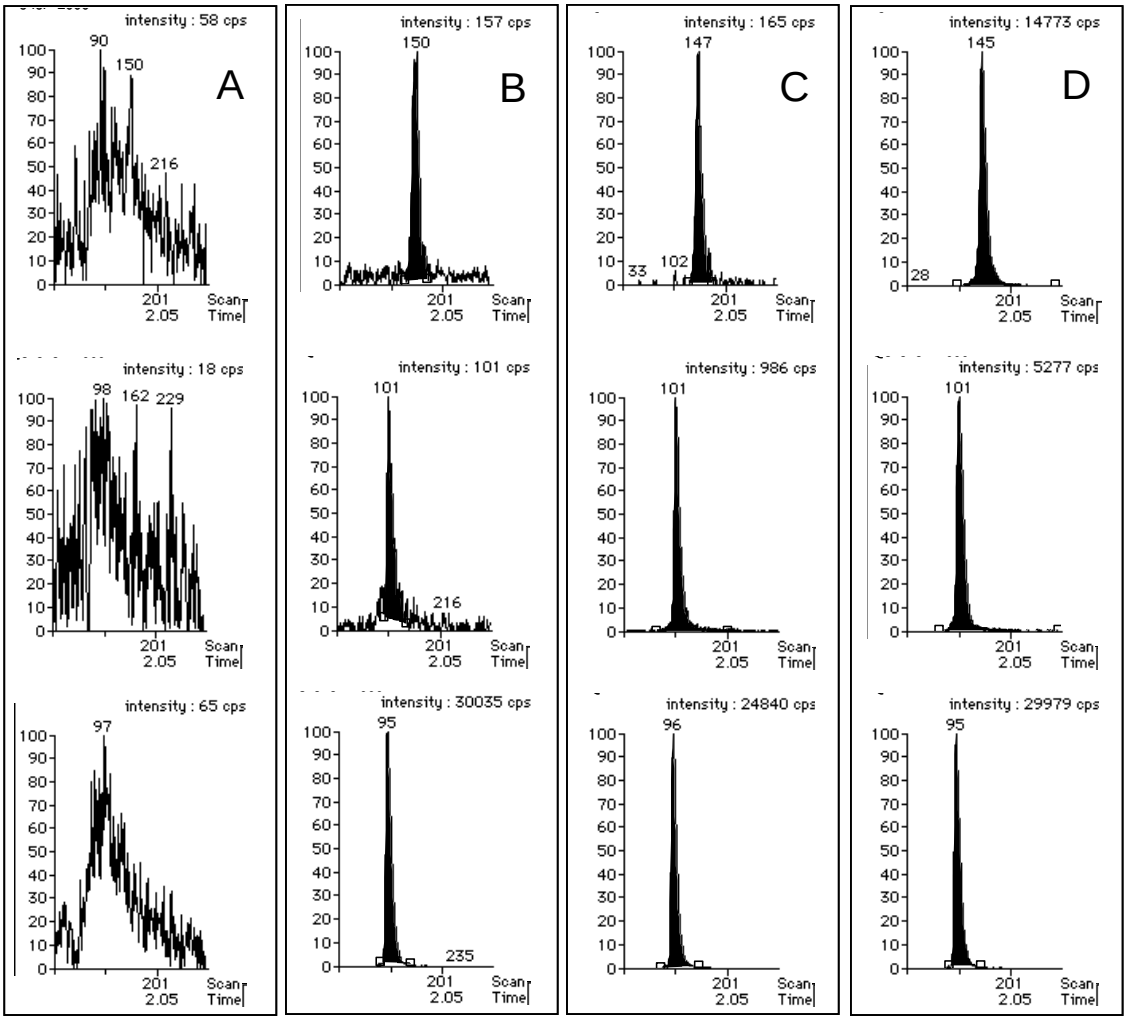
A) Blank plasma

B) LLOQ (10 ng/mL EES / 4.0 ng/mL ERY A)

C) Study sample (t = 8.0 h) containing 13.5 ng/mL EES and 44.7 ng/mL ERY A

D) Study sample (t = 0.75 h) containing 1146 ng/mL EES and 221.8 ng/mL ERY A

Figure 13 *Representative MRM-chromatograms for EES (top), ERY A (middle) and OLE (IS, bottom) in human plasma*



The back-calculated concentrations of calibration standards and the determined concentrations of spiked quality control samples were compared to the theoretical concentrations to calculate the respective accuracy. The overall precision and accuracy is summarized in Table 16.

Table 16 *Accuracy and precision of calibration standards and SQC samples during analysis of erythromycin ethylsuccinate and erythromycin A in study samples*

	Theoretical Concentration (µg/mL)	Mean Accuracy (%)	Mean Precision (%)
Calibration standards EES	5.00	100.3	1.7
	4.00	98.1	2.0
	2.00	103.6	2.9
	1.00	99.1	2.0
	0.200	100.3	1.4
	0.100	99.3	5.1
	0.0500	96.9	6.0
	0.0200	103.1	2.2
	0.0150	99.1	4.1
	0.0100	99.5	5.4
SQC samples EES	4.00	103.4	3.4
	0.400	105.8	5.0
	0.100	100.7	5.4
	0.0200	98.3	3.5
Calibration standards ERY A	2.00	98.7	2.2
	1.60	101.4	2.3
	0.800	100.2	1.9
	0.400	100.2	2.6
	0.0800	100.8	3.6
	0.0400	101.8	2.1
	0.0200	101.4	5.2
	0.00800	99.2	4.4
	0.00600	98.6	7.5
	0.00400	97.4	8.2
SQC samples ERY A	1.60	105.6	2.6
	0.160	105.2	1.6
	0.0400	104.7	5.1
	0.00800	103.8	6.0

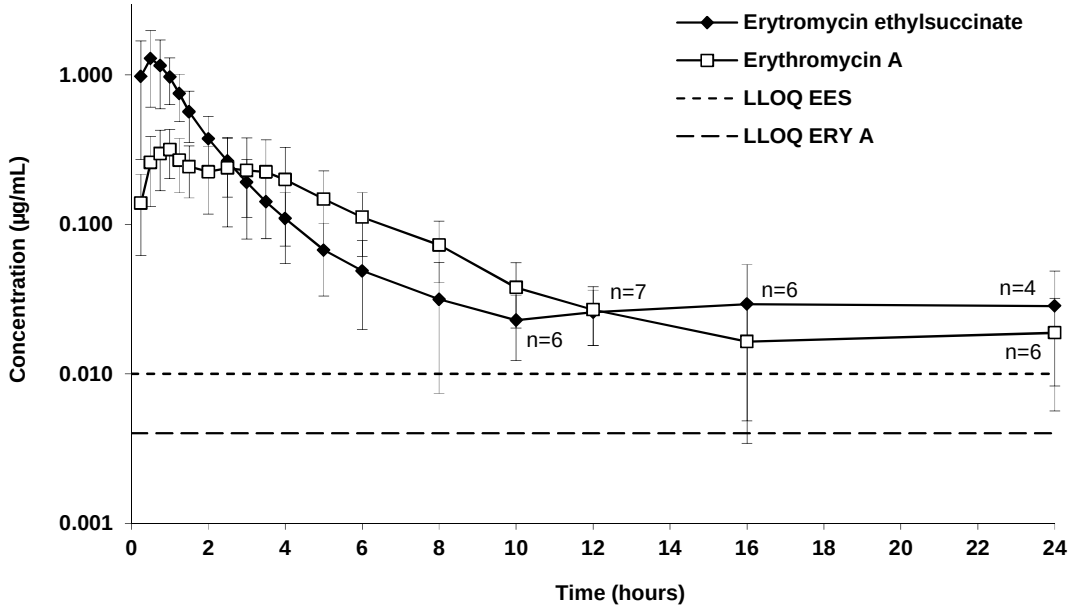
* Accepted limits by EMA and FDA: maximal 15 % for precision and 85-115 % for accuracy

The correlation coefficient of resulting linear regressions was at least 0.9994 in all sequences for both analytes. The inter-day precision of the spiked quality control samples of erythromycin ethylsuccinate and erythromycin A in human plasma analyzed within the batches of study samples was maximal 6.0 %. The mean accuracy of all calibration standards and SQC samples was within ±106.0 %, being well within the 15-% deviation limit and confirming the very high precision of this assay.

3.1.7.3 Pharmacokinetic results

The resulting concentration data was plotted versus time after oral administration of the EES suspension (587.25-mg EES suspension equivalent to 500 mg erythromycin base). The mean plasma concentration profiles are displayed in Figure 14.

Figure 14 *Plasma profiles (mean±SD) of EES and ERY A concentrations vs. time following an oral dose of EES suspension (eq. to 500 mg ERY base) to healthy volunteers (n=10)*



The mean peak concentration of the pro-drug EES (1.4 µg/mL) is reached after 36 minutes on average, whereas the mean peak concentration of its active metabolite (0.34 µg/mL) is attained on average 44 minutes later, at 1.3 hours. While the EES plasma concentrations curve is steeply sloping ($t_{1/2}$ = 5.2 h), the metabolite concentrations are decreasing more slowly ($t_{1/2}$ = 10.0 h), surpassing the EES concentrations on average 3 hours post-dose. From approximately 10 hours post-dose, the mean plasma concentrations of EES are slightly re-ascending, resulting in a second intersection with the metabolite curve. This derives from the fact that part of the samples at the time points 10, 12, 16 and 24 hours had no detectable concentration below the lower limit of quantification, not contributing to the mean values, which are used in the plasma profile. This is also reflected by the error bars in Figure 14, especially at the time points 16 and 24, which are spread below the

lower limits of quantification (LLOQ) indicating that the elimination of ERY A does not significantly overtake the elimination of EES.

In Table 17 the pharmacokinetic parameters of EES and ERY A from this study are compared to pharmacokinetic parameters reported in previously published studies.

Very few data are available on the pharmacokinetics of erythromycin ethylsuccinate. Only one paper [91] was found that describes data on a 500-mg oral dose, but without providing further information on the formulation, the higher AUC and C_{max} values can hardly be explained. Croteau et al. [106] compared EES tablets, equivalent to 600 mg ERY base, to erythromycin estolate and concluded pharmacokinetic advantages of the estolate over the ethylsuccinate ester. The time to reach maximum plasma concentrations is shorter for the suspensions (current study, [107]) than for the tablets ([106], [76]) reflecting that the tablets need additional time for dissolution before absorption can take place. It seems, that, in addition to faster absorption, the suspension could also be characterized by more complete absorption of EES. This may be concluded from the observation that the EES AUC of the suspension equivalent to 500 mg ERY A is higher than the EES AUC of the tablet equivalent to 600 mg ERY A. Comparing the ERY A AUC of the two formulations, virtually no difference can be observed. Probably, this can be ascribed to the fact that EES as a suspension is more supposed to the acidic environment in the gut than EES in the tablet. However, these comparisons should be handled carefully since erythromycin is characterized by non-linear pharmacokinetics.

In addition, the half-lives of EES (5 h) and ERY A (10 h) from the EES suspension in this study are much longer than the half-lives of the EES tablets (1.1 h EES, 1.5 h ERY A) reported by Croteau et al. [106]. The longer half-life of the studied suspension is an advantage, because longer dosing intervals can lead to more patient compliance with the dosing regime. The observed difference may be explained in two different ways:

Since the formulation of a drug mainly influences its absorption, which is probably not the rate-limiting process here (t_{max} suspension: 0.6 h/ 1.3 h EES/ERY A), the difference in the half-lives can be related more likely to the different doses (500 mg suspension versus 600 mg tablet) and non-linear behaviour of volume of distribution or elimination. As a basic compound, erythromycin binds to the saturable α-1-acid glycoprotein (protein binding: 84 % [1]), resulting in higher free plasma concentrations and a higher volume of distribution at higher doses. Erythromycin is

Table 17 *Pharmacokinetic parameters (mean±SD) of erythromycin ethylsuccinate (EES) and erythromycin base in healthy subjects after administration of a single oral dose of erythromycin ethylsuccinate*

Reference	No. of Subj./ Gender	Dose / Formulation	t_{last} [h]		$AUC_{0\rightarrow last}$ [µg·h/mL]	$AUC_{0\rightarrow\infty}$ [µg·h/mL]	C_{max} [µg/mL]	t_{max} [h]	$t_{1/2}$ [h]	Analytical method
Current study	10 / m,f	587.25 mg EES suspension (eq. to 500 mg ERY base)	24	EES	2.49 ± 1.11	2.63 ± 1.15	1.41 ± 0.63	0.60 ± 0.24	5.21 ± 3.95	LC-MS/MS
Gu 2006 [91]	4 / m	500 mg oral / n.r.	36		2.18	2.47	0.87	0.50	n.r.	LC-MS/MS
Croteau 1988 [106]	12 / m,f	EES tablets (eq. to 600 mg ERY base)	12		n.r.	1.88±1.20	0.91±0.69	1.27±0.49	1.12±0.90	LC/ED
Current study	10/m,f	587.25 mg EES suspension (eq. to 500 mg ERY base)	24	Erythromycin base from EES	1.76 ± 0.84	2.12 ± 1.42	0.34 ± 0.13	1.33 ± 0.95	10.0 ± 10.6	LC-MS/MS
Gu 2006 [91]	4 / m	500 mg oral / n.r.	36		3.16	3.39	0.71	0.50	n.r.	LC-MS/MS
Croteau 1988 [106]	12 / m,f	EES tablets (eq. to 600 mg ERY base)	12		n.r.	2.03 ± 1.93	0.58 ± 0.57	2.5 ± 1.4	1.54 ± 1.05	LC/ED
Thompson 1980 [76]	18 / m,f	EES 2x400-mg film tablets Immediately before food Immediately after food 1h after food	8		 7.48 ± 3.98 4.85 ± 3.28 5.51 ± 3.06	n.r.	 2.71 ± 1.34 1.54 ± 1.05 1.72 ± 0.89	 1.28 ± 0.79 2.39 ± 0.93 2.97 ± 0.81	n.r.	Microbiol.
Griffith 1969 [107]	9	EES suspension (eq to 200 mg ERY base)	8		n.r.	n.r.	0.32 0.26	0.5	n.r.	Microbiol

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

concentrated in the liver and excreted in the bile [1], whereas the main route of elimination is metabolism [9]. Higher free drug concentrations would increase hepatic clearance. However, given that $t_{1/2}$ behaves proportional to V/CL, the hepatic clearance needs to increase four times higher than V to achieve the observed difference in $t_{1/2}$, which is considered unlikely for an increase of the dose from 500 mg to 600 mg.

A further, probably easier explanation targets the data itself. Since the elimination half-life is determined from the latest values of each concentration-time profile, it strongly depends on the lower limit of quantification. If the concentrations of the last time points of some volunteers are detectable, but below the limit of quantification, it cannot contribute to the mean concentration of these time points. Then, the mean of all detectable concentrations appears higher and so does the elimination half-life.

3.1.8 Summary: Erythromycin ethylsuccinate

The LC-MS/MS assay for EES and ERY A in human plasma is simple, fast, reliable, sensitive, precise and accurate. The method employed simple protein precipitation of 100 µL of human plasma, and is linear in the range from 0.156 to 5000 ng/mL erythromycin ethylsuccinate and 0.098 to 2100 ng/mL erythromycin A. The principal advantage of the LC-MS/MS method described here is the simultaneous achievement of high absolute recovery (>96 %), high sensitivity on all three mass spectrometers (LLOQ from 0.049 to 10.0 ng/mL), high inter-day precision (≤12.3 %) and accuracy (within ±109.6 %) for LLOQ as well as excellent linearity (r^2>0.997) with a short run time of only 3.0 minutes. These characteristics make the method suitable for the precise and accurate measurement of low concentrations of EES and ERY A in the context of pharmacokinetic studies. The method was successfully applied to the analysis of human plasma samples during a pharmacokinetic study of an erythromycin ethylsuccinate suspension equivalent to 500 mg erythromycin base.

Stability for erythromycin ethylsuccinate and erythromycin A in native human plasma was proven for at least 2 months at approximately -20 °C and approximately -70 °C, for at least 1.5 hours at 4 °C and during three freeze-thaw cycles. No instability was observed after the sample preparation procedure for 24 hours at autosampler temperature and at approximately -70 °C.

The structural analogue oleandomycin is adequate as internal standard for erythromycin, shown by comparable recoveries of erythromycin ethylsuccinate,

erythromycin base and oleandomycin, as well as the absence of a matrix effect (erythromycin A) or reduction of the matrix factor (erythromycin ethylsuccinate) with and without oleandomycin.

The API 5000™ LC-MS/MS system significantly improves sensitivity for erythromycin ethylsuccinate and erythromycin A down to 0.16 ng/mL and 0.098 ng/mL, respectively. Moreover, it is possible to transfer the assay onto three different LC-MS/MS systems, differing in technical implementation of ionization, ion inlet and focussing. This transfer showed the good robustness of the method, and furthermore, that it can be expanded into the pg/mL range without loosing quality (accuracy and precision).

During study sample analysis the accuracy was between 96.9 and 105.8 % and precision ranged from 1.4 to 8.2 %, indicating that the quality of the obtained data was very high (allowed limits: 85-115% and maximal 15%). Only few pharmacokinetic data on erythromycin ethylsuccinate had been previously published. The current study adds data on a suspension formulation equivalent to 500 mg erythromycin A. Compared to other erythromycin formulations it can be distinguished by a pleasant flavour and a rather long half-life, which can help to increase patients' compliance with the dosing regime and by implication to increase the quality of their lives.

3.2 Roxithromycin

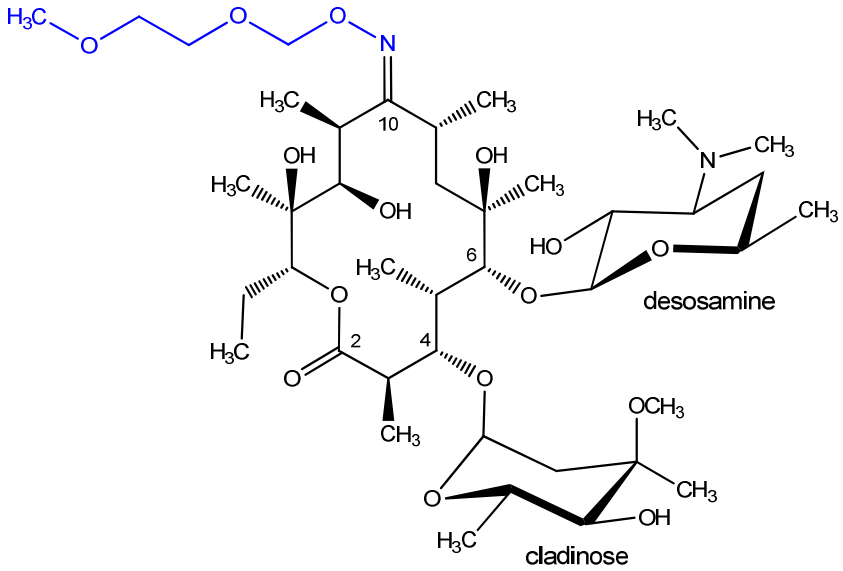
3.2.1 Chemical and pharmaceutical properties

Roxithromycin is a semi-synthetic macrolide antibiotic, which is very similar in composition, chemical structure and mechanism of action to erythromycin. Due to the conversion of the keto group in position 10 to an oxime roxithromycin is chemically more stable to acids than erythromycin and former macrolides, because a ketal formation is not possible. The stability against acids allows enhanced bioavailability and rapid absorption following oral application, as well as high serum concentrations and a longer serum half-life compared to erythromycin [108].

Roxithromycin is only partially metabolised, more than half of the parent compound being excreted unchanged [109]. As a basic compound (pKa 9.2 [110] and logP 2.75

[73]), roxithromycin binds to a small extent to albumin, mainly to α-1-acid glycoprotein, which is saturable already at clinically relevant roxithromycin concentrations above about 4 µg/mL [111]. At clinical relevant concentrations plasma protein binding ranges between 80 and 96 %. Due to the relatively high plasma protein binding of roxithromycin to the saturable α-1-acid glycoprotein, roxithromycin is characterized by non-linear pharmacokinetics, i.e. by a substantial increase in free plasma concentration from 4.3 to 13.4 % when the total concentration increases from 3.3 to 8.4 µg/mL [111].

Scheme 5 *Chemical structure of roxithromycin (ROX)*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{41}H_{76}N_2O_{15}$	837.0 g/mol	836.5 amu	9.2	2.75

IUPAC Name: (3R,4S,5S,6R,7R,9R,11S,12R,13S,14R,Z)-6-(((2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-14-ethyl-7,12,13-trihydroxy-4-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-10-(((2-methoxyethoxy)methoxy)imino)-3,5,7,9,11,13-hexamethyloxacyclotetradecan-2-one

Like erythromycin, roxithromycin is classified as a bacteriostatic agent, but, depending upon its concentration or the micro-organism, it can act bactericidal [108]. It is the least active of the 14-membered macrolides having activity against gram-positive and gram-negative cocci, gram-positive bacilli and some gram-negative bacilli [9, 112-113].

3.2.2 Published analytical methods

The majority of the published methods to quantify roxithromycin in human serum or plasma include liquid chromatography with different detection methods, for example electrochemical detection (LC/ED) [114-117] or fluorescence detection (LC/FD) in combination with derivatisation [118-119]. Although macrolides exhibit low UV absorbance due to the lack of a strong chromophore, some spectrophotometric methods (LC/UV) to determine macrolide antibiotics in a variety of matrices have been reported [120-122]. Many of these approaches have been applied in pharmacokinetic studies [116-117, 119-120].

Previously reported LC/ED, LC/FD and LC/UV methods to quantify roxithromycin present relatively low sensitivity with lower limits of quantification (LLOQ) in the range of 65 to 500 ng/mL, extensive and time-consuming sample preparation procedures [114] using relatively high sample volumes [118-120] and long run times between 6 and 25 minutes [114-116, 118-121].

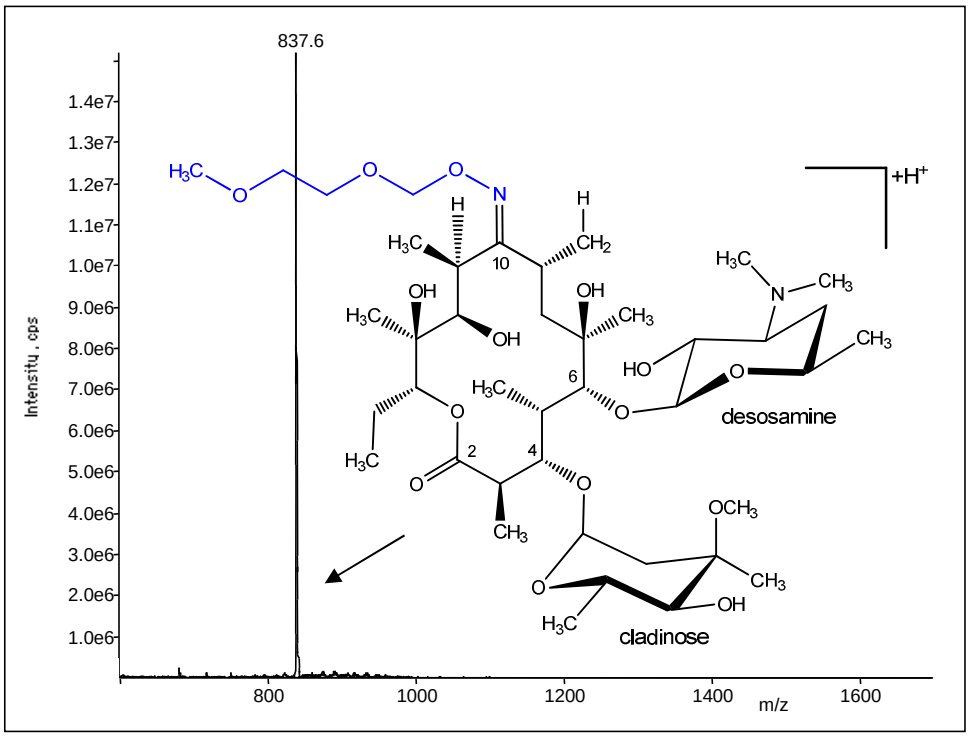
Several LC-MS/MS methods have been reported to determine macrolide antibiotics in a series of matrices like milk and yogurt [122-124], honey [125], human plasma [80, 126], rat lung tissue [127] and waste water [128-129].

However, very few LC-MS/MS methods have been published for roxithromycin in human plasma [49-51] to evaluate bioequivalence of roxithromycin formulations. The methods presented either a somewhat high LLOQ of 50 ng/mL [49-50], did not report precision and accuracy data for their LLOQ [51], needed relatively long run times of five to approximately seven minutes [49, 51], which is not suitable for large sample quantities.

3.2.3 LC-MS/MS method

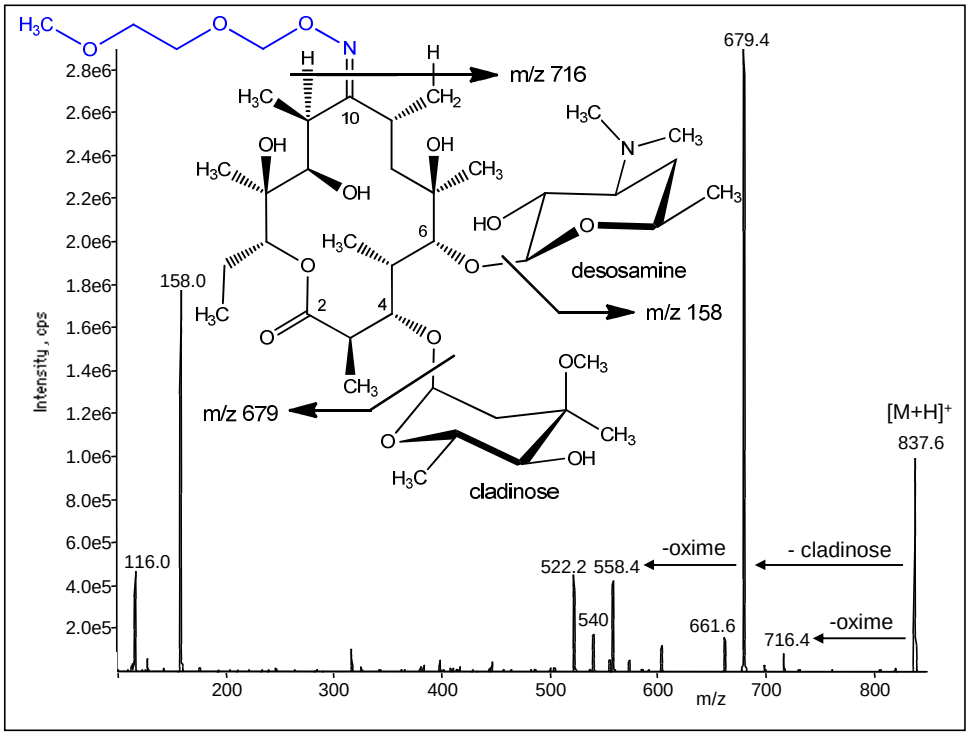
The original LC-MS/MS method was provided by Professor Dr Fritz Sörgel and is discussed in detail in the following chapter. Mass spectra of roxithromycin were acquired by use of atmospheric pressure chemical ionisation (APCI) in the positive ion mode. The precursor ion spectrum (Q1) contains an intensive peak correspondent to the protonated molecule $[M+H]^+$ with m/z 837.6 (exact mass 836.5 amu), as depicted in Figure 15.

Figure 15 *Positive precursor ion (Q1) spectrum of roxithromycin*



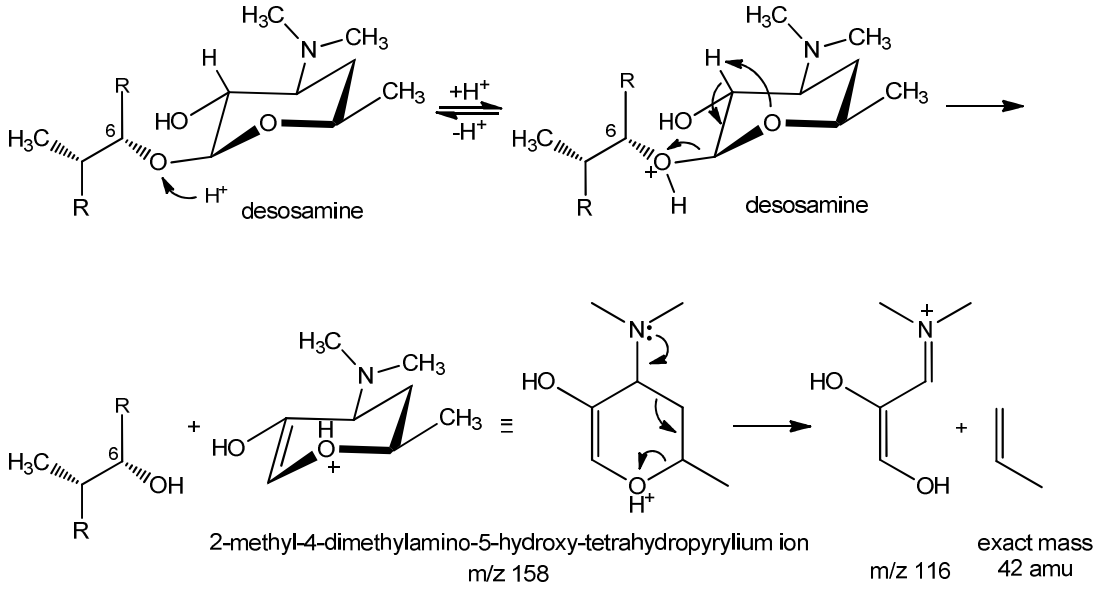
The protonated molecule of roxithromycin (m/z 837.6) is fragmented by collision-activated dissociation resulting in the MS/MS product ion mass spectrum in Figure 16.

Figure 16 *Product ion mass spectrum and fragmentation scheme of roxithromycin*



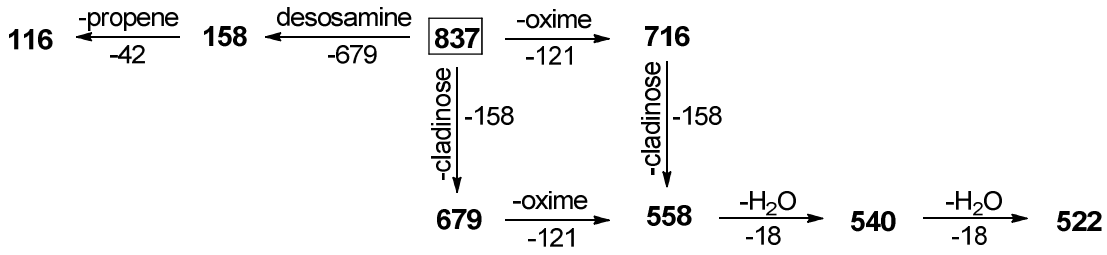
The MS/MS spectrum contains the same fragments as reported by Kearney et al. [130], namely the loss of the cladinose sugar (-158.1 amu) that leads to m/z 679.4, which looses the oxime substituent group on the 10-position (-121.1 amu) to produce m/z 558.3 and further losses of H_2O leading to m/z 540.3 and m/z 522.3. If the oxime substituent group (-121.1 amu) demerges first from the protonated molecule $[M+H]^+$ m/z 716.4 is produced, which can further loose the cladinose sugar (-158.1 amu) to form the product ion m/z 558.3.

Scheme 6 *Fragmentation mechanism of the desosamine moiety of macrolides*



The product ion with m/z 158.1 is generated by charge-site cleavage across the O-C6' bond of the D-desosamine substituent with subsequent release of the 2-methyl-4-dimethylamino-5-hydroxy-tetrahydropyrylium ion. This daughter ion may undergo a further fragmentation step to m/z 116.1 by releasing propene (42.0 amu).

Scheme 7 *Summary of the fragmentations for roxithromycin*



The system parameters for roxithromycin are optimized for the most intensive fragment signal, which is m/z 679.4. The acquired spectra of the internal standard clarithromycin (CLA) contained the protonated molecule $[M+H]^+$ with m/z 748.6 and

the most abundant fragment with m/z 158.2. Clarithromycin spectra are included in chapter 3.3 Clarithromycin in Figure 19.

As with erythromycin, the mass spectrometric conditions for roxithromycin are using APCI as ionization technique in the positive-ion mode and MRM of the following transitions:

m/z 837.5 → m/z 679.4 (ROX) and m/z 748.5 → m/z 158.1 (CLA, IS).

System parameters were optimized for each system during spectra acquisition. These settings were chosen to obtain the highest analytical sensitivity for roxithromycin. They are summarized in Table 18.

Table 18 *Optimized MS/MS parameters for roxithromycin*

	API III Plus	**API 3000™**	**API 5000™**
Probe temperature	500 °C	500 °C	350 °C
Discharge needle current	3 µA	3 µA	5 µA
Orifice voltage / Declustering potential	50 V (OR)	30 V (OR)	88 V (DP)
Collision energy	25 eV	28 eV	32 eV
Nebuliser gas (APCI)	80 psi	8 (NEB)	50 psi (GS1)
Auxiliary gas (APCI)	-	2 L/min	50 psi (GS2)
Nebuliser, auxiliary and curtain gas	High purity nitrogen	High purity nitrogen	High purity nitrogen
Collision gas	High purity argon	High purity nitrogen	High purity nitrogen
Collision gas thickness	$240 \cdot 10^{13}$ atoms/cm^2	6 ($2.7 \cdot 10^{-5}$ torr)	6 ($1.9 \cdot 10^{-5}$ torr)

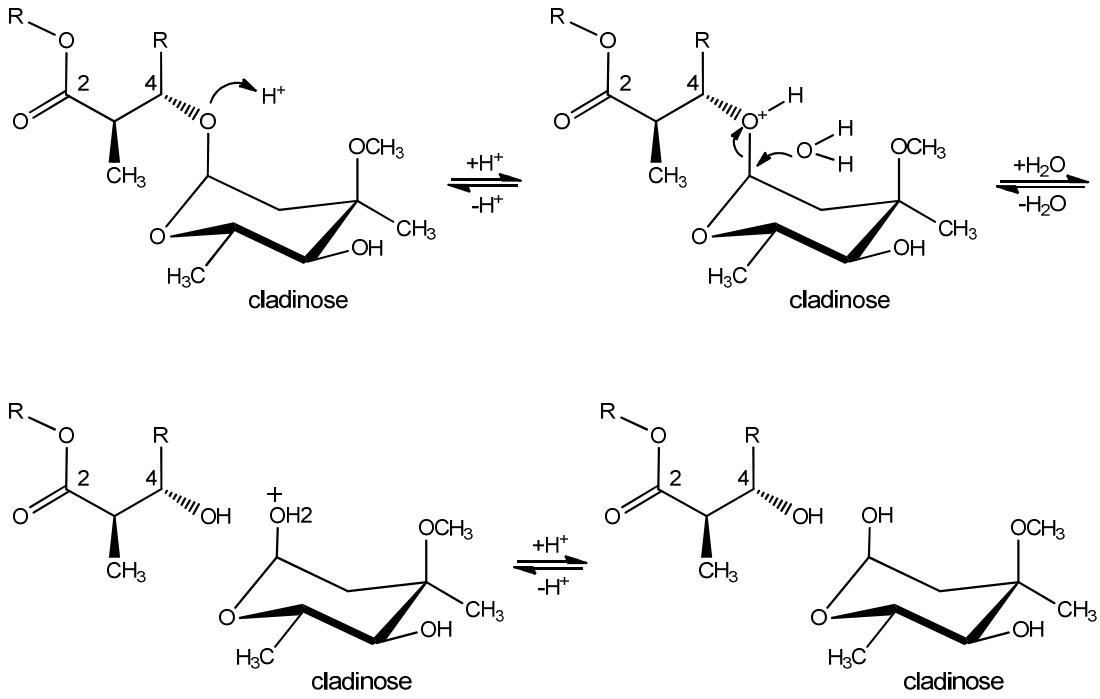
Chromatographic separation of the weak base roxithromycin is done on a reversed phase C18 column. The YMC ODS-AM, 3 µm (50 x 4.6 mm I.D.) column combined with a mobile phase consisting of ammonium acetate buffer (5 mM, pH 5.0) and acetonitrile (50/50, v/v), delivered at a flow rate of 1.0 mL/min. This combination was suitable and fast enough for routine drug analysis: Applying these conditions, roxithromycin elutes within a run time of 2.2 minutes. Additionally, at pH 5 roxithromycin is fully protonated and prepared for MS detection in the positive ion mode.

The expected concentration range in plasma for roxithromycin following administration of a 300-mg oral dose can be determined by C_{max}, which reaches concentrations between 7 and 12 µg/mL (see comparison of PK parameters from literature in Table 24). In order to be able to detect about 5 % of C_{max}, the LLOQ of

roxithromycin should be lower than 350 ng/mL. Due to the high system sensitivity, the calibration range can be set from 10.0 to 4000 ng/mL.

Roxithromycin is stable regarding intramolecular ketal formation, but it may undergo hydrolysis in aqueous and acidic conditions [131], see Scheme 8. Though human plasma has neutral to slight basic pH, all sample handling as well as the sample preparation procedure was done in an ice-water bath to assure the stability of roxithromycin.

Scheme 8 *Hydrolysis of the cladinose moiety of roxithromycin*



After thawing the human plasma samples, calibration standards and SQC samples in a cold water bath, the samples are mixed thoroughly and centrifuged for 5 minutes at approximately 4 °C to separate eventually present solid components. An aliquot (100 µL) of the plasma is deproteinised by adding 250 µL of acetonitrile. Ammonium acetate buffer (0.1 M, pH 4.6), containing the IS clarithromycin, is added before precipitation to ensure ionisation in solution. The IS working solution is prepared by diluting a stock solution of clarithromycin by factor 1000 with ammonium acetate buffer (pH 4.6) to give a clarithromycin concentration of 2 µg/mL. For the more sensitive MS systems, the IS working solution is further diluted by a factor of 10. The precipitated sample, containing the IS, is mixed thoroughly to facilitate deproteinisation. The precipitate is separated by centrifuging the samples for 5 minutes at 3600 rpm (3280 g) at approximately 4 °C. An aliquot (100 µL) of the

supernatant is diluted with 100 µL of ammonium acetate buffer (5 mM, pH 5) to lower the ion strength and raising the water content of the sample giving more constant and sharper peaks. After mixing, 15 µL of each sample is injected onto the LC-MS/MS system. In Table 19 the optimized sample preparation procedure and LC-MS/MS conditions are summarized.

Table 19 *Summary of the LC-MS/MS method for roxithromycin in human plasma*

Characteristic	***Details***
Sample preparation	100 µL plasma + 50 µL NH_4CH_3COO /IS (0.1 M, pH 4.6) + 250 µL CH_3CN 100 µL supernatant + 100 µL NH_4CH_3COO (5 mM, pH 5.0)
Internal standard	Clarithromycin (CLA)
Column	YMC ODS-AM, 3 µm (50 x 4.6 mm I.D.)
Mobile phase	50 % CH_3CN 50 % NH_4CH_3COO (5 mM, pH 5.0)
Flow rate	1.0 mL/min
Run time	2.2 min
Ionisation	APCI – atmospheric pressure chemical ionisation
Polarity	Positive ion mode
Mass transitions	m/z 837.5 → m/z 679.4 (ROX) m/z 748.5 → m/z 158.1 (CLA)

NH_4CH_3COO /IS: ammonium acetate buffer containing the internal standard clarithromycin (2 µg/mL)

3.2.4 Validation

The data were obtained from a previous validation on the API III Plus and the results are explained in this chapter.

The specificity of the LC-MS/MS method was tested in ten different batches of drug-free human plasma (five males and five females). In none of the tested plasma samples matrix components eluted at the same retention time as roxithromycin or the IS (clarithromycin).

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range of 9.21 to 3950 ng/mL roxithromycin in human plasma. The mean linear regression equation of the calibration curves (N = 8) analyzed at the beginning and at the end of each of four validation sequences was calculated:

$y = 0.0018\ (\pm\ 0.0016) + 0.0013\ (\pm\ 0.0001) \cdot x$

The correlation coefficients of the weighted ($1/x^2$) calibration curves were ≥ 0.998. The inter-day precision and accuracy of the back-calculated calibration standards of roxithromycin in human plasma ranged from 0.8 to 6.1 % and from 94.8 to 103.6 %, respectively. Using 100 µL of plasma, the LLOQ was 9.21 ng/mL, which is characterized by an inter-day precision and accuracy of 2.6 and 101.0 %, respectively.

For the evaluation of the intra-day and the inter-day precision and accuracy of the assay, five replicates of each SQC concentration were prepared and analyzed on each of four validation days. The concentrations of the SQC samples were calculated by both calibration curves for the inter-day evaluation and by the first calibration curve for the intra-day evaluation, see Table 20.

Table 20 *Intra- and inter-day precision and accuracy of roxithromycin in human plasma (four days, five replicates per day)*

Nominal concentrations [ng/mL]	Intra-day Precision [%]	Intra-day Accuracy [%]	Inter-day Precision [%]	Inter-day Accuracy [%]
2840	2.0	102.2	4.3	98.4
940	3.1	104.5	2.8	103.2
86.6	2.2	103.3	2.8	102.0
20.7	4.2	97.9	6.1	102.8

The intra-day precision and accuracy of roxithromycin in human plasma ranged from 2.0 to 4.2 % and from 97.9 to 104.5 %, respectively. The inter-day precision of the SQC samples ranged from 2.8 to 6.1 % with an accuracy from 98.4 to 103.2 %. The obtained results were within the acceptance criteria of not more than 15 %.

The mean absolute extraction recovery of roxithromycin in five replicates at each of four concentration levels between 19.2 and 2750 ng/mL was determined to be 98.2 ± 2.3 % (mean ± SD). The mean absolute extraction recovery of the IS (clarithromycin) at the working concentration of 2000 ng/mL was determined as 104.0 ± 1.8 % (mean ± SD). The precision throughout the entire concentration range was better than 2.4 %, showing good consistency and reproducibility. Therefore, the simple one-step protein precipitation procedure introduced to extract analytes from plasma showed satisfactory recovery for roxithromycin in plasma.

Stability of roxithromycin in human plasma in five replicates of an SQC sample at 940 ng/mL was proven for at least 4 hours at 4 °C, for at least 3 months at -20 °C and at -70 °C, and for at least three freeze-thaw cycles from -70 °C to room temperature. In Table 21 shows a summary of the stability data. The 95-% confidence intervals indicate no evidence of instability since the roxithromycin concentrations in the spiked test samples did not decrease by more than 10 % under the tested conditions.

Therefore, roxithromycin containing plasma samples can be frozen and thawed three times and can be kept for 4 hours in an ice-water bath for precipitation. Additionally, already precipitated samples can be kept in the refrigerator or autosampler at 4 °C for at least 120 hours until analysis.

Table 21 *Stability data on roxithromycin at 940 ng/mL in human plasma*

Stability experiment	Time	Point Estimator [%]	Lower Limit [%]	Upper Limit [%]
Short-term stability (approximately 4 °C)	2 hours	103.5	98.8	108.4
	4 hours	103.3	100.7	106.0
Post-preparative stability (autosampler, approximately 4 °C)	24 hours	99.9	95.8	104.3
	48 hours	102.6	99.1	106.1
	120 hours	101.9	98.8	105.1
Post-preparative stability (approximately -70 °C)	24 hours	100.6	98.2	103.0
	48 hours	101.5	99.2	103.8
	120 hours	101.9	101.0	102.9
Freeze-thaw stability	Once	98.0	95.9	100.1
	Twice	97.3	95.3	99.4
	Thrice	109.1	106.5	111.7
Long-term stability (approximately -20 °C)	1day	109.9	106.6	113.4
	9 days	102.9	100.5	105.4
	1 month	101.2	99.0	103.5
	3 months	110.9	109.7	112.1
Long-term stability (approximately -70 °C)	1 day	110.0	106.3	113.9
	9 days	102.4	100.8	103.9
	1 month	98.6	95.6	101.8
	3 months	109.6	108.6	110.5

No influence of haemolysed plasma on the accuracy and precision of the method was observed. The mean concentration value for haemolysed plasma was within ±15 % of the nominal value (940 ng/mL) with precision and accuracy of 3.4 % and 96.9 %, respectively.

Plasma samples containing up to 9630 ng/mL roxithromycin can be pre-diluted by a factor of 4 with drug-free human plasma. The mean concentration values were within the recommended ±15 % of the nominal value. Mean precision and accuracy of 5 replicates on each of four validation days were 4.3 % and 98.4 %, respectively and were therefore, well within the recommended ±15-% range. As a result, study samples exceeding the calibration range during the first measurement can be re-analyzed by diluting the respective sample with drug-free plasma before precipitation.

As a result, it can be concluded that the validation data meets the criteria according to the FDA guideline on BMV [33].

3.2.5 Method comparison on three different LC-MS/MS systems

As for erythromycin, absolute comparability of the roxithromycin experiments was assured by optimization of the tuning parameters on each MS and by using of the same mobile phase, column and sample preparation technique.

Due to better sensitivity of mass analyzers with a larger orifice and alternations in the ion path directly behind the orifice, possible interferences in the extended lower range may affect the selectivity of the method. On none of the three tested mass analyzers interferences between matrix components of drug-free human plasma and roxithromycin or the IS occurred in the observed concentration ranges. Even with the most sensitive instrument samples were free from interferences down to 0.395 ng/mL.

For comparison of the linearity range and the maximal sensitivity (LLOQ) on the different mass analyzers, calibration standards and SQC samples were prepared in human plasma and analyzed for roxithromycin in three validation runs.

On API III Plus the quantifiable concentrations ranged from 9.21 to 3950 ng/mL, equivalent to two orders of magnitude, whereas on API 3000™ it ranged from 3.16 to 4000 ng/mL, equivalent to three orders of magnitude.

However, in contrast to the erythromycin method, it was not possible to increase the linear range further on API 5000™: quantifiable roxithromycin concentrations reached down to 0.395 ng/mL, but only up to 1646 ng/mL, covering only three orders of magnitude. This can be ascribed to the large orifice, which enables more molecular ions to enter the mass spectrometer and saturate the detector at higher concentrations.

The linear regression of the peak area ratios versus concentrations were fitted over the mentioned concentration ranges, which were (mean±SD):

API III Plus: $y = 0.0018\ (\pm 0.0016) + 0.0013\ (\pm 0.0001) \cdot x$ ($r^2 \geq 0.998$)

API 3000™: $y = -0.0022\ (\pm 0.0015) + 0.0068\ (\pm 0.0013) \cdot x$ ($r^2 \geq 0.999$)

API 5000™: $y = 0.00001\ (\pm 0.00003) + 0.0016\ (\pm 0.0001) \cdot x$ ($r^2 \geq 0.999$)

Using 100 µL of plasma, the LLOQ for roxithromycin on API 3000™ (3.16 ng/mL) versus API III Plus (9.21 ng/mL) was lower by a factor of 3, which is a rather small step compared to ERY A (factor 5) and EES (factor 8). On the other hand, the gain in sensitivity from API 3000™ to API 5000™ (0.395 ng/mL) was a factor of 8, as also for EES and ERY A. The inter-day precision (1.0 to 2.6 %) and accuracy (98.6 to 101.0 %) obtained at the LLOQs for roxithromycin was very high on all three instruments.

The signal-to-noise ratios at the LLOQ (9.21 ng/mL) on API III Plus were greater than ten on all validation days. On API 3000™, the maximal sensitivity was tested with high precision of the LLOQ and the corresponding SQCs, what led to an LLOQ of 3.16 ng/mL. The signal-to-noise ratio at this concentration was 2, which “is generally considered acceptable” by the International Conference on Harmonization 2005 [32]. However, with a concentration of 12.6 ng/mL a signal-to-noise ratio of five was obtained, which is recommended by the FDA [132]. On API 5000™ the signal-to-noise ratios at the LLOQ (0.395 ng/mL) were greater than ten on all validation days, resulting from a very low noise. In contrast to API 3000™, the limiting point for the sensitivity on API 5000™ was linearity.

The inter-day precision and accuracy of the SQC samples over 3 validation runs measured on all three instruments ranged from 2.3 to 10.9 % and from 97.0 to 107.2 %, which is very well within the recommended acceptance criteria of <15 % for precision and 85 to 115 % for accuracy. Therefore, all three mass spectrometers showed similar precision and accuracy, even using the largest calibration range and the lowest LLOQ.

As mass analyzers with horizontal or vertical spraying ionisation sources were compared, the matrix effect was investigated by quantitative determination of the matrix factor (MF) and with post column infusion. The matrix factor (MF) calculated from roxithromycin areas was 1.00 ± 0.02 (mean ± SD) and the matrix factor calculated from roxithromycin to clarithromycin area ratios (ROX/IS) was 0.96 ± 0.01 (mean ± SD) indicating the absence of a matrix effect in the API 3000™. The same findings apply to the API 5000™ system, MF from roxithromycin areas being 1.00 ± 0.03 (mean ± SD) and MF from area ratios (ROX/IS) being 1.02 ± 0.01 (mean ± SD). Via post column infusion no increase or decrease of the signal was observed at and near the retention times of roxithromycin and clarithromycin, respectively and therefore a matrix effect was excluded for both instruments.

Data from system-dependent validation experiments on API 3000™ and API 5000™ are summarised in Table 22 by comparison to previously obtained data on API III Plus.

Table 22 *Validation data of roxithromycin from three LC-MS/MS systems*

Experiment	Unit	API III Plus™	API 3000™	API 5000™
Specificity in plasma	[-]	no interference in 10 individuals	no interference in 20 individuals	no interference in 20 individuals
Linearity range Orders of magnitude	[ng/mL]	9.21 – 3950 $4.3 \cdot 10^2$	3.16 – 4000 $1.3 \cdot 10^3$	0.395 – 1614 $4.1 \cdot 10^3$
Correlation Coefficient	[-]	≥ 0.998	≥ 0.999	≥ 0.999
LLOQ **Gain to previous API**	[ng/mL]	9.21 -	3.16 **3**	0.395 **8**
Precision of LLOQ Accuracy of LLOQ	[%] [%]	2.6 101.0	1.0 99.7	2.6 98.6
Highest SQC Lowest SQC	[ng/mL] [ng/mL]	2840 20.7	4000 3.16	1614 0.395
Precision of SQCs Accuracy of SQCs	[%] [%]	2.8 to 6.1 97.0 to 103.2	2.3 to 10.9 97.0 to 98.7	2.8 to 8.1 98.3 to 107.2
Matrix Effect ROX ROX/IS	 MF, Prec [%] MF, Prec [%]	 - -	 1.00, 2,2% 0.96, 0.9%	 1.00, 2.6% 1.02, 0.8%

SQC: spiked quality control sample, LLOQ: lower limit of quantification, ROX: Roxithromycin, IS: internal standard, Prec: Precision in plasma from 6 individuals

3.2.6 Comparison of the LC-MS/MS method with procedures reported in the literature

Table 23 shows LLOQ, run time (min) and quality control data for previously reported analytical methods for the determination of roxithromycin in human plasma in comparison to the present LC-MS/MS method.

Compared to the present assay for ROX, all previously reported methods showed less sensitivity with LLOQ values at least five times higher [49-50, 114-116, 118-121], except Hang et al. [51], who's LLOQ is comparable to the present one, but needs double sample volume and a three times longer run time. The main reason for this difference in sensitivity is presumably the detection technique, since most of them use LC/ED, LC/FD and LC/UV [114-116, 118-121]. Even after the extraction of 1.0 mL of the plasma samples [118, 120], only LLOQs of 65 and 500 ng/mL were achieved. As a result it can be said, that for the analysis of ROX in human plasma the MS/MS detection is more useful. This can be ascribed to the chemical and physical properties of ROX, since it has only few electrons suitable for UV, fluorescence or electrochemical detection, but has a tertiary amine, which is good ionisable for MS detection.

Independent from the sample preparation procedure, all methods show good and reproducible recovery data. Only one method is reported [119], where SPE is combined with a derivatisation reaction resulting in poor recovery for ROX, which is even less than for the internal standard. Clarithromycin was used by nearly all methods as internal standard, most likely due to its good acid stability. The remaining two methods used erythromycin [51, 119].

Sastre Torano et al. [118] did not report precision and accuracy data at all, whereas Taninaka et al. [116], Qin et al. [121] and Hang et al. [51] avoided precision and accuracy data at the lower limit of quantification. Overall precision and accuracy was better than ±15% for all methods. At the LLOQ Macek et al. [120] and Motta et al. [49] show a ±20% limit for both parameters.

With the exception of four methods [114-115, 118, 121] all developed and validated assays were implemented to a PK study.

Table 23 *Comparison of current method with existent methods to quantify roxithromycin in human plasma, serum or blood; grouped by analytical technique and descending years of publication*

Method	Linearity range [ng/mL]	Approx. run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of SQC samples [%]	Recovery mean ± SD [%]	Corr. coeff.	Sample Vol. [mL]	Sample preparation	Internal standard	Application
LC-MS/MS (current method)	9.21-3950	2.2	2.6 / 101.0	2.8 to 6.1 / 97.0 to 103.2	98.2*±2.3 (ROX) 104.0±1.8 (IS)	≥0.998	0.100	PPT	Clarithromycin	300 mg PK
API 3000™ (current method)	3.16-4000	2.2	1.0 / 99.7	2.3 to 10.9 / 97.0 to 98.7		≥0.999	0.100	PPT	Clarithromycin	-
API 5000™ (current method)	0.395-1614	2.2	2.6 / 98.6	2.8 to 8.1 / 98.3 to 107.2		≥0.999	0.100	PPT	Clarithromycin	-
LC-MS/MS [50] Kousoulos 2008	50-20000	1.6	6.3 / 113.0	2.7 to 6.3 / 99.3 to 113.0	84.6-86.9 (ROX) 74.1 (IS)	>0.991	0.100	automated LLE	Clarithromycin	300 mg PK
LC-MS/MS [51] Hang 2007	10-20000	7.5	n.r.	8.7 to 14.8 / 92.0 to 96.9	98.2 - 104.7	n.r.	0.200	PPT	Erythromycin	150 mg PK
LC-MS/MS [49] Motta 1999	50-5000	5.5	<20.0 / <120.0	6.6 to 9.8 / 99.8 to 114.3	n.r	≥0.997	0.040	LLE	Clarithromycin	300 mg PK
LC/FD [119] Główka 2007	500-10000	20.0	9.1 / 112.0	n.r.	38.9, 43.1*²(ROX) 83.8±2.8 (IS)	0.997	0.550	SPE+DER	Erythromycin	150 mg PK
LC/FD [118] Sastre Torano 1998	65-19500	25.0	n.r. / n.r.	n.r. / n.r.	97-102	0.9998	1.0	LLE	Clarithromycin	n.r.
LC/UV [121] Qin 2001	250-32000 (LOD: 60)	> 5.0	n.r. / n.r.	< 3.0 / n.r.	97.4	n.r.	0.500	LLE	Clarithromycin	n.r.
LC/UV [120] Macek 1999	500-29600	15.0	16.1 / 85.4	4.1 to 7.7 / 91.6 to 100.5	90±3	>0.9990	1.0	LLE	Clarithromycin	300 mg PK
LC/ED [116] Taninaka 2000	100-10000	20.0	n.r.	1.7 to 6.2 / 100.6 to102.9	112	>0.999	0.150	LLE	Clarithromycin	PK in rats
LC/ED [115] Kees 1998	100-5000	15.0	3.1 / 106.6 (50 ng/mL)	0.7 to 10.3 / 100.6 to 108.6	80-90	>0.9991	0.500	LLE	Clarithromycin	n.r.
LC/ED [114] Hedenmo 1995	418.5-20925	9.0	5.7 / n.r.	4.0 (2678 ng/mL)	89.4±5.5 (ROX) 99.0±5.3 (IS)	n.r.	0.100	SPE	Clarithromycin	n.r.

Prec: Precision, Acc: Accuracy, n.r.: not reported, PPT: protein precipitation, LLE: liquid-liquid extraction, SPE: solid phase extraction, DER.: Derivatisation, *: over the whole concentration range, *²: at 1500 and 10000 ng/mL, LLOQ: lower limit of quantification, PK: pharmacokinetics, LOD: limit of detection, S/N: signal-to-noise, ROX: roxithromycin, IS: internal standard, SD: standard deviation

3.2.7 Application of the roxithromycin assay to a pharmacokinetic study

3.2.7.1 Study design and sample handling

In the present study the pharmacokinetic profile of 300 mg roxithromycin was investigated. Therefore, 36 healthy male and female volunteers received a single film-coated tablet in the fasting state. A “pre-dose” sample was drawn from each subject prior to administration and additional samples were collected at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0, 48.0, 72.0, 96.0 and 120.0 hours after administration.

The blood samples containing ammonium heparinate as anticoagulant were cooled in an ice-water bath until centrifugation for 10 minutes, which was performed at 1500 g and approximately 4 °C. Two aliquots of each sample were transferred into different polypropylene tubes and immediately frozen at approximately -70 °C until analysis.

3.2.7.2 Determination plasma concentrations

More than 1700 human plasma samples were analyzed for roxithromycin in a total of fifteen sequences together with injection standards, calibration standards, and SQC samples (in randomized order among subject samples). Each sequence was measured within seven hours. The determined concentrations of SQC samples were compared to theoretical concentrations to assess accuracy.

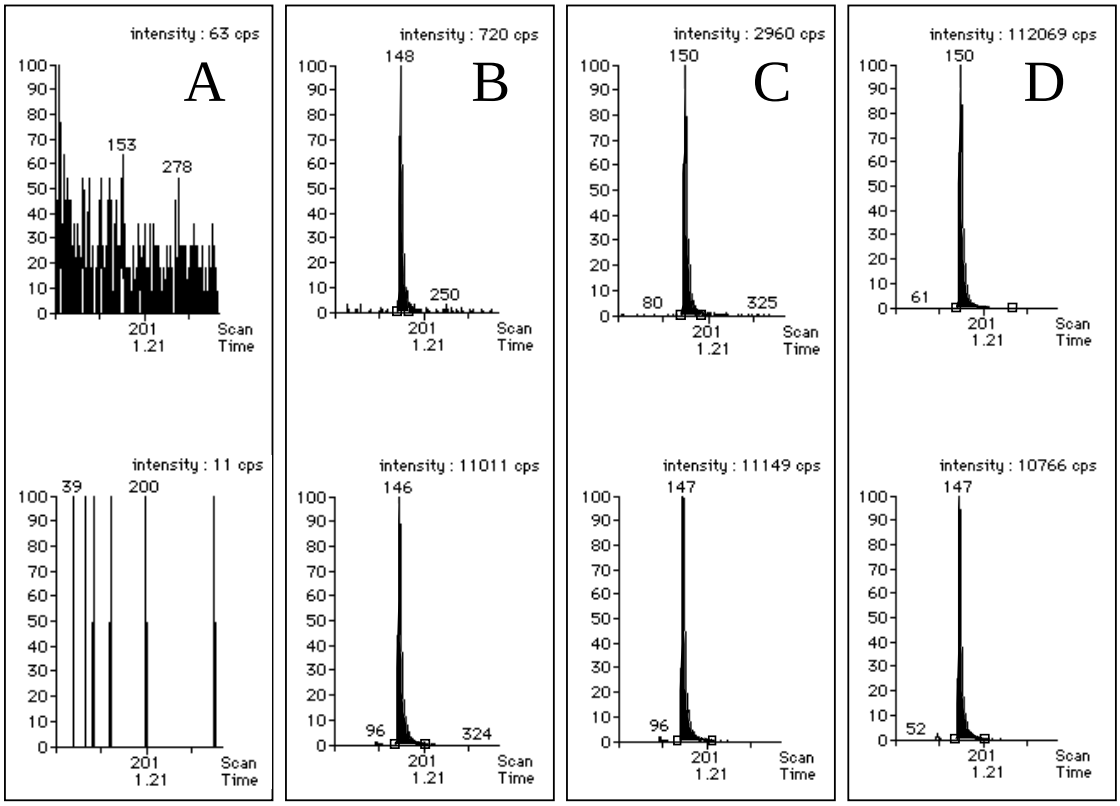
The inter-day precision and accuracy of the SQC samples of roxithromycin in human plasma analyzed with the sequences of study samples ranged from 2.9 to 5.5 % and from 97.6 to 101.8 %, respectively. The coefficient of correlation of resulting linear regressions was at least 0.999. The precision and accuracy data prove the high quality of the roxithromycin concentrations in the study samples.

For additional quality control of the assay, biological quality control (BQC) samples with three concentrations were generated by pooling subject samples of a previous study. After pooling samples with high, medium and low concentrations, the human plasma was vigorously mixed and aliquots of 150 µL were frozen at approximately -70 °C. For each sequence three BQC samples (one of each concentration) were thawed, prepared and measured together with the study samples. The precision and mean concentration of roxithromycin in the BQC samples was 4.0 % (5230 ng/mL), 4.8 % (1460 ng/mL), and 2.8 % (261 ng/mL). The excellent results obtained with

these real study samples in addition to spiked quality control (SQC) samples demonstrated the accuracy and reproducibility of the LC-MS/MS method. However, the main difference between the analyzed BQC samples and incurred sample re-analysis (ISR) recommended by the regulatory authorities (see chapter 2.5.12, page 36) is that the BQC samples were pooled samples. By pooling different samples, probable anomalous effects in those samples are diluted, minimizing the possibility to find deviating results. Therefore, it is recommended to re-analyze ISR samples “as is” for comparison of the first result with the second one. Additionally, fifteen replicates of three pools of BQC samples were analyzed, while during incurred sample re-analysis a higher number of real study samples can be re-analyzed at once, resulting in a much larger data set.

Representative MRM-chromatograms for roxithromycin (top) and the IS (bottom) in human plasma are depicted in Figure 17, showing: (A) Blank plasma, (B) the LLOQ containing 9.21 ng/mL ROX, (C) a study sample (t = 120 h) containing 47.6 ng/mL ROX, and (D) a study sample (t = 0.25 h) containing 2180 ng/mL ROX.

Figure 17 *MRM-chromatograms of roxithromycin and the IS clarithromycin*



3.2.7.3 Pharmacokinetic results

Figure 18 shows the mean plasma concentration profile of roxithromycin after oral administration of a 300 mg roxithromycin tablet to 36 healthy volunteers. The mean peak concentration (C_{max}) of 9.50 µg/mL was attained at 2.34 hours after administration of the study drug.

Figure 18 *Mean plasma profile of roxithromycin concentration vs. time following a 300 mg oral dose of roxithromycin to 36 healthy volunteers*

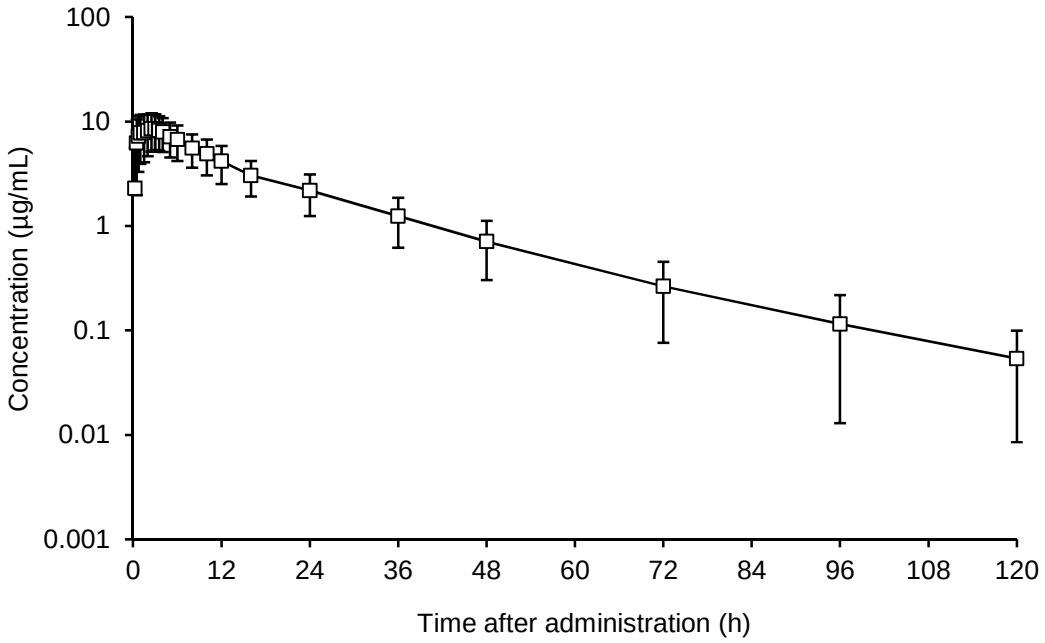


Table 24 summarizes the pharmacokinetic parameters of roxithromycin after oral administration of 300 mg in comparison to pharmacokinetic parameters reported in previously published studies [49-50, 109, 120, 133-134]. Since roxithromycin can be characterized by a dose-dependent pharmacokinetics [109, 133-134], additional PK data from the second common roxithromycin dosage, namely 150 mg [51, 117, 119, 134-135], are reported separately in the lower part of the table.

Table 24 *Pharmacokinetic parameters (mean ± SD) of roxithromycin in healthy subjects after overnight fasting and administration of a single oral dose as a tablet*

Reference	No. of subjects/ Gender	Dose / Formulation	t_{last} [h]	$AUC_{0\rightarrow last}$ [µg·h/mL]	$AUC_{0\rightarrow\infty}$ [µg·h/mL]	C_{max} [µg/mL]	t_{max} [h]	$t_{1/2}$ [h]	Analytical method
Current study	36/m,f	300 mg / film-coated tablet	120	159±65.0	160±66.1	9.50±3.41	2.34±1.74	17.0±3.34	LC-MS/MS
Kousoulos 2008 [50]	28/n.r.	300 mg / test	48	106±40.5	117±46.2	7.27±2.15	n.r.	13.3±5.1	LC-MS/MS
Kousoulos 2008 [50]	28/n.r.	300 mg / ref.	n.r.	110±41.2	122±49.0	7.49±2.23	n.r.	13.0±2.8	LC-MS/MS
Huang [136] 2007	20/m (Chinese)	300 mg / test dispersive tablet	n.r.	143.32 ±25.8	158.63 ±26.86	10.16 ±1.46	2.33±0.61	9.00±1.58	HPLC
Huang [136] 2007	20/m (Chinese)	300 mg / ref.	n.r.	138.93 ±22.49	153.77 ±24.75	10.3±1.7	2.28±0.62	8.68±1.66	HPLC
Motta 1999 [49]	24/m,f	300 mg / test suspension	72	247* (216-281)	277* (241-317)	10.8* (9.57-12.2)	3.0*² (2.0-10.0)$^{\Delta}$	18.5* (16.7-20.5)	LC-MS/MS
Motta 1999 [49]	24/m,f	300 mg / ref. tablet	n.r.	233* (199-274)	233* (199-274)	12.4* (10.9-14.1)	3.0*² (1.0-8.0)	16.7* (14.1-18.6)	LC-MS/MS
Macek 1999 [120]	26/m	300 mg / tablet	60	n.r.	n.r.	approx. 9.7	1.50	ca. 12.0	LC/UV
Nilsen 1992 [134]	12/m	300 mg / tablet	36	n.r.	215±82.5	11.0±2.19	2.25±1.62	16.2±7.76	LC/ED
Tremblay 1988 [133]	12/m,f	300 mg / tablet	72	132±17	n.r.	10.8±0.66	1.50±0.27	11.9±0.53	LC/ED
Puri 1987 [109]	21/m	300 mg film-coated tablet	48	114±23.8	117±28.6	9.1±1.7	1.9±1.3	10.5±5.2	n.r.
Puri 1987 [109]	40/m	300 mg	12	66.3±11.8	98.6±21.1	9.7±2.0	1.3±1.3	10.9±6.5	n.r.
Zheng [137] 2013	36/m (Chinese)	150 mg / test capsule	72	66.1	68.2	6.6	1.3±0.9	15.4±4.6	LC-MS/MS
Zheng [137] 2013	36/m (Chinese)	150 mg / ref.	72	70.3	72.4	7.0	1.4±0.7	16.1±5.6	LC-MS/MS
Hang 2007 [51]	12/m	150 mg / dispersible tablet	72	86.7±27.0	88.4±28.2	7.51±2.11	2.3±1.0	12.5±2.1	LC-MS/MS
Glowka 2007 [119]	26/m,f	150 mg / film-coated tablet	48	51.2±14.4	53.8±17.4	6.0±1.9	1.20±1.10	6.00±3.00	LC/FD
Sun 2005 [135]	18/m	150 mg / enteric-coated pellet	36	60.9±11.2	n.r.	5.07±0.95	2.83±0.99	n.r.	Microbiol.
Sun 2005 [135]	18/m	150 mg / dispersible tablet	36	42.7±16.3	n.r.	3.95±1.52	1.43±0.84	n.r.	Microbiol.
Nilsen 1992 [134]	12/m	150 mg / tablet	36	n.r.	107±52.9	6.68±2.58	2.54±1.42	13.0±5.69	LC/ED
Birkett 1990 [117]	12/m	150 mg / film-coated tablets	48	n.r.	76.0±30.7	5.69±2.55	2.19±2.35	12.4±3.94	LC/ED
Tremblay 1988 [133]	12/m,f	150 mg / tablet	72	81±10	n.r.	7.90±0.66	1.93±0.51	10.5±1.4	LC/ED

m: male, f: female; n.r.: not reported, *: geometric mean (90% confidence interval); *²: median (range), ref.: reference formulation, test: test formulation

The determined value for $AUC_{0\rightarrow t}$ 159 µg·h/mL is very close to the value for $AUC_{0\rightarrow\infty}$ 160 µg·h/mL, indicating that virtually no extrapolation was necessary, which was achieved by the sensitive method and sample collection until 120 hours post-dose. The determined AUC value is within the range of previously reported AUC_{∞} from 98.6 to 277 µg·h/mL. This large range of mean values can be explained by different formulations of previous studies, which influence acid stability and thus, absorption of roxithromycin. This assumption is also supported by the range of C_{max} (7.27 to 12.4 µg/mL) and of t_{max} (1.3 to 3.0 hours), whereby the determined values for C_{max} (9.50 µg/mL) and t_{max} (2.3 hours) also reside within this range. The range for $t_{1/2}$ (10.5 to 18.5 hours) indicates variability of roxithromycin elimination.

The nonlinear pharmacokinetics of roxithromycin can be seen by comparing the AUC and C_{max} of Nilsen et al. and Tremblay et al. [133-134]. They conducted studies comparing a 150-mg and a 300-mg single dose of roxithromycin in tablets. The resulting AUC and C_{max} for the 300 mg dose are lower than would be expected for linear kinetics. This can be explained by saturable plasma protein binding of roxithromycin, of which 80-96 % are bound to α-1-acid glycoprotein. The maximum total plasma concentration in the two studies increased from 6.7 and 7.9 µg/mL to 11.0 and 10.8 µg/mL, respectively. The higher concentrations can be related to an increase of free roxithromycin by more than 13% [111]. This increase causes an increase of the volume of distribution, since only free drug can cross the membranes. In addition, the renal clearance increased as a function of the dose studied by Tremblay et al. [133]. However, only 10 % of a roxithromycin dose are excreted unchanged in the urine, while about 53 % are secreted into the bile [109] and the remaining 37 % are metabolized. It seems that roxithromycin has a high hepatic extraction ratio anyway, which may not be altered significantly by an increase of the free plasma concentration. Apparently, the elimination half-lives from the 150-mg dose and the 300-mg dose appear to increase by 1 hour [134] and 3 hours [133], respectively, and therefore, it could be concluded that the volume of distribution increased by a larger factor than the elimination rate in both studies.

3.2.8 Summary: Roxithromycin

The developed and validated LC-MS/MS assay for roxithromycin in human plasma is simple, fast, reliable, sensitive, precise and accurate on three generations of mass spectrometers. The method employed acetonitrile protein precipitation, which allowed quantification of roxithromycin in 100 µL human plasma for concentrations

ranging from 9.21 ng/mL to 3950 ng/mL, extending the upper quantification limit to 15800 ng/mL, by pre-diluting samples by a factor of four.

The principal advantage of the LC-MS/MS method described here is the simultaneous achievement of high extraction recovery (98.2%), high sensitivity on all three mass spectrometers (LLOQs = 9.21 ng/mL to 0.395 ng/mL), high inter-day precision (≤2.6%) and high accuracy (98.6 to 101.0 %) for LLOQ as well as excellent linearity ($r^2 \geq 0.998$) with a short run time of only 2.2 min.

These characteristics make the method suitable for the precise and accurate measurement of low concentrations of roxithromycin in samples of pharmacokinetic studies after single dose. The method was successfully applied to the analysis of more than 1700 human plasma samples during a pharmacokinetic study of a single 300-mg dose of roxithromycin.

Due to the high sensitivity of the method, all samples had evaluable concentrations, especially the smallest concentrations at 120 hours after administration. This fact and the long sample collection interval led to a very precise determination of the area under the curve, proven by the small difference between AUC_{0-t} and AUC_{∞} and the residual area of ≤0.6 %. The pharmacokinetic parameters were in very good agreement with previously reported data for 300 mg roxithromycin. In addition, these data were compared to data from 150-mg dose studies and related to chemical and pharmaceutical properties of roxithromycin.

Roxithromycin in native human plasma is stable for at least three months at approximately -20 °C and approximately -70 °C, for at least four hours at 4 °C and during three freeze-thaw cycles. No instability was observed after the sample preparation procedure over a time period of 120 hours at autosampler temperature (approximately 4 °C) and at approximately -70 °C. The results of the 95% confidence interval calculation suggested that there is no evidence of instability.

The structural analogue clarithromycin is adequate as internal standard for roxithromycin, shown by the absence of a matrix effect of roxithromycin with and without clarithromycin in the API series, and comparable recoveries.

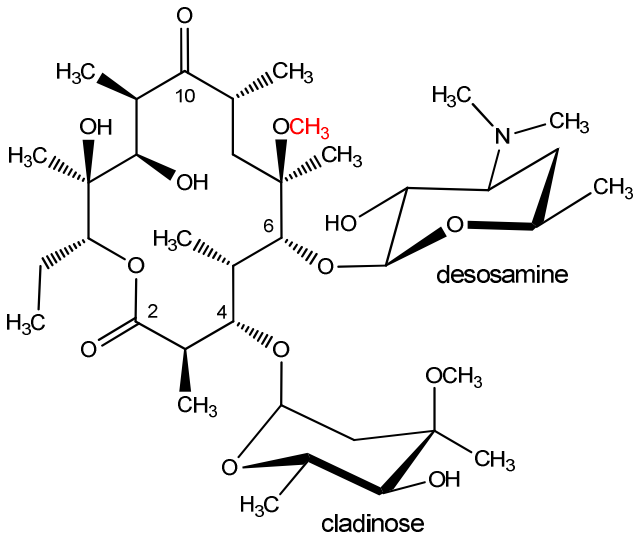
Finally, it is possible to transfer the roxithromycin method directly to three different LC-MS/MS systems and achieve a large sensitivity improvement by a factor of 23 accompanied by excellent accuracy and precision data, showing the good robustness of the method.

3.3 Clarithromycin

3.3.1 Chemical and pharmaceutical properties

Clarithromycin is composed of a 14-membered lactone ring containing ten asymmetrically substituted carbon atoms, at two of them is attached to a sugar group: cladinose to C(4) and desosamine to C(6), underlying IUPAC nomenclature. Clarithromycin, $C_{38}H_{69}NO_{13}$ (exact mass 747.5 amu) is a water-insoluble weak base with a pKa of 8.76 [104] and a logP of 3.16 [68], see Scheme 9.

Scheme 9 *Chemical structure of clarithromycin (CLA)*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{38}H_{69}NO_{13}$	748.0 g/mol	747.5 amu	8.76	3.16

IUPAC Name: (3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-6-(((2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-14-ethyl-12,13-dihydroxy-4-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-7-methoxy-3,5,7,9,11,13-hexamethyloxacyclotetradecane-2,10-dione

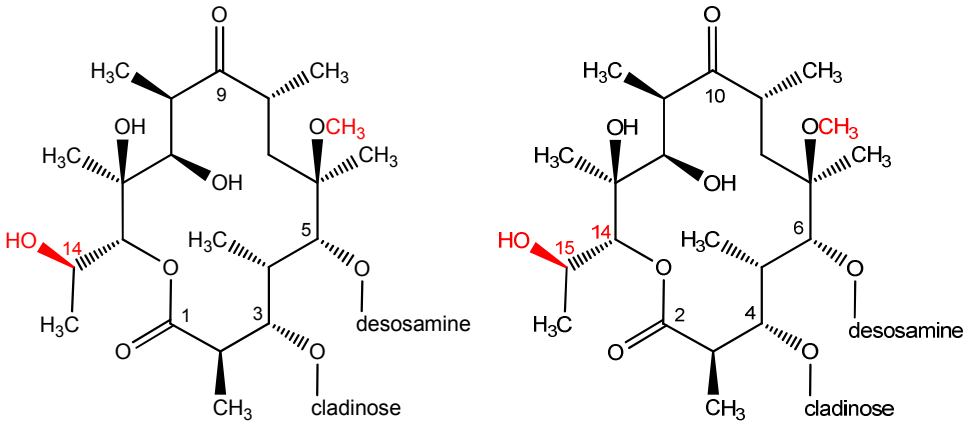
Clarithromycin is a broad-spectrum macrolide antibiotic used in the treatment of respiratory tract and other infections [138]. It's mechanism of action, blocking bacterial protein synthesis, is already described for erythromycin in chapter 3.1.1.

Clarithromycin only differs from erythromycin A by methylation of the C(7)-hydroxyl group, preventing intramolecular hemiketal formation with the C(10)-carbonyl group. This modification improves its acid stability and thus the absorption by the oral route [71]. Following oral administration of 250 mg clarithromycin, the absorption is nearly

complete [139], but first-pass metabolism causes an absolute bioavailability of about 55% [140], which is not altered significantly in the presence of food [141-142].

Clarithromycin is characterized by a nonlinear pharmacokinetic profile [143], ascribing to its auto-inhibition through CYP3A4 [77, 144]. After administering of a 250-mg clarithromycin tablet to healthy adults, C_{max} (0.72 to 1.2 µg/mL) is reached after 1.6 to 2.6 hours (t_{max}), followed by a terminal elimination half-life ($t_{1/2}$) between 1.9 and 4.9 hours and an AUC between 4.0 and 8.2 µg·h/mL [140, 145-150].

Scheme 10 *Chemical structure of 14-hydroxy clarithromycin (CLA-MET)*



Traditional numbering IUPAC numbering

Formula	Mol. weight	Exact mass	pK_a	logP
$C_{38}H_{69}NO_{14}$	764.0 g/mol	763.5 amu	-	-

IUPAC Name: (3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-6-(((2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-12,13-dihydroxy-4-(((2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-14-((R)-1-hydroxyethyl)-7-methoxy-3,5,7,9,11,13-hexamethyloxacyclotetradecane-2,10-dione

Scheme 10 compares the traditional numbering, which gave the 14-hydroxy metabolite it's name and is still used by many text books [69], with the numbering system by IUPAC [151], founded in 1919 as an international union of chemists to achieve systematic and unambiguous names for all compounds.

Metabolism of clarithromycin occurs by CYP3A4, where oxidative N-demethylation and 14-hydroxylation are the major routes. Clarithromycin is the only 14-membered macrolide that exhibits the most active 14-hydroxy metabolite [142]. Clarithromycin and 14-hydroxy clarithromycin are widely distributed into body tissues and fluids, especially respiratory tract tissues, where concentrations reach levels, which are 3 to 30-fold higher than plasma concentrations [152-154].

Like erythromycin, clarithromycin reversibly inhibits CYP3A4 [77], reducing elimination and raising plasma levels of many other drugs. This interaction may be beneficial (enhanced efficacy and cost-reduction of the immunosuppressant cyclosporine) or cause mostly adverse drug response, like QT interval prolongation (e. g. the antihistamine terfenadine) or excessive sedation (e. g. the benzodiazepine midazolam) [77]. However, clarithromycin exhibits fewer serious drug interactions than older macrolides [142].

3.3.2 Already published analytical methods

The majority of methods to quantify clarithromycin in serum or plasma include liquid chromatography with electrochemical detection (LC/ED) [114-115, 147, 150, 155-157]. Despite the lack of a large chromophore in clarithromycin, also liquid chromatography methods with spectrophotometric detection (LC/UV) have been used [145, 158]. Others reported derivatisation combined with liquid chromatography and spectrofluorometric detection (LC/FD) [159]. Many of these approaches have been applied to pharmacokinetic studies in man [145, 147, 150, 155-160] or animals [52, 161]. Previously reported LC/ED, LC/UV and LC/FD methods present long run times, and expensive or time-consuming sample preparation procedures using high sample volumes.

Several LC-MS/MS methods have been reported to determine macrolide antibiotics in a series of matrices like fish [96], eggs and milk [162], human muscle tissue [163], animal tissues [164-165], feed supplement [166], and wastewater [167-169].

However, very few LC-MS/MS methods have been published for clarithromycin in human plasma [53, 55, 170-173], reporting data from the application in a pharmacokinetic study [53, 170, 173]. The methods presented either a somewhat high LLOQ of 100 ng/mL [55, 170], had difficulties with inter-day precision [53] or did not report inter-day precision data for their LLOQ [170-171, 173]. Furthermore, they used rather cost-ineffective extraction methods [171-172] and did not report a successful application of their method [55, 172].

Only two LC-MS/MS methods for quantification of clarithromycin as well as its active 14-hydroxy metabolite in human plasma have been reported [54-55]. However, one of them was reported to have issues with carry-over and to have a high LLOQ of 100 ng/mL. Considering an expected C_{max} of approximately 0.8 µg/mL (250 mg dose

of clarithromycin), this LLOQ would cover only three half-lives of the PK profile. Moreover, no application in a pharmacokinetic study is reported for both methods.

Therefore, a highly sensitive, precise, accurate and fast LC-MS/MS quantitative method in the MRM mode for clarithromycin and its 14-hydroxy metabolite in human plasma was needed to be applied to a large pharmacokinetic study of 250 mg oral clarithromycin and to compare different generations of mass spectrometers with regard to sensitivity, selectivity and matrix effect.

3.3.3 LC-MS/MS method

The original LC-MS/MS method was provided by Professor Dr Fritz Sörgel, IMBP – Institute for Biomedical and Pharmaceutical Research in Nürnberg-Heroldsberg and is discussed in detail in the following chapter.

3.3.3.1 MS/MS spectra and tuning

The precursor ion spectra of clarithromycin and 14 hydroxy clarithromycin, recorded by scanning Q1 from m/z 600 to m/z 1700, contain intensive signals at m/z 748.6 (CLA) and m/z 764.8 (CLA-MET) correspondent to the molecular ions [M+H]+ of clarithromycin and 14 hydroxy clarithromycin. Scanning Q3 from m/z 100 to m/z 750, the protonated molecules of CLA (m/z 748.6) and CLA-MET (m/z 764.8) are fragmented by CAD resulting in the MS/MS product ion mass spectra depicted in Figure 19 and Figure 20.

The MS/MS spectra show the same fragments as reported by others [130, 174], namely the loss of a neutral species, i. e. the cladinose sugar (-158 amu) resulting in m/z 590 (CLA), m/z 606 (CLA-MET) and m/z 679 (ROX, used as IS).

The mechanism of the further fragmentation of the desosamine substituent is depicted in Scheme 6 on page 70.

The system parameters are optimized for the most intensive fragment signals, which are m/z 158.2 for CLA and CLA-MET. The acquired spectra of the internal standards roxithromycin (ROX) and deuterated clarithromycin (CLA-d_3) contained the protonated molecule $[M+H]^+$ with m/z 838 (ROX) and m/z 751.5 (CLA-d_3) as well as the most abundant fragments with m/z 680 (ROX) and m/z 161.2 (CLA-d_3). For details of ROX fragmentation see Figure 16, on page 69.

Figure 19 *Product ion scan of 14-hydroxy clarithromycin*

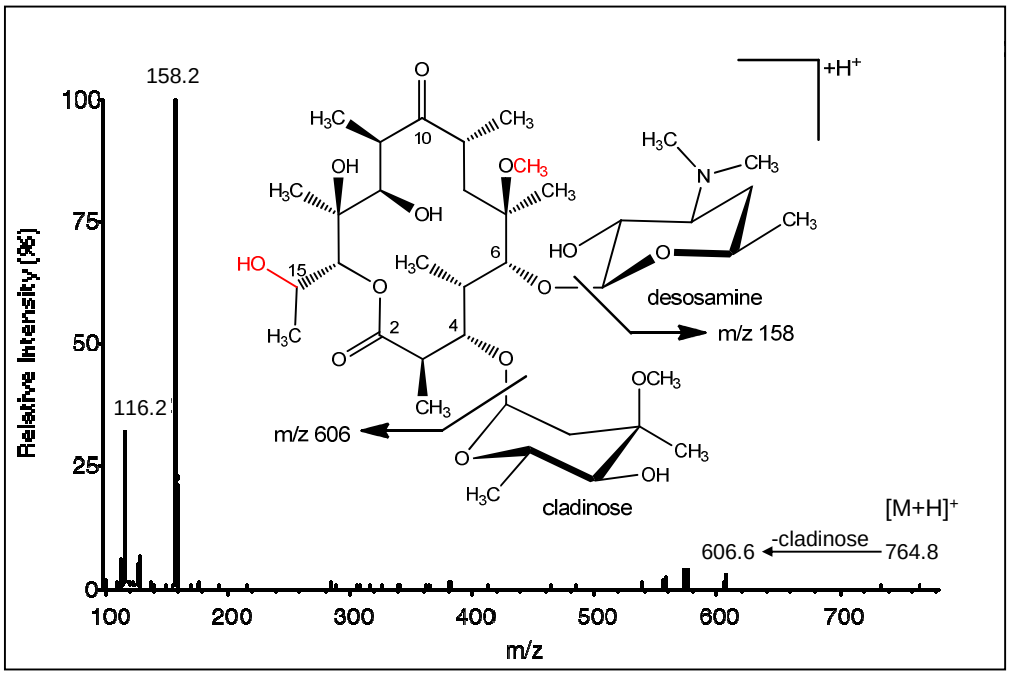
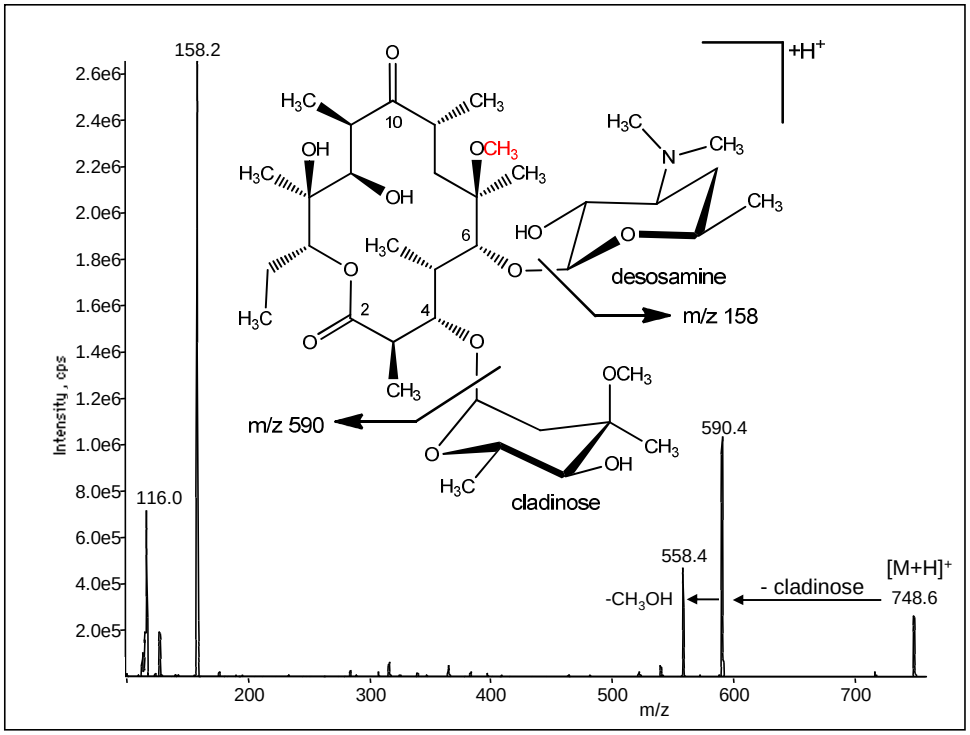


Figure 20 *Product ion mass spectrum and fragmentation scheme of clarithromycin*



Based on the observations described above, the MS detection of clarithromycin and its 14-hydroxy metabolite was performed in the positive ionization mode by using APCI and the following MRM transitions: m/z 748.6 → m/z 158.2 (CLA),

m/z 764.8 → m/z 158.2 (CLA-MET) and m/z 837.5 → m/z 679.4 (ROX), m/z 751.5 → m/z 161.2 (CLA-d_3).

The optimization of temperature, gas settings and voltages was performed during spectra acquisition to obtain the highest analytical sensitivity on each system. They are summarized in Table 25.

Table 25 *Optimized MS/MS parameters for clarithromycin (CLA) and 14-hydroxy clarithromycin (CLA-MET)*

	API III Plus	**API 3000™**	**API 5000™**
	CLA / CLA-MET	CLA / CLA-MET	CLA / CLA-MET
Probe temperature	500 °C	500 °C	350 °C
Discharge needle current	3 µA	3 µA	5 µA
Orifice voltage (OR) / Declustering potential (DP)	60 V (OR)	31 V (OR)	80 V (DP)
Collision energy	25 eV / 30 eV	42 eV / 42 eV	40 eV / 41 eV
Nebulizer gas (APCI)	80 psi	80 psi	50 psi (GS1)
Auxiliary gas (APCI)	-	2 L/min	50 psi (GS2)
Nebulizer, auxiliary and curtain gas	High purity nitrogen	High purity nitrogen	High purity nitrogen
Collision gas	High purity argon	High purity nitrogen	High purity nitrogen
Collision gas thickness	$260 \cdot 10^{13}$ atoms/cm^2	6 ($2.7 \cdot 10^{-5}$ torr)	5 ($1.7 \cdot 10^{-5}$ torr)

3.3.3.2 Chromatography and sample preparation

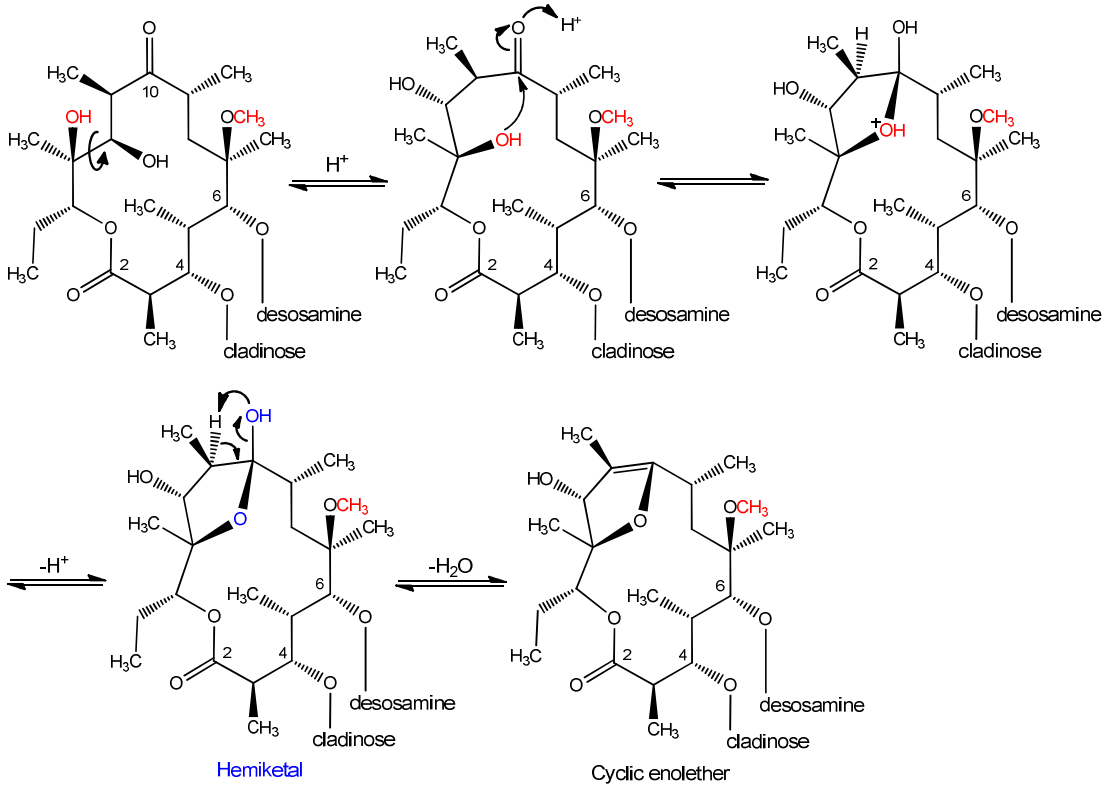
At the pH of approximately pH 4 of the mobile phase, clarithromycin (pKa 8.76), its 14-hydroxy metabolite as well as roxithromycin (pKa 9.2) will be fully protonated and can be detected in the positive ion mode. The ammonium acetate (10 mM) in the prepared sample solution and the mobile phase assists charge transition during atmospheric pressure chemical ionisation. The desolvation process of the molecular ions from the aqueous mobile phase (65 % v) is supported by a temperature of 500 °C in the APCI source. The retention time of the IS (1.7 min) is similar on a C8 column to that of clarithromycin (1.6 min) and its 14-hydroxy metabolite (0.8 min). Typical chromatograms are shown in Figure 22.

The calibration range from 10.0 to 4000 ng/mL enables analysis of plasma concentrations after administration of a single 250-mg clarithromycin tablet. Peak plasma concentrations range from 0.72 to 1.2 µg/mL (CLA) and from 0.46 to

0.76 µg/mL (CLA-MET) [140, 145-150]. Thus, at least 5% of C_{max} (36 ng/mL CLA 23 ng/mL CLA-MAT) can be detected.

Though clarithromycin is methylated at the (C7)-hydroxyl group, it may theoretically undergo hemiketal formation between the (C13)-hydroxy function and the (C10)-carbonyl group. Therefore, to prevent any degradation of CLA and CLA-MET, the plasma samples were handled and precipitated in an ice-water bath.

Scheme 11 *Acid-catalyzed degradation of clarithromycin*



After thawing the human plasma samples (calibration standards and SQC samples) in a cold water bath, the samples are mixed thoroughly and centrifuged for 5 minutes at approximately 4 °C to separate eventually present solid components. An aliquot of 100 µL of the plasma samples (calibration standards or SQCs) is deproteinised by adding 200 µL of acetonitrile containing the internal standard: For the API III Plus roxithromycin at a concentration of 1 µg/mL was used, whereas on API 3000™ and API 5000™ clarithromycin-d_3 at a concentration of 0.1 µg/mL was used.

The precipitated sample, containing the IS, is mixed thoroughly to facilitate deproteinisation. The precipitate is separated by centrifuging the samples for 10 minutes at 3600 rpm (3280 g) at approximately 4 °C. Raising the water content of the sample gives more constant and sharper peaks. Therefore, an aliquot of 100 µL

of the supernatant is diluted with 100 µL of ammonium acetate buffer (10 mM, pH 4). After mixing, 15 µL of each sample is injected onto the LC-MS/MS system. In Table 26 the optimized sample preparation procedure and LC-MS/MS conditions are summarized.

Table 26 *Summary of the clarithromycin and 14-hydroxy clarithromycin assay*

Characteristic	***Details***
Sample preparation	100 µL plasma + 200 µL CH_3CN /IS
	100 µL supernatant + 100 µL NH_4CH_3COO (10 mM, pH 4.0)
Internal standard	Roxithromycin (ROX)
	d_3-Clarithromycin (CLA-d_3)
Column	Symmetry C8, 5 µm (50 x 4.6 mm I.D.)
Mobile phase	35 % CH_3CN
	65 % NH_4CH_3COO (10 mM, pH 4.0)
Flow rate	1.0 mL/min
Run time	3.0 min
Ionisation	APCI
Polarity	Positive ion mode
Mass transitions	m/z 748.6 → m/z 158.2 (CLA)
	m/z 764.8 → m/z 158.2 (CLA-MET)
	m/z 838 → m/z 680 (ROX)
	m/z 752 → m/z 161 (CLA-d_3)

CH_3CN /IS: acetonitrile containing the IS (ROX, 1 µg/mL) or (CLA-d_3, 0.1 µg/mL)

3.3.4 Validation

The following experiments for full validation of clarithromycin and 14-hydroxy clarithromycin were performed on an API III Plus mass spectrometer and by the use of human plasma containing heparin as anticoagulant. The data were obtained from a previous validation and the results are explained in this chapter for full characterisation of the method and the analytes.

Being intended for a pharmacokinetic study, the present validation was conducted according to the guidance for industry (bioanalytical method validation) [45] by the FDA. The present LC-MS/MS method meets all criteria suggested by the guidance.

The specificity of the method was determined by analysis of drug-free plasma from six individuals (3 males and 3 females). Matrix components did not interfere with

CLA, CLA-MET or the IS, over the whole concentration range, i.e. no co-eluting peak was observed.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range from 9.58 to 3970 ng/mL for CLA and from 9.55 to 3960 ng/mL CLA-MET in human plasma. The mean linear regression equation of the calibration curves (N = 8) generated during the validation was determined:

$y = 0.0017\ (\pm 0.0013) + 0.0015\ (\pm 0.0001) \cdot x$ for clarithromycin,

$y = 0.0002\ (\pm 0.0010) + 0.0006\ (\pm 0.0001) \cdot x$ for 14-hydroxy clarithromycin

All correlation coefficients of the weighted ($1/x^2$) calibration curves were > 0.998 for CLA and > 0.997 for CLA-MET. The inter-day precision and accuracy of the back-calculated calibration standards of CLA and CLA-MET in human plasma ranged from 3.2 to 4.9 % (CLA), 3.9 to 5.7 % (CLA-MET) and from 95.7 to 102.5 % (CLA), 98.2 to 102.1 % (CLA-MET), respectively. Using 100 µL plasma, the lower limit of quantification was 9.58 ng/mL for CLA and 9.55 ng/mL for CLA-MET. The inter-day precision and accuracy obtained at the LLOQ were 3.2 % (CLA), 5.0 % (CLA-MET) and 100.7% (CLA), 99.9 % (CLA-MET), respectively.

Table 27 and Table 28 summarize the intra- and inter-day precision and accuracy for CLA and CLA-MET, evaluated by assaying five replicates of each SQC concentration on four validation days, each. The concentrations of the SQCs were calculated by using both calibration curves for the inter-day precision and by using the first calibration curve for the intra-day precision.

Table 27 *Intra- and inter-day precision and accuracy of clarithromycin (CLA) in human plasma (four days, five replicates per day)*

Nominal concentrations [ng/mL]	Intra-day Precision [%]	Intra-day Accuracy [%]	Inter-day Precision [%]	Inter-day Accuracy [%]
2960	4.2	95.2	4.8	97.1
980	2.6	98.4	4.0	99.8
94.9	2.8	101.4	3.5	102.4
24.1	7.6	99.7	5.8	100.8

Table 28 *Intra- and inter-day precision and accuracy of 14-hydroxy clarithromycin (CLA-MET) in human plasma (four days, five replicates per day)*

Nominal Concentrations [ng/mL]	Intra-day Precision [%]	Intra-day Accuracy [%]	Inter-day Precision [%]	Inter-day Accuracy [%]
2980	4.5	101.0	5.6	100.0
999	1.5	101.2	5.3	98.8
98.7	4.6	101.6	5.4	100.1
24.7	6.5	107.2	7.9	103.0

The inter-day precision of the SQC samples for CLA and CLA-MET in human plasma ranged from 3.5 to 5.8 % (CLA), 5.3 to 7.9 % (CLA-MET) and from 97.1 to 102.4 % (CLA), 98.8 to 103.0 % (CLA-MET), respectively. The obtained results fulfilled excellently the acceptance criteria of not more than 15% deviation by the above cited guidelines.

The mean absolute extraction recovery was determined to be 96.9 ± 3.6 % (CLA) and 97.7 ± 2.2 % (CLA-MET). The mean absolute extraction recovery of the IS (roxithromycin) at the working concentration was determined as 98.2 ± 2.0. All recoveries had relative standard deviations better than 3.7 % throughout the entire calibration ranges, showing good consistency and reproducibility of the simple protein precipitation for CLA and CLA-MET in human plasma.

Plasma samples containing up to 9950 ng/mL CLA and up to 9980 ng/mL CLA-MET can be pre-diluted by a factor 4 with drug-free human plasma. The mean concentration values were within ±15 % of the nominal value with mean accuracies of 96.8 % (CLA) and 99.2 % (CLA-MET) and mean precisions of 6.2 % (CLA) and 7.6 % (CLA-MET).

Stability data of CLA and CLA-MET in human plasma in five replicates of SQC samples at concentrations of 998 ng/mL CLA and 999 ng/mL CLA-MET are compiled in Table 29 and Table 30. The results of the 95% confidence interval calculation suggest that there is no evidence of instability. Therefore, CLA and CLA-MET are stable in human plasma for at least 4 hours at 4 °C, and for at least 9 weeks at -20 °C and -70 °C. Furthermore, both analytes are stable in prepared human plasma for at least 48 hours and during three freeze-thaw cycles, each longer than 12 hours.

Table 29 *Stability data on clarithromycin (CLA, 998 ng/mL) in human plasma.*

Stability experiment	Time	Point Estimator [%]	Lower Limit [%]	Upper Limit [%]
Short-term stability	2 hours	102.95	99.77	106.22
(approximately 4 °C)	4 hours	104.21	99.77	108.84
Post-preparative stability	24 hours	99.44	94.77	104.34
(autosampler, approximately 4 °C)	48 hours	102.52	99.11	106.05
Post-preparative stability	24 hours	99.17	94.03	104.58
(approximately -70 °C)	48 hours	102.81	95.86	110.26
Freeze-thaw stability	Once	103.88	97.33	110.86
(approximately -70 °C)	Twice	103.06	97.98	108.42
	Thrice	101.99	97.53	106.65
Long-term stability	2 weeks	100.84	97.44	104.36
(approximately -20 °C)	9 weeks	102.63	99.65	105.69
Long-term stability	2 weeks	100.50	99.03	101.99
(approximately -70 °C)	9 weeks	102.14	98.92	105.47

Table 30 *Stability data on 14-hydroxy clarithromycin (CLA-MET, 999 ng/mL) in plasma.*

Stability experiment	Time	Point Estimator [%]	Lower Limit [%]	Upper Limit [%]
Short-term stability	2 hours	102.97	98.77	107.35
(approximately 4 °C)	4 hours	101.92	97.23	106.83
Post-preparative stability	24 hours	97.96	89.73	106.94
(autosampler, approximately 4 °C)	48 hours	103.71	99.01	108.63
Post-preparative stability	24 hours	95.97	89.36	103.06
(approximately -70 °C)	48 hours	103.50	95.45	112.22
Freeze-thaw stability	Once	103.53	95.97	111.70
(approximately -70 °C)	Twice	104.67	99.19	110.45
	Thrice	101.30	93.79	109.40
Freezer storage stability	2 weeks	102.39	98.30	106.65
(approximately -20 °C)	9 weeks	96.52	92.13	101.13
Freezer storage stability	2 weeks	103.94	101.78	106.15
(approximately -70 °C)	9 weeks	102.17	98.17	106.34

No influence of haemolysed plasma on the accuracy and precision of the method was observed. The mean concentration values were within ±15 % of the nominal

value. Mean precision and accuracy in haemolysed plasma at 991 ng/mL CLA were 4.2 and 98.1 %, and at 997 ng/mL CLA-MET precision and accuracy were 5.1 and 102.1 %.

Determination of CLA and CLA-MET was also performed in human plasma containing EDTA as anticoagulant. Therefore, drug-free human EDTA plasma from six donors (3 males, 3 females) was analyzed to assess specificity. No interference occurred with the analytes or the IS. Additionally, calibration standards and SQC samples from 10.00 to 4000 ng/mL were prepared in EDTA plasma. The correlation coefficient obtained using $1/x^2$ linear regression analysis of the calibration curve was 0.999 for clarithromycin and 0.998 for 14-hydroxy clarithromycin. The precision and accuracy of the SQC samples (6 replicates at 4 concentration levels) ranged from 2.2 to 8.9 % and from 98.6 to 101.1 %.

3.3.5 Method comparison on three different LC-MS/MS systems

Validation experiments were chosen that show the influence of the different ionisation sources or the alternated ion paths on the quantification of clarithromycin and 14-hydroxy clarithromycin. These experiments were performed by using the same chromatographic conditions and sample work-up procedure as from a previous method on API III Plus (obtained by Professor Dr Fritz Sörgel) to assure absolute comparability to the experiments on API 3000™ and API 5000™.

The selectivity may vary between different MS instruments when small interferences become visible due to better sensitivity. No interference between matrix components in drug-free plasma from twenty individuals and CLA, CLA-MET or the IS occurred on all three tested mass analyzers in the monitored concentration ranges, even down to 0.391 ng/mL.

The calibration range for CLA on API III Plus was linear from 9.58 to 3070 ng/mL, equivalent to two orders of magnitude, whereas on API 3000™ and on API 5000™ it ranged from 0.781 to 4000 ng/mL, equivalent to three orders of magnitude. The linearity range for clarithromycin could not be extended further with the API 5000™, because the calibration function was not linear below 0.781 ng/mL. Therefore, the LLOQ had to be set to 0.781 ng/mL, despite good signal-to-noise ratios at this concentration.

For CLA-MET it was possible to extend the linearity ranges with increasing sensitivity from API III Plus, to API 3000™, to the largest range on API 5000™, covering 0.391

up to 4000 ng/mL. The large orifice sets a relatively high number of molecular ions into the MS, whereas the vertical spraying ion source permits most interfering uncharged molecules to enter the mass spectrometer.

The inter-day precision and accuracy obtained at the LLOQs on all instruments and for both analytes ranged from 2.1 to 7.0 % and from 99.0 to 102.9 %, respectively. Compared to the accepted limits of 15 % and 85-115 %, these data are showing a very high precision and accuracy.

The mean linear regression equations (peak area ratio versus concentration) of the calibration curves were established:

API III Plus, CLA: $y = 0.0017\ (\pm 0.0013) + 0.0015\ (\pm 0.0001) \cdot x$ $(r^2 \geq 0.998)$

API 3000™, CLA: $y = 0.0263\ (\pm 0.0026) + 0.0154\ (\pm 0.0004) \cdot x$ $(r^2 \geq 0.997)$

API 5000™, CLA: $y = 0.0017\ (\pm 0.0004) + 0.0032\ (\pm 0.0001) \cdot x$ $(r^2 \geq 0.998)$

API III Plus, CLA-MET: $y = 0.0002\ (\pm 0.0010) + 0.0006\ (\pm 0.0001) \cdot x$ $(r^2 \geq 0.997)$

API 3000™, CLA-MET: $y = -0.0028\ (\pm 0.0011) + 0.0087\ (\pm 0.0008) \cdot x$ $(r^2 \geq 0.995)$

API 5000™, CLA-MET: $y = -0.0002\ (\pm 0.0003) + 0.0198\ (\pm 0.0006) \cdot x$ $(r^2 \geq 0.998)$

Good linearity and correlation coefficients were obtained on all systems.

The signal-to-noise (S/N) ratios at the LLOQ on API III Plus were greater than 10 on all validation days. On API 3000™, the maximal sensitivity with high precision of the LLOQ and the SQCs was tested, what led to an LLOQ of 0.781 ng/mL (CLA) and 1.56 ng/mL (CLA-MET), respectively. The S/N ratios at these concentrations were at least 5 for both analytes which is also recommended by the FDA [132]. On API 5000™ the S/N ratios at the LLOQ were greater than 10 on all validation days, resulting from a very low noise. Comparing to the API 3000™, the limiting point for the sensitivity on API 5000™ was the linearity in the lower calibration range.

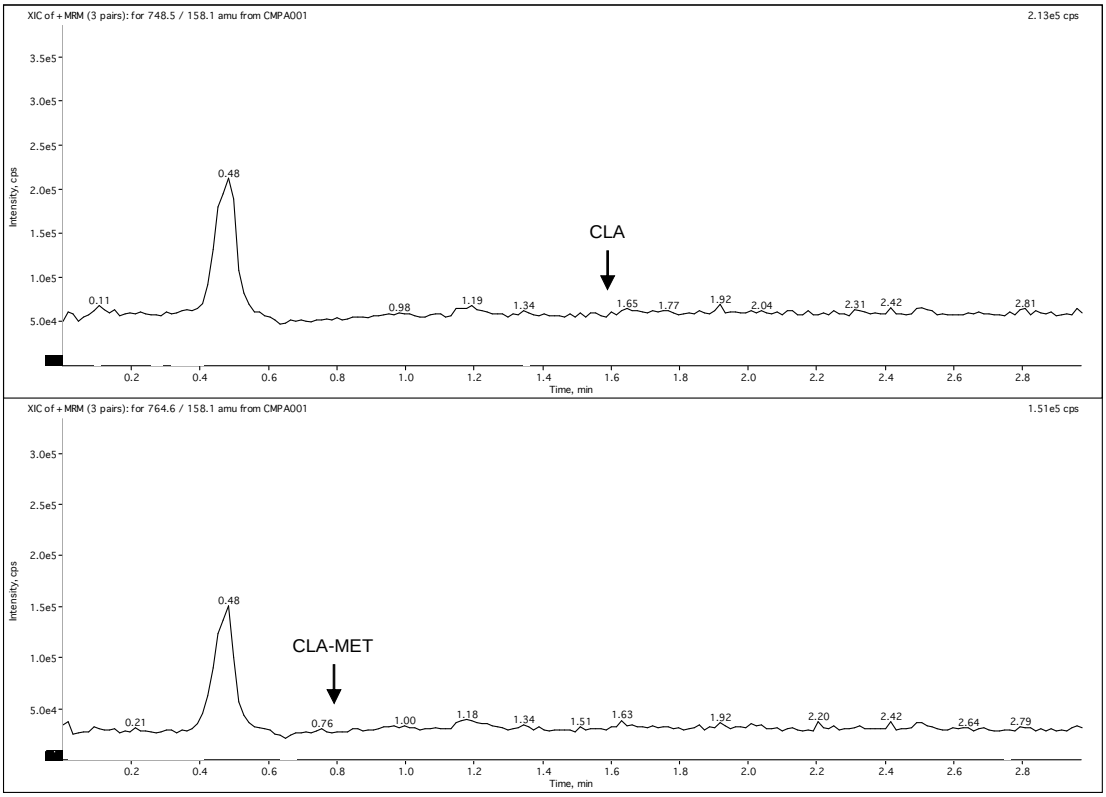
Since mass analyzers with quite different ionisation sources using horizontal or vertical spray are compared, the matrix effect may vary distinctively. The matrix factors (MF, mean ± SD) on API 3000™ calculated from peak areas were almost identical to those calculated from peak area ratios and were close to 1.0. This indicates the absence of a matrix effect in the API 3000™ analytical system.

On the API 5000**™**, matrix factors (mean ± SD) from clarithromycin were 0.85 ± 0.02 (peak area) and 1.01 ± 0.01 (area ratio CLA/IS), whereas the matrix factors from 14-hydroxy clarithromycin were 0.74 ± 0.03 (peak area) and 0.88 ± 0.04 (area ratio CLA-MET/IS). The lower MF values calculated by peak areas indicate light ion

suppression of clarithromycin and its metabolite in the API 5000™ ionisation source. However, the overall MF, calculated by the peak area ratios, shows that the IS compensates for this effect. The MF does not need to be 1.0 (see EMA [34]), but has to be constant for all analyzed matrices, which is the case here, proven by the small standard deviations across six different plasma sources. This assures no difference in response for calibration standards and different subject's samples.

Via post column infusion no increase or decrease of the signal was observed at and near the retention times of CLA, CLA-MET, see Figure 21.

Figure 21 *Matrix-effect assessment via post-column infusion of clarithromycin (top) and 14-hydroxy clarithromycin (bottom)*



Data from system-dependent validation experiments on API III Plus, API 3000™ and API 5000™ by comparison are outlined in Table 31 and Table 32. Data from API III Plus were obtained from previous experiments by IBMP – Institute for Biomedical and Pharmaceutical Research.

Table 31 *Data of clarithromycin (CLA) from three LC-MS/MS systems*

Experiment	Unit	API III Plus	API 3000™	API 5000™
Specificity in plasma	[-]	no interference in 6 individuals	no interference in 20 individuals	no interference in 20 individuals
Linearity range Orders of magnitude	[ng/mL]	9.58 – 3970 $4.1 \cdot 10^2$	0.781 – 4000 $5.1 \cdot 10^3$	0.781 – 4000 $5.1 \cdot 10^3$
Correlation Coefficient	[-]	>0.998	>0.997	>0.998
LLOQ **Gain to previous API**	[ng/mL]	9.58 -	0.781 **12**	0.781 **1**
Precision of LLOQ Accuracy of LLOQ	[%] [%]	3.2 100.7	4.9 102.3	4.3 101.8
Highest SQC Lowest SQC	[ng/mL] [ng/mL]	2960 24.1	4000 1.56	4000 1.56
Precision of SQCs Accuracy of SQCs	[%] [%]	3.5 to 5.8 97.1 to 102.4	2.9 to 14.9 101.2 to 103.0	2.2 to 12.0 96.0 to 101.4
Matrix Effect CLA CLA/IS	 MF, Prec [%] MF, Prec [%]	 - -	 1.03, 1.8% 1.01, 1.6%	 0.85, 2.9% 1.01, 1.0%

SQC: spiked quality control, LLOQ: lower limit of quantification, CLA: clarithromycin, IS: internal standard, MF: matrix factor, Prec: Precision from plasma of 6 individuals

Table 32 *14-hydroxy clarithromycin (CLA-MET) data of three LC-MS/MS systems*

Experiment	Unit	API III Plus	API 3000™	API 5000™
Specificity in plasma	[-]	no interference in 6 individuals	no interference in 20 individuals	no interference in 20 individuals
Linearity range Orders of magnitude	[ng/mL]	9.55 – 3960 $4.1 \cdot 10^2$	1.56 – 4000 $2.6 \cdot 10^3$	0.391 – 4000 $1.0 \cdot 10^4$
Correlation Coefficient	[-]	>0.997	>0.995	>0.998
LLOQ **Gain to previous API**	[ng/mL]	9.55 -	1.56 **6**	0.391 **4**
Precision of LLOQ Accuracy of LLOQ	[%] [%]	5.0 99.9	7.0 99.0	2.1 102.9
Highest SQC Lowest SQC	[ng/mL] [ng/mL]	2970 24.2	4000 3.13	4000 0.391
Precision of SQCs Accuracy of SQCs	[%] [%]	5.3 to 7.9 98.8 to 103.0	5.0 to 11.1 96.3 to 103.1	5.0 to 10.5 91.1 to 99.5
Matrix Effect CLA-MET CLA-MET/IS	 MF, Prec [%] MF, Prec [%]	 - -	 0.99, 2.8% 0.97, 3.1%	 0.74, 4.7% 0.88, 4.5%

SQC: spiked quality control, LLOQ: lower limit of quantification, CLA-MET: 14-hydroxy clarithromycin, IS: internal standard, MF: matrix factor, Prec: Precision from plasma of 6 individuals

3.3.6 Comparison of the LC-MS/MS method with procedures reported in the literature

In Table 33 the calibration range, run time, work-up procedure and quality control data for several previously reported chromatographic methods for the determination of clarithromycin and 14-hydroxy clarithromycin in human plasma is compared to the present LC-MS/MS method.

All previously reported LC/ED [114-115, 147, 150, 155-157], LC/UV [145] and LC/FD [159] methods need between 7 and 30 minutes analysis time, while previously reported LC-MS/MS methods are able to analyze CLA and CLA-MET within 4.5 minutes, except one [54], needing 9 minutes per sample. The first reason for the shorter analysis times with MS/MS detection is that, the analytes need not necessarily be baseline separated from each other since simultaneous detection is possible. Secondly, in contrast to other detection techniques, the high selectivity of MS/MS detection produces no background signals in the first minutes of the chromatograms, making shorter retention times within 1-2 minutes possible. Due to the poorer selectivity in detection, LC/ED and LC/UV methods need more cost and time-consuming sample preparation techniques. Some of them extract CLA from 0.5 mL or even 1.0 mL [145, 159] sample volume to reach LLOQs between 25 and 100 ng/mL. This new method uses 0.1 mL plasma for quantification of CLA and CLA-MET down to 0.78 and 0.39 ng/mL, respectively, within 3.0 minutes.

Five previously reported LC-MS/MS methods reach LLOQ values between 2.5 and 10 ng/mL [52, 161, 171-173], of which only one is substantiated by inter-day accuracy and precision data at the LLOQ [161]. These methods are characterised by more cost-intense and time-consuming extraction methods like µSPE [161] or LLE [52, 171-172], the latter using equal or thrice the sample volume.

The extraction recovery (97.1%) of Li et al. [172] compared to the recovery (80.5%) of van Rooyen et al. [171] indicates that extraction with methyl *tert*-butyl ether (MTBE) and reconstitution with 0.1% formic acid in water/methanol (1:1) is more efficient than shaking with hexane/ethyl acetate (1:1) and reconstitution at neutral pH (water/acetonitrile, 9:1). Both extractions were performed at pH 9.2 (and 9.5), which is near the pKa of CLA 8.86. Obviously, the water-insolubility of CLA helps during extraction, even if the protonation equilibrium is not shifted towards the neutrally charged base.

The LC-MS/MS methods, reported by Shin et al. [170] and de Velde et al. [55] report an easy to use precipitation method with a low sample volume, but are lacking sensitivity with a ten times higher LLOQ, and omitted inter-day precision data of the reported LLOQ [170]. Recently, Vu et al. [54] published a modification of the method by de Velde et al. reducing the carry-over from samples with high concentrations into samples which should not contain any analyte concentration. This had also a positive effect on the sensitivity, which was increased by a factor of two. However, to achieve this, a second gradient during the chromatographic separation of each sample was introduced, increasing the run-time per sample from 3.6 minutes to 9.0 minutes.

Besides the current method, only six previously reported methods were used for determination of both, clarithromycin and 14-hydroxy clarithromycin in plasma. These include two LC/ED methods [147, 150], of which only sparse data is given. Two further methods only estimated 14-hydroxy clarithromycin concentrations semi-quantitatively: Oswald et al. [52] used LC-MS/MS to monitor the mass transition of 14-hydroxy clarithromycin in the study samples and estimated the metabolite concentrations by comparing the peak areas to calibration standards of clarithromycin. Lerner et al. [53] cited a time-consuming bioassay technique with derivatisation, not reporting any quality data for 14-hydroxy clarithromycin. The last two methods for determination of 14-hydroxy clarithromycin were developed and modified by de Velde et al. [55] and Vu et al. [54], which were discussed a few lines above and were not reported to be applied to a pharmacokinetic study.

To the best knowledge, this is the only LC-MS/MS method which quantifies both, clarithromycin and its 14-hydroxy metabolite with high sensitivity, which is fully validated and was furthermore applied to a large pharmacokinetic study.

With the present method, clarithromycin and 14-hydroxy clarithromycin can be measured with high sensitivity using simple protein precipitation sample work up. With only 3 min runtime it is possible to measure about 400 human plasma samples per LC-MS/MS machine per day.

Table 33 *Comparison of current method with other analytical methods for the determination of clarithromycin and 14-hydroxy clarithromycin in human plasma or serum; grouped by analytical technique and descending years of publication*

Method	Matrix	Linearity range [ng/mL]		Approx. run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of QC samples [%]	Recovery mean ± SD [%]	Sample Vol. [mL]	Sample preparation	Application
LC-MS/MS	Human	CLA	9.58-3970	3.0	3.2 / 100.7	3.5 to 6.2 / 96.8 to 102.4	96.9 ± 3.6[1]	0.100	PPT	250-mg PK
(current method)	plasma	MET	9.55-3960		5.0 / 99.9	5.3 to 7.9 / 98.8 to 103.0	97.7 ± 2.2[1]			
API 3000™	Human	CLA	0.781-4000	3.0	4.9 / 102.3	2.9 to 14.9 / 101.2 to 103.0	98.2 ± 2.0 (IS)[2]	0.100	PPT	
(current method)	plasma	MET	1.56-4000		7.0 / 99.0	5.0 to 11.1 / 96.3 to 103.1				
API 5000™	Human	CLA	0.781-4000	3.0	4.3 / 101.8	2.2 to 12.0 / 96.0 to 101.4		0.100	PPT	
(current method)	plasma	MET	0.390-4000		2.1 / 102.9	5.0 to 10.5 / 91.1 to 99.5				
LC-MS/MS [54]	Human	CLA	50-10000	9.0	8.8 / n.r.	0.0 to 8.8 / 98.0 to 101.6*	n.r.	0.010	PPT (30 min)	n.r.
Vu 2013	plasma	MET	50-10000	9.0	1.9 / n.r.	0.8 to 4.8 / 98.0 to 111.0*	n.r.	0.010	PPT (30 min)	n.r.
UPLC-MS/MS [161] Wang 2012	Rat plasma	CLA nano	10-4000	2.0	4.2 / 94.1 12.2 / 104.9	3.6 to 7.4 / 94.1 to 102.0 0.1 to 12.2 / 98.9 to 104.9	Referenced accuracy data as recovery	0.050	PPT + µSPE	PK in rats
LC-MS/MS [52] Oswald 2011	Horse plasma	CLA/ MET	2.5-25 and 25-250	3.0	n.r. / n.r. n.r. / n.r.	6.2 to 10.0 / 100.1 to 106.2 5.1 to 8.9 / 99.9 to 108.6	77.4 to 82.8 78.0 to 81.0	0.200	LLE	PK in foals
LC-MS/MS [55]	Human	CLA	100-10000	3.6	4.5 / 94.6	0.0 to 4.5 / 94.6 to 100.2	100.1 to 111.4	0.010	PPT	n.r.
De Velde 2009	plasma	MET	100-10000	3.6	6.7 / 90.5	0.0 to 6.7 / 90.5 to 105.0	101.3 to 116.8	0.010	PPT	n.r.
LC-MS/MS [170] Shin 2008	Human plasma	CLA	100-5000	< 3	n.r. / n.r.	7.0 to 7.9 / 95.3 to 100.1	78.3 ± 1.4 [4], 90.5 ± 6.4 [5] 98.1 ± 5.4 (IS)[2]	0.025	PPT	500-mg PK
LC-MS/MS [173] Jiang 2007	Human plasma	CLA	10-5000	2.4	n.r. / n.r.	6.7 to 9.9 / 97.5 to 104.5	95.4 ± 3.9 to 97.0 ± 7.2	0.050	PPT	500-mg PK
LC-MS/MS [172] Li 2006	Human plasma	CLA	5-5000	3.0	n.r. / n.r.	0.8 to 5.0 / 87.4 to 108.7	97.1 to 102	0.100	LLE	n.r.
LC-MS/MS [171] Van Rooyen 2002	Human plasma	CLA	2.95-20016	2.6	n.r. / n.r.	0.8 to 6.8 / 94.6 to 105.4 6	80.5-90.8 1	0.300	LLE	500-mg (no data)

Continued on the next page

Table 33 (continued) *Comparison of current method with other analytical methods for the determination of clarithromycin and 14-hydroxy clarithromycin in human plasma or serum; grouped by analytical technique and descending years of publication*

Method	Matrix	Linearity range [ng/mL]		Approx. run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of QC samples [%]	Recovery mean ± SD [%]	Sample Vol. [mL]	Sample preparation	Application
LC-MS/MS [53]	Human	CLA	50-10000	4.5	10.2 / 86.0	14.8 to 54.1 / 90.6 to 112.8	n.r.	0.200	LLE	500-mg PK
Lerner 2000	plasma	MET	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	DER + BIO	
LC/FD [159] Bahrami 2007	Human serum	CLA	25-10000	>6.6	16.0 / 94.7	2.6 to 16.0 / 94.7 to 99.7	93±4	1.0	LLE and DER	500-mg PK
LC/UV [145] Amini 2005	Human plasma	CLA	31.3-2000	<11	7.3 / 109.5	2.8 to 7.3 / 96.5 to 109.5	92.3 ± 4.3 to 104 ± 8	1.0	LLE	250-mg PK
LC/ED [157] Zaater 2012	Human plasma	CLA	50-5000	15	3.9 / 102.0	3.0 to 3.9 / 99.0 to 100.1	95.7 ± 5.2	0.500	LLE	500-mg PK
LC/ED [150]	Human	CLA	55.7-3421	n.r.	n.r.	n.r.	n.r.	0.500	LLE	250-mg PK
Koytchev 2004	plasma	MET		n.r.	n.r.	n.r.	n.r.			
LC/ED [155] Niopas 2001	Human plasma	CLA	100-2000	11.0	n.r. / n.r.	2.1 to 4.7 / 97.3 to 100.2	84.3 ± 4.5 [3], 86.2 ± 3.4 (IS)[2]	0.500	LLE	500-mg PK
LC/ED [156] Choi 2001	Human plasma	CLA	100-4000	30	3.2 / 100.0	1.4 to 3.2 / 98.0 to 102.0	86.7 ± 4.1	0.100	DIL + CS	500-mg PK
LC/ED [115] Kees 1998	Human plasma	CLA	100-5000	15-20	4.1 / 115.7 (30ng/mL)	0.3 to 9.1 / 100.6 to 107.3	80-90	0.500	LLE	n.r.
LC/ED [114] Hedenmo 1995	Human plasma	CLA	0.5-25µM	9.0	1.2 / n.r.	5.8 / n.r.(3.0 µM)	99.0 ± 5.3	0.100	automated SPE	n.r.
LC/ED [147]	Human	CLA	30-	> 17.2	n.r. / n.r.	<10 / n.r.	70-75	0.500	PPT, SPE	250 mg /
Kees 1995	plasma	MET	50-							500 mg PK

Prec: Precision, Acc: Accuracy, SD: standard deviation, *: intra-day data for accuracy, 1: over the whole concentration range, 2: internal standard roxithromycin, 3: based on direct comparison of peak heights, 4: at 200 ng/mL clarithromycin, 5: at 4000 ng/mL clarithromycin, 6: during study sample analysis (n=6), n.r.: not reported, PPT: protein precipitation, LLE: liquid-liquid extraction, SPE: solid phase extraction, DER.: derivatisation, BIO: bioassay, DIL + CS: dilution followed by column-switching

3.3.7 Application to a pharmacokinetic study of oral clarithromycin

3.3.7.1 Study design and sample handling

The pharmacokinetic profiles of clarithromycin and its active metabolite 14-hydroxy clarithromycin were investigated by administering a single 250-mg film-coated tablet of clarithromycin to 36 healthy male and female volunteers in the fasting state. Blood was drawn immediately before administration and at 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 12.00, 16.00, 24.00, and 36.00 hours after oral administration of the study drug.

Shortly after collecting the human blood samples into monovettes containing ammonium heparinate, the samples are centrifuged for 10 minutes at approximately 1500g in a cooled centrifuge. Each plasma sample is immediately transferred into at least two storage tubes and frozen at -70 °C until analysis.

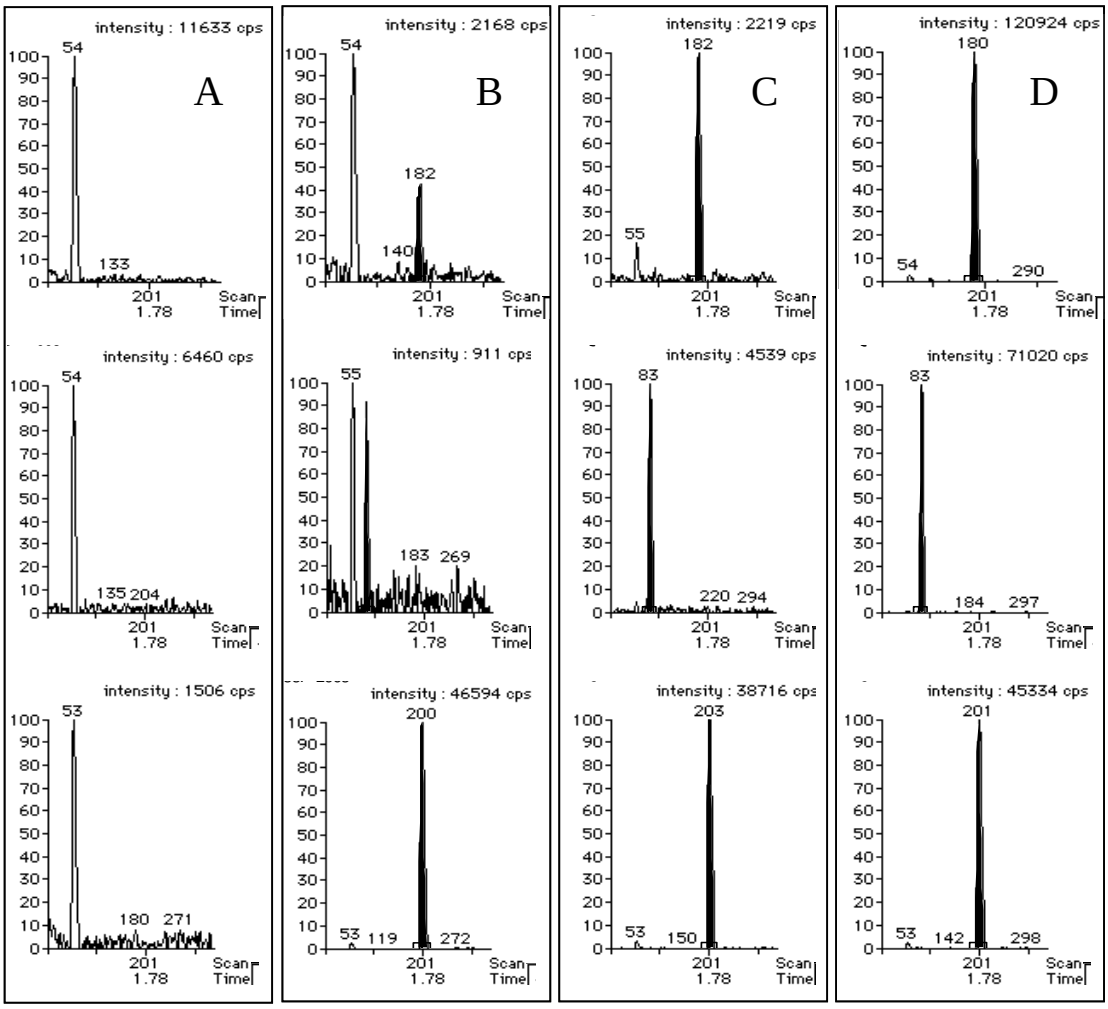
3.3.7.2 Determination of plasma concentrations

A total of 1839 human plasma samples were analyzed for clarithromycin and 14-hydroxy clarithromycin in a total of thirteen sequences, which were measured within eight hours respectively. Each sequence, consisted of injection standards, calibration standards (low to high concentrations), subject samples and 3 SQC samples at each of four concentration levels (in randomized order among subject samples).

Representative MRM-chromatograms of CLA, CLA-MET and the IS are depicted in Figure 22: (A) a blank sample, (B) the LLOQ containing 9.81 ng/mL CLA and 9.69 ng/mL CLA-MET, (C) a high study sample 16 h after administration containing 33.0 ng/mL CLA and 63.2 ng/mL CLA-MET and (D) a low study sample 1.0 h after administration containing1721 ng/mL CLA and 778.0 ng/mL CLA-MET.

Both, clarithromycin and 14-hydroxy clarithromycin give intense peaks with a high signal-to-noise ratio, being separated from matrix components.

Figure 22 *Chromatograms of CLA (top), CLA-MET (middle) and ROX (IS, bottom)*



In order to control the precision and accuracy of the assay during the measurement of study samples calibration standards and SQC samples were analyzed together with the study samples in each sequence. The determined concentrations of SQC samples and the back-calculated concentrations of the calibration standards were compared to the theoretical concentrations for accuracy. The accuracy and precision for each concentration level is summarized in Table 34.

The coefficient of correlation of resulting linear regressions was at least 0.996 for both analytes. The mean precision and accuracy of the SQC samples (N = 4 x 39) of clarithromycin in human plasma analyzed within the batches of study samples ranged from 6.4 % to 7.4 % and from 98.5 % to 101.8 % and for 14-hydroxy clarithromycin (N = 4 x 39) 6.3 % to 8.4 % and from 97.7% to 101.5%, respectively, demonstrating very high precision and accuracy of the assay as well as the concentration data of the study samples.

Table 34 *Accuracy and precision of calibration standards and spiked quality control samples during analysis of CLA and CLA-MET in study samples*

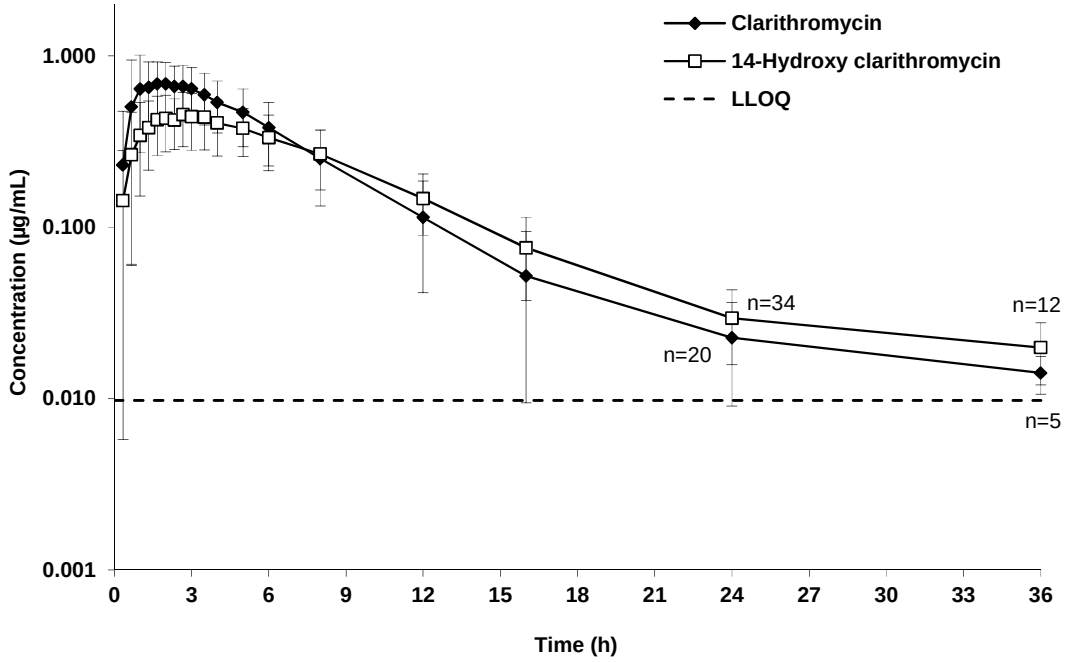
	Theoretical Concentration (µg/mL)	Mean Accuracy* (%)	Mean Precision* (%)
Calibration standards CLA	4.03	97.4	3.9
	3.02	98.0	2.4
	2.02	100.7	2.4
	1.02	100.3	1.9
	0.509	100.7	2.4
	0.214	100.7	3.9
	0.106	101.6	2.1
	0.0527	100.4	4.1
	0.0196	99.6	6.2
	0.0147	102.4	6.8
	0.00981	98.7	5.4
SQC samples CLA	3.00	98.5	6.6
	1.01	99.9	6.4
	0.0910	101.2	7.4
	0.0258	101.8	7.2
Calibration standards CLA-MET	3.98	98.9	4.7
	2.98	101.4	3.2
	1.99	103.4	3.2
	1.01	101.9	2.6
	0.503	98.8	4.1
	0.212	94.9	5.5
	0.105	101.7	3.4
	0.0520	100.0	6.2
	0.0193	98.6	7.2
	0.0145	97.9	8.0
	0.00969	102.2	4.9
SQC samples CLA-MET	2.94	101.3	6.3
	0.990	101.5	6.9
	0.0889	97.7	8.4
	0.0252	98.9	8.3

* Accepted limits by EMA and FDA: maximal 15 % for precision and 85-115 % for accuracy

3.3.7.3 Pharmacokinetic results

The measured concentration data of clarithromycin and 14-hydroxy clarithromycin is plotted versus the time after oral administration of a 250-mg clarithromycin tablet to 36 healthy volunteers. Figure 23 displays the mean plasma concentration profile after oral administration of the 250-mg clarithromycin tablet.

Figure 23 *Plasma profiles (mean±SD) of clarithromycin and 14-hydroxy clarithromycin concentrations versus time after administration of a 250-mg oral clarithromycin dose to 36 healthy volunteers*



The mean peak concentrations (C_{max}) of clarithromycin (0.83±0.33 µg/mL, mean±SD) and 14-hydroxy clarithromycin (0.51±0.16 µg/mL, mean±SD) were attained at 1.6±1.0 hours and 2.4±1.1 hours (mean±SD) after administration, respectively.

Table 35 and Table 36 show the comparison of the pharmacokinetic parameters of clarithromycin and its 14-hydroxy metabolite after oral administration of a 250-mg tablet to pharmacokinetic parameters reported in previously published studies. Since clarithromycin is characterised by dose-dependent pharmacokinetics [143-144], only PK data from other 250-mg studies are collated.

The data for C_{max} and t_{max} of clarithromycin and its 14-hydroxy metabolite are in very good agreement with the data reported by others. The elimination half-life of CLA and CLA-MET was found to be 4.33±2.57 hours (mean±SD) and 6.29±2.83 hours (mean±SD), which is in good agreement to Goodman & Gilman [1] (3-7 hours for CLA and 5-9 hours for CLA-MET) and within the range of reported data from 1.9±0.6 hours up to 4.88±2.97 hours for clarithromycin and from 3.9 hours up to 6.20±3.82 hours for its 14-hydroxy metabolite. Also the data for $AUC_{0\rightarrow\infty}$ of 5.29±2.13 h·µg/mL for CLA and 4.82±1.63 h·µg/mL for CLA-MET are comparable to the data reported by others [140, 145-148].

Only one author [150] reported higher values for $AUC_{0\rightarrow\infty}$ (8.2 and 7.4 h·µg/mL for CLA and CLA-MET, respectively). Especially, by comparing their 14-hydroxy clarithromycin $AUC_{0\rightarrow\infty}$ (7.4 h·µg/mL) with their $AUC_{0\rightarrow last}$ (5.9 h·µg/mL) it seems as if they had to extrapolate about 25% of their AUC. This may indicate some issues with study design or sensitivity of their bioanalytical method. Like other authors, they collected PK samples until 24 hours post dose, covering most of the concentration-time curve. However, collecting until 36 hours post dose, allows for a more precise determination of the total AUC (2.5 % and 3.9 % were extrapolated for CLA and CLA-MET in the present study). Having a closer look at the bioanalytical method, which was used to quantify the samples of their study, it seems as if it was not sensitive enough (LLOQ 55 ng/mL, present method: 10 ng/mL), which may be concluded by the plasma profile of 14-hydroxy clarithromycin stopping at 14 hours post dose. As a consequence, concentration data at 24 hours post dose are missing.

Table 35 *Pharmacokinetic parameters (mean±SD) of clarithromycin in healthy subjects after overnight fasting and administration of a single oral dose as a tablet*

Reference	No. of subjects / Gender	Dose / Formulation	t_{last} [h]	$AUC_{0\rightarrow t}$ [h·µg/mL]	$AUC_{0\rightarrow\infty}$ [h·µg/mL]	C_{max} [µg/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current study	36 / m,f	250 mg / film-coated tablet	36	5.16±2.12	5.29±2.13	0.83±0.33	1.59±1.01	4.33±2.5 7
Lappin 2011 [175]	6 / m	250 mg tablet	24	n.r.	4.905 (36)*	0.958 (34)*	0.96 (85)*	3.4 (46)*
Traunmüller 2007 [149]	6 / n.r.	250 mg tablet	24	4.45±1.94	n.r.	1.09±0.35	2.6±0.5	1.9±0.6
Amini 2005 [145]	14 / n.r.	250 mg tablet	24	n.r.	6.34±1.62	1.07±0.20	1.64±0.49	4.31±0.87
Koytchev 2004 [150]	24	250 mg tablet / test	24	7.13±2.56	7.92±2.97	1.21±4.13	1.78 ± 2.70	4.35 ± 2.08
Koytchev 2004 [150]	24	250 mg tablet / reference	24	7.36±3.02	8.16±3.32	1.17±3.62	1.63 ± 1.29	4.88 ± 2.97
Kim 2001	24 / m (Korean)	250 mg tablet / test	12	n.r.	7.015	1.260	1.85 ± 0.43	n.r.
Kim 2001	24 / m (Korean)	250 mg tablet / reference	12	n.r.	6.807	1.238	1.79 ± 0.69	n.r.
Kees 1995 [147]	12 / m	250 mg / film-sealed tablets	12	5.21±1.31	5.80±1.31	0.94±0.33	2.07±1.03	3.33±0.4 6
Chu 1993 [146]	17 / m	250mg / film-coated tablets	24	n.r.	4.36±1.51	0.78±0.25	1.8±0.7	2.7 (1.8-6.5)
Guay 1993 [148]	22 / m	250 mg tablet	24	6.07±1.97	n.r.	1.10±0.33	1.70±0.60	n.r.
Chu 1992 [140]	19 / m	250 mg tablet / formul. 1	24	4.21±1.52	4.27±1.52	0.76±0.24	1.72±0.71	2.7 (1.8-6.5)
Chu 1992 [140]	19 / m	250 mg tablet / formul. 2	24	3.96±1.55	4.03±1.56	0.72±0.25	1.92±0.97	2.6 (1.6-9.5)

*: CV in parentheses

Table 36 *Pharmacokinetic parameters (mean ± SD) of 14-hydroxy clarithromycin in healthy subjects after overnight fasting and administration of a single oral tablet*

Reference	No. of subjects / Gender	Dosis / Formulation	t_{last} [h]	$AUC_{0\rightarrow last}$ [µg·h/mL]	$AUC_{0\rightarrow\infty}$ [µg·h/mL]	C_{max} [µg/mL]	t_{max} [h]	$t_{1/2}$ [h]
Current study	36 / m,f	250mg / film-coated tablets	36	4.63±1.59	4.82±1.63	0.51±0.16	2.39±1.09	6.29±2.83
Koytchev 2004 [150]	24	250 mg tablet / test	24	5.98±1.35	7.39±1.48	0.76±0.23	2.59±2.83	6.20±3.82
Koytchev 2004 [150]	24	250 mg tablet / reference	24	5.86±1.55	7.38±1.62	0.75±0.21	2.33±1.03	5.90±2.88
Kees 1995 [147]	12 / m	250 mg / film-sealed tablets	12	3.24±1.03	4.59±1.42	0.46±0.17	2.22±0.81	6.17±2.97
Chu 1993 [146]	17 / m	250mg / film-coated tablets	24	n.r.	4.97±1.16	0.65±0.19	2.3±0.7	4.1 (2.7-10.1)
Guay 1993 [148]	22 / m	250 mg tablet (study 1)	24	4.28±1.16	n.r.	0.55±0.17	1.90±0.60	n.r.
Chu 1992 [140]	19 / m	250 mg tablet / formul. 1	24	4.91±1.12	4.91±1.12	0.65±0.19	2.17±0.77	4.2 (2.7-10.1)
Chu 1992 [140]	19 / m	250 mg tablet / formul. 2	24	4.58±1.16	4.63±1.17	0.64±0.20	2.32±1.17	3.9 (1.6-12.3)

3.3.8 Summary: clarithromycin

With the developed and validated method clarithromycin and its 14-hydroxy metabolite can be quantified from 9.6 to 15,800 ng/mL using 100 µL of human plasma. Roxithromycin was found to be the ideal candidate as internal standard by showing a comparable extraction and chromatographic behaviour as clarithromycin and its active metabolite.

High absolute recovery (96.9 % CLA, 97.7 % CLA-MET), high sensitivity on all three mass spectrometers (down to 0.391 ng/mL on the API 5000™), high inter-day precision (≤7.0 %) and high accuracy (within ±102.9 %) for LLOQ as well as excellent linearity (r2 ³ 0.995) with a short run time of only 3.0 minutes make the method suitable for high throughput sample measurement during clinical trials. The method was successfully applied to the analysis of more than 1800 human plasma samples from healthy volunteers dosed with a single 250-mg clarithromycin tablet.

The high precision of the assay contributed to the precise determination of C_{max} (0.83±0.33 µg/mL CLA, 0.51±0.16 µg/mL CLA-MET) and the high assay sensitivity allowed the determination of concentrations until 36 hours post-dose. Therefore, only

2.6 % (CLA) and 4.2 % (CLA-MET) of the total AUC had to be extrapolated. The obtained PK parameters are in good agreement with data from comparable studies.

3.4 Summary and conclusions: Macrolides

3.4.1 LC-MS/MS Assays

Three assays for five macrolides were developed and validated for the determination of plasma concentrations of erythromycin ethylsuccinate, erythromycin A, roxithromycin, clarithromycin and 14-hydroxy clarithromycin. Probably, all macrolides could have been analyzed simultaneously, since they are characterised by similar chemical properties. For example, their logP values range from 2.1 (EES) to 3.2 (CLA), while their pKa values range from 7.1 (EES) to 9.2 (ROX). Especially for residue analysis in food or water, where screening of a large number of antibiotics is favoured [128, 169, 176-180], simultaneous detection by LC-MS/MS can save a lot of time. However, compromises with respect to optimal extraction conditions or optimal detection conditions have to be accepted, which may lead to issues with sensitivity or accuracy and precision.

In the present work, high accuracy and precision, as well as the shortest possible analysis time for each analyte was crucial for the intended application in pharmacokinetic trials, bearing a later drug application for human beings in mind. In the final validated methods the retention times of all macrolides were shorter than 2 minutes. Clarithromycin and roxithromycin retention times were almost identical when analyzed by means of the ROX assay (0.9 minutes) or with the CLA/CLA-MET assay (1.6 minutes). However, with the chromatographic conditions of the ROX assay, 14-hydroxy clarithromycin could not be analyzed, since it would elute too early. While ROX was separated on a C-18 column (17% carbon load, low silanol activity), CLA and CLA-MET were separated on a C-8 column (12% carbon load, low silanol activity), resulting in more symmetrical peaks. This can be ascribed to the shorter C-8 chains with lower steric hindrance, allowing a more effective endcapping process of remaining silanol groups and therefore, less peak tailing of the basic macrolides. Erythromycin ethylsuccinate is the most “polar” of the analyzed macrolides with two additional keto-functions within the ethylsuccinate moiety. It was therefore, separated from erythromycin A by using a CN column, which profits from

the dipole-dipole interactions between the CN function and the 4 CO functions of EES.

While most previously reported methods for the determination of CLA, ROX or ERY use neutral (phosphate) buffers, here all validated assays use buffers for sample preparation and mobile phase with a weakly acidic pH 4-5, supporting fast RP chromatography and ionization in the MS. Previously reported stability data [104-105] support this procedure, indicating stability of CLA above pH 3 and of ERY above pH 5.

All analytes were ionized by APCI in the positive ionization mode, whereas ionization on the desosamine nitrogen is most likely. Since all macrolides had intensive signals in the MS, precipitation was the sample work-up procedure of choice and concentrating the analytes by expensive techniques was not necessary.

Table 37 *Validated concentration ranges (ng/mL), range of linearity and sensitivity gain compared to prior API.*

Analyte	API III Plus	API 3000™	API 5000™
ERY A	4.0 – 2000 **10^2** **-**	0.781 – 2100 **10^3** **5**	0.0977 – 2100 **10^4** **8**
EES	10 – 5000 **10^2** **-**	1.25 – 4002 **10^3** **8**	0.156 – 4002 **10^4** **8**
ROX	9.21 – 3950 **10^2** **-**	3.16 – 4000 **10^3** **3**	0.395 – 1614 **10^3** **8**
CLA	9.58 – 3970 **10^2** **-**	0.781 – 4000 **10^3** **12**	0.781 – 4000 **10^3** **1**
CLA-MET	9.55 – 3960 **10^2** **-**	1.56 – 4000 **10^3** **6**	0.391 – 4000 **10^4** **4**

Moreover, the LC-MS/MS assays of the three macrolides and the 14-hydroxy metabolite of clarithromycin were validated on three different mass analyzers. The innovations in the ion source (horizontal vs. vertical ESI), the ionpath (replacement of the skimmer by the QJet) and the larger orifice enabled a better sensitivity and a larger linearity range within this mass analyzer series. All methods were extended by larger calibration ranges. However, only the EES / ERY A assay allowed a larger linearity range and a better LLOQ at the same time, when transferred from API III to API 3000™ to API 5000™. When transferring the ROX method to the latter system, an increase in sensitivity was possible, but the upper limit of quantification had to be

dropped due to linearity. This can be ascribed to the detector being saturated at higher concentrations or by matrix effects during ionization.

All three assays were tested for matrix effects: None was noted for the assays of ROX and ERY A on both MS systems and for CLA and CLA-MET on API 3000, while slight suppression of CLA (MF 0.85, API 5000), CLA-MET (MF 0.74, API 5000), and EES (MF 0.84, both MS) were observed, whereas, the respective IS compensated for these effects. However, it is remarkable that especially EES and CLA-MET are the most polar molecules of the tested compounds. This can be explained by a reduced efficiency of proton transfer during APCI from charged solvent ions in the gaseous phase.

The LLOQ for ROX on API 3000™ (3.16 ng/mL) versus API III Plus (9.21 ng/mL) was lower by a factor of 3, which is a rather small step compared to ERY A (factor 5) and EES (factor 8). On the other hand, the gain in sensitivity from API 3000™ to API 5000™ (0.395 ng/mL) was a factor of 8, as also for EES and ERY A. The inter-day precision (1.0 to 2.6 %) and accuracy (98.6 to 101.0 %) obtained at the LLOQs for roxithromycin was very high on all three instruments.

The improved sensitivity did not affect quality or precision data on the MS analyzers. Whereas with the API III Plus linearity ranges over 2 orders of magnitude were common, on the API 5000™ linearity ranges of 4 orders of magnitude became possible. This is especially advantageous when samples from clinical trials should be analysed, that are designed to evaluate reliable data from a low-dosed or bronchially-administered drug.

3.4.2 Pharmacokinetics and Pharmacodynamics

Each macrolide was studied in a clinical trial and the respective validated assay was used to determine the plasma concentration-time profile. The highest concentrations (C_{max} 9.5±3.4 µg/mL) were achieved after application of 300 mg roxithromycin, which was a lower dose than studied for ERY (500 mg) and comparable to that of CLA (250 mg). The reason is the good bioavailability of ROX (60 %) compared to ERY (25-50 %), deriving from improved acid stability. The bioavailability of CLA (52-55 %) is limited, despite its good acid stability, because of a fast first-pass metabolism by CYP3A4.

Additionally, CLA inhibits its own metabolism by CYP3A4, counteracting the first-pass-effect and inhibiting its own metabolism. As a consequence, the elimination

half-life of CLA is dose-dependent and time-dependent, increasing from 2.27 hours to 5.98 hours at doses from 100 mg to 1200 mg. [142]. The elimination half-life for the 250-mg dose of CLA determined here ($t_{1/2}$ 4.33 ± 2.57 hours), fits well into this range. The auto-inhibition was also reported for ERY and ROX, [77, 133]. However, for ERY at therapeutical concentrations it is not reflected in a non-linear PK, but plays an important role with regard to drug-drug interactions. The non-linear PK of ROX is less ascribed to its auto-inhibition, but rather to its high and saturable PPB to α_1-acid glycoprotein, causing higher free ROX concentrations above 4 µg/mL and therefore, higher renal elimination rates at times of higher plasma concentrations. Additionally, under-proportional increase of AUC from 81±34 to 170±69 µg·h/mL is observed with increasing ROX doses from 150 to 450 mg. The determined AUC of 160±66.1 µg·h/mL lies within this range.

Table 38 *Summary of the macrolides' chemical and pharmacokinetic properties*

	ERY A (from EES)	**EES (prodrug)**	**ROX**	**CLA**	**CLA-MET (active)**
pKa	8.8	7.1	9.2	8.4 - 8.99	-
logP	3.06	2.1	2.75	3.16	-
Acid stability	low	low	good	improved	-
Bioavailability (%)	25-50	25-35	60	52-55 (first-pass-e.)	-
PPB (%)	54-74	-	73-97	41-72	-
Standard Doses, adults (mg)	-	588 tid – 1175 qid	150 bid, 300 qd	250 bid, 500 bid	-
Studied Dose (mg)	500	587.25	300	250	-
Formulation	suspension		film-coated tablet	film coated tablet	
C_{max} (µg/mL)	0.34±0.13	1.41±0.63	9.50±3.41	0.83±0.33	0.51±0.16
t_{max} (hours)	1.33±0.95	0.60±0.24	2.34±1.74	1.59±1.01	2.39±1.09
AUC (µg·h/mL)	2.12±1.42	2.63±1.15	160±66.1	5.29±2.13	4.82±1.63
$t_{1/2}$ (hours)	10.1±10.6	5.21±3.95	17.0±3.34	4.33±2.57	6.29±2.83
Non-linear PK	negligible at therap. conc.	-	saturable PPB >4µg/mL	autoinhibition CYP3A4	-

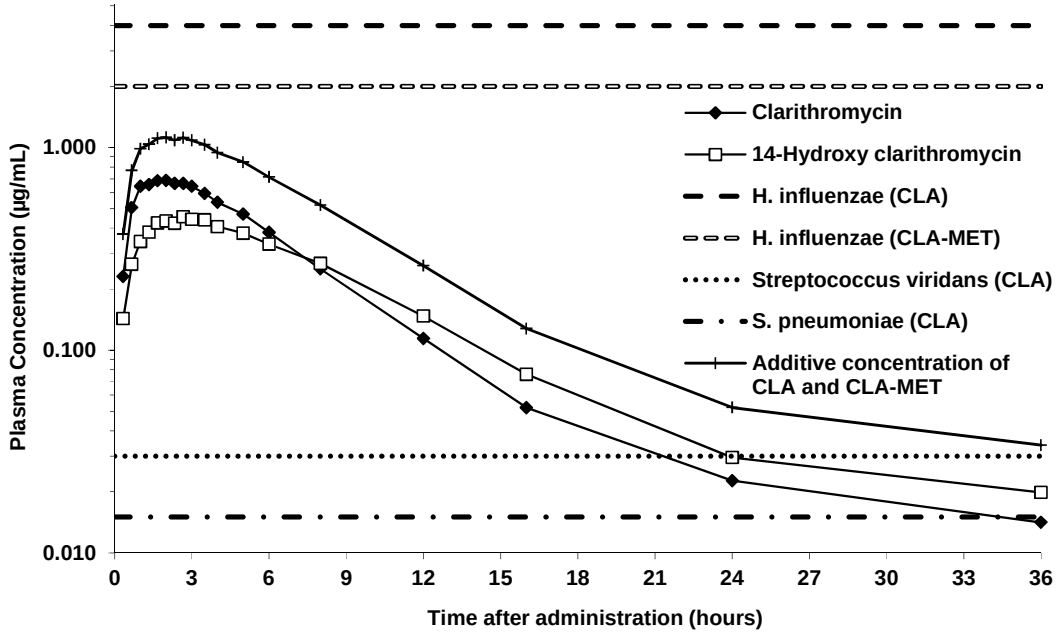
However, comparing PK data from different clinical trials of macrolides is hardly possible, since the outcome of macrolide studies is strongly depending on the composition and schedule of food intake. Additionally, due to the low bioavailability,

many different formulations have been developed to improve acid stability, which makes comparability of PK data virtually impossible.

The greatest variability of oral formulations is available for erythromycin. These are film-coated or enteric-coated tablets, pellets, and powders for suspension, of course containing different excipients. Additionally, erythromycin is modified by different esters, like ethylsuccinate, stearate, estolate, and acistrate. Comparison of different erythromycin ethylsuccinate suspensions creates the impression that even if the shaking intensity during re-dispersing is standardised, marketed preparations differ significantly [88]. However, the different shaking procedures used in clinical trials and therefore, the rather large range of the resulting PK data most likely mimic the diversity in real life patients.

Clarithromycin is the only macrolide, which is converted to the active 14-hydroxy metabolite, which has almost half the activity as clarithromycin. However, it is even more active against *H. influenzae* than clarithromycin. This is illustrated in Figure 24 (MIC_{90} from [181-182]), where the concentration-time curves of CLA and CLA-MET exceed the MICs of most pathogens, i.e. T>MIC is a possible measure for its activities. However, this is not the case for *H. influenzae,* indicating a lack of efficacy against this pathogen. Indeed, clinical data are evidence for the activity of CLA and CLA-MET against *H. influenzae*. Synergistic effects of CLA and CLA-MET could be responsible for this observation, but simple addition of the CLA and CLA-MET concentration-time profile is not sufficient as explanation (see Figure 24). Knowledge on the site of infection, which means extracellular or intracellular localization of the respective micro-organisms and macrolide concentrations at the respective target location, is necessary for in-depth understanding and predicting of macrolide pharmacodynamics.

Figure 24 *Plasma concentration-time profile of a 250-mg oral dose of clarithromycin to healthy subjects in comparison to different MIC_{90} values*



Therefore, the macrolides cannot be classified clearly to one of the three PK/PD parameters AUC_{0-24}/MIC, C_{max}/MIC or T>MIC, i. e. to concentration-dependent or –independent behaviour [108, 141]. Some authors tend to conclude that macrolides can be classified to T>MIC [13, 183], whereas others distinguish between the traditional erythromycin and the newer macrolides, and find, depending on the susceptible organism, that macrolide efficacy is also influenced by AUC_{0-24}/MIC [11, 141].

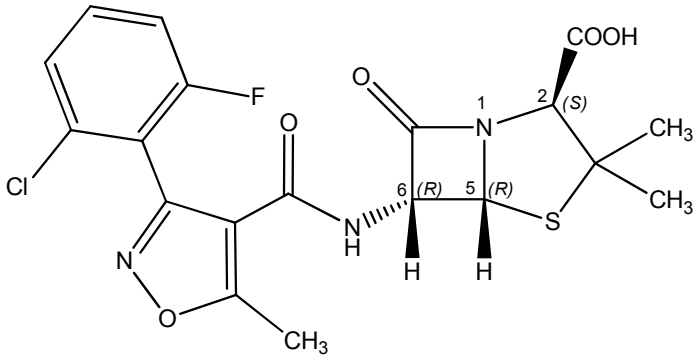
4 BETA-LACTAMS

4.1 Flucloxacillin

4.1.1 Chemical and pharmaceutical properties

Flucloxacillin is a narrow-spectrum isoxazolyl penicillin antibiotic used in the treatment of penicillin-resistant *Staphylococcus aureus* infections [1]. It is approved in European countries for use against infections of the respiratory tract, skin and soft tissues, as well as bone and bone marrow infections. Like other isoxazolyl penicillins, flucloxacillin is not active against MRSA [9]. Flucloxacillin is pharmacologically similar to oxacillin, cloxacillin and dicloxacillin [184], differing only by the halogen substituents on the benzene ring.

Scheme 12 *Chemical structure of flucloxacillin (FLU)*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{19}H_{17}ClFN_3O_5S$	453.9 g/mol	453.1 amu	2.7	2.58

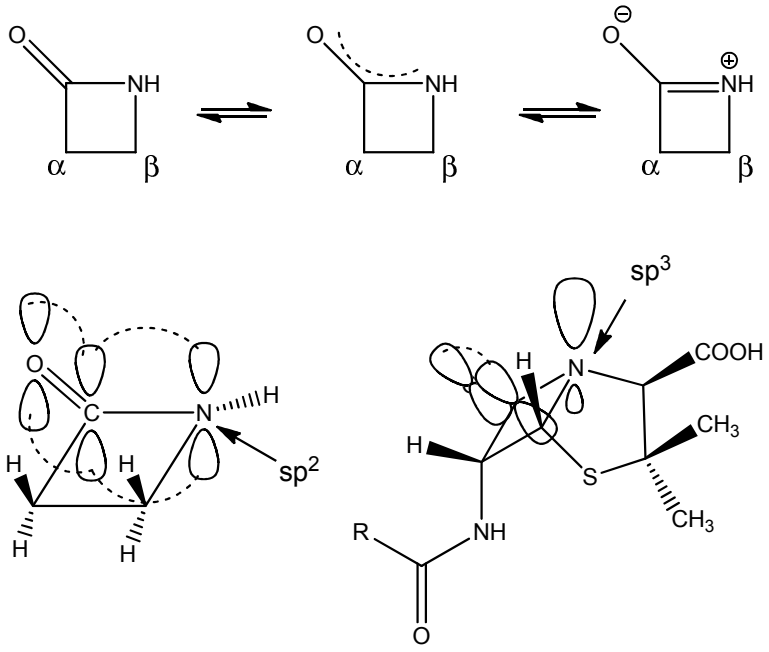
IUPAC Name: (2S,5R,6R)-6-(5-(2-chloro-6-fluorophenyl)-3-methyl-2H-pyrrole-4-carboxamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid

Due to the electron withdrawing side-chain flucloxacillin is stable to acids (see Scheme 14) and can, thus, be orally administered. It is well absorbed, although absorption is reduced in the presence of food [184]. Like most other isoxazolyl penicillins, flucloxacillin is highly bound to serum proteins (95%) and has a short elimination half-life of 45 to 60 minutes [185-186]. Since the major route of flucloxacillin excretion is tubular renal secretion [187], the half-life may be augmented in case of renal failure or due to a combined administration with probenecid, which was used for a long time to prolong the dosage interval and to

increase the serum level of penicillins [186, 188]. This wanted drug-drug interaction may be helpful when flucloxacillin resistance is an issue [1]. Probenecid is a lipophilic (logP 3.2) benzoic acid derivative (pKa 3.4), inhibitiing tubular secretion transporters in the kidney. This mechanism is also responsible for elevated flucloxacillin levels when administered together with piperacillin [187]. Important clinical adverse effects of flucloxacillin are hepatotoxicity and bone-marrow depression [186].

The bactericidal activity of flucloxacillin derives from the irreversible inhibition of cell wall synthesis by covalently binding to the transpeptidases in proliferating bacterial cells, leading to cell lysis [69]. Important for this reaction is the three-dimensional structure of flucloxacillin resulting in a highly reactive β-lactam ring (see Scheme 13). The cyclic amide containing four atoms in the ring, which is called a β-lactam, is stabilized by resonance of the nitrogen with its carbonyl group [69]. This is possible since the nitrogen in the planar molecule is sp^2-hybridised and its orbitals can therefore overlap easily with the ones of the adjacent carbonyl group. However, in β-lactam antibiotics, the β-lactam ring is fused to a thiazolidine ring, which forces the amide nitrogen to sp^3 hybridisation and thus, to a highly reactive "V"-shaped molecule sensitive to nucleophilic attack.

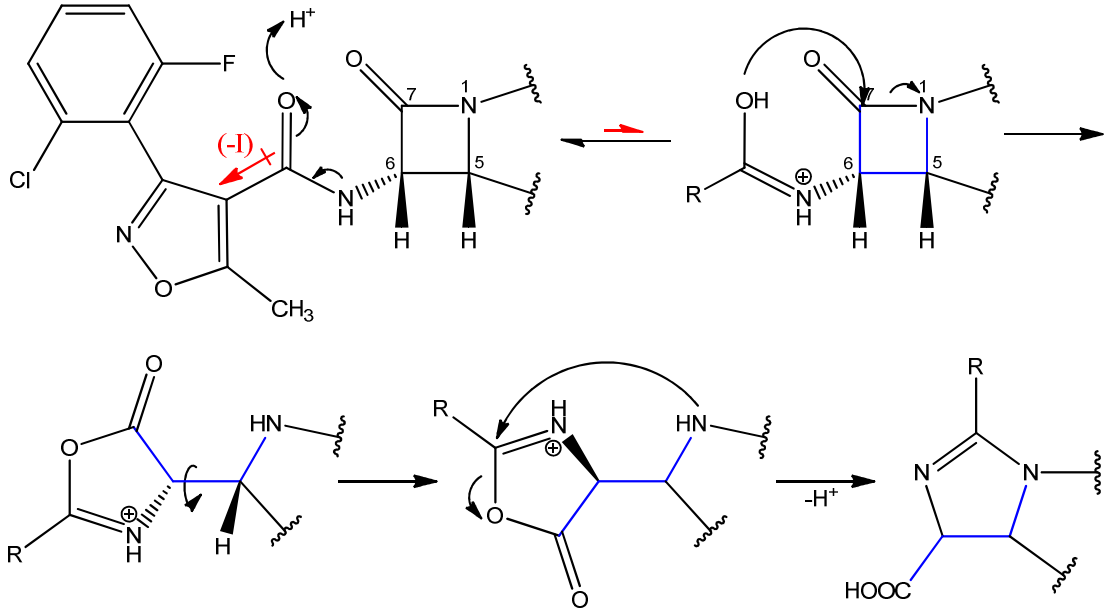
Scheme 13 *Beta-lactam structure compared to beta-lactam antibiotics*



Flucloxacillin, 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolylpenicillin is a weak acid with a pKa of 2.7 [69] (and logP 2.58 [189]). It contains a large phenyl-

substituted isoxazolyl moiety which protects the beta-lactam bond (N1-C7) by steric hindrance from hydrolysis by a variety of beta-lactamases, including penicillinases, cephalosporinases and extended spectrum beta-lactamases.

Scheme 14 *Acid stability of flucloxacillin*



Due to the inductive effect of the electron-withdrawing chloro-fluoro phenyl substituted isoxazolyl moiety, the amid-enol interconversion is shifted towards the amid, increasing acid stability of flucloxacillin compared to other penicillins. However, at basic pH, cleavage of the β-lactam ring occurs. The mechanism is depicted in Scheme 18 in the piperacillin chapter 4.2.1.

4.1.2 Current state of published analytical methods for flucloxacillin

Typical methods to quantify flucloxacillin in human serum or plasma include microbiological [185, 190-191] and chromatographic techniques [58, 192-196]. Many of these approaches have been applied in pharmacokinetic [58, 185, 191-194, 196-198] and therapeutic drug monitoring studies [190].

Previously reported LC/UV methods present expensive and time-consuming sample preparation procedures and long run times of 8 to 16 minutes [192, 194-195, 197], and relatively high lower limits of quantification (LLOQs) in the range from 0.200 to 2.700 µg/mL [58, 192, 194-197], not suitable for the intended low-dose flucloxacillin clinical trial.

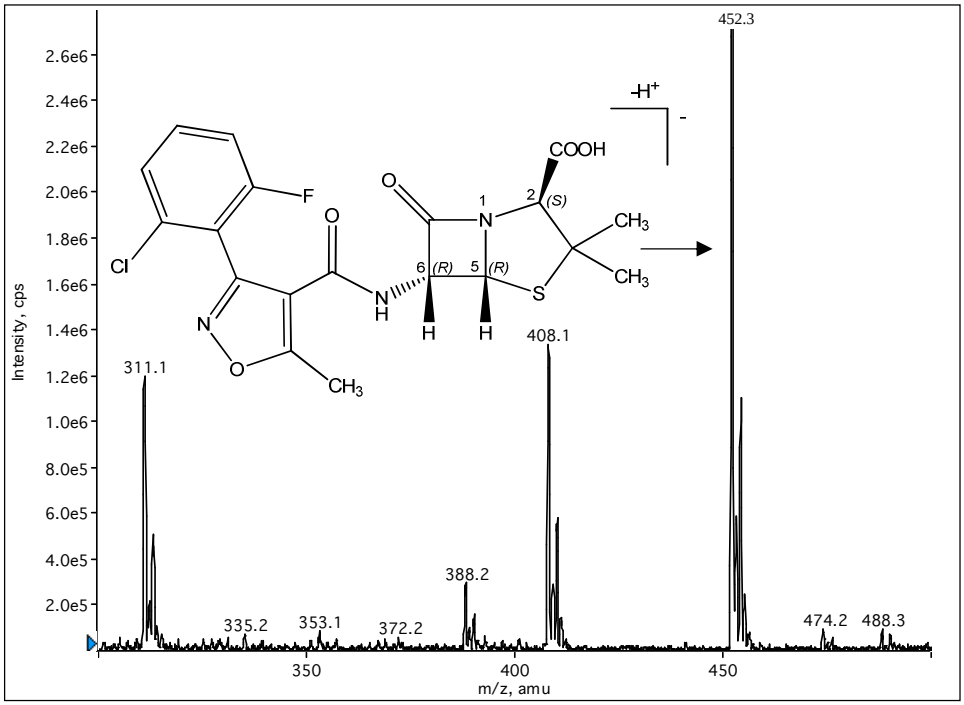
Two LC-MS/MS methods have been previously published [57-58]: one to quantify trace amounts of penicillin contaminants in commercially available drug products and the other to quantify flucloxacillin and ampicillin in human plasma samples of a PK study with intravenous flucloxacillin doses (1.5 to 5 g). Both were not suitable for the low-dose (250 mg) clinical trial.

Therefore, the aim was to develop and validate a highly sensitive, precise and fast LC-MS/MS quantitative method for the determination of low-dose flucloxacillin in human plasma.

4.1.3 LC-MS/MS method development

The first step in method development is acquisition of mass spectra and optimizing the MS/MS parameters. The optimization procedure on a triple quadrupole mass spectrometer (API 3000™) resulted in the use of a TurboIonSpray® interface for electrospray ionisation in the negative ion mode.

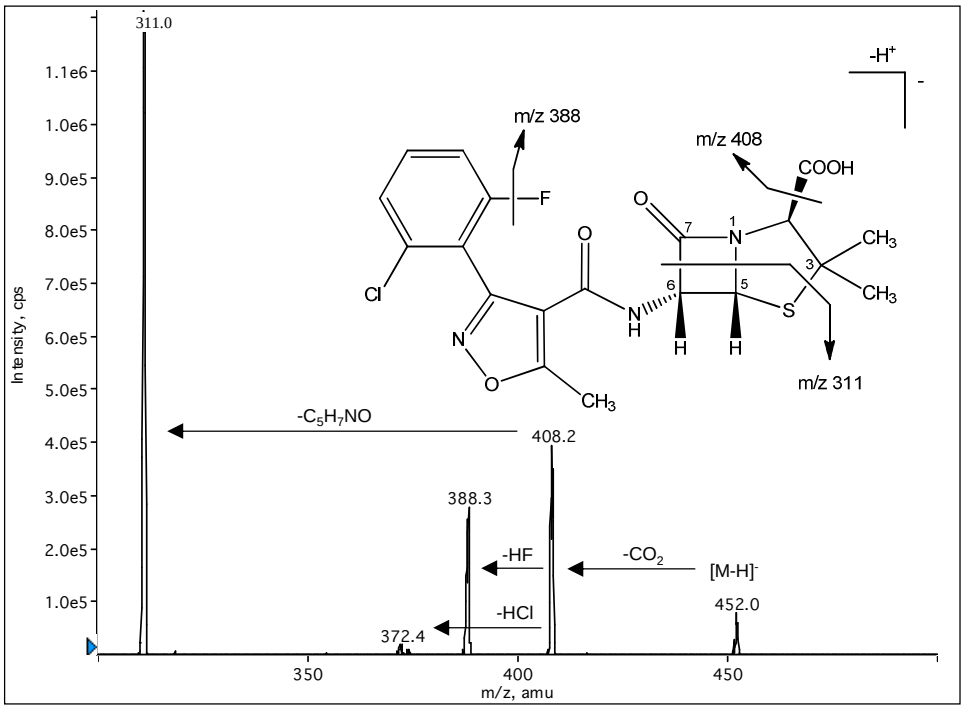
Figure 25 *Negative precursor ion (Q1) spectrum of flucloxacillin*



The most abundant peak in the negative precursor ion spectrum of flucloxacillin at m/z 452 corresponds to the deprotonated molecule $[M-H]^-$ of flucloxacillin (exact mass 453.1 amu) as can be seen in Figure 25. The signals with m/z 408 and

m/z 311 derive from flucloxacillin, which already dissociates to a small extent in the electrospray ionisation source by CID. The fragment m/z 311 is predominantly formed by CAD, as can be seen from the product ion spectrum, recorded in the Q3 of an API 3000™ (Figure 26).

Figure 26 *Product ion mass spectrum and fragmentation scheme of flucloxacillin in the negative ion mode*

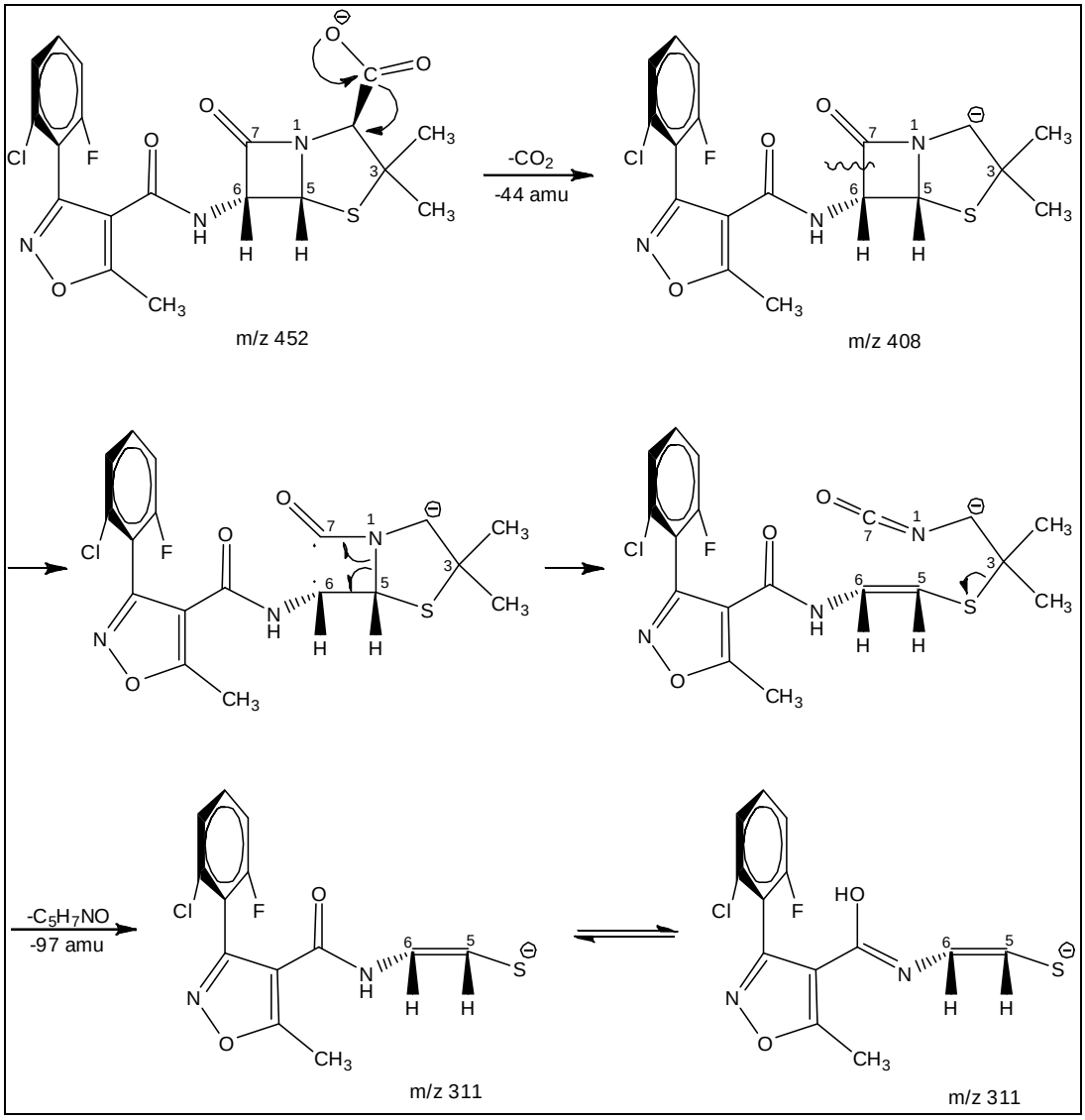


The deprotonated flucloxacillin molecule $[M-H]^-$ with m/z 452 may easily loose two neutral species. The loss of CO_2 (-44 amu) results in the fragment ion with m/z 408, which further looses HF (-20 amu) resulting in the fragment ion with m/z 388.

But the main fragmentation pathway of the deprotonated flucloxacillin molecule $[M-H]^-$ leads to the most abundant ion in the product ion spectrum (see Figure 26) with m/z 311. It corresponds to the loss of carbon dioxide and further loss of a neutral species with 97 amu (C_5H_7NO) originating from the β-lactam ring as previously suggested by Rabbolini et al. [199].

The cleavage of the C(6)-C(7) bond, the C(5)-N(1) bond, and the C(3)-S(4) bond is typical for the fragmentation of penicillins in the negative-ion mode. The proposed mechanism by Rabbolini et al. [199] involving intermediate radical anions in Scheme 15 is adopted to the example of flucloxacillin.

Scheme 15 *Mechanism of flucloxacillin fragmentation in the negative-ion mode (adopted to the example of flucloxacillin from [199])*



Mass spectra of tolbutamide (IS) are recorded under the same conditions (pneumatically assisted electrospray, negative-ion mode) to evaluate the most intense mass transition for the MRM mode. The Q1 spectrum shows the deprotonated molecular ion $[M-H]^-$ of tolbutamide (exact mass 270.1 amu) with m/z 269. The most abundant ion in the negative-mode product ion spectrum of tolbutamide is m/z 170, which is selected for the MRM transition and corresponds to the loss of a species of 99 amu (C_5H_9NO) from the deprotonated molecule.

Figure 27 *Precursor ion scan of tolbutamide*

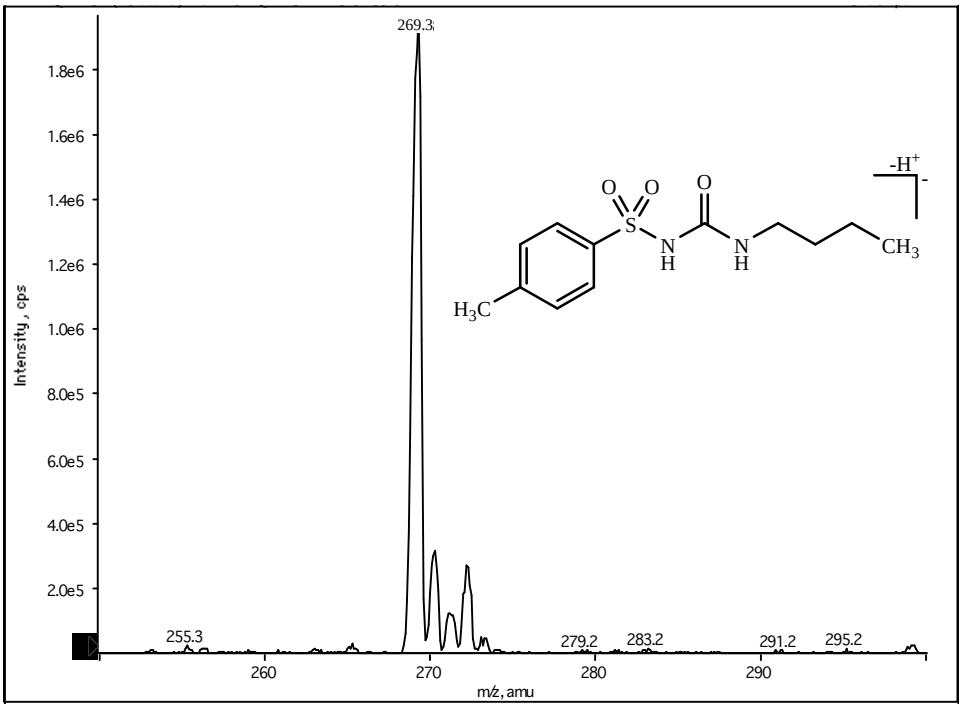
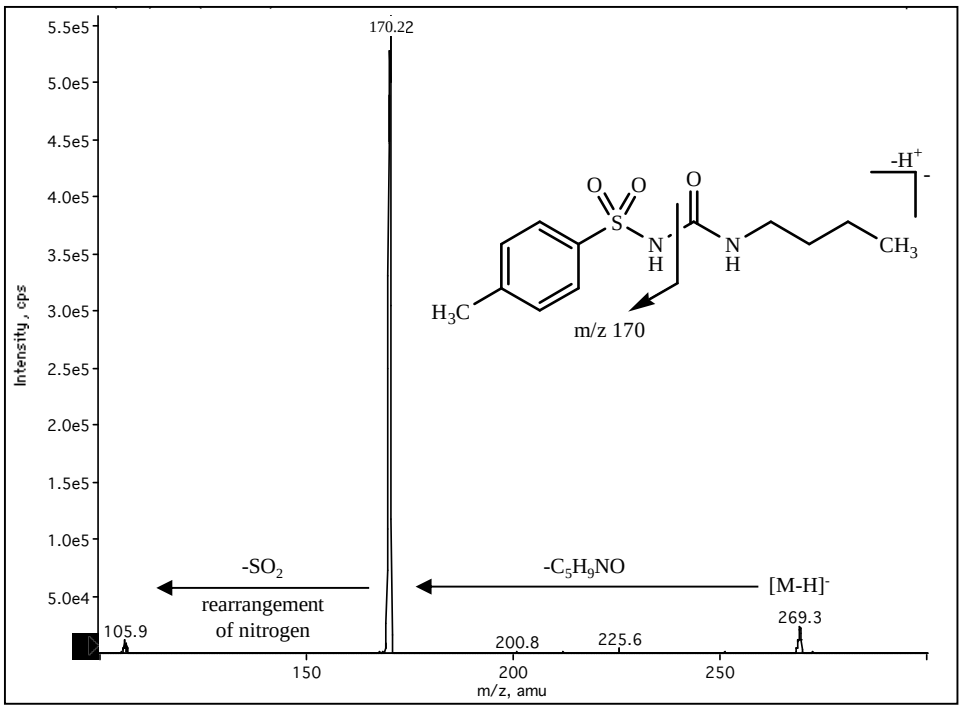


Figure 28 *Product ion scan of tolbutamide*



Thus, the mass spectrometer is equipped with an electrospray interface and is operated in the negative ion mode using Multiple-reaction-monitoring (MRM) of the following transitions: m/z 452 → m/z 311 (FLU) and m/z 269 → m/z 170 (TOL, IS).

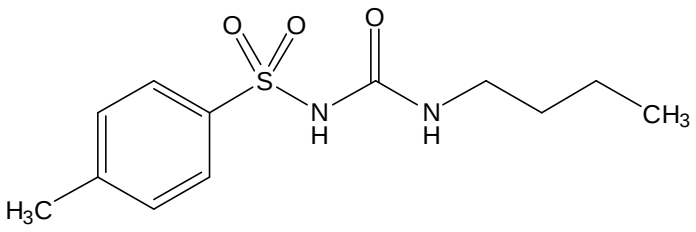
System parameters were optimized during spectra acquisition to obtain the highest analytical sensitivity for flucloxacillin. They are summarized in Table 39.

Table 39 *Optimized MS/MS parameters for flucloxacillin*

Parameter	API 3000™
Probe temperature	350 °C
Ionspray voltage	-5 kV
Orifice voltage	-21 V
Collision energy	16 eV
Nebulizer gas (TISP)	80 psi
Auxiliary gas (TISP)	2 L/min
Nebulizer, auxiliary and curtain gas	High purity nitrogen
Collision gas	High purity nitrogen
Collision gas thickness	6 ($2.7 \cdot 10^{-5}$ torr)

Chromatographic conditions: Isocratic conditions on the reversed phase column (Betasil C18, 5µm, 50x4.6mm i.d.) support the high specificity of the method. The weak acid flucloxacillin with pKa 2.7 was deprotonated in the mobile phase at approximately pH 7 to increase the number of negatively ionized flucloxacillin molecules to be detected in the negative ion mode. The ammonium acetate (5 mM) in the mobile phase assisted charge transition during electrospray ionisation. The desolvation process of the molecular ions from the aqueous mobile phase (67% v) was assisted by heated nitrogen gas (350 °C) in the TurboIonSpray® source.

Scheme 16 *Chemical structure of tolbutamide (TOL)*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{12}H_{18}N_2O_3S$	270.3 g/mol	270.1 amu	5.2	2.34

IUPAC Name: N-(butylcarbamoyl)-4-methylbenzenesulfonamide .

Experience from other methods has shown, that the weak acid tolbutamide (pKa 5.2, logP 2.34 [200]), which is also deprotonated at pH 7, has a similar retention time on a C18 column with water – acetonitrile (67:33, v/v) and was therefore used as internal standard. The retention times of flucloxacillin and tolbutamide were 1.1 and 1.5 min, respectively. Typical chromatograms are depicted in Figure 29 on page 133.

The expected concentration range was estimated as follows: Administration of a 250-mg flucloxacillin capsule to healthy adults, results in mean peak plasma concentrations in the range from 7.4 to 14.5 µg/mL [193-194, 197-198]. In order to be able to detect about 5 % of C_{max}, the LLOQ of flucloxacillin should be lower than 350 ng/mL, which is the case even with the precipitation method down to 10 ng/mL.

In order to assure the stability of the β-lactam flucloxacillin, all sample handling as well as the sample preparation procedure were done in an ice-water bath at approximately 4 °C.

After thawing the human plasma samples, calibration standards and SQC samples in a cold water bath, the samples are mixed thoroughly and centrifuged for 5 minutes at approximately 4 °C to separate eventually present solid components.

As discussed in chapter 1.4.3 “Sample preparation techniques”, the most economic sample preparation technique (material costs and time) is protein precipitation. As flucloxacillin plasma concentrations are expected to be sufficient high, this technique was tested first: An aliquot of 100 µL of the spiked human plasma samples is deproteinised by adding 200 µL of acetonitrile containing the internal standard tolbutamide. Different IS working solutions are tested to find a concentration that results in a peak area ratio (highest analyte area/IS) of approximately 5 revealing the following result: The IS working solution is prepared by diluting a stock solution of tolbutamide by factor 200 with acetonitrile to give a concentration of 10 µg/mL.

The precipitated sample, containing the IS, is mixed thoroughly to facilitate deproteinisation. The precipitate is separated by centrifuging the samples for 10 minutes at 3600 rpm (3280 g) at approximately 4 °C. Addition of 200 µL of ammonium acetate solution (5 mM, pH 7) to 50 µL of the supernatant promoted neutral pH in the sample and thus, prevented flucloxacillin from degradation. In addition, this increased the water content of the sample, decreasing plasma matrix content and improving the peak shape on the C18 reversed phase column. After mixing, 15 µL of each sample is injected onto the LC-MS/MS system. In Table 40 the optimized sample preparation procedure and LC-MS/MS conditions are summarized.

Table 40 *Summary of the LC-MS/MS method for flucloxacillin in human plasma*

Characteristic	***Details***
Sample preparation	100 µL plasma + 200 µL CH_3CN /IS 50 µL supernatant + 200 µL NH_4CH_3COO (5 mM, pH 7.0)
Internal standard	Tolbutamide (TOL)
Column	Betasil C18, 5 µm (50 x 4.6 mm i.d.)
Mobile phase	33 % CH_3CN 67 % NH_4CH_3COO (5 mM, pH 7.0)
Flow rate	1.0 mL/min
Run time	2.2 min
Ionisation	Electrospray Ionisation (TurboIonSpray®)
Polarity	Negative ion mode
Mass transitions	m/z 452 → m/z 311 (FLU) m/z 269 → m/z 170 (TOL, IS)

CH_3CN /IS: acetonitrile containing the internal standard tolbutamide (10 µg/mL)

4.1.4 Validation

Being intended for a human pharmacokinetic study, the method was validated according to the guideline on bioanalytical method validation by the FDA [33].

The selectivity of the flucloxacillin method was determined by preparing 12 (6 males and 6 females) different batches of drug-free human plasma with and without IS. Matrix components did not interfere with flucloxacillin and the IS, i.e. no co-eluting peak was observed.

Additionally, no influence on the analysis of flucloxacillin was observed for six different batches of human plasma: all (N = 18) test samples with the high flucloxacillin concentration (15 µg/mL) had a mean accuracy of 97.6 % ± 3.2 % and a precision of 3.3 %. All (N = 18) test samples with the low flucloxacillin concentration (0.0375 µg/mL) had a mean accuracy of 96.8 % ± 4.9 % and a precision of 5.0 %.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range from 0.0100 to 15.0 µg/mL in human plasma. The mean linear regression equation of the calibration curves on five validation days was established: $y = 0.0001\ (\pm 0.0001) + 0.0950\ (\pm 0.0179) \cdot x$. All correlation coefficients

of the weighted calibration curves were ≥ 0.997. Using 100 µL of plasma, the lower limit of quantification for flucloxacillin was 0.0100 µg/mL. The signal-to-noise ratio of flucloxacillin at the LLOQ (10 ng/mL) was at least five on all five validation days. The inter-day precision and accuracy of all back-calculated calibration standards ranged from 1.1 to 6.5 % and from 97.8 to 103.1 %, respectively.

Table 41 summarizes the intra- and inter-day precision and accuracy for flucloxacillin, evaluated by assaying the SQC samples. The inter-day precision of the SQC samples for flucloxacillin in human plasma ranged from 4.0 to 8.6 % with an accuracy from 97.6 to 101.4 %. The obtained results were well within the acceptance criteria of the above cited guidelines.

Table 41 *Intra- and inter-day precision and accuracy of the analysis of flucloxacillin in human plasma (five days, five replicates per day)*

Theoretical concentrations [µg/mL]	Intra-day Precision [%]	Intra-day Accuracy [%]	Inter-day Precision [%]	Inter-day Accuracy [%]
15.0	5.0	103.9	4.5	101.4
7.50	5.3	97.6	4.0	97.6
0.375	6.0	95.8	4.2	99.2
0.0375	8.3	94.1	7.1	99.8
0.0100	9.1	93.2	8.6	101.0

The mean absolute extraction recovery of flucloxacillin was determined to be 92.8 ± 2.6 % over the whole concentration range from 0.0100 to 15.0 µg/mL. The mean absolute extraction recovery of the IS (tolbutamide) at the working concentration was found to be 88.4 ± 4.0 %. All recoveries had relative standard deviations better than 4.5 % throughout the entire standard concentration ranges, showing good consistency and reproducibility of the simple one-step protein precipitation procedure for flucloxacillin in human plasma.

Stability data of flucloxacillin at concentrations of 15 µg/mL and 0.0375 µg/mL in human plasma is compiled in Table 42. The results of the 95 % confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes. Therefore, flucloxacillin can be regarded as stable in human ammonium heparin plasma for at least 4 hours at 4 °C, for at least 2 months at -20 °C, and for at least three freeze-thaw cycles from -70 °C to room temperature. In processed human plasma, flucloxacillin is stable for at least 72 hours at 4 °C (autosampler temperature) and at -70 °C. Flucloxacillin and the IS were stable in

stock solutions at concentrations of 1000 µg/mL flucloxacillin or 2000 µg/mL tolbutamide for at least 6 hours at 4 °C.

Table 42 *Statistical evaluation of the stability experiments for flucloxacillin in human ammonium heparin plasma*

Stability experiment	Conc. [µg/mL]	Time	Point estimator [%]	Lower Limit [%]	Upper Limit [%]
Short-term stability (approximately 4 °C)	15.0	2 hours	103.56	100.97	106.22
		4 hours	103.62	102.92	104.32
	0.0375	2 hours	102.80	98.60	107.19
		4 hours	103.41	96.82	110.45
Post-preparative stability (autosampler temperature, approximately 4 °C)	15.0	24 hours	106.10	104.67	107.54
		48 hours	95.21	93.75	96.70
		72 hours	107.83	106.46	109.21
	0.0375	24 hours	102.96	98.38	107.75
		48 hours	92.22	90.12	94.36
		72 hours	106.47	103.80	109.22
Post-preparative stability (approximately -70 °C)	15.0	24 hours	105.64	104.25	107.05
		48 hours	95.59	94.22	96.98
		72 hours	107.33	106.97	107.69
	0.0375	24 hours	100.29	96.23	104.51
		48 hours	94.82	91.03	98.76
		72 hours	104.69	101.78	107.69
Freeze-thaw stability (approximately -70 °C)	15.0	Once	103.47	101.83	105.13
		Twice	94.83	93.71	95.96
		Thrice	107.67	105.95	109.42
	0.0375	Once	100.98	94.92	107.42
		Twice	96.33	92.45	100.36
		Thrice	105.24	102.13	108.45
Long-term stability (approximately -20 °C)	15.0	2 days	94.34	92.51	96.21
		1 month	97.81	95.79	99.87
		2 months	91.88	90.98	92.79
	0.0375	2 days	94.53	93.06	96.03
		1 month	105.91	103.82	108.05
		2 months	94.86	91.84	97.97
Long-term stability (approximately -70 °C)	15.0	2 days	94.05	91.56	96.60
		1 month	108.20	106.96	109.45
		2 months	100.97	99.41	105.55
	0.0375	2 days	92.76	90.01	95.60
		1 month	101.88	96.14	107.96
		2 months	93.84	91.31	96.45

No influence of haemolysed or hyperlipidaemic human plasma on the accuracy and precision of the method was observed in five replicates of two concentration levels in each type of plasma. The mean concentration values were within ±15 % of the

nominal value. Mean precision and accuracy for flucloxacillin in haemolysed / hyperlipidaemic plasma at 15 µg/mL were 1.5 % / 1.9 % and 104.8 % / 90.2 %, whereas mean precision and accuracy at 0.0375 µg/mL were 3.1 % / 5.5 % and 101.4 % / 94.5 %, respectively.

Flucloxacillin plasma samples can be pre-diluted by factor 5. The mean concentration values of five replicates were within ±15 % of the nominal value with a mean accuracy of 102.6 %. The precision for the 1:5 diluted human plasma samples at 60.0 µg/mL was 2.9 %.

4.1.5 Comparison of the LC-MS/MS method with procedures reported in the literature

In Table 43 the LLOQ, run time and quality control data for several previously reported analytical methods for the determination of flucloxacillin in human plasma are displayed in comparison to the present LC-MS/MS method.

All previously reported LC/UV methods needed longer run times (8-30 min compared to the present 2.2 min) and reached less sensitivity with LLOQ values at least 20 times higher than the LLOQ value achieved in the present study [192, 194-197, 201].

Additionally, the paper published by Thijssen [192] did not report linearity, recovery, precision and accuracy data and the authors of three methods [194, 196-197] avoided the precision and accuracy data for LLOQ. An accurate and precise LC/UV method was reported by Charles et al. [195] and Zhou et al. [197]. However, compared with the actual LC-MS/MS method, they were more time-consuming in terms of sample preparation procedure [195] and run time [197] and therefore less suitable for high-throughput analysis of large clinical and pharmacokinetic studies.

The LC-MS/MS method of Huang et al. is characterized by similar precision data as the present method. However, their reported recovery for FLU and the IS is 115% and 135%, respectively. Together with the high CV values, this indicates that very different individual results were obtained.

Furthermore, most of the previously reported methods were to the best knowledge not reported to be applied to a clinical setting [195-196] or were applied to PK studies with higher dosages [192, 194, 201]. Only one method [197] was also applied to a 250-mg oral dose study, but the run time of 10 minutes is relatively long and due to less sensitivity, the AUC had to be extrapolated by 4% (compared to 0.4% herein).

Table 43 *Comparison of developed method to existent methods to quantify flucloxacillin in human plasma or serum; grouped by analytical technique and descending years of publication*

Method	Linearity range [µg/mL]	Approx. run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of SQCs [%]	Recovery mean ± SD [%]	Corr. coeff.	Sample Vol. [mL]	Sample preparation	Application
LC-MS/MS (current method)	0.010 - 15.0	2.2	8.6 / 1.0	4.0 to 8.6 / 97.6 to 101.4	92.8 ± 2.6 (FLU) 88.4 ± 4.0 (IS)	≥ 0.997	0.100	PPT	250-mg p.o. PK
LC-MS/MS [58] Huang et al. 2012	0.20 - 500	3.5	12.1 / 100.0 (one day)	3.7 to 11.2 / 95.1 to 102.0	115.1±9.6 to 135.6±18.2 %	0.998	0.050	PPT	i.v. PK 1.5g – 5g
LC/UV [201] Mc Whinney et al. 2010	5 - 500	30	2.5 / n.r.	1.9 to 4.4 / 92.0 to 97.0	n.r.	0.995	0.200	PPT+LLE	TDM
LC/UV [196] Pullen et al. 2007	5.0 – 100 (calc. LLOQ: 2.7)	n.r.	6.8 / 112.2 (at 5 µg/mL)	4.7 / 98.4 (at 25µg/mL)	101.4, 100.6 (50; 100 µg/mL)	> 0.996	0.020	LLE	-
LC/UV [197] Zhou et al. 2007	0.20 - 40.0	10.0	n.r. / n.r.	5.1 to 10.5 / 100.3 to 105.0 (0.4-30 µg/mL)	87.2	0.9999 (mean)	0.100	PPT	250-mg p.o. PK
LC/UV [195] Charles et al. 1994	0.20 - 40.0 (LLOQ: 0.3)	8.0	6.8 / 93.2	3.0 to 6.8 / 93.2 to 107.7 (0.3-30 µg/mL)	60 - 70	> 0.999	0.100	LLE	-
LC/UV [194] Hung et al. 1988	0.20 - 50.0	15.0	n.r. / n.r.	n.r. / n.r.	> 90	≥ 0.980	0.250	PPT+ SPE	500-mg p.o. PK
LC/UV [192] Thijssen et al. 1980	0.40 - n.r. 1.0 – n.r.	16.0	n.r. / n.r.	n.r. / 106.0 n.r. / 116.0	n.r.	n.r.	n.r.	PPT + LLE	1.5-g p.o. PK

Prec: precision, Acc: accuracy, n.r.: not reported, PPT: protein precipitation, LLE: liquid-liquid-extraction, SPE: solid phase extraction

4.1.6 Application to a pharmacokinetic study

4.1.6.1 Study design and sample handling

The clinical trial studied a 250-mg capsule of flucloxacillin as a single oral dose to 24 healthy male and female subjects in the fasting state. Blood was collected into ammonium heparinate monovettes at the following time points: immediately before administration and at 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, 12.00 and 16.00 hours after administration of the study drug.

The monovettes were cooled immediately in an ice-water bath until centrifugation for 10 minutes at 4 °C and 1500 g. The obtained plasma is subsequently transferred into polypropylene tubes and immediately frozen at approximately -70 °C until analysis.

The concentrations of flucloxacillin are determined using the developed and validated LC-MS/MS method as summarized in Table 40 (page 127).

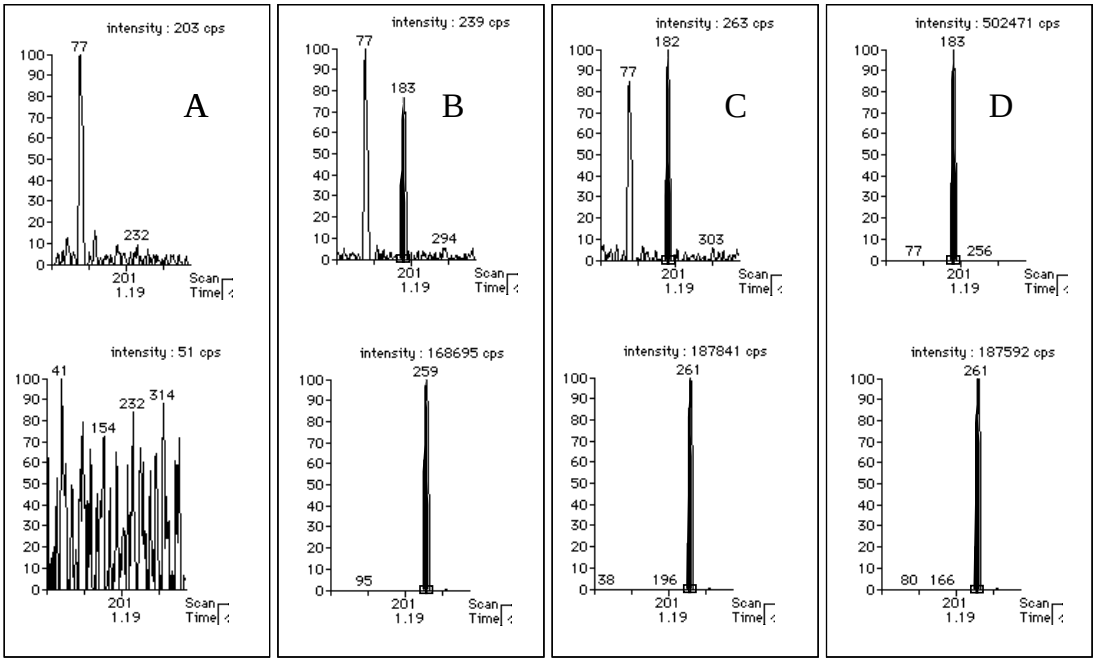
4.1.6.2 Determination of flucloxacillin concentrations in human plasma

Flucloxacillin concentrations of more than 1300 human plasma samples were measured in a total of fourteen sequences together with ten injection standards, twelve calibration standards and twelve SQCs. Each sequence, consisting of calibration standards (low to high concentrations), subject samples and the quality control standards (in randomized order among subject samples) were measured within 5.5 hours. The determined concentration of the SQC samples was compared to theoretical concentrations to account for accuracy.

The coefficient of correlation of resulting linear regressions was at least 0.999. The inter-day precision and accuracy of the spiked quality control standards of flucloxacillin in human plasma analyzed with the batches of study samples ranged from 3.9 to 7.7 % and from 97.7 to 101.3 %, respectively and were therefore of very good quality.

All sample concentrations were detectable and within the calibration range. In particular, no BQL samples with concentrations below the quantification limit occurred, indicating the very good sensitivity of the developed method. Typical MRM-chromatograms are depicted in Figure 29.

Figure 29 *Representative MRM-chromatograms for flucloxacillin (top) and tolbutamide (IS, bottom) in human plasma: (A) Blank plasma sample, (B) LLOQ (0.0100 µg/mL), (C) Subject sample (16 hours post-dose, 0.0129 µg/mL) and (D) Subject sample (0.75 hours post-dose, 24.37 µg/mL).*

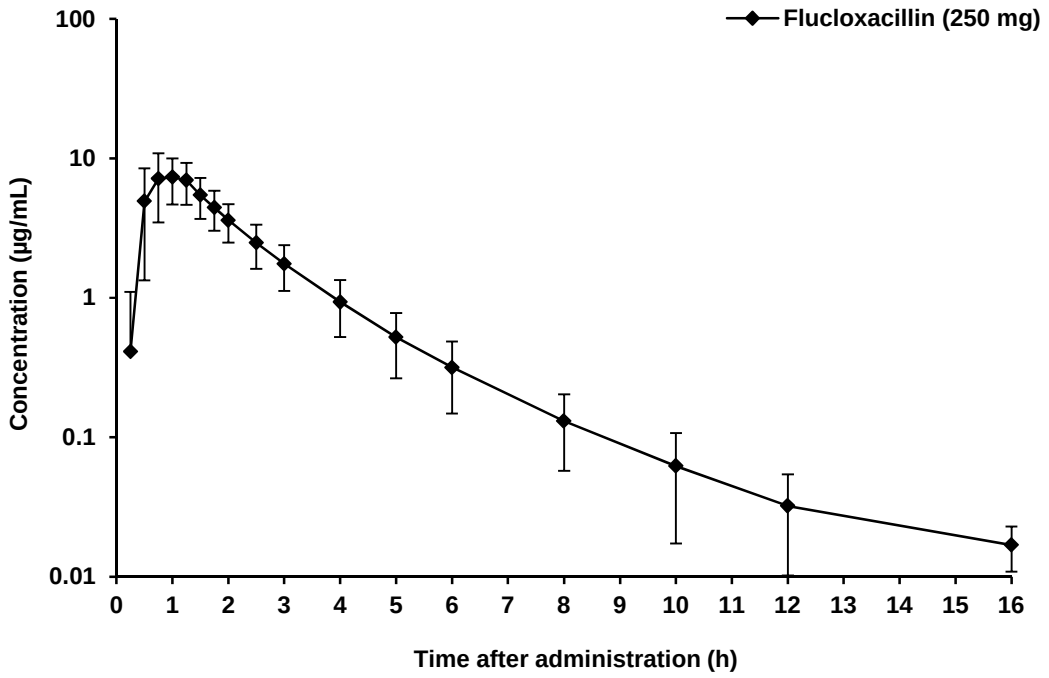


4.1.6.3 Pharmacokinetic results

Figure 30 shows the mean plasma concentration profile of flucloxacillin obtained after oral administration of a 250-mg capsule of flucloxacillin to 24 healthy volunteers. The mean peak concentration (C_{max}) of 8.33 ± 2.88 µg/mL (mean±SD) was attained after 0.95 ± 0.23 hours (mean±SD) followed by an elimination half-life of 1.89 ± 0.42 hours (mean±SD). The mean total area under the curve ($AUC_{0\rightarrow\infty}$) was 15.5 ± 4.92 h·µg/mL (mean±SD), of which only 0.4 % had to be extrapolated.

Table 44 shows the pharmacokinetic parameters of flucloxacillin after oral administration of 250 mg in comparison to pharmacokinetic parameters reported in previously studies. In order to put the pharmacokinetics of flucloxacillin side by side, given in different doses, the calculations of C_{max}, $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow\infty}$ were dose-corrected. Dose linearity of flucloxacillin in doses up to 1000 mg was demonstrated by Landersdorfer et al. [202].

Figure 30 *Mean plasma concentration profile of flucloxacillin following a single oral 250-mg flucloxacillin capsule to 24 healthy volunteers.*



The pharmacokinetic data are in good agreement with the three data sets reported by Paton et al. [193]. The elimination half-lives and times to peak concentration reported by other authors are also comparable to the data from this study. However, Zhou et al. [197] and Hung et al. [194] reported higher peak concentrations and AUC values in male subjects only.

This relatively high inter-study variability observed for AUC and C_{max}, can be explained by non-specific effects like dosage accuracy, adherence to dietary restrictions and drug assay accuracy. Other effects, like subject gender and sample size may also contribute to a considerable intra- and inter-individual variation [185]. In the present study, non-specific variability for pharmacokinetic parameters was minimised by using a randomized crossover design, strict adherence to dietary restrictions and the development of a highly accurate drug assay.

Table 44 *Reported values for pharmacokinetic parameters (mean ± standard deviation) of flucloxacillin in healthy subjects after overnight fasting and administration of a single oral dose as capsule*

Reference	No. of subjects/ Gender	Dose / Formulation	t_{last} [h]	$AUC_{0\rightarrow t}$* [µg·h/mL]	$AUC_{0\rightarrow\infty}$* [µg·h/mL]	$AUC_{t\rightarrow\infty}$ [%]	C_{max}* [µg/mL]	t_{max} [h]	$t_{1/2}$ [h]	Analytical Method
Current study	24/m,f	250 mg p.o.	16	15.4 ± 4.91	15.5 ± 4.92	0.4 ± 0.2	8.33 ± 2.88	0.95 ± 0.23	1.89 ± 0.42	LC-MS/MS
[197] Zhou 2007	20/m (Chinese)	250 mg / A	8	28.7 ± 1.3*²	29.8 ± 1.3*²	4 ± 2*²	14.5 ± 1.5*²	0.7 ± 0.1*²	1.9 ± 0.6*²	LC/UV
		250 mg / B		27.9 ± 1.4*²	28.8 ± 1.3*²	3 ± 3*²	13.9 ± 1.6*²	0.8 ± 0.4*²	1.7 ± 0.4*²	LC/UV
[185] Røder 1995	12/m,f	750 mg	8	n.r.	22.6*³ (17.4-27.2)	n.r.	11.5*³ (7.6-9.1)	0.75*³ (0.5-1.0)	1.27*³ (1.0-1.45)	Microbiol. Assay
[194] Hung 1988	10/m	500 mg	10	approx. 24	n.r.	n.r.	approx. 11	approx. 1	approx. 1.4	LC/UV
[198] Paton 1986	9/m,f	250 mg	8	18.8 ± 2.0	n.r.	n.r.	7.7 ± 1.3	0.9 ± 0.1	1.5 ± 0.2	LC/UV
[193] Paton 1982	8/m,f	250 mg / A	8	15.85 ± 1.98	n.r.	n.r.	7.37 ± 1.38	0.9 ± 0.1	1.39 ± 0.15	LC/UV
		250 mg / B		16.96 ± 2.08	n.r.	n.r.	7.98 ± 1.60	1.1 ± 0.2	1.31 ± 0.06	LC/UV

m: male, f: female, n.r.: not reported, *: if necessary, dose-corrected to 250 mg, *²: geometric mean ± SD, *³: median (first-third quartile).

4.1.7 Summary: flucloxacillin

This is the first LC-MS/MS assay validated for determination of very low flucloxacillin concentrations after oral administration of a 250-mg dose. The method allows simple, fast, reliable, sensitive, precise and accurate determination of flucloxacillin in human plasma. The method employed acetonitrile protein precipitation, which permits quantification of flucloxacillin in human plasma for concentrations ranging from 15.0 down to 0.0100 µg/mL using 100 µL of plasma only.

The principal advantage of the LC-MS/MS method described here is the simultaneous achievement of high absolute recovery (92.8 %), high sensitivity (LLOQ = 0.0100 µg/mL), high inter-day precision (8.6 %) and high accuracy (101.0 %) for LLOQ as well as excellent linearity ($r^2 \geq 0.997$) with a short run time of only 2.2 minutes. These characteristics make the method suitable for the precise and accurate measurement of low concentrations of flucloxacillin in the context of pharmacokinetic studies at low single dose concentrations. The method was successfully applied to the analysis of more than 1300 samples during a pharmacokinetic study.

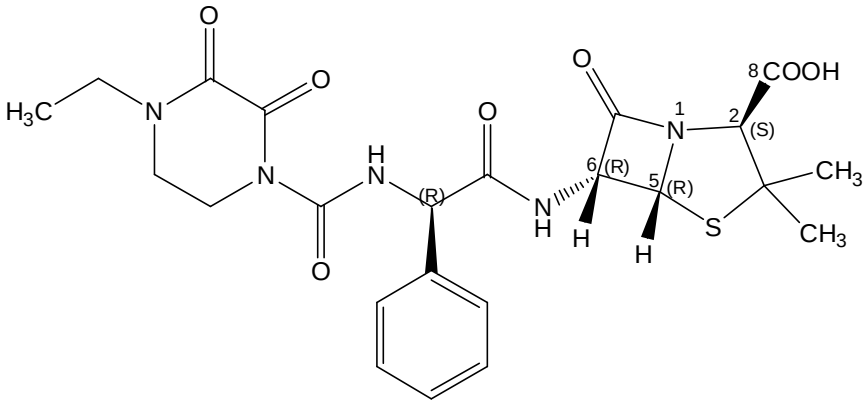
Short sampling intervals and high assay sensitivity led to a very precise estimation of the total AUC, whereas the mean residual area was only 0.4 %.

4.2 Piperacillin

4.2.1 Chemical and pharmaceutical properties

Piperacillin is a broad-spectrum acylureido-penicillin used in the treatment of severe infections like respiratory tract infections, ear, nose and throat infections, severe systemic infections, including sepsis, intra-abdominal infections, skin and skin-structure infections, as well as bone and joint infections, including osteomyelitis. Piperacillin as a mono-therapy is recommended only for use in patients with known resistance profile of antimicrobial strains as it is susceptible to β-lactamases. Applied together with tazobactam (acting as a β-lactamase inhibitor) it has the broadest antibacterial spectrum of the penicillins, playing an important role in the treatment of infections caused by gram-negative bacteria [1].

Scheme 17 *Chemical structure of piperacillin (PIP)*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{23}H_{27}N_5O_7S$	517.6 g/mol	517.2 amu	2.9	0.22

IUPAC Name: (2S,5R,6R)-6-((R)-2-(4-ethyl-2,3-dioxopiperazine-1-carboxamido)-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid

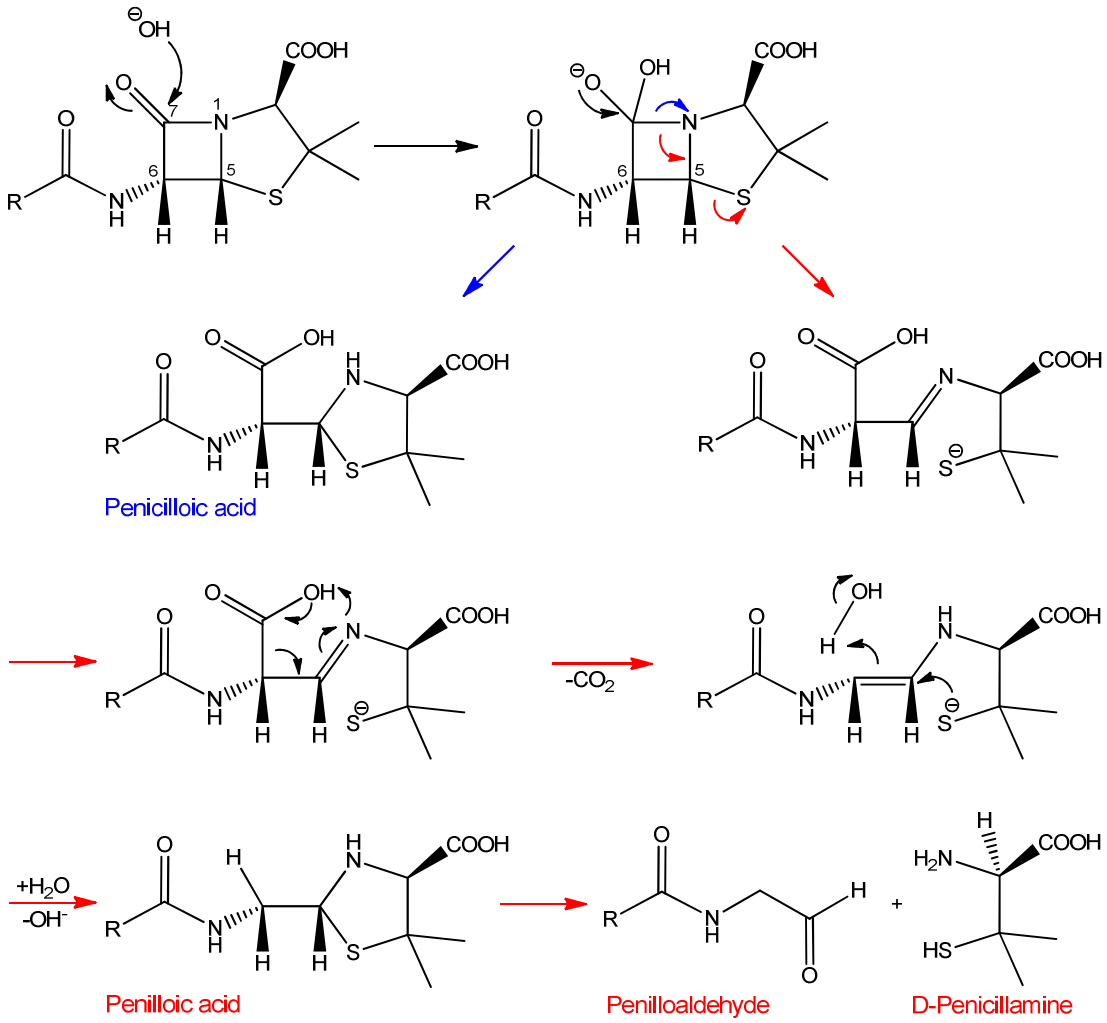
Like flucloxacillin, piperacillin inhibits bacterial cell wall synthesis by its highly reactive β-lactam ring, see Scheme 13 in chapter 4.1.1. Due to its dioxo-piperazin moiety, piperacillin is especially effective against gram-negative bacteria, but at the same time it is instable towards acids. Therefore, piperacillin is poorly absorbed from the GI tract and has to be administered parenterally. Following a 30-min infusion of 4g piperacillin, maximum concentrations between 220 and 280 µg/mL can be reached, while approximately 30 % higher peak concentrations are reached after bolus injection [203-208]. Different dosing regimens and intermittent versus continuous infusion were studied by several authors [209-214]. Piperacillin levels remain above the MIC_{90} for 2 hours post-administration [206, 215]. The plasma protein binding is 20-30 % [216] and due to its hydrophilic character piperacillin distributes well into water-containing tissues, especially into skin and the gastrointestinal tract [206, 217].

Piperacillin is eliminated rapidly by glomerular filtration and tubular secretion. This has several consequences: at first, a short half-live of about 1 hour and second, high concentrations in urine. Third, drug-interactions between piperacillin and other drugs mainly depend on extend and mechanism of their renal excretion, e.g. flucloxacillin, tobramycin [187, 216, 218-219]. Non-linear pharmacokinetics, i.e. saturable clearance at clinically used doses, could be caused by capacity limited tubular

secretion [220-223]. However, also some PK studies found linear or probably "pseudolinear" behaviour of elimination [211, 224-225]

The biliary route of piperacillin excretion in humans is negligible [226-227] and very little is known about hepatic metabolism, although some metabolites have been identified in urine [228] and bile [229].

Scheme 18 *Mechanism of beta-lactam degradation at basic pH*



Like other penicillins, piperacillin is a weak acid with a pKa of 2.9 [230] (and logP of 0.22 [231]), which derives from the carboxylic acid group adjacent to the electronegative sulphur atom and the β-lactam peptide bond [232], see Scheme 17. In aqueous solutions piperacillin is most stable at approximately pH 5 [230, 233]. Below pH 3 the β-lactam ring is hydrolysed [230]. The mechanism of the β-lactam degradation in acidic environment is depicted in Scheme 14 (see chapter 4.1.1). At pH 8-9 piperazinyl ring cleavage occurs [230] as well as cleavage of the β-lactam ring (see Scheme 18).

4.2.2 Previously published analytical methods

Most previously reported methods to quantify piperacillin in human plasma or serum used liquid chromatography with UV detection (LC/UV) [201, 211, 226, 234-241] or with MS/MS detection (LC-MS/MS) [62], of which some were applied to therapeutic drug monitoring [62, 201, 235, 237, 241] or pharmacokinetic studies [211, 226, 238-239]. Most of those methods need long run times of 10 to 35 minutes per sample, reducing the daily sample number significantly. Only three methods [62, 234, 239] are characterized by run times between 5 and 8 minutes, but were not suitable for the intended application for the following reasons: low sensitivity (10µg/mL) combined with expensive and time-consuming sample preparation techniques like liquid-liquid extraction, solid phase extraction or derivatisation.

Very few methods for piperacillin have been reported using LC-MS/MS as technique of choice. Piperacillin was determined in waste-water [242], ground and surface water [243], in cell culture medium [244], and in bile [226]. Only four LC-MS/MS methods were reported for determination of piperacillin in serum [62] or plasma [59-61], but none was validated for analysis of urine samples. All previously reported LC-MS/MS methods were designed for simultaneous determination of several β-lactam antibiotics, needing relatively long run times (5.5 to 8.0 minutes) or reported issues with poor precision [59]. All of them were characterized by either costly and time-intense sample preparation techniques (SPE, LLE), or were less sensitive (LLOQs of 0.3 to 1.5 µg/mL).

Therefore, the aim was to develop and fully validate a fast and highly sensitive LC-MS/MS method that allows easy and cost-effective sample preparation and high-throughput analysis of piperacillin in human plasma and urine samples and to investigate the pharmacokinetics of a 4.5 g single intravenous dose of piperacillin/tazobactam (4:1).

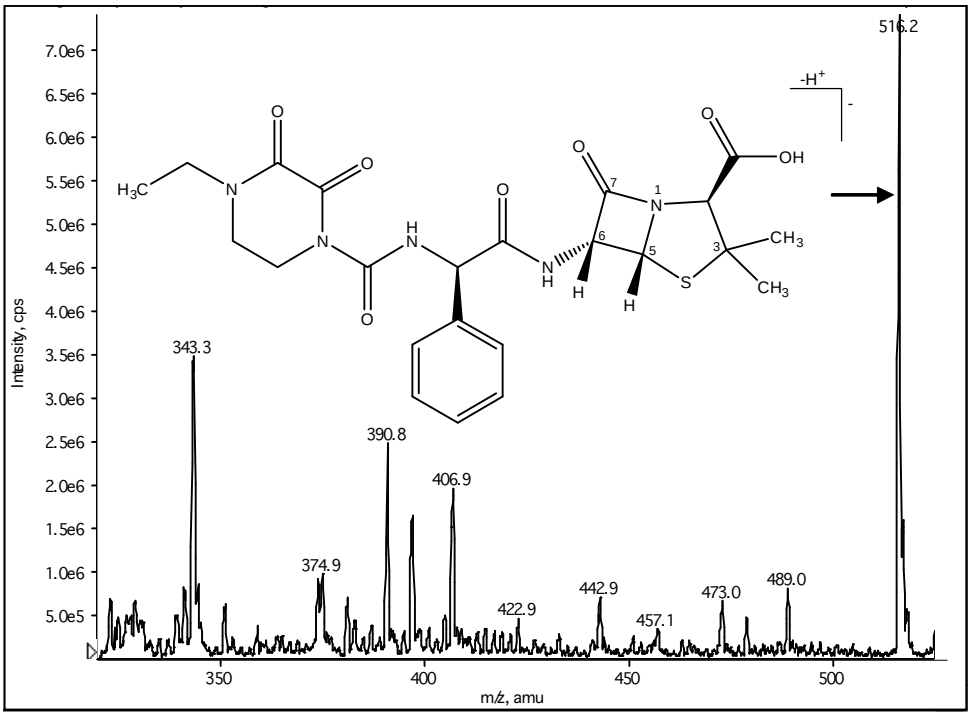
4.2.3 LC-MS/MS method development

4.2.3.1 Mass spectra and tuning

Mass spectra of piperacillin are acquired by continuous infusion into the mass spectrometer. Testing different ionisation sources in both polarities showed that electrospray ionisation (TurboIonSpray®) in the negative ion mode gives the best results, what is reasonable for carboxylic acids.

An abundant peak correspondent to the deprotonated piperacillin molecule $[M-H]^-$ with m/z 516 (exact mass 517.2 amu) is observed in the negative ion mass spectrum of piperacillin recorded by scanning Q1 from m/z 320 to m/z 520 (see Figure 31).

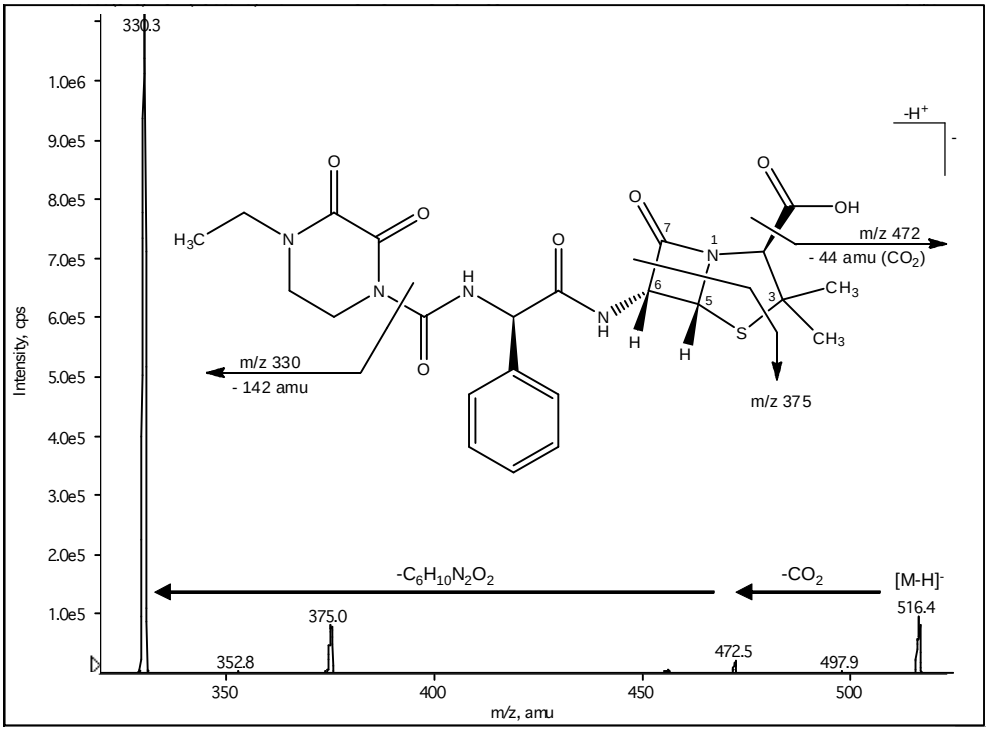
Figure 31 *Precursor ion (Q1) spectrum of piperacillin (negative-ion mode)*



An MS/MS product ion mass spectrum was obtained on collision-activated dissociation of the deprotonated molecule of piperacillin by scanning Q3 from m/z 320 to m/z 520 in the negative-ion mode (see Figure 32).

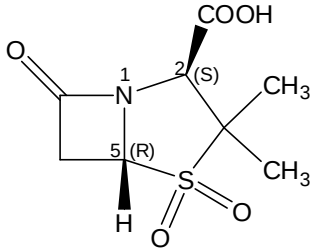
The most intensive peak has m/z 330 and is produced by the loss of carbon dioxide (44 amu) from the deprotonated molecule subsequently followed by the loss of the diketopiperazine residue (142 amu). Similar fragmentation occurs in the positive ionisation mode from the protonated piperacillin molecule, but the positive charge remains on the diketopiperazine moiety (m/z 143). This fragment was used for quantification by other authors [229, 244-246], but due to the lower background noise in the negative ionisation mode of the mass spectrometer, the signal-to-noise ratio of the m/z 330 peak in the negative mode was better than of the m/z 143 peak in the positive-ion mass spectrum. Additionally, using the 330-amu part of the molecule in the negative mode is supposed to be more specific for the determination of piperacillin in human matrices, as plasma and urine.

Figure 32 *Product ion spectrum and fragmentation scheme of piperacillin in the negative-ion mode*



The less abundant fragment ion in the negative product ion mass spectrum of piperacillin with m/z 375 is produced by the loss of CO_2 and subsequent loss of a neutral molecule with 97 amu, which originates from the β-lactam ring and is therefore typical for the fragmentation of penicillins in the negative-ion mode. The same fragments were observed by Casy et al. [247]. The fragmentation pathway was also examined by Rabbolini et al. [199] and is further detailed in chapter 4.1.3.

Scheme 19 *Chemical structure of sulbactam (SUL, IS)*

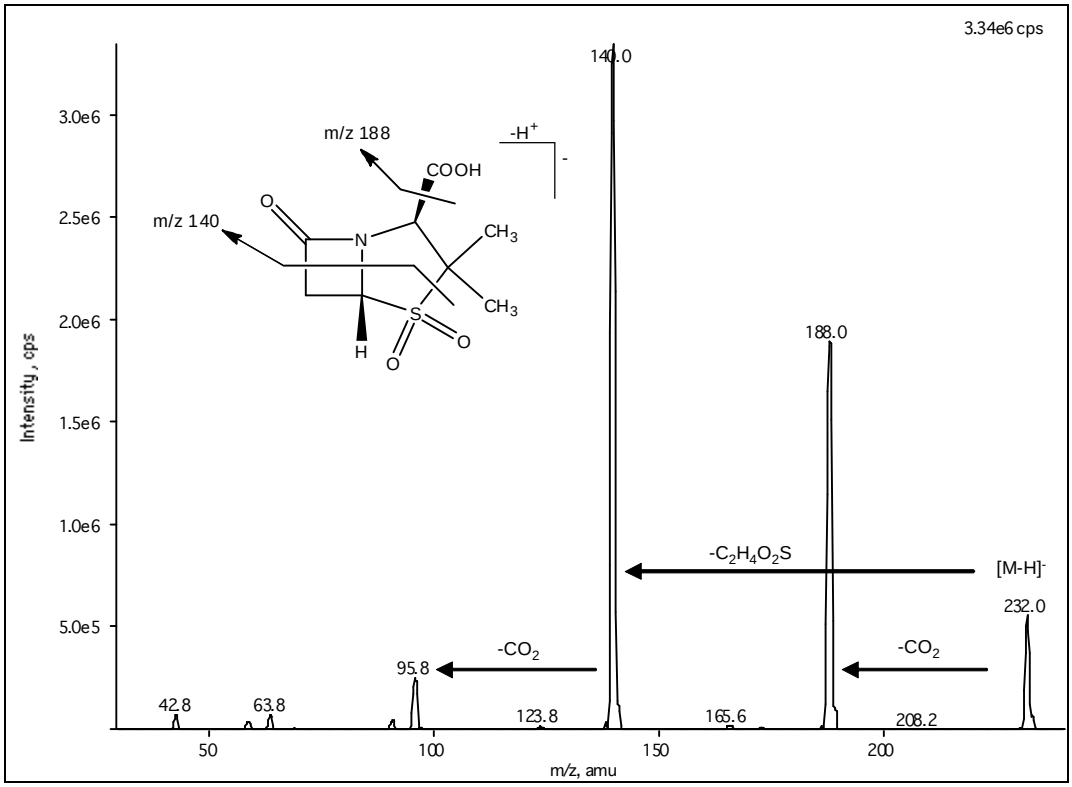


Formula	Mol. weight	Exact mass	pK_a	logP
$C_8H_{11}NO_5S$	233.2 g/mol	233.0 amu	2.5	3.16

IUPAC Name: (2S,5R)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide

Sulbactam was tested as IS, because its chemical structure is also based on the penicillin scaffold and has similar chemical properties.

Figure 33 *Product ion mass spectrum and fragmentation scheme of sulbactam*



The spectrum of sulbactam (IS, exact mass 233.0 amu), recorded by scanning Q1 from m/z 150 to m/z 500 in the same ionisation mode as piperacillin, contains the deprotonated molecule $[M-H]^-$ at m/z 232. The MS/MS product ion mass spectrum of sulbactam was obtained from the deprotonated molecule (m/z 232) by scanning Q3 from m/z 30 to m/z 250 (see Figure 33). The most abundant fragment ion with m/z 140 is produced by the typical negative-ion fragmentation pattern for penicillins as discussed for flucloxacillin (see Figure 26). The only difference to flucloxacillin is the charge site cleavage, as sulbactam can be left by a small neutral molecule, probably sulfonylethane, with m/z 92 amu.

In conclusion, the mass spectrometer is equipped with a TurboIonSpray® interface for pneumatically-assisted electrospray ionisation and is operated in the negative ion mode using Multiple-reaction-monitoring (MRM) of the following transitions: m/z 516 → m/z 330 (PIP) and m/z 232 → m/z 140 (SUL; IS).

The system parameters, which were optimized during spectra acquisition to obtain the highest analytical sensitivity for piperacillin, are summarized in Table 45.

Table 45 *Optimized MS/MS parameters for piperacillin in the negative-ion mode*

Parameter	API 3000™
Probe temperature	350 °C
Ionspray voltage	-5 kV
Orifice voltage	-26 V
Collision energy	18 eV
Nebulizer gas (ESI)	8 L/min
Auxiliary gas (ESI)	10
Nebulizer, auxiliary and curtain gas	High purity nitrogen
Collision gas	High purity nitrogen
Collision gas thickness	4 ($2.4 \cdot 10^{-5}$ torr)

4.2.3.2 Chromatography and sample preparation

Yamana et al. [230] found that piperacillin is most stable in aqueous solutions at approximately pH 5. Therefore, the samples were buffered at pH 5.0 during sample preparation. As β-lactams are also known for hydrolysis and ring-opening, two actions were taken: first, all sample preparation procedures were performed in an ice-water bath at 4 °C and second, acetonitrile instead of methanol was used for precipitation and as organic modifier in the mobile phase.

Previously published methods used C18 columns for reversed phase chromatography [201, 211, 226, 234, 236, 238, 241]. PIP is an organic acid, that can be ionized in the mobile phase prior to electrospray ionisation, interaction with a nonpolar stationary phase is moderate and allows only limited amount of organic modifier. As organic modifiers facilitate the ionisation process in LC-MS and increase thereby the sensitivity, the aim of the present work was to use a high amount of organic modifier. Due to its carboxylic acid moiety, PIP is a possible candidate for polar interactions and ion-exchange interactions with a Nucleosil amino column, which was tested for its chromatographic behavior by using an acetate buffer (0.01 M) and acetonitrile in varying compositions. It was observed that retention on this column is much stronger than on a C18 column, which allows an acetonitrile content of 60 % in the mobile phase. Starting from an ammonium acetate buffer (0.01 M) at pH 5.0, which caused only poor retention and chromatographic

resolution, the ammonium acetate buffer was adjusted with acetic acid to pH 3.0 and mixed with acetonitrile through the binary HPLC pump.

Although some authors report piperacillin methods using methanol in the mobile phase [226, 243], acetonitrile was used for the present method, because β-lactams are known to be susceptible to degradation in methanol by hydrolysis and ring opening [244, 248]. With the conditions described (ammonium acetate (0.01 M, pH 3.0) - acetonitrile (40:60, v/v)), PIP elutes after 1.1 minutes and the IS after 2.9 minutes from the analytical column.

The expected peak concentration of piperacillin in human plasma resulting from administration of a 4 g intravenous dose to healthy adults is in the range of 220-320 µg/mL [203, 205, 217, 249]. The LLOQ should at least be lower than 5 % of C_{max}, which is below 11 µg/mL. As piperacillin has a short half-live of approx. 1 hour and collection of plasma samples was planned up to 24 hours, an even more sensitive method was necessary. Using simple protein precipitation and dilution of the samples, concentrations down to 0.050 µg/mL were detectable with a high precision.

In order to assure the stability of the β-lactam piperacillin, all sample handling as well as the sample preparation procedures for plasma and for urine was done in an ice-water bath at approximately 4 °C.

The human plasma samples (spiked calibration standards or spiked quality control standards) are thawed in a cold water bath, mixed thoroughly and centrifuged for 5 minutes at approximately 4 °C to separate eventually present solid components.

The precipitation method produced sufficient sensitivity. An aliquot of 100 µL of the human plasma samples, calibration standards, or quality control samples are mixed with 100 µL of ammonium acetate buffer (10 mM, pH 5.0 to support stability) containing the internal standard sulbactam (10 µg/mL) and precipitated with 400 µL of acetonitrile.

The precipitated sample, containing the IS, is mixed thoroughly to facilitate deproteinisation. The precipitate is separated by centrifuging the samples for 5 minutes at 3600 rpm (3280 g) at approximately 4 °C. Raising the water content of the sample gave more constant and sharper peaks. Therefore, a 50-µL aliquot of the supernatant is diluted with 250 µL of ammonium acetate buffer (10 mM, pH 5.0) and after mixing, 10 µL of each sample is injected into the LC-MS/MS system.

Human urine samples are thawed in a cold water bath and mixed for 30 seconds directly before an aliquot is used. The 20 µL aliquot is diluted with 1980 µL of ammonium acetate buffer (10 mM, pH 5.0) containing the internal standard sulbactam (10 µg/mL). In a second dilution step, 200 µL of the dilution is mixed with 200 µL of ammonium acetate buffer (10 mM, pH 5.0). After mixing, 10 µL of each sample are injected into the LC-MS/MS system. In Table 46 the optimized sample preparation procedures and LC-MS/MS conditions are summarized.

Table 46 *Summary of the LC-MS/MS method for piperacillin in human plasma*

Characteristic	***Details***
Plasma sample preparation	100 µL plasma + 100 µL NH_4CH_3COO /IS (pH 5.0) + 400 µL CH_3CN 50 µL supernatant + 250 µL NH_4CH_3COO (10 mM, pH 5.0)
Urine sample preparation	20 µL urine + 1980 µL NH_4CH_3COO /IS (pH 5.0) 200 µL dilution + 200 µL NH_4CH_3COO (10 mM, pH 5.0)
Internal standard (IS)	Sulbactam (SUL)
Column	Nucleosil 100 NH_2 5 µm (40 × 4.6 mm I.D.)
Mobile phase	60 % CH_3CN 40 % NH_4CH_3COO (10 mM, pH 3.0)
Flow rate	1.0 mL/min
Run time	3.2 min
Ionisation	TurboIonSpray® - pneumatically assisted electrospray ionisation
Polarity	Negative-ion mode
Mass transitions	m/z 516 → m/z 330 (PIP) m/z 232 → m/z 140 (SUL; IS).

NH_4CH_3COO/IS: ammonium acetate buffer (pH 5.0) containing the IS (sulbactam, 10 µg/mL)

4.2.4 Validation in human plasma and urine

The validation was conducted according to the FDA guidance for industry on bioanalytical method validation [20].

Fresh stock solutions, calibration standards and quality control samples were prepared on each of five validation days to allow proper analysis of stability experiments.

The selectivity was proven, which can be seen from the analysis of drug-free human plasma from different individuals (N = 6) and drug-free human urine (N = 6), where no peaks from matrix components occurred at the retention times of piperacillin and the IS (sulbactam).

Additionally, no interference with piperacillin was observed in the plasma from six individuals and in the urine from six individuals. All test samples in human plasma with the high piperacillin concentration (125 µg/mL, N = 18) had an accuracy of 97.3±6.2 % (mean±SD) and a precision of 6.4 %, whereas at the low piperacillin concentration (0.125 µg/mL, N = 18) an accuracy of 96.2±7.8 % (mean±SD) and a precision of 8.1 % was achieved. All test samples in human urine with the high concentration (1250 µg/mL, N = 18) were analyzed with an accuracy of 93.3±5.2 % (mean±SD) and a precision of 5.5 %, whereas at the low concentration (3.50 µg/mL, N = 18) an accuracy of 99.0±7.1 % (mean±SD) and a precision of 7.2 % was measured.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range of 0.0500 to 125 µg/mL in human plasma and 1.25 to 1250 µg/mL in human urine. The mean linear regression equations of the calibration curves (N=5) were determined:

$y = 0.0011 (\pm 0.0004) + 0.1212 (\pm 0.0261) \cdot x$ (human plasma)

$y = 0.0003 (\pm 0.0014) + 0.0048 (\pm 0.0008) \cdot x$ (human urine).

All correlation coefficients of the weighted ($1/x^2$) calibration curves were ≥ 0.997 for plasma and ≥ 0.995 for urine. The inter-day precision and accuracy of the back-calculated calibration standards of piperacillin in human plasma ranged from 2.0 to 7.1 % and from 96.7 to 102.4 %, respectively. The inter-day precision and accuracy obtained at the plasma LLOQ were 3.0 % and 100.2 %, respectively. The inter-day precision and accuracy of the back-calculated calibration standards of piperacillin in human urine ranged from 2.6 to 9.5 % and from 92.6 to 108.0 %, respectively. The inter-day precision and accuracy obtained at the urine LLOQ were 5.1 % and 101.4 %, respectively. The signal-to-noise ratio of piperacillin at the LLOQs of 0.0500 µg/mL (plasma) and 1.25 µg/mL (urine) was at least five in all five validation sequences.

Table 47 summarizes the intra- and inter-batch precision and accuracy for piperacillin in human plasma and in human urine, respectively, evaluated by assaying the SQC samples. The obtained results are proving the high accuracy and

precision of the bioanalytical method, because the obtained data were well within the acceptance criteria of maximal 20 % deviation at the LLOQ (0.0500 µg/mL) and no more than 15 % at all other concentrations.

Table 47 *Intra- and inter-day precision and accuracy for the analysis of piperacillin in human plasma and urine (five days, five replicates per day)*

Nominal concentrations [µg/mL]	Matrix	Intra-day Precision [%]	Intra-day Accuracy [%]	Inter-day Precision [%]	Inter-day Accuracy [%]
125	Plasma	1.2	99.6	3.7	99.5
12.5	Plasma	6.9	92.7	7.5	101.0
1.25	Plasma	2.7	92.6	7.4	100.5
0.125	Plasma	6.6	93.2	10.7	99.8
0.0500	Plasma	9.5	96.2	10.1	93.0
1250	Urine	2.4	94.5	5.8	95.0
350	Urine	2.9	102.5	7.0	104.3
3.50	Urine	3.0	103.3	5.9	102.7
3.50	Urine	9.4	106.5	9.1	102.2
1.25	Urine	5.9	107.6	10.5	100.7

The mean absolute extraction recovery of piperacillin in five concentration levels (0.0500 to 125 µg/mL in plasma and 1.25 to 1250 µg/mL in urine) was determined to be 86.2±5.2 % (mean±SD) in human plasma and 92.3±3.5 % (mean±SD) in human urine. The mean absolute extraction recovery of the IS (sulbactam) at the working concentration was found to be 104.7±0.8 % (mean±SD) in human plasma and at the working concentration in human urine 99.4±1.6 % (mean±SD). The simple one-step protein precipitation procedure for plasma samples and the simple urine dilution procedure showed satisfactory recoveries for piperacillin.

Stability data of piperacillin at concentrations of 0.125 and 125 µg/mL in ammonium heparin plasma and at concentrations of 3.50 and 1250 µg/mL in human urine is summarised in Table 48 and Table 49. No evidence of instability during chromatography, extraction and sample storage processes for piperacillin in human plasma samples or in human urine samples was observed. Furthermore, piperacillin and the IS were stable in stock solutions at concentrations of 12.5 mg/mL and 1.00 mg/mL piperacillin in methanol and at 0.100 mg/mL sulbactam in water for at least 6 hours at 4 °C.

Table 48 *Stability data of piperacillin in human ammonium heparin plasma*

Stability experiment	Time	Nominal Concentration [µg/mL]	Mean Accuracy [%]	Mean Precision [%]
Short term stability	2 hours	125	103.8	2.1
(room temperature)		0.125	104.1	7.6
	4 hours	125	104.5	1.1
		0.125	101.7	7.0
Post-preparative stability	24 hours	125	105.3	1.7
(autosampler, approx. 4 °C)		0.125	103.6	2.5
Post-preparative stability	24 hours	125	106.3	1.3
(approx. -70 °C)		0.125	100.1	9.1
Freeze-thaw stability	once	125	103.7	2.8
(approx. -70 °C)		0.125	99.9	7.0
	twice	125	105.4	1.2
		0.125	99.3	4.2
	thrice	125	103.3	1.0
		0.125	94.6	3.5
Long-term stability	3 days	125	104.6	3.1
(approx. -20 °C)		0.125	100.0	6.3
	1 month	125	92.6	2.2
		0.125	104.4	6.0
	2.5 months	125	100.1	2.6
		0.125	102.7	7.3
Long-term stability	3 days	125	103.5	0.9
(approx. -70 °C)		0.125	97.0	5.8
	1 month	125	94.5	2.2
		0.125	107.7	9.2
	2.5 months	125	104.5	1.3
		0.125	104.1	6.8

Piperacillin plasma samples can be pre-diluted by factor 5 up to a concentration of 500 µg/mL. The mean concentration value was within ±15 % of the nominal value with a mean accuracy of 107.8 % and a precision of 5.3 %. Human urine samples containing piperacillin can be additionally pre-diluted by a factor of 10. Mean precision and accuracy over five validation runs were 7.5 % and 105.1 %, respectively.

Table 49 *Stability data on piperacillin in human urine*

Stability experiment	Time	Nominal Concentration [µg/mL]	Mean Accuracy [%]	Mean Precision [%]
Short term stability	2 hours	1250	97.0	2.4
(room temperature)		3.50	105.9	3.2
	4 hours	1250	97.7	1.7
		3.50	105.1	5.4
Post-preparative stability	24 hours	1250	96.4	5.0
(autosampler, approx. 4 °C)		3.50	102.9	7.0
Post-preparative stability	24 hours	1250	99.1	6.5
(approx. -70 °C)		3.50	103.4	7.1
Freeze-thaw stability	once	1250	93.7	2.3
(approx. -70 °C)		3.50	98.2	7.0
	twice	1250	92.0	1.4
		3.50	95.3	5.7
	thrice	1250	93.3	1.6
		3.50	98.9	8.4
Long-term stability	2 days	1250	97.5	10.1
(approx. -20 °C)		3.50	103.8	5.6
	3 months	1250	92.5	3.4
		3.50	97.7	5.1
Long-term stability	2 days	1250	97.4	7.2
(approx. -70 °C)		3.50	102.0	4.0
	3 months	1250	93.7	3.8
		3.50	99.2	3.8

No influence of haemolysed plasma on the determination of piperacillin was observed. Mean precision and accuracy (N=5) at 125 µg/mL were 2.3 % and 99.4 %, respectively, whereas precision and accuracy at 0.125 µg/mL were 5.7 % and 99.3 %, respectively.

In addition, the method was tested towards analytical interference with telavancin, an anti-infective that is excreted by the same route as piperacillin, since it was intended to apply the method to a drug-drug-interaction study (see chapter 4.2.6, p. 156). The data indicates no interference of telavancin with the determination of piperacillin in human plasma or urine, as no peaks near the piperacillin peak were observed in the matrix from 5 individuals, spiked with 120 µg/mL telavancin. Mean precision and accuracy of piperacillin in human plasma containing 125 µg/mL piperacillin and

120 µg/mL telavancin were 1.4 % and 92.0 %, whereas in human plasma containing 0.125 µg/mL piperacillin and 120 µg/mL telavancin mean precision and accuracy were 9.9 % and 100.4 %, respectively.

In human urine containing 1250 µg/mL piperacillin and 1200 µg/mL telavancin, the mean precision and accuracy were 12.0 % and 94.3 %, whereas at concentrations of 3.50 µg/mL piperacillin and 1200 µg/mL telavancin in human urine, mean precision and accuracy were 12.7 % and 96.1 %, respectively.

The analysis of 36 incurred human urine samples (see chapter 2.5.12 for definition) demonstrated a high precision of the analysis of piperacillin in human urine. The mean absolute difference between the first and the second measurement was 8.0 % and 35 of the 36 samples were within the 20-% acceptance range, and succeeding with a 97% success rate.

4.2.5 Comparison of the LC-MS/MS method with procedures reported in the literature

In Table 50 the characteristic parameters of the developed method and of several previously reported methods for the determination of piperacillin in human plasma and urine are compared to the present LC-MS/MS method.

All previously reported methods need longer LC run times than this method (3.2 minutes), most of them between 10 and 35 minutes, reducing the daily sample number to at least one third compared to this method. Only three previous publications report run times up to 5 minutes, but were not suitable for the planned application due to other reasons: expensive and time-consuming sample preparation techniques (derivatisation, SPE and PPT followed by LLE) combined with lower sensitivity [59, 234, 239].

The high sensitivity of the developed LC-MS/MS method (LLOQ 0.05 µg/mL plasma) allows easy sample preparation by plasma protein precipitation and there is no need of expensive and time-consuming SPE [59, 62, 237-238] and LLE [60, 201, 211, 226, 235-236, 239] techniques, or even derivatisation [234]. All other methods are characterized by LLOQs between 0.1 and 10 µg/mL, whereas the LC/UV methods are characterized by less sensitive LLOQs between 0.25 and 10 µg/mL.

Most previously published methods used LC/UV for analysis of piperacillin in human plasma, serum or urine samples. Only four LC-MS/MS methods were reported for this purpose to date [59-62]. All were intended for use in therapeutical drug monitoring, but only two authors report their methods were actually applied for determination of piperacillin. All TDM methods were designed for simultaneous determination of several β-lactam antibiotics, needing longer run times (4-8 min) and often more time-consuming sample preparation (SPE, LLE), which was not suitable for the high-throughput analysis of a high sample number from PK studies.

Simultaneous determination of a high number of analytes bears the risk that compromises have to be done. These are typically needed during sample preparation or chromatography, for example concerning the optimal pH for extraction efficiency or chromatographic separation from matrix components. The result can be poor extraction recovery [59-60] and thus poor sensitivity, or the result can be poor selectivity.

Colin et al. published the most recent method for 13 β-lactams, reporting issues with poor precision. This may result from the choice of phenoxymethyl-penicillin-d_5 as internal standard, which is in fact a stable isotopic labelled IS as recommended by relevant guidelines. However, since the molecular structure of this compound is only similar to piperacillin and not identical (beside the deuterium atoms), it may not compensate for variations during the washing and elution steps of the solid phase extraction procedure. Another reason for poor precision, especially at the LLOQ, may be poor sensitivity, because the instrument noise has a remarkable impact on the integration of very small peaks. Overall, the method was reported to be precise enough, meeting a 20% criterion.

Furthermore, the authors of two LC-MS/MS methods did not assess the stability of the analytes during their sample preparation procedure [60, 62].

Table 51 complements Table 50 by additional validation experiments, which were done for some of the previously reported methods. Only four of eleven previously reported methods performed the full spectrum of required stability experiments. Comparing those data, it seems that the results are not consistent. Xia et al. [238] report that piperacillin (1.00, 10.0 and 160 µg/mL) was stable in human plasma for 24 hours at room temperature (data not shown), while Augey et al. [235] found piperacillin stability at low concentrations (1.5 µg/mL) for 1 hour at room temperature, but already a decrease of 16% at 70 µg/mL piperacillin in plasma. However, Denooz et al. [237] conclude that piperacillin at 20 µg/mL plasma could stay at room

temperature for not more than 6 hours, which is in good agreement with the data by Arzuaga et al. [241] as well as the stability data in Table 48 (plasma data) and Table 49 (urine data). The current data provide evidence that piperacillin is stable in urine at room temperature for at least 4 hours, while Augey et al. even confirmed stability in urine after 8 days at room temperature. The latter data is probably less meaningful for laboratory work, but interesting for theory, since piperacillin seems to be more stable in urine than in plasma. One reason could be that urine does not contain large molecules like β-lactamases. The second reason is that piperacillin rapidly degrades below pH 3 and is most stable at pH 5, while the pH of urine typically ranges between 4.8 and 7.5 [1, 9]. However, during bacterial decomposition of urine ammonia is produced, shifting the pH more into the alkaline region. Since at pH 8-9 cleavage of the piperazinyl ring [230] and the β-lactam ring occurs, piperacillin may not be stable indefinitely in urine, even if kept as abacterially as possible.

The present LC-MS/MS method is one of the first methods which is confirmed by the re-analysis of incurred samples.

Additionally, two previous methods were developed for determining piperacillin in human serum [62, 240], which is not very common, because the stability of β-lactams can be better ensured by plasma production directly after blood collection in a cooled centrifuge rather than keeping the serum tube for 15 to 20 minutes at room temperature until clotting is completed.

Table 50 *Comparison of bioanalytical methods for piperacillin in human plasma, serum and urine; grouped by analytical technique and descending years of publication*

Method	Matrix	Linearity range [µg/mL]	Approx run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of QC samples [%]	Recovery [%]	Corr. Coeff.	Sample Vol. [µL]	Sample preparation	Internal standard	Additional validation experiments	Application
LC-MS/MS (current method)	P	0.050-125	3.2	3.0 / 100.2	3.7 to 10.7 / 93.0 to 101.0	86.2±5.2 (PIP) 104.7±0.8 (IS)	≥ 0.997	100	PPT	sulbactam	1, 2, 3a-e, 4, 5, 7	sd-PK [204] (4.5g PIP/TAZ)
	U	1.25-1250	3.2	5.1 / 101.4	5.8 to 10.5 / 95.0 to 104.3	92.3±3.5 (PIP) 99.4±1.6 (IS)	≥ 0.995	20	DIL	sulbactam	1, 2, 3a-e, 5, 8	
LC-MS/MS [59] Colin 2013	P	0.32-38.06	4	n.r.	4.9 to 15.5 / n.r.	67.4±3.5	n.r.	n.r.	SPE	Phenoxymethyl-penicillin-d_5	1,2,3c,e,6	TDM (no PIP samples)
LC-MS/MS [60] Carlier 2012	P	1.5 - 100	5.5	12.8 / 98.5	3.1 to 12.8 / 94.3 to 98.5	67.2±9.7	n.r.	20	PPT + LLE	Piperacillin-d_5	1,2,6	TDM (no PIP samples)
LC-MS/MS [61] Cohen-Wolkowiez 2011	P	0.300-150	6	6.4 / 107.9	5.7 to 8.2 / 99.5 to 107.9	97.6	0.9984	50	PPT	dicloxacillin	1, 3a-d, 5, 6	TDM (36 samples)
LC-MS/MS [62] Ohmori 2011	S	0.1-50	8	6.3 / 101.2	6.3 to 7.8 / 98.7 to 109.3	90.5 to 98.4	1.00	50	SPE	ethylparaben	6, 7	TDM (14 samples PIP)
LC/UV [201] McWhinney 2010	P	10-1000	30	3.2 / n.r.	2.3 to 3.2 / 97.0 to 100.0	n.r.	0.9999	200	PPT + LLE	oxacillin	1, 3b,c, 8	TDM (data n.r.) (400 samples)
LC/UV [236] Di Giovamberardino 2009	P	0.49-500	11	9.4 / n.r.	6.9 to 9.4 / n.r.	100.4 to 103.0	> 0.999	100	PPT + LLE	n.r.	1	n.r.
LC/UV [237] Denooz 2008	P	2.5-60	30	n.r. / n.r.	3.3 to 5.6 / 98.3 to 100.0	78.3 to 81.1 (5.0-30µg/mL)	≥ 0.994	500	SPE	ceforanid	3a-e, 6	TDM (4 patients PIP)
LC/UV [238] Xia 2007	P	0.25-320	10	n.r. / n.r.	3.5 to 6.3 / 97.0 to 106.6 §	80.8 to 83.3 (1.0-100µg/mL)	0.995	800	SPE	benzylpenicillin	1, 3a-e	sd-PK (PIP/TAZ) 1.25g, 2.5g, 3.75g

Continued on the next page

Table 50 (continued) *Comparison of bioanalytical methods for piperacillin in human plasma, serum and urine; grouped by analytical technique and descending years of publication*

Method	Matrix	Linearity range [µg/mL]	Approx run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of QC samples [%]	Recovery [%]	Corr. Coeff.	Sample Vol. [µL]	Sample preparation	Internal standard	Additional validation experiments	Application
LC/UV [226] Ghibellini 2006	P	1-200 (dynamic range)	14	n.r. / n.r.	≤11 / 83 to 119	n.r.	n.r.	300	PPT + LLE	cefoxitin	n.r.	sd-PK (2g PIP) (fat, also in bile)
	U		14	n.r. / n.r.	≤11 / 83 to 119	n.r.	n.r.	n.r.	DIL	cefoxitin	n.r.	
LC/UV [241] Arzuaga 2005	P	2-500	20	4.9 / 105.6	1.9 to 5.9 / 101.5 to 105.6	n.r.	>0.999	100	PPT	penicillin G	1, 3a-e	md-PK [250] (4.5g PIP/TAZ)
LC/UV [240] Trittler 2002	S	1-16	35	2.6 / 102.6	n.r. / n.r.	n.r.	>0.999	n.r.	DIL + Filtr	none	2,7	n.r.
LC/UV [234] García-Gonzalez 1998	P	0.4-100	5	n.r. / n.r.	4.2; 4.6 / 104; 102	98.2 and 96.9	≥ 0.998	400	DEV	n.r.	n.r.	n.r.
	U	4-500	5	n.r. / n.r.	5.2; 6.9 / 99; 102	102 and 95.3	≥ 0.998	n.r.	DEV	n.r.	n.r.	n.r.
LC/UV [211] Occhipinti 1997	P	0.50-200	>15	n.r.	0.38 to 3.16 / n.r.	n.r.	n.r.	200	PPT + LLE	benzylpenicillin	n.r.	md-PK (PIP/TAZ) (3.375g / 4.5g)
	U	50-10000	>15	n.r.	2.76 to 2.94 / n.r.	n.r.	n.r.	40	DIL	benzylpenicillin	n.r.	
LC/UV [235] Augey 1996	P	1.0-100	16	13.7 / 98.5	7.6 to 10.7 / 101.3 to 116.7	90.0±8.8 (PIP) 90.4± 7.1 (IS)	≥ 0.993	500	PPT + LLE	benzylpenicillin	1,3a-d	TDM (data n.r.)
	U	1.0-100	16	14.7 / 102.0	3.5 to 17.7 / 97.7 to 103.3	83.5±8.2	≥ 0.993	50	Online SPE	none	1,3a-d	
LC/UV [239] Gautier 1991	P	10-100 50-500	5	n.r. / n.r.	1.6 to 5.7 / 96.2 to 102.0	77.5±1.6 (50µg/mL)	0.997 0.993	100	PPT + LLE	cephalothin	1, 3a-c,e	sd-PK (4g PIP) (data n.r.)

P: Plasma, U: Urine, S: Serum, Prec: Precision, Acc: Accuracy, SD: standard deviation, LLOQ: lower limit of quanification, PPT: protein precipitation, DIL: Dilution, SPE: solid phase extraction, LLE: liquid-liquid extraction, DEV: Derivatisation, n.r.: not reported, TDM: therapeutical drug monitoring; s.d.: single dose, m.d.: multiple dose, 1: Specificity, 2: Selectivity, 3: Stability (a) short-term, (b) long-term, (c) post-preparative, (d) freeze-thaw, (e) stock solution, 4: hemolyzed plasma, 5: Dilution, 6: Matrix Effect, 7: Signal-to-noise, 8: Incurred Samples

Table 51 *Comparison of validation procedures for piperacillin in human plasma and urine.*

Method	Matrix	Freeze-thaw (3 cycles)	Short-term stability	Post-prep stability	Long-term stability	SL stability	Specificity: interference in blank matrix	Influence of matrix	Incurred Samples (N / Prec)	Dilution
LC-MS/MS (current method)	plasma	✓	4h (RT)	24h (4 °C)	2.5m (-20 °C) 2.5m (-70 °C)	6h (RT) (methanol)	✓ (6 individuals)	6 individuals 2 concentrations 3 replicates	-	1:5
	urine	✓	4h (RT)	24h (4 °C)	3m (-20 °C) 3m (-70 °C)	6h (RT) (methanol)	✓ (6 individuals)	6 individuals 2 concentrations 3 replicates	36 / 8.0%	1:10
LC-MS/MS [59] Colin 2013	plasma	n.r.	n.r.	20h (5 °C)	4d (-20 °C)	35d (-20 °C) (water)	✓ (8 sources)	8 individuals 3 concentrations 4 replicates	n.r.	n.r.
LC-MS/MS [61] Cohen-Wolkowiez 2011	plasma	✓	24h (4 °C)	24h (8 °C)	1m (-80 °C)	n.r.	✓ (7 plasma lots)	7 plasma lots 1 concentration 3 replicates	n.r.	“1:1”, 1:3, 1:9
LC/UV [201] McWhinney 2010	plasma	n.r.	n.r.	24h (RT)	8m (-70 °C)	n.r.	CoMed	n.r.	5 / 10 %	n.r
LC/UV [237] Denooz 2008	plasma	✓	6h (20 °C) 24h (4 °C)	5h (RT) 24h (4 °C)	2m (-80 °C)	6h (RT) 2m (-80 °C)	chromatograms with/without IS	Dilution with serum	n.r.	n.r.
LC/UV [238] Xia 2007	plasma	✓	24h (RT)	24h (n.r.)	1m (-70 °C)	24h (RT) 2w (-20 °C)	chromatograms with/without IS	n.r.	n.r.	n.r.
LC/UV [241] Arzuaga 2005	plasma	✓	4h (RT) 24h (4 °C)	24h (RT) 24h (4 °C)	2m (-20 °C) 2m (-80 °C)	n.r.	✓ (6 individuals) CoMed	n.r.	n.r.	n.r.
LC/UV [235] Augey 1996	plasma	✓	1h (RT) 3h (4 °C)	<6h (RT) <6h (4 °C)	3d (-30 °C) 3m (-80 °C)	12h (4 °C) (aqueous; 50% loss after 14d)	CoMed	n.r.	n.r.	n.r.
	urine	✓	8d (RT) 8d (4 °C)	<6h (RT) <6h (4 °C)	8d (-30 °C) 3m (-80 °C)			n.r.	n.r.	n.r.
LC/UV [239] Gautier 1991	plasma	n.r.	3h (4 °C) 3h (-20 °C)	5h (RT)	16d (-20 °C)	12m (-20 °C) (methanol)	CoMed	n.r.	n.r.	n.r.

CoMed: influence of co-medications on the specificity

4.2.6 Application to a drug-drug interaction study of intravenous piperacillin/tazobactam with telavancin

4.2.6.1 Study design and sample handling

The present method was used to analyze piperacillin concentrations in human plasma and urine of a drug-drug interaction study of piperacillin/tazobactam with telavancin. A single intravenous dose of 4.5 g piperacillin/tazobactam was infused during 30 minutes to twelve healthy male and female volunteers in the fasting state. In three study periods, the pharmacokinetics of piperacillin/tazobactam alone, of a 48-h infusion of 10 mg/kg telavancin alone, and of a combination of both treatments was studied. Here, the piperacillin part will be described, as the concentrations were determined by the developed and validated LC-MS/MS method.

Blood samples were drawn immediately before administration and at 0.5, 0.58, 0.67, 0.75, 0.83, 1.0, 1.25, 1.5, 2.0, 2.5, 3.5, 4.5, 6.5, 8.5, 12.5, and 24.5 hours after the intravenous administration of piperacillin/tazobactam.

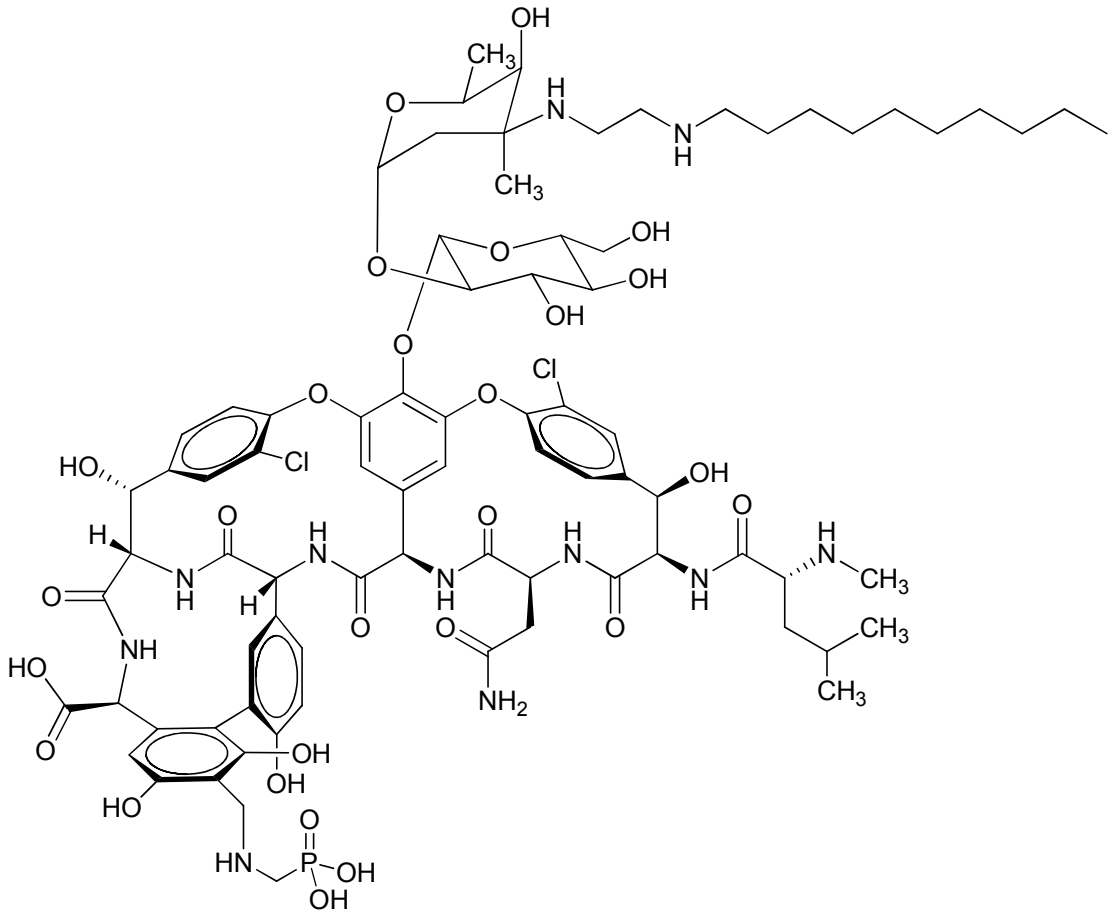
Urine collection was performed 30 minutes before the start of infusion, 0 to 7 hours, 7 to 12 hours, 12 to 24 hours and 24 to 48 hours after administration of the piperacillin/tazobactam treatment.

The ammonium heparinate monovettes were cooled in an ice-water bath until plasma production. The blood samples are centrifuged for 10 minutes at 2-8 °C and 3000 rpm. The plasma is subsequently transferred into polypropylene tubes and immediately frozen at approximately -70 °C until analysis.

4.2.6.2 Chemical and pharmaceutical properties of telavancin

Telavancin is a lipoglycopeptide, derived semisynthetically from the glycopeptide vancomycin. It was approved by the FDA in 2009 and by the EMA in 2011. Since 2012, the EMA suspended the use of Telavancin in the European Union due to a withdrawal of the GMP authorization of the manufacturing site following an inspection by MHRA, AFSSAPS and FDA. This suspension can only be lifted if the Marketing Authorization Holder (MAH) provides evidence that there is an authorized manufacturing site. Since then, in 2013, the MAH changed twice and the FDA expanded the approved use, which was originally to treat complicated skin and skin structure infections (cSSSI), and is now to treat also patients with hospital acquired bacterial pneumonia caused by *Staphylococcus aureus*.

Scheme 20 *Chemical structure of telavancin*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{80}H_{106}Cl_2N_{11}O_{27}P$	3547.7 g/mol	3543.3 amu	1.6-10.0*	2.3*

*predicted value from DrugBank [251]

Telavancin acts bactericidal against a wide range of Gram-positive bacteria, like the anaerobe *Clostridium*, *Lactobacillus*, and *Corynebacterium* species, but also penicillin-resistant pneumococci, methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus [252-254]*. Today, both nosocomial and community-acquired MRSA strains have been isolated. Nosocomial MRSA can cause pneumonia after ventilatory assistance and is associated with increased mortality among hospitalised patients. Community-acquired MRSA is most commonly related to skin and soft tissue infections and toxic shock syndrome by *S. aureus* toxins [252]. Therefore, telavancin is reserved for severe infections, like nosocomial pneumonia caused by MRSA and when other anti-infectives are not suitable.

The rapid bactericidal effect of telavancin against MRSA derives from two mechanisms. At first, telavancin, like vancomycin, inhibits the cell wall synthesis by

binding to the D-alanyl-D-alanine terminus of peptidoglycan precursors [1, 252, 255]. The second mechanism is attributed to the decylaminoethyl side chain, which is supposed to anchor in the bacterial cell wall resulting in increased membrane permeability and leakage of intracellular ATP and K^+ [252, 255]. This second mechanism explains why telavancin is active against *S. aureus* already resistant against vancomycin.

Telavancin, like other lipoglycopeptides, is poorly absorbed after oral administration, which can be assigned to its molecular size limiting the intestinal permeability. The peak plasma concentrations after infusing a 10 mg/kg dose of telavancin range between 91.9 to 95.7 µg/mL [204]. Telavancin is highly bound to plasma proteins (94-96 % [256]) and distributes well into body tissues, including skin blister fluid [257] and epithelial lining fluid [258] proving high enough concentrations at the site of infection.

The pharmacokinetics of telavancin appears to be linear in healthy volunteers between 0.25 and 15 mg/kg [259]. The half-live of 6.1 to 7.9 hours in healthy subjects allows once-daily dosing [204, 260].

Telavancin is mostly excreted unchanged via renal elimination [204, 259], whereas renal clearance accounts for approximately 50 % of the total clearance (CL_R was 7 mL/h/kg after a 10 mg/kg dose [204]). This makes telavancin a potential candidate for drug-drug interaction with piperacillin.

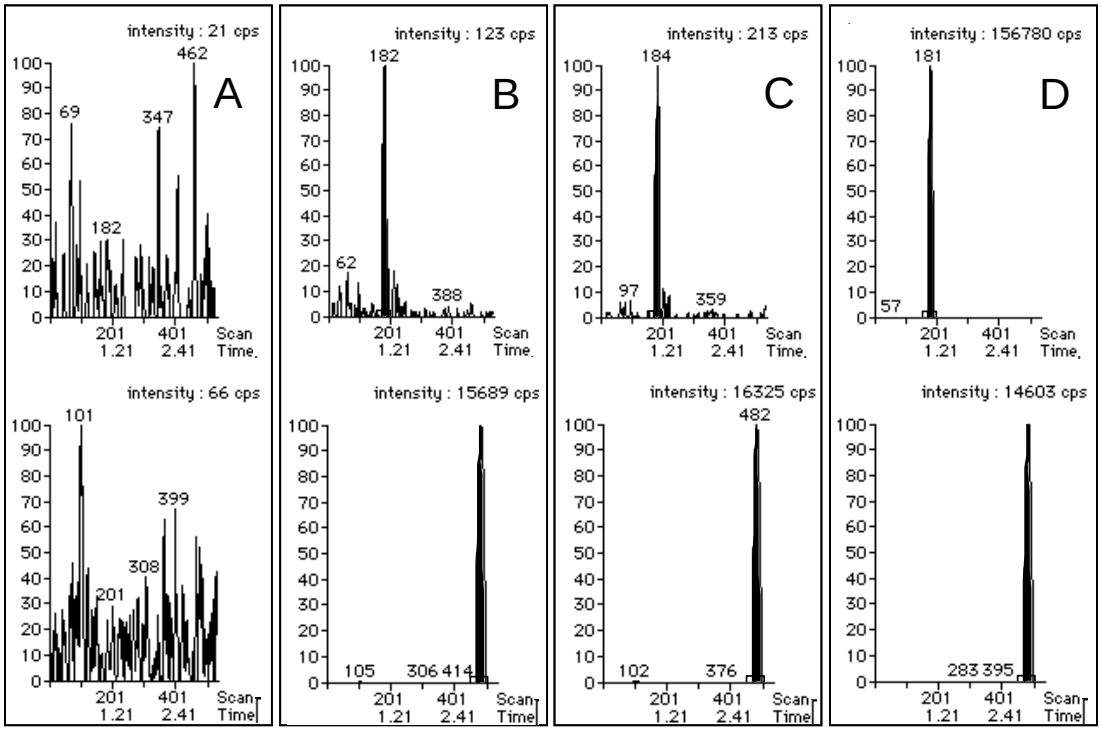
4.2.6.3 Determination of piperacillin concentrations in plasma and urine

A total of 408 human plasma samples and 120 human urine samples were analyzed for piperacillin together with injection standards, calibration standards (low to high concentrations) and SQC samples (in randomized order among subject samples). Human plasma samples were analyzed in a total of six sequences, each completed within eight hours, whereas human urine samples were measured in two sequences within five hours, each. The coefficient of correlation of resulting linear regressions was at least 0.995 for both matrices and the determined concentrations of SQC samples were compared to the theoretical concentrations for accuracy. The precision and accuracy of the SQC samples of piperacillin in human plasma analysed together with the study samples ranged from 3.8 to 6.1 % and from 92.0 to 103.4 %, respectively. Precision and accuracy of the SQC samples in human urine

ranged from 4.7 to 8.2 % and from 89.1 to 106.7 %, respectively. These support the high precision of the study sample concentration data.

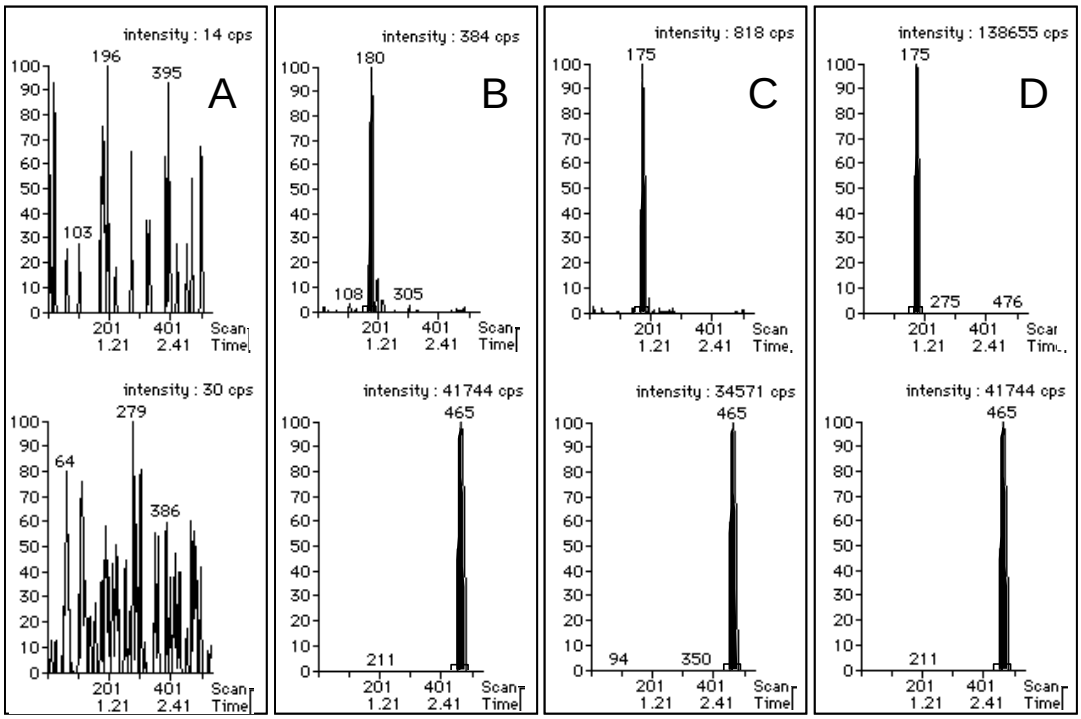
Representative MRM-chromatograms for piperacillin (top) and sulbactam (IS, bottom) in human **plasma** are depicted in Figure 34
(A) blank plasma sample;
(B) blank plasma sample spiked with piperacillin (at LLOQ = 0.0487 µg/mL);
(C) plasma sample from a volunteer 12 h after the administration of piperacillin (piperacillin concentration= 0.0995 µg/mL) and
(D) plasma sample from a volunteer 10 min after the administration of piperacillin (piperacillin concentration= 96.6 µg/mL).

Figure 34 *Representative MRM-chromatograms of piperacillin in human plasma*



Representative MRM-chromatograms for piperacillin (top) and sulbactam (IS, bottom) in human **urine** are plotted in Figure 35
(A) blank urine sample;
(B) blank urine sample spiked with piperacillin (at LLOQ = 1.25 µg/mL);
(C) urine sample from a volunteer 12-24 h after the administration of piperacillin (PIP concentration= 2.69 µg/mL) and
(D) urine sample from a volunteer 7-12 h after the administration of piperacillin (PIP concentration= 523.9 µg/mL).

Figure 35 *Representative MRM-chromatograms of piperacillin in human urine*



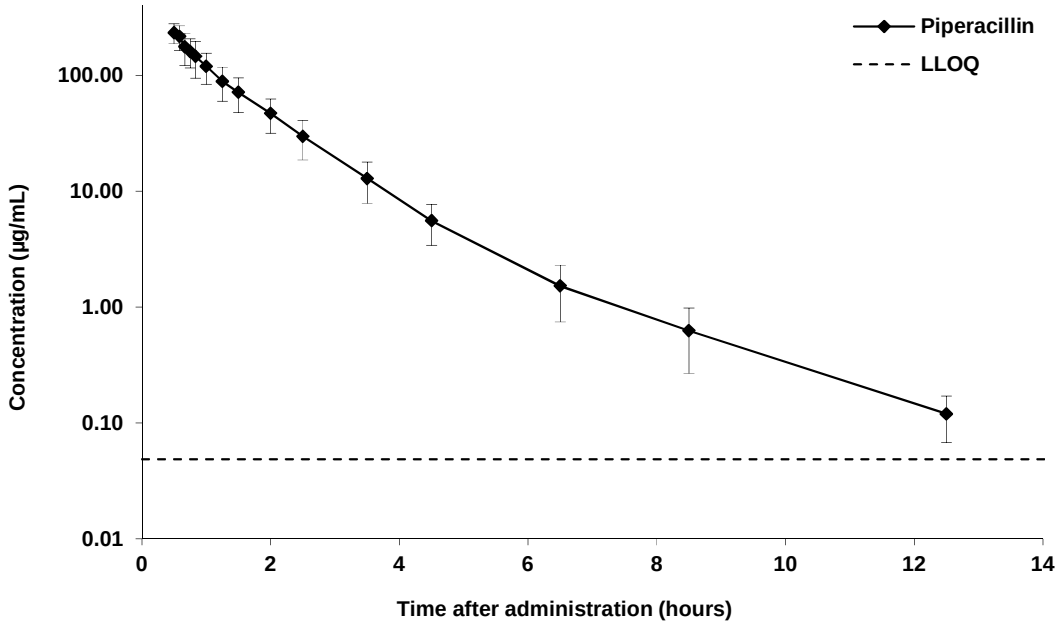
The lowest analyzed piperacillin concentration in human urine of all samples was 1.341 µg/mL (the 24 samples collected before drug administration and 20 samples collected after drug administration were below the lower limit of quantification). The highest piperacillin concentration of all urine samples was 4339.8 µg/mL. Samples with concentrations above the upper quantification limit were pre-diluted (1:10) with drug-free human urine.

4.2.6.4 Pharmacokinetic results

Here, only the pharmacokinetic data of piperacillin will be described, because the developed and validated LC-MS/MS method was applied to determine the piperacillin concentrations of this study.

Figure 36 shows the mean plasma concentration profile of piperacillin after a 30-minute infusion of 4 g piperacillin and 0.5 g tazobactam to 12 healthy volunteers. The mean peak concentration of 236.9±46.8 µg/mL (mean±SD) for piperacillin was attained at the end of the 30-minutes infusion and the elimination half-life ($t_{1/2}$) was determined to be 1.2±0.2 hours. The mean total area under the curve ($AUC_{0\rightarrow\infty}$) was determined as 278.2±74.4 µg·h/mL (mean±SD), of which only 0.07% had to be extrapolated.

Figure 36 *Plasma profile (mean±SD) of piperacillin concentrations versus time following a 4 g intravenous dose of piperacillin to healthy volunteers (n = 12)*



In Table 52 the pharmacokinetic data of piperacillin are compared to literature data. It has been shown that tazobactam has no influence on piperacillin pharmacokinetics [206, 217, 261]. Therefore, the pharmacokinetic data of piperacillin alone and of piperacillin in combination with tazobactam are presented side by side. As piperacillin pharmacokinetics are suspected to follow a non-linear behaviour [220] at higher doses, only data from 4-g piperacillin studies are compared to each other.

When piperacillin/tazobactam was given together with 10 mg/kg telavancin, C_{max} and $t_{1/2}$ remained the same, whereas total AUC increased slightly. At the same time the total CL dropped slightly, which is in good agreement with the observed change in AUC. Since $t_{1/2}$ remained the same and the total CL decreased slightly, one could conclude that the volume of distribution of piperacillin increased slightly in the presence of telavancin. This could also be explained by the protein binding behaviour of the two drugs: Telavancin seems to have a higher affinity to plasma proteins (PPB 95-96 % [256]) than piperacillin (PPB 20-30 % [216]) and may probably replace piperacillin from part of its binding sites. A higher free fraction of piperacillin would also lead to an increased renal clearance of piperacillin, which was indeed observed in this study. Obviously, the renal elimination pathway was not yet saturated at these dose levels. Finally, the decrease of the total clearance and the increase of the renal clearance at the same time, can only be explained, if the hepatic clearance of piperacillin was further decreased by telavancin. However,

these variations are not considered to be clinical relevant at these dose levels. More details on the study and pharmacokinetic data on tazobactam and telavancin are published elsewhere [204].

When piperacillin was given as a bolus infusion (lower part of Table 52), mean maximal plasma concentrations were approximately 30% higher than when given as a 30-min infusion.

Additionally, three data sets were added, which include pharmacokinetic data from patients, whose creatinine clearance was categorized as normal (given beyond the table). Comparison of data from patients versus healthy volunteers shows an elevation of AUC values and concomitant decrease of clearances in patients, which is expected as piperacillin is eliminated predominantly renally. However, two data sets are conspicuous as the AUC values of the healthy subjects and patients with normal renal clearance of approx. 120 mL/min [217, 249] exceed even the AUCs of the elderly patients (CL_R approx. 74 mL/min and 84 mL/min [205, 207]) while renal clearance is unexpected low. The current data fits logically well into the data of other previously reported pharmacokinetic data in healthy subjects and patients.

4.2.7 Summary: Piperacillin

To the best knowledge, this is the first fully validated LC-MS/MS method for high-throughput analysis of piperacillin in human plasma and urine. The developed and validated LC-MS/MS assay for piperacillin in human plasma and urine is simple, fast, reliable, sensitive, precise and accurate. The method employs acetonitrile protein precipitation, which allows quantification of piperacillin for concentrations between 0.0500 and 125.0 µg/mL from 100 µL of human plasma, and concentrations between 1.25 and 1250 µg/mL from 20 µL of urine. The principal advantage of the LC-MS/MS method described here is the high sensitivity (LLOQ = 0.0500 µg/mL plasma and 1.25 µg/mL urine), high precision at the LLOQ in plasma and urine (3.0 and 5.1 %) and high accuracy in plasma and urine (100.2 and 101.4 %) with a short run time of only 3.2 minutes. These characteristics make the method suitable for the precise and accurate measurement of piperacillin in high sample numbers of plasma and urine from pharmacokinetic studies. Indeed, the method was successfully applied to the analysis of more than 500 samples from a pharmacokinetic study of 4 g piperacillin and 0.5 g tazobactam as a single intravenous dose in healthy volunteers.

Table 52 *Pharmacokinetic parameters (mean±SD or mean (CV)) for piperacillin of a single intravenous dose of 4 g piperacillin (PIP) and 0.5 g tazobactam (TAZ) to healthy volunteers or patients with normal renal function (upper part: 30-min infusion; lower part: bolus infusion)*

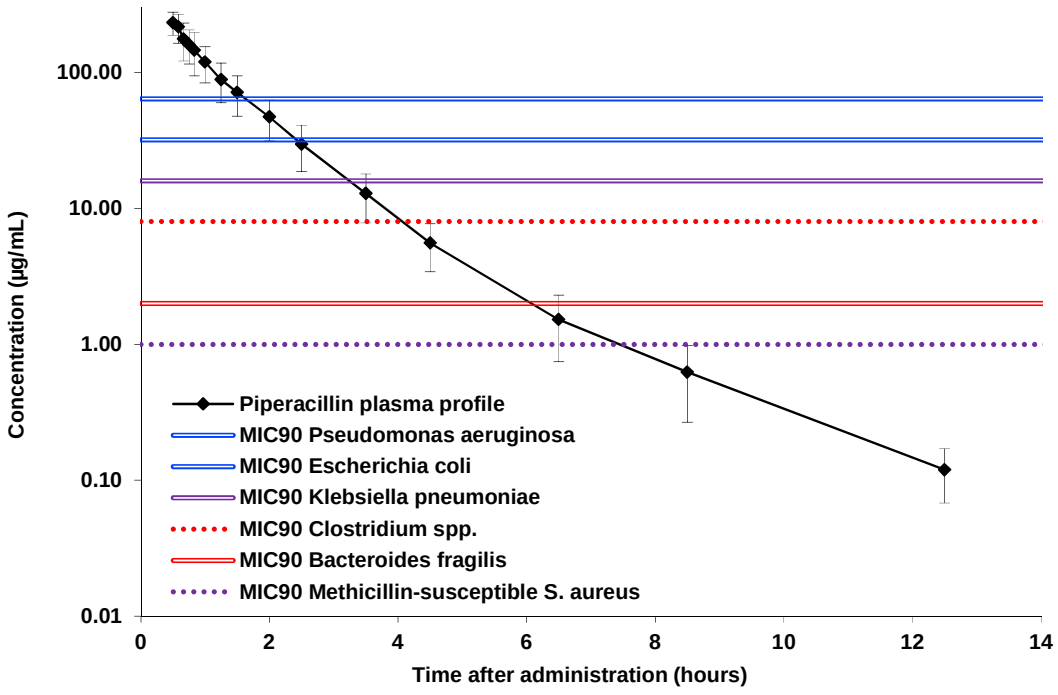
Reference	No. and gender of subjects	Dosage (Infusion Time)	t_{last} [h]	C_{max} [µg/mL]	$AUC_{0\rightarrow last}$ [µg·h/mL]	$AUC_{0\rightarrow\infty}$ [µg·h/mL]	$t_{1/2}$ [h]	CL_{tot} [mL/min]	CL_{renal} [mL/min]	Analytical method
Current study [204]	12 m,f (healthy)	4g PIP, 0.5 g TAZ (30 min)	24	236.9±46.8	278.0±74.4	278.2±74.4	1.2±0.2	291.2±92.1	153.0±69.8	LC-MS/MS (plasma, urine)
Current study [204]	12 m,f (healthy)	4g PIP, 0.5 g TAZ and 10mg/kg TLV	24	235.3±49.7	289.0±51.9	289.3±51.9	1.1±0.2	237.8±45.5	167.8±58.6	LC-MS/MS (plasma, urine)
Cheung 1989 [203]	6 (healthy)	4g PIP, 0.5 g TAZ (30 min)	n.r.	277 (14)	n.r.	278 (9)	0.83 (14)	242 (8)	109 (22)	n.r.
Cheung 1989 [203]	6 (healthy)	4g PIP (30 min)	n.r.	284 (15)	n.r.	286 (18)	0.87 (9)	240 (17)	116 (15)	n.r.
Wise 1991 [217]	6 m (healthy)	4g PIP, 0.5 g TAZ (30 min)	10	223.7±49.7	n.r.	485±82.1	1.0±0.15	145±22.7	73.1±6.5 mg/min[$]	Microbiol. (serum, urine)
Kinzig 1992 [205]	18 m,f (surgery patients*[1])	4g PIP, 0.5 g TAZ (30 min)	6	259±81.8	n.r.	361±80.3	1.27±0.24	194±42.9	n.r.	LC/UV (plasma, tissue)
Bourget 1996 [249]	10 m,f (burn patients*[2])	4g PIP, 0.5 g TAZ (30 min)	6	322.2±39.0	n.r.	640.3±122.9	1.8±0.3	140.5±22.8	75.9±16.2	Microbiol. (serum, urine)
Sörgel 1994 [206]	6 (healthy)	4g PIP, 0.5 g TAZ (5 min)	n.r.	380 (27)	n.r.	294 (11)	1.14 (20)	229 (8)	125 (16)	n.r.
Welling 1983 [207]	5 m (patients*[3])	4g PIP (2 min)	12	329	n.r.	384±72	1.4±0.4	153±25	95±8	Microbiol. (serum, urine)
Schepper 1982 [208]	5 m (healthy)	4g PIP (3 min)	8	330.7±68	n.r.	250.3±12.5	1.02±0.05	258.4±6.2	213.8±19.6	Microbiol. (serum, urine)
Tjandramaga 1978 [221]	5 m (healthy)	4g PIP (3 min)	6	330.7±67.8	n.r.	250.3±12.5	1.02±0.05	254.2±19.3	203.7±19.5	Microbiol. (serum, urine)

n.r.: not reported, m: male, f: female, *[1]: creatinine clearance 72.4±21.3 mL/min (mean±SD), *[2]: creatinine clearance 119.8 mL/min (82-186 mL/min), *3: creatinine clearance; 84 mL/min (71-111), $ cumulative renal excretion divided by extrapolated AUC, TLV: telavancin, coadministered at a dose of 10 mg/kg

4.3 Summary: β-Lactams

The β-lactams are clearly assigned to the PK/PD parameter T>MIC, wherefore piperacillin efficacy can be improved by dosing more frequently or continuously [11]. In Figure 37, the determined plasma concentrations of the broad-spectrum penicillin piperacillin is related to several MIC_{90} values of gram-negative aerobe bacteria (E. *coli*, P. *aeruginosa*, *Klebsiella pneumoniae* [262]) and anaerobes (B. *fragilis*, *Clostridium spp.* [263]), as well as the gram-positive Methicillin-susceptible S. *aureus [264]*. It seems that anaerobe bacteria could be more susceptible to piperacillin/tazobactam than aerobe bacteria. However, more data need to be studied to examine this thesis. For example, for the given dose of piperacillin/tazobactam, it would be necessary to give the next dose after 6 hours to keep the piperacillin levels above MIC_{90} of B. *fragilis* as long as possible to achieve an efficient therapy. However, other antimicrobials may be more efficient against this bacterium.

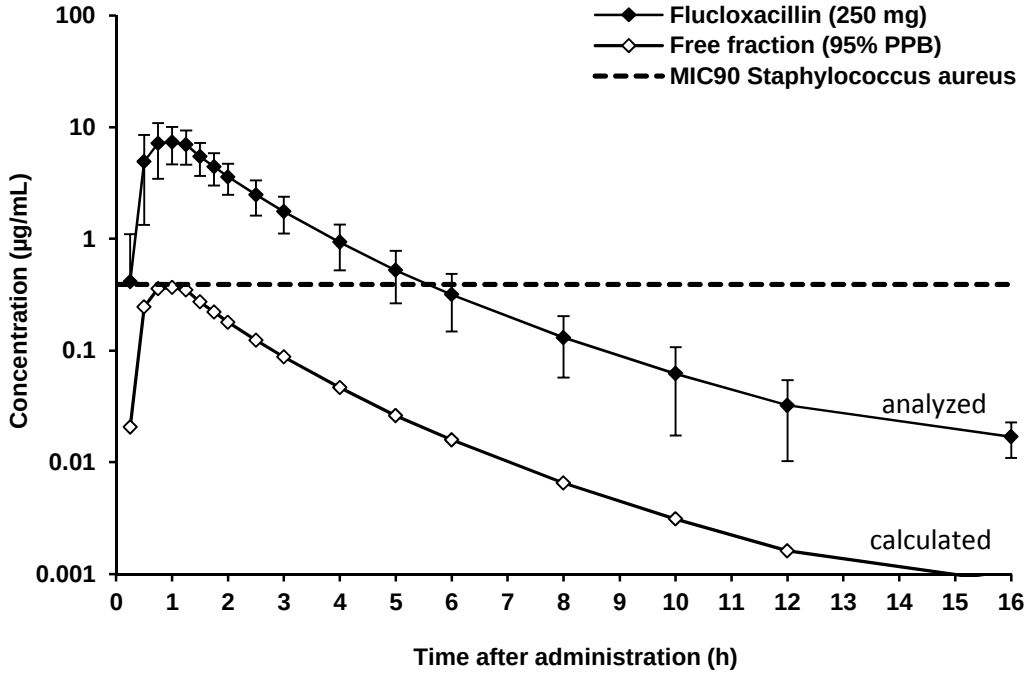
Figure 37 *Plasma concentration-time profile of a 30 min intravenous dose of 4 g piperacillin and 0.5 g tazobactam in relation to MIC_{90} data of aerobe, facultative anaerobe and anaerobe gram-negative (empty line) or gram-positive (dotted line) bacteria*



Though the MIC_{90} of P. aeruginosa is higher compared to other miroorganisms, piperacillin/tazobactam is the most active antimicrobial (>86 % susceptibility in Asia/Pacific Rim, Europe, and North and South America).

In Figure 38, flucloxacillin concentrations after a 250-mg dose remain above MIC_{90} of *Staphylococcus aureus* [265] for approximately 5 hours. The concentration data obtained by the present bioanalytical method are total plasma concentrations since flucloxacillin is released from the reversible protein-interaction during protein-precipitation with acetonitrile. Therefore, the free fraction of flucloxacillin was calculated by assuming 95 % plasma protein binding of flucloxacillin. Apparently, the curve of the free fraction never reaches effective concentrations. Therefore, higher flucloxacillin doses may be recommended in severe infections. However, for efficacy of a given drug, the concentration at the site of infection, i. e. respiratory tract, skin, soft tissues, bone, is more important than plasma concentrations. To maximise efficacy of the narrow spectrum isoxazolyl-penicillin flucloxacillin, it has been shown that continuous infusion of 8-12g flucloxacillin per day was more effective against MSSA than standard dosing [266].

Figure 38 *Plasma concentration-time profile of a 250-g tablet of flucloxacillin in relation to the MIC_{90} value of S. aureus*



5 FLUOROQUINOLONES

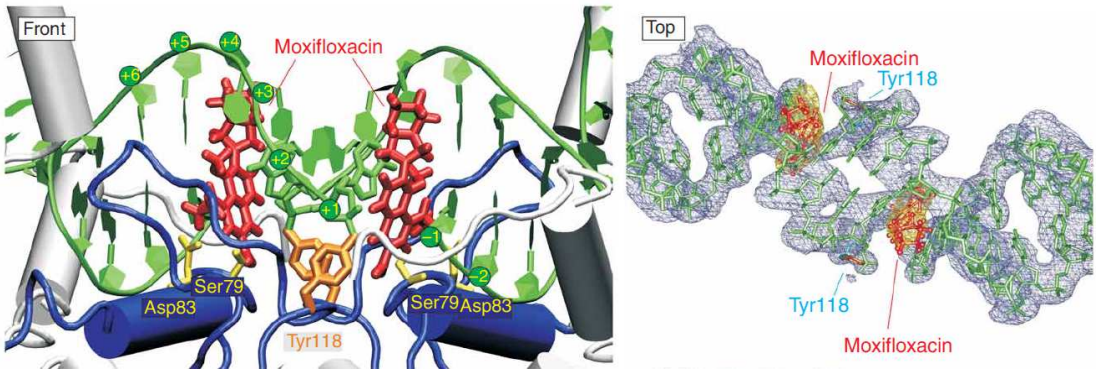
5.1 Moxifloxacin

5.1.1 Chemical and pharmaceutical properties

Moxifloxacin belongs to the fluoroquinolone class of antibacterials with a broad antimicrobial spectrum against Gram-positive and Gram-negative pathogens including multi-drug resistant strains. Originally, it was developed for respiratory tract infections including community acquired pneumonia, acute bacterial sinusitis, and acute bacterial exacerbation of chronic bronchitis [267], but over the years moxifloxacin received approval for additional indications, like uncomplicated and complicated skin and skin-structure infections, complicated intra-abdominal infections, and ocular infections like bacterial conjunctivitis [268-272]. Still, further applications are being studied, for example the use in bone diseases like osteomyelitis [273-274]. It is the most recent quinolones playing a major role in antibiotic therapy.

The bactericidal activity of moxifloxacin derives from the inhibition of the bacterial DNA conformation process by interacting with DNA gyrase (primarily in gram-negative bacteria) and with topoisomerase IV in important gram-positive bacteria. The interaction takes place by stabilizing the DNA-enzyme cleavage complex with Mg^{2+} and two moxifloxacin molecules [275-276].

Figure 39 *Moxifloxacin-topoisomerase IV cleavage complex, reprinted with permission from Macmillan Publishers Ltd: Nature structural & molecular biology [275], copyright 2009*



The moxifloxacin-topoisomerase IV cleavage complex is depicted in Figure 39. The front view of the active site is shown on the left side, including moxifloxacin (red), the

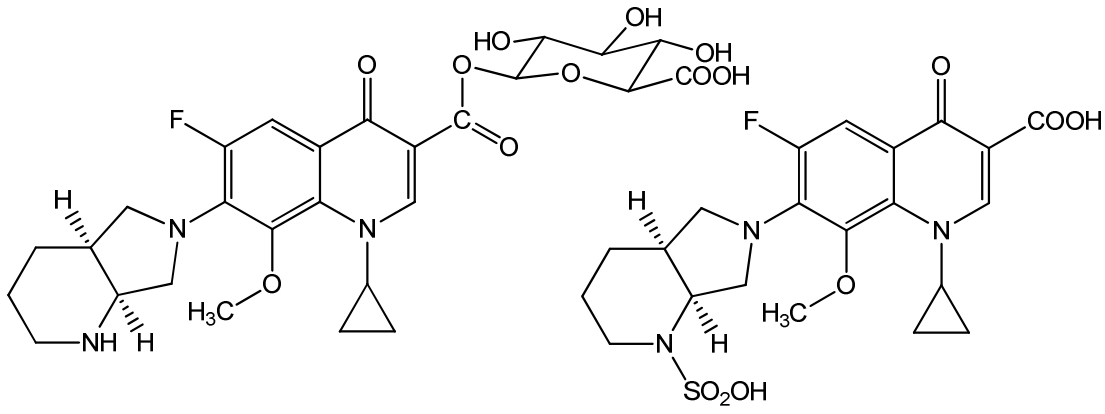
active site tyrosines (orange), the DNA G-segment (green) and the residues responsible for resistance upon mutation (yellow). The Mg^{2+}, which should be located between the two moxifloxacin molecules, is not shown. The right part of Figure 39 contains the top view of the cleaved bacterial DNA intercalated with the moxifloxacin molecules.

Moxifloxacin is known to have an effect on cardiac repolarisation, which can clinically be detected by QT interval measurement. Excessive prolongation of this interval can result in life-threatening cardiac arrhythmias. Therefore, moxifloxacin should be administered carefully in patients with known cardiac diseases [277-280].

In 2008, a so-called Red-Hand-Letter [281] was published, warning against serious hepatotoxicity and blistering skin disease, which are both potentially life-threatening conditions. Since then, the European prescribing information is stating that moxifloxacin is contraindicated for patients with impaired liver function (Child Pugh C) and cases of unwanted effects should be reported to the health authorities.

Recently, a population-based study of diabetic patients was published, where moxifloxacin was found to increase the risk of severe dysglycemia [282-283]. This was also observed in patients receiving moxifloxacin concomitantly with insulin [283]. Individual case reports associate moxifloxacin also with rhabdomyolysis [284], acute interstitial nephritis [285], acute iris depigmentation [286], hepatotoxicity [63-64], and abdominal wall hematoma if combined with warfarin [287]. However, a review of the moxifloxacin safety in the past 14 years concluded that the safety of moxifloxacin is essentially comparable to standard therapies since it can be characterized by a favourable safety profile including fatal but rare toxicities [288].

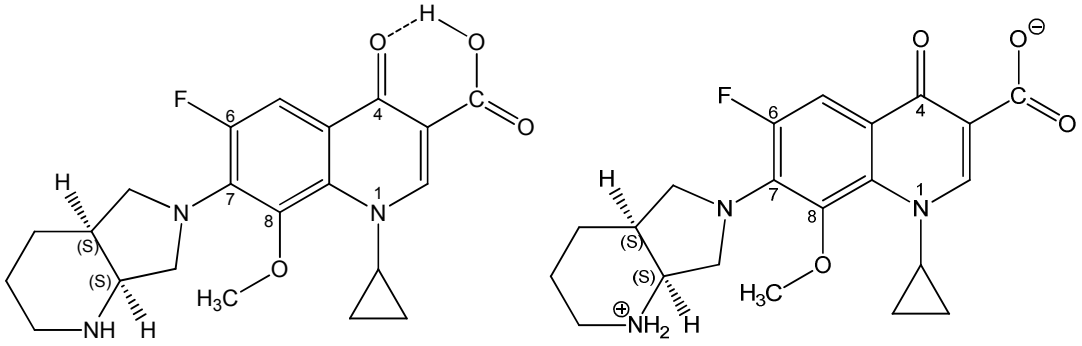
Scheme 21 *Chemical structures of the N-sulfate conjugate and the acyl-glucuronide metabolites of moxifloxacin*



acyl-glucuronide N-sulfate conjugate

The bioavailability after oral administration of moxifloxacin is high (82 to 92 % [289-291]), therefore no dosage adjustment is necessary when patients switch from i.v. to oral therapy. After a single oral dose of 400 mg moxifloxacin mean peak plasma levels can range from 2.5 µg/mL to 4.98 µg/mL [292-298]. The elimination half-life (8.3 hours to 15.6 hours [292-298]) may allow once-daily administration. While most quinolones are cleared predominantly by the kidney making dosage adjustment necessary for renal failure, moxifloxacin is metabolized predominantly by the liver and should not be used in patients with hepatic failure [1]. Moxifloxacin is metabolized to an N-sulfate conjugate and an acyl glucuronide in humans (see Scheme 21). The N-sulfate conjugate can be found in plasma, urine and feces, while the acyl glucuronide can only be found in plasma and urine, but not in feces [299]. This may derive from enterohepatic recirculation of the acyl glucuronide. Moxifloxacin is excreted unchanged in urine by 19-22% of a given oral dose [299]. Data of an SAD study of six oral moxifloxacin doses from 50 mg to 800 mg indicate that pharmacokinetics are independent from the dose [293].

Scheme 22 *Chemical structure of moxifloxacin (MOX)*



non-ionic / neutral form zwitterionic form

Formula	Mol. weight	Exact mass	pK_a	logP
$C_{21}H_{24}FN_3O_4$	401.4 g/mol	401.2 amu	6.25 / 9.29	0.832

IUPAC Name: 7-[(4aS,7aS)-1,2,3,4,4a,5,7,7a-octahydropyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-4-oxoquinoline-3-carboxylic acid

Moxifloxacin is a weak acid with pKa 6.3 and a weak base with a pKa of 9.3 at the same time [300]. At physiological pH, zwitterionic and neutral forms are predominating, where the neutral from has a share of approximately 10 %. Compared to other fluoroquinolones this is a rather high number of molecules in the most lipophilic form, contributing to an overall logP value of 0.832. Therefore,

moxifloxacin can be considered a lipophilic fluoroquinolone [300-301], which is also confirmed clinically by many reports studying moxifloxacin in a variety of human body fluids [302-307], tissues [308-311], and bone [273-274, 312-314].

5.1.2 Current state of published analytical methods

Consequently, a high number of analytical methods have been reported, determining moxifloxacin concentrations not only in human samples, but also in growth media [315], in formulation samples [316-317], in animal body fluids and tissues [318].

Some methods are used to determine moxifloxacin among other fluoroquinolones [319-325], as was also reviewed recently [326].

Most common methods to quantify moxifloxacin in human plasma or serum use liquid chromatography with fluorescence detection (LC/FD) [321-322, 327-333] or with ultraviolet detection (LC/UV) [319, 323, 334-337] or both detection techniques [320, 338]. Application to pharmacokinetic studies for 400 mg moxifloxacin doses was reported for most LC/FD [327-329, 331, 333] and LC/UV methods [335-336]. To date, only four previously reported methods for moxifloxacin use liquid chromatography with mass spectrometric detection (LC MS/MS) [65-66, 339-340], whereas one is designed for the determination of moxifloxacin in dried blot spots [339], one for rat plasma [340] and two methods for human plasma [65-66]. The latter were not useful for our application, since they were characterised by either a very high sample volume (1.0 mL plasma) associated with a laborious sample preparation technique or less sensitivity (LLOQ of 0.050 µg/mL). Recently, an LC-MS/MS method was published with the same sensitivity as the present method, down to 0.005 µg/mL [340], but it is characterised by a higher sample volume and longer run-time. The aim was to develop and validate a highly sensitive, precise and cost-effective LC-MS/MS method, requiring only small plasma volumes from pharmacokinetic studies.

5.1.3 LC-MS/MS method development

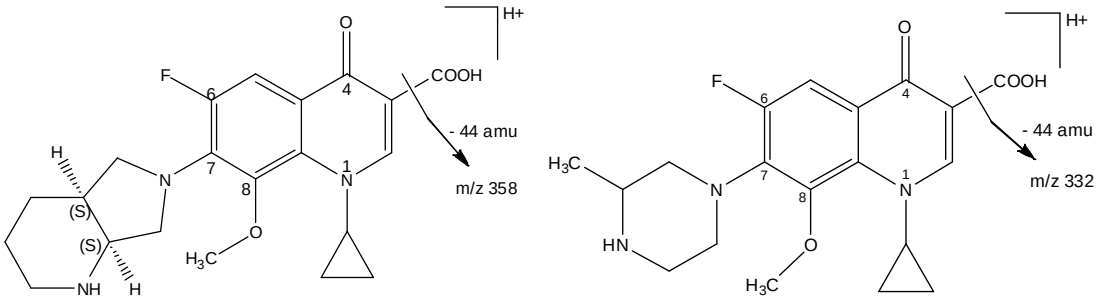
5.1.3.1 MS/MS spectra and tuning

The mass spectrum of moxifloxacin was recorded in the first quadrupole (Q1) of an API 3000^{TM} mass spectrometer operated in the positive ion mode. The latter was selected, because more intensive signals were obtained and, additionally, due to its

two pKa values, moxifloxacin should be ionized below pH 4 as $[M+H]^+$ or above pH 11 as $[M-H]^-$. For acidic conditions, a larger assortment of analytical columns is available. As moxifloxacin is easily protonated in solution, the TurboIonSpray® interface was used for electrospray ionisation. An abundant peak with m/z 402, correspondent to the protonated molecule $[M+H]^+$ of moxifloxacin (exact mass: 401.2 amu), was used for the MRM transition.

MS/MS product ion mass spectra were obtained on collision induced dissociation of the protonated molecule of moxifloxacin (m/z 402) by scanning Q3. The only intensive fragment with m/z 358 corresponds to the loss of carbon dioxide (44 amu) from the protonated molecule and was also selected by other authors [65-66] for the MRM transition. The fragmentation scheme is depicted in Scheme 23.

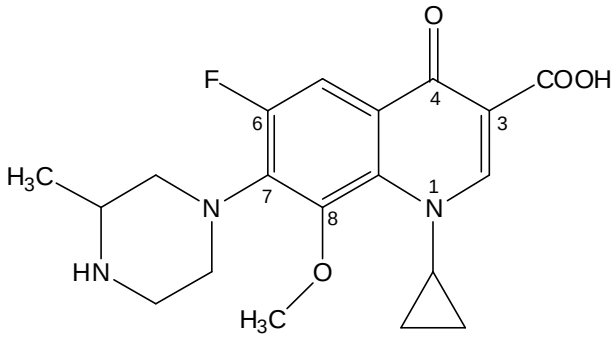
Scheme 23 *Fragmentation scheme of moxifloxacin and gatifloxacin (IS) following collision induced dissociation in the positive ionisation mode*



Gatifloxacin was chosen as IS, because it has similar chemical properties as moxifloxacin and is unlike to be available in plasma at the same time with moxifloxacin (Since 2006 gatifloxacin is only marketed as ophthalmic solution.), but is still available as reference substance.

The precursor ion mass spectrum of gatifloxacin contains the protonated molecular ion $[M+H]^+$ with m/z 376 (exact mass: 375.2 amu). The most abundant ion in the product ion spectrum of gatifloxacin in the positive-ion mode with m/z 332, also derives from the neutral loss of CO_2 (-44 amu).

Scheme 24 *Chemical structure of gatifloxacin (GAT)*



Formula	Mol. weight	Exact mass	pK_a	logP
$C_{19}H_{22}FN_3O_4$	375.4 g/mol	375.2 amu	6.0	2.6

IUPAC Name: 1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid

Thus, the mass spectrometer is equipped with a TurboIonSpray® interface for electrospray ionisation and is operated in the positive-ion mode using Multiple-reaction-monitoring (MRM) of the following transitions: m/z 402 → m/z 358 (MOX) and m/z 376 → m/z 332 (GAT, IS). System parameters were optimized during spectra acquisition to obtain the highest analytical sensitivity for moxifloxacin summarized in Table 53.

Table 53 *Optimized MS/MS parameters for moxifloxacin*

Parameter	API 3000™
Probe temperature	350 °C
Ionspray voltage	5 kV
Orifice voltage	31 V (OR)
Collision energy	26 eV
Nebulizer gas (TISP)	8 L/min
Auxiliary gas (TISP)	5 L/min
Nebulizer, auxiliary and curtain gas	High purity nitrogen
Collision gas	High purity nitrogen
Collision gas thickness	8 ($2.9 \cdot 10^{-5}$ torr)

5.1.3.2 Chromatography

As moxifloxacin was suspected to be instable under day-light [333], the sample preparation procedure was done in an ice-water bath at approximately 4 °C and under day-light protection. Plasma samples (100 µL) were precipitated with two parts of acetonitrile (200 µL) for nearly complete protein removal and good vaporization in the MS. The precipitated sample, containing the IS, is mixed thoroughly to facilitate deproteinisation. The precipitate is separated by centrifuging the samples for 10 minutes at 3,000 rpm (approx. 3280 g) at approximately 4 °C.

Plasma samples were diluted (1:2) after precipitation with ammonium formate buffer (1mM, pH 3.0), which decreased the organic solvent content of the sample, brought it to the “right” pH conditions and improved peak shape. At pH 3 moxifloxacin (pKa 6.25) is completely protonated and therefore well prepared for analysis in the positive ion mode.

The electrospray ionisation is supported by the mobile phase, which is also composed of acetonitrile and formate buffer (1 mM, pH 3.0). Gradient elution from low to high acetonitrile content on a standard C18 column showed that moxifloxacin is already eluted at low acetonitrile volumes. Therefore, the isocratic solvent system was optimized to a buffer content of 75 % on a Phenomenex Aqua C18 column, which is especially stable in aqueous mobile phases. Heated nitrogen gas at 350 °C supported vaporisation of the relatively high water content of the mobile phase.

The IS gatifloxacin (pKa 6.0) is also fully protonated at pH 3 and has a similar retention time in the chromatographic retention time in this method, which is 0.9 and 1.1 minutes for the IS and moxifloxacin, respectively. Typical chromatograms are depicted in Figure 40.

The expected plasma concentration range following administration of a 400-mg oral dose of moxifloxacin to healthy adults is determined by C_{max}, which is between 2.5 µg/mL and 4.98 µg/mL [292-298]. Accordingly, 5 % of C_{max} lies between 0.125 µg/mL and 0.250 µg/mL. However, the LLOQ was set to 0.005 µg/mL, as the assay was already very sensitive with sample dilution by precipitation. For further applications, like measurement of plasma concentrations after ophthalmic use of moxifloxacin, the present method is a good starting point for scaling down the LLOQ by a sample enrichment technique, like SPE. Currently, the calibration range is set from 0.005 to 5.00 µg/mL.

In the first test run of the prepared calibration curve, the IS working solution is prepared at a concentration that equals one half of the highest calibration level of the analyte to check the resulting peak area ratio (MOX/IS). Tests with different IS concentrations revealed the following result: The IS working solution is prepared by diluting a stock solution of gatifloxacin by factor 400 with acetonitrile to give a concentration of 0.5 µg/mL. Thus, only one pipetting step was needed for IS addition and precipitation.

All pipetting steps were carried out in 96-well plates using the Multimek^{TM} Automated 96-Channel Pipettor, which is able to pipette 96 samples at the same time. Together with the short chromatographic run time of only 2.0 minutes, this method is best prepared for high-throughput analysis of biological samples.

In Table 54 the optimized sample preparation procedure and LC-MS/MS conditions are summarized.

Table 54 *Summary of the LC-MS/MS method for moxifloxacin in human plasma*

Characteristic	***Details***
Sample preparation	100 µL plasma + 200 µL CH_3CN /IS 100 µL supernatant + 200 µL NH_4HCOO (1 mM, pH 3.0)
Internal standard	Gatifloxacin (GAT)
Column	Aqua C18, 5 µm (50 x 4.6 mm I.D.)
Mobile phase	25 % CH_3CN 75 % NH_4HCOO (1 mM, pH 3.0)
Flow rate	1.0 mL/min
Run time	2.0 min
Ionisation	Electrospray Ionisation (TurboIonSpray®)
Polarity	Positive-ion mode
Mass transitions	m/z 402 → m/z 358 (MOX) m/z 376 → m/z 332 (GAT, IS)

CH_3CN /IS: acetonitrile containing the internal standard gatifloxacin (0.5 µg/mL)

5.1.4 Validation

The method was validated according to the FDA guidance for industry on BMV [20].

Analysis of drug-free human plasma from 12 individuals (6 males, 6 females) proved the selectivity of the method for MOX and the IS. No co-eluting peaks with areas above 20 % of the LLOQ peak were observed.

Additionally, no influence on the analysis of MOX was observed for 6 different batches of human plasma. All 18 test samples with the high moxifloxacin concentration (5.00 µg/mL) had an accuracy of 92.5 ± 3.4 % (mean±SD) with a precision of 3.6 %. Accuracy and precision at the low MOX concentration (0.0125 µg/mL) were 103.7 ± 9.1 % (mean±SD) and 8.8 %.

The linear regression of the peak area ratios versus concentrations were fitted over the concentration range of 0.00500 to 5.00 µg/mL MOX in human plasma. The mean linear regression equation of the calibration curves (N = 5) was determined:

$y = 0.00129\ (\pm 0.00118) + 1.30225\ (\pm 0.32435) \cdot x.$

All correlation coefficients of the weighted calibration curves ($1/x^2$) were ≥ 0.995. The inter-day precision and accuracy of all back-calculated calibration standards ranged from 1.8 to 5.3 % and from 91.1 to 107.6 %. Using 0.1 mL of plasma, the LLOQ for moxifloxacin was 0.00500 µg/mL, analyzed with a perfect inter-day precision and accuracy of 1.8 % and 100.0 %, respectively.

Table 55 *Intra- and inter-day precision and accuracy of moxifloxacin in human plasma (five days, five replicates per day*

Theoretical concentrations [µg/mL]	Intra-day Precision [%]	Intra-day Accuracy [%]	Inter-day Precision [%]	Inter-day Accuracy [%]
5.00	7.9	90.2	6.1	92.4
1.00	4.5	106.1	4.4	105.6
0.125	4.2	106.9	4.6	108.0
0.0125	9.7	106.8	7.7	106.6
0.00500	5.6	98.8	7.9	99.0

Table 55 summarizes the inter- and intra-run precision and accuracy for assaying five replicates of each SQC concentration on each of five validation days. The inter-day precision and accuracy ranged from 4.4 to 7.9 % and from 92.4 and 108.0 %, whereas the intra-day precision and accuracy ranged 4.2 to 9.7 % and from 90.2 to

106.9 %. The obtained results were well within the acceptance criteria of no more than 20% deviation at LLOQ and no more than 15% above LLOQ.

Table 56 *Stability data on moxifloxacin in human plasma*

Stability experiment	Conc. [µg/mL]	Time	Point Estimator [%]	Lower Limit [%]	Upper Limit [%]
Short-term stability (room-temperature)	5.00	2 hours	94.6	93.2	96.0
		4 hours	96.7	94.4	99.1
	0.0125	2 hours	101.2	93.9	108.9
		4 hours	100.5	93.9	107.4
Post-preparative stability (autosampler, approx. 4 °C)	5.00	48 hours	92.7	90.5	94.9
	0.0125	48 hours	103.4	100.2	106.7
Post-preparative stability (approximately -70 °C)	5.00	48 hours	93.9	91.9	96.0
	0.0125	48 hours	99.6	93.6	105.9
Freeze-thaw stability (approximately -70 °C)	5.00	Once	98.5	97.1	99.8
		Twice	95.1	91.6	98.7
		Thrice	99.9	96.7	103.1
	0.0125	Once	98.5	93.1	104.2
		Twice	105.8	101.0	110.8
		Thrice	98.5	92.2	105.2
Long-term stability (approximately -20 °C)	5.00	4 days	93.7	88.7	98.9
		1 month	92.8	89.8	95.9
	0.0125	4 days	102.4	97.5	107.6
		1 month	101.7	94.4	109.5
Long-term stability (approximately -70 °C)	5.00	4 days	93.8	90.6	97.1
		1 month	93.5	92.2	94.7
	0.0125	4 days	96.7	90.0	103.7
		1 month	97.7	93.0	102.7

Stability data of MOX at concentrations of 0.0125 µg/mL and 5.00 µg/mL in human plasma is summarised in Table 56. The results of the 95% confidence interval calculation showed no evidence of instability during chromatography, extraction and sample storage processes. Therefore, MOX can be regarded as stable in human plasma for at least 4 hours at room temperature, for at least one month at -20 °C, and for at least three freeze/thaw cycles form -70 °C to room temperature. Additionally, MOX is stable in processed human plasma for at least 48 hours at autosampler temperature (4 °C) and at -70 °C.

The stock solutions of MOX and the IS gatifloxacin were stable for at least 6 hours at room temperature. Additionally, no instability was observed for moxifloxacin in human plasma and human whole blood for at least 6 hours exposed to the dark, to artificial light or daylight.

The absolute extraction recovery of MOX from human plasma was determined to be 77.6 ± 7.9 % (mean±SD) over the whole concentration range of 0.00500 to 5.00 µg/mL. The absolute extraction recovery of the IS at the working concentration was found to be 49.4 ± 3.3 % (mean±SD). The precision of 10 % shows good consistency and reproducibility of the acetonitrile precipitation procedure introduced to extract MOX from human plasma.

No influence of haemolysed or hyperlipidaemic human plasma on the accurate and precise determination of moxifloxacin was observed. Mean accuracy and precision of moxifloxacin in haemolysed / hyperlipidaemic plasma at 5.00 µg/mL were 94.0% / 89.6% and 5.4% / 5.5%, whereas accuracy and precision at 0.0125 µg/mL were 99.5% / 101.2% and 12.9% / 11.3%, respectively.

Human plasma samples that exceed the calibration range up to 20.0 µg/mL can be pre-diluted with blank human plasma by a dilution factor of 5. Accuracy and precision of the pre-diluted human plasma samples (five replicates) were 98.4% and 5.9%.

5.1.5 Comparison of the LC-MS/MS method with procedures reported in the literature

A direct comparison of the current LC-MS/MS assay to validated methods from the literature is summarized in Table 57. All previously reported methods for the determination of moxifloxacin in human plasma or serum had longer run times per sample, whereas previously reported LC-MS/MS methods [65-66, 340] remain below 5 minutes per sample and three newer LC/FD methods remain below 10 minutes [327, 329, 331]. But all other liquid chromatography methods need up to 30 minutes analysis time per sample. Methods with shorter run times were applied tendentially more often in pharmacokinetic studies [327, 329, 331, 333, 335-336] than methods without reported application, indicating that shorter run times are more useful for routine analysis.

A key factor for ethics is the blood volume taken from volunteers or patients during pharmacokinetic studies. Thus, analytical methods should be sensitive enough to keep the needed volume of plasma or serum low. The developed method and four

previously reported methods [65, 327] need only 100 µL of plasma or less, but only the present method is very sensitive at the same time (LLOQ 0.005 µg/mL). All other literature methods use more than twice the plasma volume, whereas two assays even use 1.0 mL [66] and 1.2 mL [334]. Extracting moxifloxacin from a very high volume of plasma can lead to a very low quantification limit [66], but together with the rather expensive and time-consuming SPE sample work-up, this method was not the first choice for the application to high sample through-put in a pharmacokinetic study.

Some authors reference the FDA guideline on bioanalytical method validation [33]. However, one should be aware that not only the type of experiments, but also the way how to conduct them is requested there. That is, accuracy and precision data “representing the entire range of the standard curve should be studied” [33], which was not reported during two validations [323, 325]. Validation according to FDA includes also several stability assessments, which were not reported for another method [336].

With the developed and validated LC-MS/MS method, moxifloxacin can be measured with high sensitivity using simple protein precipitation sample work-up. Due to the cost and time effective sample preparation procedure and the 2-minutes runtime, it is possible to measure over 500 human plasma samples per LC-MS/MS machine per day. Additionally, the assay is characterized by high precision and accuracy data down to the LLOQ at 5 ng/mL (CV: 1.8%). Together with the low sample volume of 100 µL of plasma, the method is especially useful for large pharmacokinetic studies.

Table 57 *Validated bioanalytical methods for the determination of moxifloxacin in human plasma or serum; grouped by analytical technique and descending years of publication*

Method	Matrix	Linearity range [µg/mL]	Approx. run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of QC samples [%]	Recovery (mean±SD) [%]	Corr. Coeff.	Sample Vol. [µL]	Sample Prep.	Internal standard	Additional validation experiments	Application
LC-MS/MS (current method)	P	0.005 - 5.00	2.0	1.8 / 100.0 (n=25)	4.4 to 7.9 / 92.4 to 108.0 (n=5x25)	77.6±7.9 (MOX, n=5) 49.4±3.3 (IS, n=5)	≥0.995 (n=5)	100	Automated PPT	gatifloxacin	1,2,3a-f, 4,5,6	400-mg p.o., single-dose PK
LC-MS/MS [340] Raju 2012	P (rat)	0.005 – 0.100	4	3.2 / 100.3	1.0 to 1.8 / 96.7 to 97.6	>75 (MOX, n=6) >89 (IS, n=6)	0.998 (mean)	200	PPT	gemifloxacin	1,2,3a-d,6,7	single-dose PK in rats
LC-MS/MS [65] Pranger 2010	P	0.050 - 5.00	2.5	4.8 / 107.1	1.9 to 3.7 / 102.7 to 107.1	114.2 to 122.2 96.5 (IS)	0.990 (n=3)	100	PPT	Cyanoimipramine “internal control”	1,2,3a,c,d,f,6, 7	TDM
LC-MS/MS [66] Vishwanathan 2002	P	0.001 - 1.00	4	11.3 / 110.0 (n=18)	2.3 to 6.8 / 97.5 to 100.5 (n=18)	90.6, 92.7 (MOX, n=5) 97.4±1.5 (IS, n=5)	>0.999 (n=3)	1000	SPE	lomefloxacin	n.r.	n.r.
LC/UV + LC/FD [338] Cavazos-Rocha 2014	P	0.2 - 10 (UV) 0.04 - 10 (FD)	30	9.2 / n.r. 12.3 / n.r.	5.0 to 15.0 / n.r.	102 to 111	0.997 0.998	50	PPT	none	2, 3b,c	Mouse plasma samples
LC/UV [337] Helmy 2013	P	0.100 – 10.0	6	11.2 / 98.3	1.6 to 8.4 / 97.9 to 102.9	99.6 to 103.5	>0.999	250	PPT	metronidazole	1, 2, 3b,c,d,e,8	TDM
LC/UV [336] Abdelaziz 2012	P	0.125 - 16	5.0	3.5 / 107.6	n.r. / 99.5 to 105.7	n.r.	≥0.999 (n=6)	250	PPT	tinidazole	1	400-mg p.o., single-dose PK
LC/UV [335] Xu 2010	P	0.050 - 5.00	10	<15 / <115	4.5 to 9.6 / 100.4 to 108.2 (n=6)	80.5 to 91.2	0.999 (n=3)	500	LLE	gatifloxacin	1,2,3a-d	400-mg p.o. Single dose PK
LC/UV [323] Baietto 2009	P	0.160 - 10.0	28	n.r.	5.5 to 14.8 / 93.1 to 114.9 (n=3x5)	75.9±5.8 (MOX, n=5) 77.9±4.1 (IS, n=5)	>0.998	300	PPT + Evaporation	quinoxaline	1, 2	TDM
LC/FD [321] De Smet 2009	P	0.020 - 7.50	17	4.8 / 102.5 (n=5)	3.0 to 5.6 / 98.3 to 100.0 (n=3x16)	92.4 (n=3x3)	0.998 (mean)	400	PPT + Evaporation	sarafloxacin	1,3c,d	400-mg i.v. (results n.r.)
LC/FD [327] Hemanth Kumar 2009	P	0.125 - 10.0	8	9.9 / 94.4 (n=6)	1.1 to 6.0 / 96.5 to 106.0 (n=3x6)	96 to 105 (n=3x6)	0.999 (mean)	100	PPT	ofloxacin	1,2	400-mg p.o. Single dose PK

Continued on the next page.

Table 57 (continued) *Validated bioanalytical methods for the determination of moxifloxacin in human plasma or serum; grouped by analytical technique and descending years of publication*

Method	Matrix	Linearity range [µg/mL]	Approx. run time [min]	Inter-day Prec / Acc at LLOQ [%]	Inter-day Prec / Acc of QC samples [%]	Recovery (mean±SD) [%]	Corr. Coeff.	Sample Vol. [µL]	Sample Prep.	Internal standard	Additional validation experiments	Application
LC/UV [319] Srinivas 2008	P	0.100 - 10.0	18	<20 / ≤104.4	1.7 to 9.0 / 94.7 to 107.0 (n=4x18)	62.5 to 67.9 63.5 (IS)	≥0.999 (n=3)	500	LLE	levofloxacin	1,2,3a-e	n.r.
LC/FD [325] Nemutlu 2007	P	0.035 - 30.0	20	n.r.	3.9 to 7.9 / 100.7 to 101.2	96.5±1.0 (absolute) 98.3±2.1 (relative)	0.9991	500	SPE	marbofloxacin	1, 2, 3a, c, d (at 1 µg/mL)	TDM
LC/FD [329] Tatar Ulu 2007	P	0.015 - 2.70	8	4.8 / 106.0 (n=6)	0.05 to 4.8 / 103.1 to 106.0 (n=6)	95.7 (n=6)	>0.999	500	4xLLE	norfloxacin	1,3a,b,d	400-mg p.o. Single dose PK
LC/FD [331] 2006 Laban-Djurdjevic	P	0.003 - 1.30	9	4.5 / 105.4	n.r. / n.r.	92.5	>0.999	n.r.	Filtration	ofloxacin	1,3b,d	400-mg p.o.
LC/FD [328] Schulte 2006	P	0.200 - 3.50	18	1.0 / 100.0 (n=18)	1.0 to 4.1 / 97.0 to 101.0 (n=4x18)	97±1.7 (MOX, n=6) 106±1.4 (IS, n=8)	0.9995	400	PPT	levofloxacin	3b, c	400-mg p.o. of one volunteer
LC/FD [322] Nguyen 2004	S	0.125 - 4.00	17	12.9 / 114.3 (n=10)	1.6 to 5.3 / 95.8 to 96.9 (n=10)	91.5 to 94.7 (n=10)	≥0.999 (n=10)	5	"on-line extraction"	none	3a,b,d	n.r.
LC/UV, LC/FD [320] Liang 2002	P	0.100-10 (UV) 0.020-5.0 (FD)	15	n.r.	n.r.	n.r.	n.r.	500	UF	ciprofloxacin	none for MOX	n.r.
LC/UV [334] Lemoine 2000	P	0.025 - 3.20	13	<15 / <115	1.1 to 9.1 / 100.2 to 101.8 (n=3x36)	97.4 (0.03-2.5µg/mL)	n.r.	1200	Automated SPE	enrofloxacin	1,2	n.r.
LC/FD [332] Tobin 1998	S	0.500 - 4.00	30	n.r.	n.r. / 100.0 to 104.4	100.0	0.998	n.r.	2xLLE	n.r.	1	n.r.
LC/FD [333] Stass 1997	P	0.0025 - 1.00	10	4.5 / 114.6	2.1 to 4.5 / 104.3 to 114.6	n.r.	0.993	250	PPT	none	3a,b,d-f	PK studies 200-mg / 400-mg

P: plasma, S: serum, Acc: Accuracy, Prec: Precision, SD: standard deviation, LLOQ: lower limit of quanification, PPT: protein precipitation, SPE: solid phase extraction, LLE: liquid-liquid extraction, Dil: Dilution, Deriv: Derivatisation, n.r.: not reported, TDM: therapeutical drug monitoring, UF: ultrafiltration
1: Specificity, 2: Selectivity, 3: Stability (a) short-term, (b) long-term, (c) post-preparative, (d) freeze-thaw, (e) stock solution, (f) light, 4: hemolyzed plasma, 5: lipemic plasma, 6: Dilution, 7: Matrix Effect, 8: Incurred Samples

5.1.6 Application to a pharmacokinetic study

5.1.6.1 Study design and sample handling

The study was designed to investigate the pharmacokinetics of 400 mg moxifloxacin as a film-coated tablet, given as a single oral dose to nine healthy male volunteers under fasting conditions. Blood was collected into ammonium heparinate monovettes according to the following schedule: immediately before administration and at 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 4.50, 5.00, 6.00, 8.00, 12.00, 24.00, and 48.00 hours after oral administration of the study drug.

The blood samples were cooled in an ice-water bath until centrifugation for 10 minutes at 4 °C and 1500 g. The obtained plasma was subsequently transferred into polypropylene tubes and immediately frozen at approximately -70 °C until analysis.

The concentrations of moxifloxacin in the human plasma samples were determined using the developed and validated LC-MS/MS method summarized in Table 54 (page 173).

5.1.6.2 Determination of moxifloxacin concentrations in human plasma

In the present study moxifloxacin concentrations of more than 700 human plasma samples were measured in a total of five sequences. The coefficient of correlation of resulting linear regressions was at least 0.995. The inter-day precision and accuracy of the SQC samples of moxifloxacin in human plasma analyzed with the batches of study samples ranged from 6.4 to 9.9 % and from 99.0 to 104.3 %, respectively and support the high precision and accuracy of the concentration data of the study samples (see Table 58).

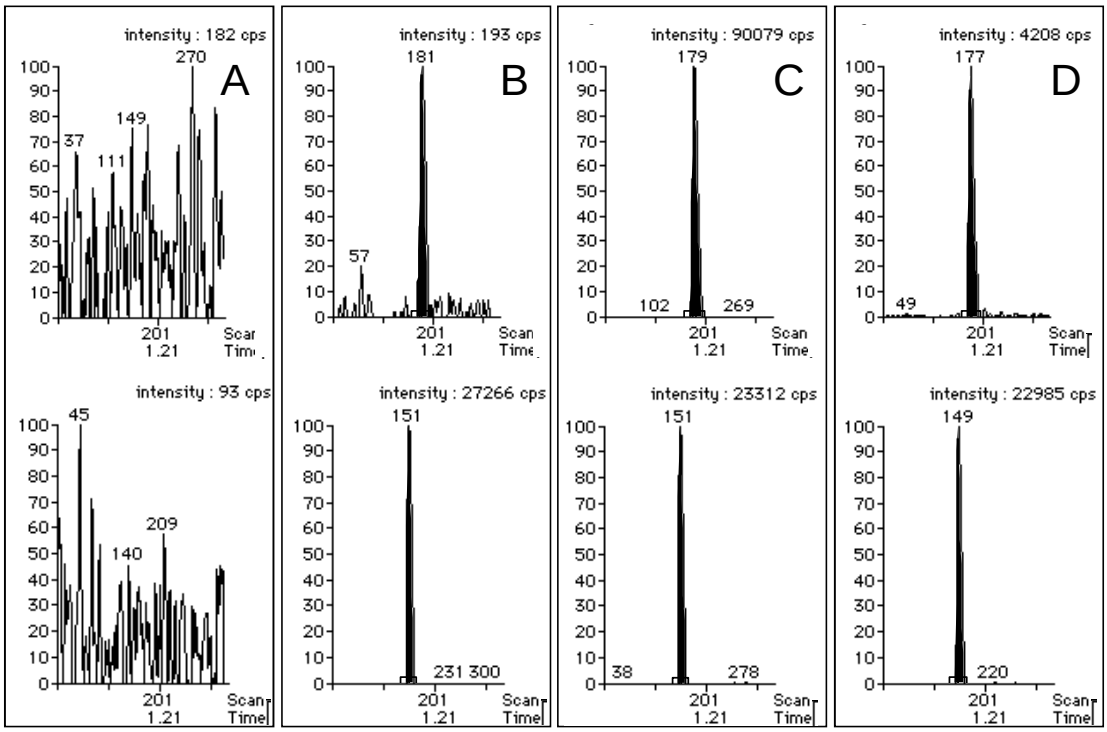
Representative MRM-chromatograms are depicted in Figure 40: (A) Blank plasma sample (B) LLOQ, containing 0.00514 µg/mL moxifloxacin (C) Subject plasma sample (t=0.67h) containing 4.31 µg/mL moxifloxacin (D) Subject plasma sample (t=48h) containing 0.204 µg/mL moxifloxacin

Table 58 *Accuracy and precision of calibration standards and SQC samples during analysis of moxifloxacin in study samples*

	Theoretical Concentration (µg/mL)	Mean Accuracy* (%)	Mean Precision* (%)
Calibration standards	4.97	99.2	2.4
	3.73	101.3	2.7
	1.02	101.8	2.7
	0.255	100.6	5.4
	0.0509	102.9	4.8
	0.0255	93.5	8.4
	0.0102	98.2	8.2
	0.00816	101.5	7.2
	0.00514	100.9	5.9
SQC samples	3.81	99.0	6.8
	1.09	99.8	8.0
	0.108	104.3	6.4
	0.0135	101.9	9.9

* Accepted limits by EMA and FDA: maximal 15 % for precision and 85-115 % for accuracy

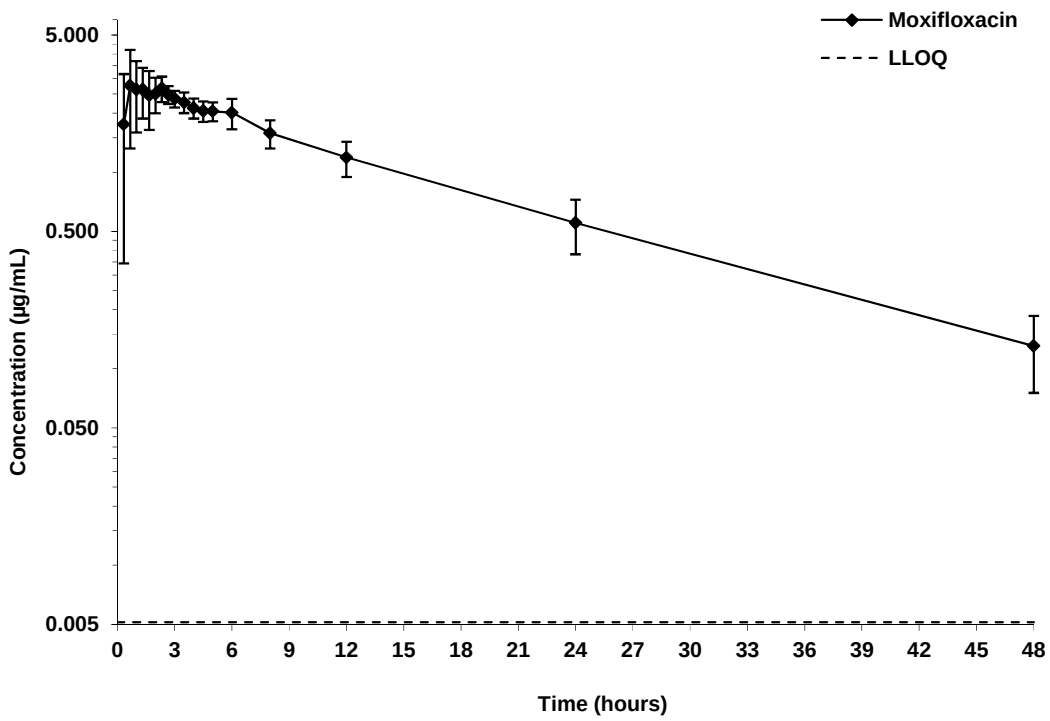
Figure 40 *MRM-chromatograms of moxifloxacin in human plasma: (A) Blank plasma sample, (B) LLOQ (0.00514 µg/mL), (C) Subject plasma sample (t=0.67h, 4.31 µg/mL), (D) Subject plasma sample (t=48h, 0.204 µg/mL)*



5.1.6.3 Pharmacokinetic results

Figure 41 shows the mean plasma concentration profile of moxifloxacin after oral administration of a 400-mg moxifloxacin tablet to 9 healthy volunteers. The mean peak concentration (C_{max}) of 3.47 ± 0.72 µg/mL was attained at 1.37 ± 0.89 hours after administration. Table 4 shows the pharmacokinetic parameters of moxifloxacin after oral administration in comparison to pharmacokinetic parameters reported in previously published studies [292-298].

Figure 41 *Plasma profile (mean±SD) of moxifloxacin concentrations versus time after administration of a 400-mg oral moxifloxacin dose to healthy volunteers (n=9)*



The pharmacokinetic data from the current study are in good agreement with previously reported values for a 400-mg oral moxifloxacin dose. The maximum concentration of 3.47 ± 0.72 µg/mL lies within the range of other studies, i.e. from 2.50 ± 1.31 [293] up to 4.98 ± 1.01 µg/mL [297], which may derive from different tablet formulations. The mean t_{max} in the present study of 1.37 ± 0.89 hours is also comparable with other data between 0.5 hours [295] and 2.0 hours [294]. The present AUC_{∞} (42.59 ± 8.59 µg·h/mL) is slightly higher than the AUC_{∞} from Stass et al. [293-295], i.e. between 26.9 ± 1.18 µg·h/mL and 30.9 ± 1.12 µg·h/mL, but the present data is in good agreement with the data reported for four independent other studies [292, 296-298]. In healthy volunteers with a gastric bypass the AUC_{∞} of moxifloxacin (46.2 ± 1.4 µg·h/mL) was higher compared to data from other studies.

The authors suppose that due to the gastric bypass the bile is secreted more distally influencing the enterohepatic recirculation of moxifloxacin [341-342]. This would also explain the longer half-life of 14 h compared to most other studies [292, 295-298, 343].

5.1.7 Summary: Moxifloxacin

The developed and validated LC-MS/MS assay for moxifloxacin in human plasma is simple and fast (precipitation of plasma and 2.0 min run-time), reliable, sensitive, precise and accurate. The method employes simple protein precipitation, which allows quantification of moxifloxacin in human plasma for concentrations ranging from 0.005 to 5.00 µg/mL. The principal advantage of the LC-MS/MS method described here is the simultaneous achievement of high sensitivity (LLOQ 0.005 µg/mL) and a low sample volume (100 µL), and high inter-day precision (7.9 %) and accuracy (99.0 %) at the LLOQ as well as excellent linearity within a short run time of 2.0 minutes. These characteristics make the method suitable for the precise and accurate measurement of low moxifloxacin concentrations in human plasma samples from pharmacokinetic studies. Similarly, the method should be feasible for samples from animals. The method was successfully applied to the analysis of more than 700 human plasma samples during a pharmacokinetic study, of which the results were in good agreement with comparable studies. The mean peak concentration of 3.47 ± 0.72 µg/mL was attained at 1.37 ± 0.89 hours after administration. The area under the curve was 42.59 ± 8.59 µg·h/mL and the terminal elimination half-life was determined to be 11.2 hours. The efficacy of fluoroquinolones can be described by the C_{max}/MIC and AUC/MIC [13], whereas an AUC/MIC of 125 and a C_{max}/MIC of is assumed be necessary for efficient bacterial killing rates. The C_{max}/MIC is given in Table 59 for different micro-organisms.

Table 59 *C_{max}/MIC for a 400-mg oral moxifloxacin dose and different micro-organisms*

Micro-organism	**MIC_{90}, µg/mL**	**C_{max}/MIC**
Streptococcus pneumoniae [344]	0.125	28
Fusobacterium spp. (anaerobe) [263]	2	1.75
Clostridium spp. (anaerobe) [263]	32	0.1

MIC: minimal inhibitory concentration of a given micro-organism, C_{max}: maximal observed plasma concentration

Table 60 *Pharmacokinetic parameters (mean±SD) of moxifloxacin in healthy subjects after administration of a single oral dose*

Reference	Subjects / Gender BMI [kg/m²]	Dosage / Formulation	t_{last} [h]	$AUC_{0\rightarrow last}$ [µg h/mL]	$AUC_{0\rightarrow\infty}$ [µg h/mL]	C_{max} [µg/mL]	t_{max} [h]	$t_{1/2}$ [h]	Analytical Method
Current study	9/m	400 mg / film-coated tablet	48	40.53 ± 7.61	42.59 ± 8.59	3.47 ± 0.72	1.37 ± 0.89	11.2	LC-MS/MS
[345] Kanjanawart 2013	20 (Thai)	400 mg reference tablet 400 mg test tablet	34	51.93 49.73	54.46 51.87	4.21 4.04	2.0 (0.25-8.00)[a] 2.0 (0.25-6.00)[a]	n.r.	LC/FD
[341] De Smet 2012	12 / m,f / 23-38 with gastric bypass	400 mg / film-coated tablet	72	n.r.	46.2 ± 1.4	3.38 ± 1.41	1.75 (0.75-4.00)[a]	14.1 ± 1.3	LC/FD
[308] 2011 Majcher-Peszynska	28 / m,f diabetic elderly	400 mg / tablet	24	29.36 (steady state)	n.r.	2.69	2.0 (1.0-8.0)[a]	n.r.	n.r.
[343] Burkhardt 2005	12 / m,f / 22.2 (m), 25.9 (f)	400 mg / tablet (intact)	48	n.r.	39.6 ± 1.13	3.20 ± 1.12	1.75 (0.50-4.00)[a]	9.86	LC/FD
[298] Burkhardt 2002	12 / m / 24.2 (mean)	400 mg / tablet	24	28.2 ± 4.1	35.6 ± 6.5	3.10 ± 0.60	1.67 ± 0.96	10.6	LC/FD
[295] Stass 2001	8 / m / n.r.	400 mg / n.r.	24	n.r.	30.9 ± 1.12	3.10 ± 1.34	0.5	9.6 ± 1.12	LC/FD
[292] Lubasch 2000	12 / m,f / 67.4 kg,1.81 m²	400 mg / n.r.	48	n.r.	39.3 ± 5.35	4.34 ± 1.61	1.02 ± 0.72	9.15 ± 1.62	LC/FD
[289] Ballow 1999	10 / m / within 15% of normal	100 mg / two 50mg film-coated round tablets	48	39.44[b]	43.00[b]	4.6[b]	0.86	13.5	LC/FD
[296] Sullivan 1999	15 / m,f / 24.5 (m), 24.7 (f)	400 mg / encapsuled tablet	24	30.24 (14.2)[c]	36.68 (13.2)[c]	3.36 (21.5)[c]	1.49 (62.2)[c]	9.30 (12.1)[c]	LC/FD
[297] Wise 1999	8 / m / 23.5 (mean)	400 mg / tablet	24	39.0±2.16	45.49 ± 4.68	4.98 ± 1.01	1.0 ± 0.91	8.32 ± 1.70	Micro
[294] Stass 1999	12 / m / 25.7 (mean)	400 mg / tablet	96	n.r.	29.8 ± 1.25	2.50 ± 1.29	2.0	15.6 ± 1.15	LC/FD
[293] Stass 1998	7 / m / 24.6 (mean)	400 mg / n.r.	72	n.r.	26.9 ± 1.18	2.50 ± 1.31	1.50	13.1 ± 1.06	LC/FD

m: male, f: female, n.r.: not reported, [a]: median, range in parentheses, [b]: dose-corrected to 400 mg, [c]: percent CV in parentheses, LC-MS/MS: liquid chromatography tandem mass spectrometry, LC/FD: liquid chromatography with fluorescence detection, Micro: Microbiological assay

6 SUMMARY

In the present thesis the development and validation of bioanalytical LC-MS/MS methods for the quantification of erythromycin A, erythromycin ethylsuccinate, roxithromycin, clarithromycin, 14-hydroxy clarithromycin, flucloxacillin, piperacillin and moxifloxacin in human plasma and human urine (piperacillin) is introduced. All methods were applied to analyze human plasma and urine samples from clinical trials and therefore, have been validated according to international guidelines. The methods were reliable in these studies and fulfilled all regulatory requirements known at the time of the study conduct.

Table 61 *Summary of the properties of the drugs and the developed LC-MS/MS methods*

	ERY A	**EES**	**ROX**	**CLA**	**14-OH CLA**	**FLU**	**PIP**	**MOX**
pKa	8.8	7.1	9.2	8.4 - 8.99	-	2.7	2.9	6.3 / 9.3
logP	3.06	2.1	2.75	3.16	-	2.58	0.22	0.83
Ionisation	APCI(+)	APCI(+)	APCI(+)	APCI(+)	APCI(+)	ESI(-)	ESI(-)	ESI(+)
LLOQ (ng/mL)	0.156	0.098	0.395	0.781	0.390	10.0	50.0	5.0
Run time (min)	3.0	3.0	2.2	3.0	3.0	2.2	3.2	2.0
Sample vol. (mL)	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Inter-day Precision (%)	3.9 to 12.4	1.6 to 9.7	2.8 to 8.1	2.2 to 12.0	5.0 to 10.5	4.0 to 8.6	3.7 to 10.7	4.4 to 7.9
Inter-day Accuracy (%)	96.9 to 104.5	100.8 to 104.2	98.3 to 107.2	96.0 to 101.4	91.1 to 99.5	97.6 to 101.4	93.0 to 101.0	92.4 to 108.0

-: not known; no available data in the literature, pKa: logarithmic acid dissociation constant, logP: partition-coefficient in octanol-water, LLOQ: lower limit of quantification, APCI(+): atmospheric pressure chemical ionisation in the positive-ion mode, ESI(-): electrospray ionisation in the negative-ion mode, ERY A: erythromycin A, EES: erythromycin ethylsuccinate, ROX: roxithromycin, CLA: clarithromycin, 14-OH CLA: 14-hydroxy clarithromycin, FLU: flucloxacillin, PIP: piperacillin, MOX: moxifloxacin

Moreover, the validation data of the macrolides were compared on three different mass spectrometers (API III Plus, API 3000™, API 5000™). The new innovations in the ion source (horizontal versus vertical electrospray), the ionpath (skimmer, QJet) and the diameter of the orifice resulted in better sensitivity and a larger linearity range for the majority of the analytes. Sensitivity was improved up to a factor of 12 (for clarithromycin) between API III Plus to API 3000™ and up to a factor of 8 (for erythromycin and roxithromycin) between API 3000™ and API 5000™, keeping the accuracy and precision data at about the same level. The high sensitivity was a

benefit for example for the flucloxacillin study, because concentrations from all subject samples were detectable up to approximately eight half-lives, i.e. no concentrations needed to be reported below the quantification limit. Also the linearity range were extended from two orders of magnitude to up to four orders of magnitude, which increases the likelihood to allow to analyze all samples from a pharmacokinetic study in the same run.

This is especially useful if a large concentration range needs to be analysed, for example, if the method shall be applied in an ascending dose study. Then, all low concentrations from the beginning of the study can be determined, as well as all high concentrations, without the need to dilute and analyse single samples repeatedly.

The pharmacokinetic data were compared to previously reported literature data and correlated graphically with MIC values of popular microorganisms which might be a starting point for further PK/PD investigations.

Table 62 *Summary of chemical and pharmacokinetic properties*

	ERY A (EES)	**ROX**	**CLA**	**14-OH CLA**	**FLU**	**PIP**	**MOX**
pKa	8.8	9.2	8.4 - 8.99	-	2.7	2.9	6.3 / 9.3
logP	3.06	2.75	3.16	-	2.58	0.22	0.83
Acid stability	low	improved	good	-	improved	no	good
Bioavailability (%)	25-50	60	52-55 (first-pass)	-	50	Low	86
PPB (%)	54-74	73-97	41-72	-	95	16-21	47
*Dose (mg)	500 po	300 po	250 po	-	250 po	4000 iv	400 po
*C_{max} (µg/mL)	0.34-0.71	7.3-12	0.72-1.21	0.46-0.76	7.4-14	223-322	2.5-5.0
*t_{max} (hours)	0.5-1.3	1.3-3.0	1.6-2.6	1.9-2.6	0.7-0.9	-	1.0-1.7
*AUC_{∞} (µg·h/mL)	2.1-3.4	98-277	4.0-8.2	3.2-6.0	15.4-29.8	278-640	29.8-45.5
*$t_{1/2}$ (hours)	1-2	10-18	3-5	3-6	1.3-1.9	0.8-1.8	8.3-15.6
Non-linear PK	negligible at therap. conc.	saturable PPB >4µg/mL	Autoinhib. CYP3A4	-	Linear up to 1g	saturable renal elimin.	-

*: investigated here, -: not known; no available data in the literature, pKa: logarithmic acid dissociation constant, logP: partition-coefficient in octanol-water, PPB: plasma protein binding, C_{max}: maximal observed plasma concentration, t_{max}: time to peak concentration, AUC_{∞}: area under the curve from time of administration up to time infinity, $t_{1/2}$: half-life, PK: pharmacokinetics, ERY A: erythromycin A, EES: erythromycin ethylsuccinate, ROX: roxithromycin, CLA: clarithromycin, 14-OH CLA: 14-hydroxy clarithromycin, FLU: flucloxacillin, PIP: piperacillin, MOX: moxifloxacin

The PK/PD theory is a very helpful tool for prediction of the efficacy of given drugs against certain micro-organisms. Depending on the pharmacodynamic processes, e. g. the mode of action, three classes of drugs have been identified:

Table 63 *Classes of antimicrobial agents related to PK/PD parameters*

Type	PK/PD parameter	Antibiotics	How to maximise efficacy
I	T>MIC	β-Lactams Macrolides	dose more frequently, or prolong $t_{1/2}$, or infuse continuously
II	C_{max}/MIC	Fluoroquinolones	Maximise concentrations, e. g. intravenously
III	AUC_{0-24}/MIC	Macrolides	Maximise cumulative dose, i. e. prolong $t_{1/2}$

PK: pharmacokinetic, PD: pharmacodynamic, T>MIC: Duration of time when the drug concentration exceeds the minimal inhibitory concentration of a given micro-organism, C_{max}: maximal observed plasma concentration, AUC_{0-24}: area under the curve from time of administration up to 24 hours, $t_{1/2}$: half-life

In the same way this applies to adverse effects, which need to be minimised by reducing plasma concentrations. These coherences are not well-investigated, yet, and are not discussed further in this thesis.

Still, a lot of research has to be done in this interdisciplinary field to minimise uncertainty in single values, like an AUC/MIC. These include:

Improve accuracy and precision of bioanalytical methods determining total and free concentration data in biological matrices for calculation of AUC and C_{max}

These parameters are related to the MIC in pharmacodynamic considerations. Since the determination of the MIC often underlies significant variations and also differences between microbiological laboratories, the determination of concentrations of anti-infectives is particular important, being achievable by scientific exact techniques. Finally, from the volume of distribution of antibiotics can be used to derive information about intracellular concentrations and effectivity of antiinfectives.

7 ZUSAMMENFASSUNG

In der vorliegenden Arbeit wird die Entwicklung und Validierung von bioanalytischen LC-MS/MS-Methoden zur Quantifizierung von Erythromycin A, Erythromycin-ethylsuccinat, Roxithromycin, Clarithromycin, 14-Hydroxy-clarithromycin, Flucloxacillin, Piperacillin und Moxifloxacin in Humanplasma und Humanurin (Piperacillin) vorgestellt. Alle Methoden wurden für die Analyse von Plasma- und Urinproben aus klinischen Studien beim Menschen eingesetzt und deshalb nach international anerkannten Richtlinien validiert. In diesen Studien haben sich die Methoden bewährt und alle Anforderungen der zum Zeitpunkt der Studie bekannten behördlichen Ansprüche erfüllt.

Table 64 *Übersicht über die Wirkstoffeigenschaften und die LC-MS/MS Methoden*

	ERY A	**EES**	**ROX**	**CLA**	**14-OH CLA**	**FLU**	**PIP**	**MOX**
pKs	8.8	7.1	9.2	8.4 - 8.99	-	2.7	2.9	6.3 / 9.3
logP	3.06	2.1	2.75	3.16	-	2.58	0.22	0.83
Ionisierung	APCI(+)	APCI(+)	APCI(+)	APCI(+)	APCI(+)	ESI(-)	ESI(-)	ESI(+)
LLOQ (ng/mL)	0.156	0.098	0.395	0.781	0.390	10.0	50.0	5.0
Messzeit (min)	3.0	3.0	2.2	3.0	3.0	2.2	3.2	2.0
Probenvol. (mL)	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100
Inter-day Präzision (%)	3.9 to 12.4	1.6 to 9.7	2.8 to 8.1	2.2 to 12.0	5.0 to 10.5	4.0 to 8.6	3.7 to 10.7	4.4 to 7.9
Inter-day Richtigkeit (%)	96.9 to 104.5	100.8 to 104.2	98.3 to 107.2	96.0 to 101.4	91.1 to 99.5	97.6 to 101.4	93.0 to 101.0	92.4 to 108.0

-: nicht bekannt; keine Daten in der Literatur verfügbar, pKs: Logarithmus der Säuredissoziationskonstante, logP: Oktanol-Wasser Verteilungs-Koeffizient, LLOQ: Untere Bestimmungsgrenze, APCI(+): Chemische Ionisierung bei Atmosphärendruck im Positiv-Ionen-Modus, ESI(-): Elektrospray Ionisierung im Negativ-Ionen-Modus, ERY A: Erythromycin A, EES: Erythromycin Ethylsuccinate, ROX: Roxithromycin, CLA: Clarithromycin, 14-OH CLA: 14-Hydroxy clarithromycin, FLU: Flucloxacillin, PIP: Piperacillin, MOX: Moxifloxacin

Die Validierungsdaten der Makrolid-Methoden konnten zudem an drei Massenspektrometern der selben Baureihe verglichen werden. Dabei zeigte sich, dass die Neuerungen an der Ionenquelle (horizontale versus vertikale ESI), dem Ionenpfad (Skimmer, QJet), sowie das immer größere Orifice in der Baureihe, nicht nur stetig verbesserte Sensitivität ermöglichen, sondern auch immer größere Linearitätsbereiche. So konnte ein Sensitivitätsgewinn bis zu Faktor 12 (Clarithromycin) von API III Plus auf API 3000™ und bis zu Faktor 8 (Erythromycin

und Roxithromycin) von API 3000™ auf API 5000™ bei etwa gleichen Werten für die Präzision erreicht werden. Die hohe Sensitivität zeigte sich zum Beispiel bei der Flucloxacillin Studie von Vorteil, da alle Konzentrationen bis circa acht Halbwertszeiten nach Wirkstoffgabe bestimmt werden konnten, ohne dass einzelne Proben als „BLOQ“ (unter dem Quantifizierungslimit) berichtet werden mussten.

Während früher noch Linearitätsbereiche um zwei Größenordnungen üblich waren, sind jetzt am API 5000™ Konzentrationsbereiche über bis zu vier Größenordnungen reproduzierbar linear. Das ist insbesondere von Nutzen, wenn bei einer Substanz ein größerer Konzentrationsbereich gemessen werden muss. Das ist beispielsweise dann der Fall, wenn die Methode für eine Dosissteigerungsstudie eingesetzt werden soll. Dann können sowohl alle kleinen Konzentrationen zu Beginn der Studie gemessen werden, als auch alle hohen Konzentrationen zum Ende der Studie, und zwar ohne, dass einzelne Proben gesondert verdünnt und wiederholt gemessen werden müssen.

Table 65 *Übersicht der chemischen und pharmakokinetischen Eigenschaften*

	ERY A (EES)	**ROX**	**CLA**	**14-OH CLA**	**FLU**	**PIP**	**MOX**
pKs	8.8	9.2	8.4 - 8.99	-	2.7	2.9	6.3 / 9.3
logP	3.06	2.75	3.16	-	2.58	0.22	0.83
Säurestabilität	niedrig	besser	gut	-	besser	keine	gut
Bioverfügbarkeit (%)	25-50	60	52-55 (first-pass)	-	50	niedrig	86
PPB (%)	54-74	73-97	41-72		95	16-21	47
*Dosis (mg)	500 po	300 po	250 po	-	250 po	4000 iv	400 po
*C_{max} (µg/mL)	0.34-0.71	7.3-12	0.72-1.21	0.46-0.76	7.4-14	223-322	2.5-5.0
*t_{max} (hours)	0.5-1.3	1.3-3.0	1.6-2.6	1.9-2.6	0.7-0.9	-	1.0-1.7
*AUC_{∞} (µg·h/mL)	2.1-3.4	98-277	4.0-8.2	3.2-6.0	15.4-29.8	278-640	29.8-45.5
*$t_{1/2}$ (hours)	1-2	10-18	3-5	3-6	1.3-1.9	0.8-1.8	8.3-15.6
Nicht lineare PK	Vernach-lässigbar bei therap. Konz.	Sättigb. PPB >4µg/mL	Autoinhib. CYP3A4	-	Linear bis 1g	Sättigb. renale Elimin.	-

*: hier untersucht, -: nicht bekannt; keine Daten in der Literatur verfügbar, pKs: Logarithmus der Säuredissoziationskonstante, logP: Oktanol-Wasser Verteilungs-Koeffizient, PPB: Plasmaprotein-bindung, C_{max}: höchste gemessene Plasmakonzentration, t_{max}: Zeitpunkt der höchsten Plasmakonzentration, AUC_{∞}: Fläche unter der Konzentrations-Zeit-Kurve vom Zeitpunkte der Gabe bis Unendlich, $t_{1/2}$: Halbwertszeit, PK: Pharmacokinetik, ERY A: Erythromycin A, EES: Erythromycin Ethylsuccinate, ROX: Roxithromycin, CLA: Clarithromycin, 14-OH CLA: 14-Hydroxy clarithromycin, FLU: Flucloxacillin, PIP: Piperacillin, MOX: Moxifloxacin

Die pharmakokinetischen Daten der Antibiotika wurden in den Kontext mit Literaturdaten gestellt und graphisch mit MHK-Werten für gängige Mikroorganismen verglichen, was den Ausgangspunkt für spätere PK/PD-Untersuchungen darstellt.

Für die Vorhersage der Wirksamkeit einer verabreichten Substanz gegenüber bestimmten Mikroorganismen ist die PK/PD-Korrelation ein gutes Hilfsmittel. Anhand der pharmakodynamischen Parameter, wie der Wirkungsweise, können drei Antibiotikaklassen gebildet werden.

Table 66 *Einteilung von Antiinfektiva in PK/PD Parameter*

Klasse	PK/PD Parameter	Antibiotika	Wirksamkeit maximieren durch
I	T>MHK	β-Lactame Makrolide	Häufiger dosieren, oder $t_{1/2}$verlängern oder Dauerinfusion
II	C_{max}/MHK	Fluoroquinolone	Konzentrationen maximieren, z. B. intravenöse Verabreichung
III	AUC_{0-24}/MHK	Makrolide	Dosis erhöhen oder $t_{1/2}$ verlängern

PK: Pharmacokinetik, PD: Pharmacodynamik, T>MIC: Zeitdauer wenn die Antibiotikakonzentration über der minimalen Hemmkonzentration eines bestimmten Bakteriums liegt, C_{max}: höchste gemessene Plasmakonzentration, AUC_{0-24}: Fläche unter der Konzentrations-Zeit-Kurve vom Zeitpunkt der Wirkstoff-Gabe bis 24 Stunden danach, $t_{1/2}$: Halbwertszeit

In analoger Weise gilt für die Nebenwirkungen, dass die Plasma-Konzentrationen verringert werden müssen. Diese Zusammenhänge sind jedoch weniger gut untersucht und werden in dieser Dissertation nicht näher diskutiert.

In diesem interdisziplinären Feld ist immer noch viel Forschung nötig um beispielsweise Unsicherheiten bei einzelnen Werten wie z.B. AUC/MIC zu minimieren. Diese beinhalten:

Verbesserung der Genauigkeit und Präzision von bioanalytischen Methoden welche für die Bestimmung der Gesamtkonzentration und der freien Konzentration in biologischen Matrices verwendet werden um AUC und C_{max} zu bestimmen.

Diese Parameter werden in pharmakodynamischen Überlegungen in Beziehung zur MHK gesetzt. Da die MHK-Bestimmung oft erheblichen Schwankungen und auch Unterschieden zwischen verschiedenen mikrobiologischen Laboratorien unterliegt, kommt der Konzentrationsbestimmung der Antibiotika besondere Bedeutung zu, weil sie mit naturwissenschaftlich exakten Methoden möglich ist. Aus den Verteilungsvolumina von Antibiotika lassen sich schließlich auch noch Informationen über die intrazelluläre Konzentration und Wirksamkeit von Antibiotika ableiten.

List of abbreviations

AAPS	American Association of Pharmaceutical Scientists
AC	Alternating current
Ae	Amount (of drug) excreted
AFSSAPS	Agence française de sécurité sanitaire des produits de santé (French Drug and Health Products Safety Agency, until 05/2012)
amu	Atomic mass units
ANOVA	Analysis of variance
ANSM	Agence nationale de sécurité du médicament et des produits de santé (National Drug and Health Products Safety Agency, France)
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photo ionisation
ATP	Adenosine triphosphate
AUC	Area under the curve
$AUC_{0\text{-last}}$	AUC from time of administration up to the time of the last quantifiable concentration
$AUC_{0\text{-}\infty}$	AUC from time of administration up to time infinity
BfArM	Bundesinstitut für Arzneimittel und Medizinprodukte (Federal Institute for Drugs and Medical Devices Germany)
bid	Bis in die (twice daily)
BMV	Bioanalytical method validation
BQC	Biological quality control
CAD	Collision-activated dissociation (MS)
CEM	Channel electron multiplier (MS)
CHMP	Committee for Medicinal Products for Human Use (committee of the EMA)
CID	Collision-induced dissociation
CL	Clearance

CLA	Clarithromycin
CLA-MET	14-hydroxy metabolite of clarithromycin
C_{last}	Last quantifiable plasma concentration
C_{max}	Maximal observed plasma concentration
CPMP	Committee for Proprietary Medicinal Products (committee of the EMA, since 2004 named CHMP)
CR	Calibration row
CS	Column switching
cSSSI	Complicated skin and skin structure infections
CV	Coefficient of variation
CYP	Cytochrome P450
D	Dose
DC	Direct current
Dil	Dilution
DP	Declustering potential
EBF	European Bioanalysis Forum
EDTA	Ethylene diamine tetra acetic acid
EES	Erythromycin ethylsuccinate
EI	Electron ionisation
ELF	Epithelial lining fluid
EMA	European Medicines Agency
ERY A	Erythromycin A
ESI	Electrospray ionisation
FDA	US Food and Drug Administration
FLU	Flucloxacillin
FT	Freeze-thaw (stability)
g	Gravitational acceleration
GBC	Global Bioanalysis Forum

GC	Gas chromatography
GCP	Good clinical practice
GLP	Good laboratory practice
GMP	Good manufacturing practice
HN	Heated Nebulizer® (APCI source)
HPLC	High performance liquid chromatography
IBMP	Institute for Biomedical and Pharmaceutical Research
IUPAC	International Union of Pure and Applied Chemistry
ICH	International Conference on Harmonization (of technical requirements for registration of pharmaceuticals for human use within the EU, Japan and the US)
IS	Internal standard
IUPAC	International union of pure and applied chemistry
iv	Intravenous
JBC	Japanese Bioanalysis Forum
k_{el}	Elimination rate constant
LC	Liquid chromatography
LC/ED	Liquid chromatography with electrochemical detection
LC/FD	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
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
ln	Natural logarithm
LOD	Limit of detection
log	Logarithm
logP	Partition-coefficient in octanol-water

LT	Long-term (stability)
MAH	Marketing authorisation holder
MALDI	Matrix-assisted laser desorption ionisation (MS)
MCA	Multi channel acquisition
ME	Matrix effect
MF	Matrix factor
MHLW	Ministry of Health, Labour and Welfare, Japan
MHRA	Medicines and Healthcare Products Regulatory Agency, UK
MIC	Minimum inhibitory concentration
MOX	Moxifloxacin
MRM	Multiple-reaction-monitoring
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
m/z	Mass to charge ratio
OECD	Organisation for Economic Cooperation and Development
OR	Orifice voltage
PD	Pharmacodynamics
PIP	Piperacillin
PK	Pharmacokinetics
pKa	Logarithmic acid dissociation constant
po	Peroral
PPB	Plasma protein binding
PPT	Protein precipitation
Q	Blood flow
Q1	First quadrupole of a triple-quadrupole mass spectrometer
qd	Quaque die (every day, once a day)
qid	Quarter in die (four times daily)
rcf	Relative centrifugal force (in multiples of g)

RE	Relative error
RF	Radio frequency
RNA	Ribonucleic acid
ROX	Roxithromycin
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SL	Stock solution
S/N	Signal-to-noise ratio
SOP	Standard operating procedure
SPE	Solid phase extraction
SQC	Spiked quality control
$t_{1/2}$	Half-life
tid	Ter in die (three times daily)
TISP	TurboIonSpray® (ESI source)
t_{max}	Time to peak concentration
TOF	Time of flight
ULOQ	Upper limit of quantification
VAL	Validation
V	Volume of distribution

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Full Papers

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