Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

an der medizinischen Fakultät der

Ludwig-Maximilians-Universität München

Development and application of a stability-indicating assay method for meropenem and its open-ring metabolite

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List of abbreviations

API	atmospheric pressure ionization
EMA	European Medicines Agency
ESI	electrospray ionization
FDA	Food and Drug Administration
GFR	glomerular filtration rate
ICH	International Conference on Harmonization
LC-MS/MS	liquid chromatography tandem mass spectrometry
MIC	minimal inhibitory concentration
m/z	mass-to-charge-ratio
ORM	open-ring metabolite
PBP	penicillin-binding protein
PD	pharmacodynamics
PK	pharmacokinetics
SIAM	stability-indicating assay method
SID	stable-isotope dilution
SIL-IS	stable isotopically labeled internal standard
TAT	total turnaround time
TDM	therapeutic drug monitoring

Publications

Publication I

S. Rakete, C. Schuster, M. Paal, M. Vogeser, An isotope-dilution LC-MS/MS method for the simultaneous quantification of meropenem and its open-ring metabolite in serum, Journal of Pharmaceutical and Biomedical Analysis 197 (2021) 113944.

Publication II

U. Liebchen, S. Rakete, M. Vogeser, F.M. Arend, C. Kinast, C. Scharf, M. Zoller, U. Schönermarck, M. Paal, The Role of Non-Enzymatic Degradation of Meropenem—Insights from the Bottle to the Body, Antibiotics 10(6) (2021) 715.

1. Contribution to publications

1.1 Contribution to publication I

The author of this doctoral thesis was responsible for all parts of the described publication: project planning and conceptualization, experimental laboratory work (e.g. ISD-HPLC-MS/MS method development including all aspects of sample preparation), planning and performing the method validation according to the “Guideline on bioanalytical method validation” from the European Medicines Agency [1], data acquisition, analysis and evaluation, manuscript writing, submission and editing.

1.2 Contribution to publication II

The doctoral candidate contributed substantially in the processes of literature research, methodology, formal evaluation and analysis of data and writing of this publication.

2. Introduction

2.1 Background

2.1.1 Therapeutic drug monitoring of antibiotics

With a history of more than forty years, the measurement of drug concentrations in biological samples called therapeutic drug monitoring (TDM) is still a developing field and is increasingly important for the administration of pharmaceuticals, e.g. antibiotics [2, 3]. In this context, the initial objective of TDM was, specifically for aminoglycosides, the prevention of toxic effects and increased mortality risks due to narrow therapeutic ranges, i.e. the gap between the minimum effective and the minimum toxic concentration [4]. Nowadays, the paradigm has changed towards the avoidance of treatment failure caused by sub-therapeutic drug concentrations. Further reasons for TDM have emerged, e.g. understanding drug-drug interactions in multidrug therapy, monitoring alternate administration of antibiotics including continuous infusion or preventing the formation of resistance [5-7]. Low antibiotic susceptibilities or high inter-and intra-patient pharmacokinetic heterogeneities may impair therapeutic outcomes. Particularly in critically ill patients, the latter plays an important role, as deviating blood concentrations may occur due to unpredictable pathophysiological changes. This includes, among others, an altered renal clearance or volume of distribution, which can be affected by a systemic inflammatory response [8]. In this case, imminent adjustment of the dosage is critical to prevent adverse effects such as resistances or therapy failure.

In this context, the concept of personalized medicine is drawing attention, providing the potential for individualized dosage adjustments [9]. Generally, the effect of a drug can be described by its pathway through the body, known as the pharmacokinetics (PK), and its biochemical and physiological impact on the body, known as the pharmacodynamics (PD) [10]. **Figure 1** illustrates this relationship, which is known as the PK/PD target. Besides pharmacogenomics (age, gender or weight), pathophysiological changes influence PK. A successful outcome also depends on the selection of a suitable antibiotic. TDM can help to monitor and achieve therapeutic drug levels defined by the minimal inhibitory concentration (MIC) of a pathogen, which depends on the PD [7, 11]. All factors must be identified and addressed to achieve an optimal outcome with low toxicity and high efficacy. The modeling of PK/PD parameters by means of TDM is a supportive and commonly used tool to address the individual characteristics of a patient and therefore ensure an efficient and safe administration of antibiotics [12, 13].

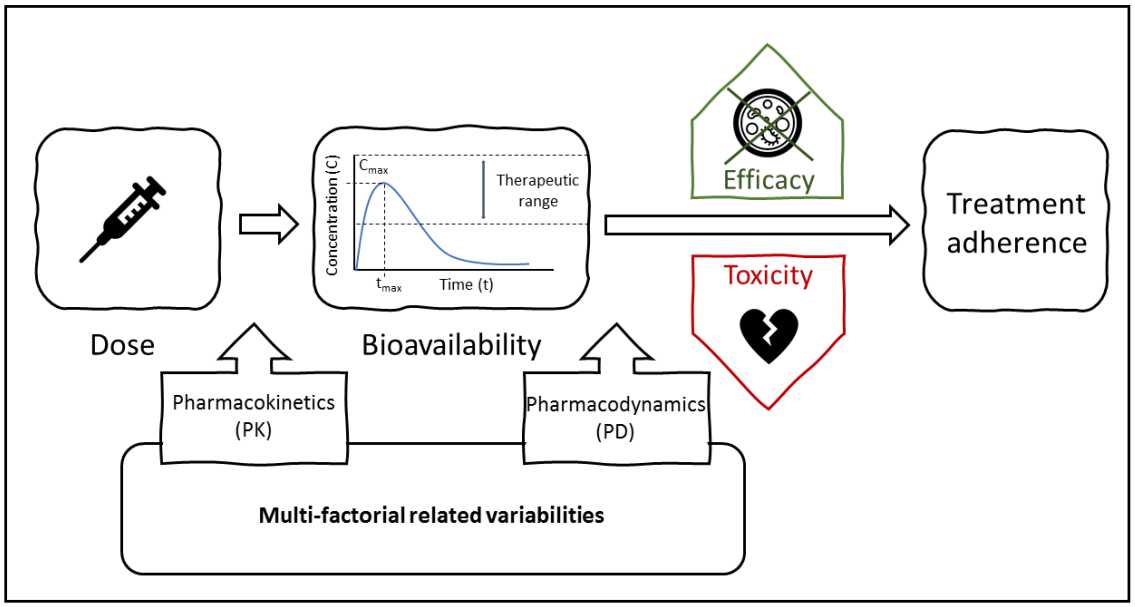

Figure 1 Procedure for establishing a dosing regimen considering the dose-concentration/exposure and concentration/exposure-effect relationship and influencing factors with the aim of maintaining a treatment adherence with high efficacy and low toxicity (illustration based on [14]).

Short total turnaround times (TAT), i.e. the time between sampling and the analytical result, are required for efficient TDM. Keeping the TAT to a minimum enables faster dose adjustments, which is enormously important for critically ill patients. In addition to pre-analytics, particularly the consideration of the stability of the target analytes, the selection of a suitable measurement technique is an essential aspect. Since the early 2000s, liquid chromatography tandem mass spectrometry (LC-MS/MS), a mainly matrix-independent technique, is increasingly important in routine laboratory diagnostics of small molecules. Advantages compared to commonly used immunoassays include high selectivity and sensitivity, the possibility for (on-demand) multi-analyte methods including metabolite analysis, short acquisition times and an excellent accuracy by stable-isotope dilution (SID) [15].

In this process, liquid chromatography allows molecules, such as those contained in human serum, to be separated by interactions of varying strength with a stationary phase [16]. In the ion source of the mass spectrometer, reached after specific retention times, the analytes are ionized to positively or negatively charged ions, typically by electrospray (ESI) or atmospheric pressure ionization (API). For the technique of MS/MS, triple quadrupole systems are used almost exclusively in laboratory diagnostics [17]. Here, three quadrupoles are connected in series, each consisting of four metal rods arranged in parallel to which periodic voltages can be applied. This allows ions with selected specific mass-to-charge ratios (*m/z*) to pass through the quadrupole on stable paths. In this way, the first quadrupole functions as a mass analyzer. In contrast, the second quadrupole acts as a collision cell in which the pre-selected precursor ions fragment due to the collision with an inert gas and the application of specific collision voltages. The

fragments, also called product ions, are again selected in the third quadrupole and subsequently detected, e.g. by a photomultiplier [18, 19].

2.1.2 Efficacy and stability of beta-lactam antibiotics

Beta-lactam antibiotics, which represent about 65 % of the global market of antibiotic drugs [20], are a class with high tolerability and broad-spectrum activity, particularly against certain critical pathogens (e.g. *P. aeruginosa*) [21, 22]. Pharmacodynamically, this class belongs to the time-dependent bactericides. Therefore, a free drug concentration above a specific level at the site of action over a certain time period for therapeutic effects is required [23]. Currently, the general recommendation, specifically for patients with severe infections, aims to achieve high PK/PD targets with levels of 4-5 times the MIC (expressed as fT ≥ 4-5x MIC = 100 %) during antibiotic treatment [24]. In this context, the beta-lactam ring has a significant impact. Structurally, it mimics the terminal D-alanyl-D-alanine residue of the glycan chains, which is necessary for cell wall assembly [25]. As structure analogues, they can block the active center of the bacterial transpeptidases, an enzyme, which is responsible for cross-linking of the glycan chains given structure and stability of the cell wall. Consequently, bacterial replication and cell growth is irreversible inhibited, resulting in cell death [26]. Consequently, beta-lactam antibiotics do not affect cell wall-deficient pathogens. In general, antibacterial efficacy depends on three factors [27]:

- Dosing and penetration rate: How and in what time can the cell wall and periplasmic space be passed to reach the target enzyme?
- Site of action affinity: To which extent can specific target structures, e.g. penicillin-binding proteins (PBP), be linked?
- Stability: How stable are the active substances, especially against beta-lactamases, which represent the predominant resistance mechanism?

Carbapenems, a sub-group of beta-lactams, fulfill these criteria very well. They were introduced in the mid-1980s as a further development of penicillin by replacing the sulfur atom with a methylene group in the thiazolidine moiety and introducing a C2-C3 double bond [28]. The administration needs to be intravenous due to a low oral bioavailability. They are predominantly eliminated renally, unaltered and with short half-lives [29]. Carbapenems bind to a variety of PBPs and have great potency against key pathogens and severe nosocomial infections, e.g. sepsis caused by *Enterobacteriaceae*. Hence, they are known as “last-line agents” mainly used in intensive care medicine [30]. In addition, most carbapenems are very stable towards beta-lactamases and human dehydropeptidase-1, structurally based on a hydroxyethyl moiety and a 1-ß-methyl group (**Figure 2**) [31, 32]. However, carbapenem resistance is on the rise as a result of excessive administration, partly as a result of insufficient drug levels [30].

The fusion of the beta-lactam ring and a thiazolidine ring is critical for the stability of carbapenems since the non-planarity of the molecule and its high ring tension makes it extremely susceptible for nucleophilic attacks [33]. **Figure 2** exemplifies a hydrolytic

cleavage (R = H) of the carbapenem structure leading to the open beta-lactam ring product according to a pseudo-first order reaction. Instead of water, other nucleophiles can attack and the degradation can be affected by temperature, pH, buffers or oxidizing factors [34].

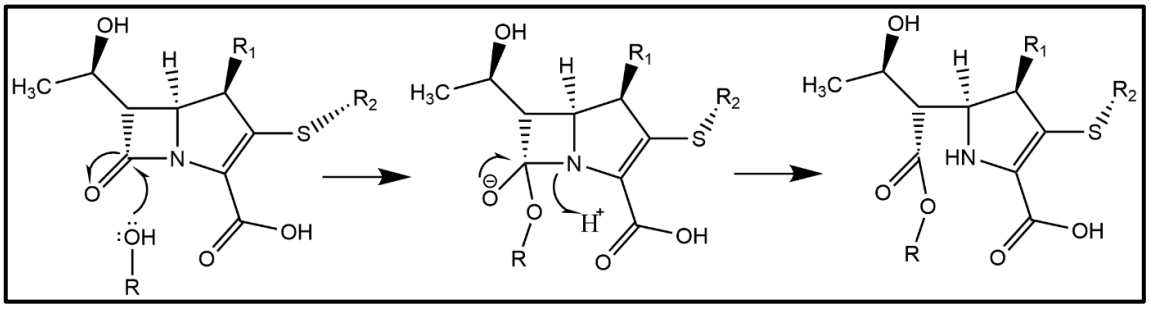

Figure 2 Degradation pathway of carbapenems such as meropenem by nucleophilic attack of the beta-lactam ring resulting in the open-ring product.

2.1.3 Stability studies

Stability studies of drugs are extremely important because degradation can occur both *in vitro* and *in vivo*. This concerns the entire pharmaceutical spectrum, from the need for market launch through medication and evaluation of pharmacokinetic properties up to TDM [33]. With regard to potential drug instabilities, degradation products have to be analyzed and evaluated, too. These studies can be applied for the investigation of impurities in the solid drug, instabilities in the infusion solution, metabolization and degradation in the body and pre-analytical stabilities of clinical samples and standards and reference materials [34]. The lack of this knowledge can result in insufficient concentrations in the patient, impairing therapeutic outcomes and increasing the risk of resistance.

International regulatory agencies, e.g. the International Conference on Harmonization (ICH), the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) have established guidelines for the requirement of developed and validated stability-indicating assay methods (SIAM). This involves methods that can quantitatively measure the change in an active ingredient and its degradation products over time accurately and without interference. [35-38] It is recommended to perform the SIAMs as accelerated decomposition studies, e.g. under the influence of pH, heat or light [35]. Nowadays, SIAMs without stress testing are also common [39]. In addition, the concept of isochronous stability studies is gaining importance, especially in industry and for candidate certified reference material [40]. This concept is based on the principle that all samples are measured at the same time after storage for different periods and temperatures. Following the different conditions, it is also important to store them at a reference temperature, at which stability is ensured, until analysis. As a result, day-to-day batch variations can be minimized, resulting in lower variability and higher significance of the data [41]. In general, -80 °C can be used as the reference temperature, e.g. for antibiotics, which generally show good stability at this condition. Although mainly

used in pharmaceutical settings, this concept is perfectly suitable for stability studies in the field of laboratory medicine.

2.1.4 Aim and scope

The main objective of this thesis was the investigation of the stability of meropenem and the establishment of a stability-indicating assay method for meropenem and its main degradation product applicable in hospital and industrial laboratories. A method for the simultaneous quantification of the active drug and the main degradation product can improve therapeutic efficacy by providing enhanced TDM and valuable information on drug stability and individual pharmacokinetics. This is an essential contribution both to the concept of personalized medicine and to the identification and prevention of pre-analytical challenges.

The aim of the first project was the development and validation of a quantitative and precise analytical method for the simultaneous analysis of meropenem and its open-ring metabolite (ORM), which is the main degradation product, suitable for routine diagnostics. Consequently, SID-LC-MS/MS was selected to ensure the highest metrological standard for TDM requirements. Understanding the degradation mechanisms of meropenem and measure the degradation by the assay was an essential part of this work. Additionally, a considerable technical aspect was the prevention of the degradation of meropenem throughout the TAT.

The objectives of the second project were (isochronous) *in vitro* and *in vivo* stability studies using the aforementioned validated SID-LC-MS/MS method. This involved the investigation of meropenem degradation and ORM formation from the beginning of the treatment, the antibiotic administration, to the end, the physiological concentrations. Furthermore, the percentage of the non-enzymatic degradation and the applicability of the method were evaluated.

2.2 An isotope-dilution LC-MS/MS method for the simultaneous quantification of meropenem and its open-ring metabolite in serum

The stability of meropenem in neat solutions, e.g. infusion solutions in particular, and in matrix-based samples was the subject of multiple studies under various conditions [42-47]. This includes investigations that qualitatively characterized possible degradation products. Mendez et al. identified a pyrrole derivative as a thermal degradation product and the open-ring derivative under alkaline and acidic conditions in aqueous solutions [48]. At higher concentrations of meropenem, a dimer may be formed as a result of intermolecular aminolysis caused by a nucleophilic attack of a second meropenem molecule [49]. Overall, the inactive open ring metabolite (ORM) is described as the main degradation product, mainly caused by hydrolysis as shown in section 2.1.2. This has been recently confirmed by direct infusion ESI-MS/MS [50].

Several technical aspects played a major role in the method development for the simultaneous quantification of meropenem and the ORM by SID-LC-MS/MS. Initially, the

availability of a target analogue with similar physicochemical properties, applied as an internal standard, is a requirement for robust and precise measurement results. Stable isotopically labeled internal standards (SIL-IS) are preferentially used. After the selection and tuning of the MS parameters for each target compound and its SIL-IS, chromatographic baseline separation of meropenem and the ORM needed to be achieved. A sufficient separation of both substances is extremely important, as so-called in-source transformation can occur in the ion source [51]. As a result, the ORM (*m/z* 402.3) may yield the same precursor mass as meropenem (*m/z* 384.2) by loss of water (-18 Da), leading to incorrect results. Several columns and mobile phases were tested for this purpose. Sufficient separation with a resolution of 2.5 [52] was obtained by using an XSelect HSS PFP column and 10 mM ammonium formate in water-formic acid (99.8/0.2, v/v) and methanol as mobile phases.

Furthermore, an essential part of the development was that meropenem remains stable during the TAT in order to exclude false results. For this purpose, the time from sampling to analysis must be kept as short as possible and optimal measurement conditions, e.g. temperature, must be ensured. Therefore, a fast and simple LC-MS/MS method was developed in human serum with an acquisition time of only 5.5 minutes. Protein precipitation was used as a fast sample preparation technique. For this purpose, different solvents and compositions were tested and methanol was selected at a ratio of 7.5:1 (v:v). In addition, the samples were stored between 4 and 10°C for most of the time. The stability of the stock solutions used for the preparation of the calibrator and quality control samples was guaranteed by using 0.9% NaCl solution according to Patel and Cook [43]. This was confirmed by measuring freshly prepared quality control samples of meropenem and ORM individually and in combination.

There is a lack for standardized validation protocols of LC-MS/MS methods of xenobiotics in the field of clinical laboratory diagnostics. Therefore, the method was validated according to the *Guideline on bioanalytical method validation* from the EMA commonly used for antibiotics [1]. All specifications were met for the clinically relevant range of 1-100 mg/l for meropenem and 0.62-62.30 mg/l for ORM. Accuracy and precision were ≥ 93.1 % for all quality control samples tested with an exception only for the lowest concentration of the ORM (precision ≥ 90.7 %). No significant matrix effects were noticed. This was supported by the aforementioned SIL-IS, a high sample dilution and low injection volume which contributed to an increased method robustness.

In addition, 35 anonymized intensive care patients' serum samples from routine meropenem TDM were analyzed for method comparison. Linear regression analysis showed a high correlation between both methods with a Pearson`s correlation coefficient of 0.99 and a slope of 0.85. Additionally, the ratio of ORM and meropenem was calculated, ranging from 19.7 % to 186.7%. These inter-individual patient ratios may be of interest for further clinical studies. Additionally, this method can be used to evaluate stability issues regarding pre-application or pre-analytics and external quality samples for inter-laboratory tests.

2.3 The Role of Non-Enzymatic Degradation of Meropenem – Insights from the Bottle to the Body

To maximize treatment outcomes, meropenem is usually administered by continuous infusion after a loading dose, especially in patients with severe illness [53]. Considerations about stability are a frequent concern, as degradation of meropenem in the infusion solution prior to administration may result in treatment failure when stored for too long [43]. *In vivo*, about 70 % of meropenem is renally excreted via the kidneys in its native form (renal elimination). The rest is metabolized to ORM (non-renal degradation) [54]. However, this percentage can rise up to 50% for patients with renal impairment [55]. Therefore, TDM-guided personalized medicine will become increasingly important for unpredictable PK and essential dose adjustments. To gain a deeper understanding of the degradation and metabolism of meropenem, stability was studied across all stages of antibiotic therapy. The precise and robust SID-LC-MS/MS method described before was perfectly suitable to evaluate the degradation of meropenem and the formation of ORM in different matrices.

For an optimal treatment success, the concentration of an antibiotic administered to the patient is already of utmost importance. Alongside, the initial concentration is needed for calculations regarding individual PK. Therefore, a 24-hour isochronous stability experiment was applied for *in vitro* infusate solutions of meropenem (2 %, w/v). From the regulatory perspective, the maximum acceptable degradation of 10 % was reached after 17.5 hours with a degradation rate of 0.6 % decay per hour [56, 57].

A 2.5 % decay per hour was found for buffered human serum at 37°C. The idea behind this 24-hour isochronous stability experiment was to mimic physiological conditions in the body *in vitro* and to investigate the spontaneous non-enzymatic degradation. Based on a population pharmacokinetic modelling and the investigated degradation rate, a modest influence of only 6 % was found for the spontaneous non-enzymatic degradation. As some studies reported concentration-dependent degradations of meropenem [47, 49], a further task was to investigate whether the blood concentration affect this result. This was debunked as three tested concentrations covering the therapeutic range (2, 10 and 50 mg/l) showed the same degradation pattern over the study time. The analyzed ORM concentrations were not quantitatively recovered for both isochronous stability experiments. This supports the theory that other degradation products can still be formed in small amounts, e.g. by decarboxylation of the ORM [50].

Metabolic ratios ($c_{ORM}/c_{ORM}+c_{meropenem}$) of six critically ill patients receiving continuous meropenem infusion were investigated over several days in a third experiment to apply the method for native samples and to obtain a deeper understanding in individual *in vivo* degradation. All analyzed concentrations were in the calibration range of the method. Meropenem serum concentrations ranged from 7.25 – 31.25 mg/l and the ORM concentrations were slightly lower, ranging from 2.71 – 23.37 mg/L. Patient`s metabolic ratios were highly individualized and correlated with the glomerular filtration rate (GFR). As no significant day-to-day variations were visible for each patient, a one-time

determination of the metabolic ratio during routine TDM might be helpful in defining non-renal degradation and consequently provide personalized dose adjustments. This is of particular interest for patients with sub-therapeutic drug levels.

Overall, the utilization of the validated method as stability-indicating assay for meropenem and TDM applicability was proven by the performed experiments. Furthermore, calculated degradation rates can be used for future pharmacokinetic studies.

3. Zusammenfassung

Ausgeprägte inter- und intra-individuelle pharmakokinetische Varianzen erfordern eine personalisierte Dosis-Anpassung von Antibiotika, insbesondere bei kritisch kranken Patienten. Das therapeutische Drug-Monitoring (TDM) wird eingesetzt, um eine Therapiekontrolle zu realisieren und Zielspiegel zu gewährleisten. Insbesondere die ergänzende Messung von Metaboliten erlaubt eine fundiertere Aussage über die Eliminierung der Antibiotika. Aufgrund von erheblichen Stabilitätsproblemen bei beta-Laktam-Antibiotika ist die Forderung nach sogenannten „*Stability-indicating assay methods*“ sowohl für klinische als auch industrielle Anwendungen von sehr hoher Bedeutung.

Die Entwicklung einer neuartigen Stabilisotopenverdünnungsmethode auf Basis der LC-MS/MS zur simultanen Quantifizierung von Meropenem und dessen Hauptabbau-metaboliten, dem offenen beta-lactam Ring (ORM), bildete den ersten Teil des Promotionsprojektes. Eine kurze Gesamt-Turnaround Zeit wurde durch eine einfache, aber effektive Proteinfällung mittels Methanol und anschließender analytischer Trennung und interferenzfreier Detektion innerhalb von 5,5 Minuten erreicht. Des Weiteren wurde die Stabilität von Meropenem sowohl während der Probenaufarbeitung als auch der gesamten Messzeit gewährleistet. Im Zuge einer ausführlichen Validierung wurde die Anwendbarkeit der Methode, auch im Intensivmedizinischen Bereich, durch eine präzise und robuste Quantifizierung beider Analyte gezeigt.

Der zweite Teil des Promotionsprojektes konnte die Eignung dieser LC-MS/MS-Methode als „stability-indicating assay method“ beweisen. Dies wurde anhand von Stabilitätsprüfungen in allen Stufen der antimikrobiellen Therapie, in vitro und in vivo, gezeigt. Ein bemerkenswertes Ergebnis war, dass der spontane, nicht-enzymatische Abbau nur 6 % des Gesamtabbaus ausmacht, und zwar unabhängig von der Konzentration. Darüber hinaus haben die Erkenntnisse über die patientenindividuelle metabolische Ratio, die mit der individuellen PK korrelierten, das Interesse an einer simultanen Bestimmung der Serumkonzentrationen beider Wirkstoffe im Rahmen eines routinemäßigen TDM erhöht. Dies könnte eine personalisierte Dosisanpassung unterstützen.

Die entwickelte und validierte Methode, welche international akzeptierten analytischen Anforderungen entspricht, kann zukünftig sowohl für weitere Stabilitätsstudien, pharmakokinetische Untersuchungen und zur Qualitätskontrolle eingesetzt werden, als auch als Grundlage für verwandte Strukturen dienen.

4. Abstract

Considerable pharmacokinetic variability, especially in critically ill patients, requires personalized dose adjustment of antibiotics. Therapeutic drug monitoring (TDM) is used to achieve therapeutic control and to ensure target levels. In particular, the additional measurement of metabolites allows a more profound statement about the elimination of antibiotics. Based on tremendous stability problems with beta-lactam antibiotics, the demand for so-called "stability-indicating assay methods" is of high interest for clinical as well as industrial applications.

The development of a stable-isotope dilution LC-MS/MS method for the simultaneous quantification of meropenem and its main degradation product, the open-ring metabolite (ORM), was of novelty and is the first part of the work. A short overall turnaround time was achieved by a simple but effective protein precipitation using methanol followed by analytical separation and interference-free detection within 5.5 min. Furthermore, the stability of meropenem during sample preparation and throughout the total measurement time was ensured. An in-depth validation demonstrated the applicability of the method, also in critical care settings, by precise and robust quantification of both analytes.

The second part of this doctoral thesis demonstrated the suitability of the LC-MS/MS method as a "stability-indicating assay method". This was proved by performing stability testing at all stages of antibiotic therapy, *in vitro* as well as *in vivo*. An important result was that the spontaneous non-enzymatic degradation accounts for only 6 % of total degradation, regardless of concentration. Furthermore, the determination of patients` individual metabolic ratios, which were correlated to individual PK, increased the interest of a single determination of both compounds simultaneously within routine TDM. This may support personalized dose adjustment.

In conclusion, this developed and validated method, which meets national and international requirements, can be used in the future for further stability and pharmacokinetic studies, quality control as well as provide a first step for related structures.

5. Original articles

5.1 S. Rakete, C. Schuster, M. Paal, M. Vogeser, An isotope-dilution LC-MS/MS method for the simultaneous quantification of meropenem and its open-ring metabolite in serum, Journal of Pharmaceutical and Biomedical Analysis 197 (2021) 113944.

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An isotope-dilution LC-MS/MS method for the simultaneous quantification of meropenem and its open-ring metabolite in serum

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ABSTRACT

Background: Therapeutic drug monitoring (TDM) of beta-lactam antibiotics and, among them, especially meropenem gains importance in the field of laboratory medicine. Meropenem is known to be unstable, resulting in a degradation product with an open beta-lactam ring. For a more comprehensive TDM of meropenem, the aim was to develop a LC–MS/MS method for the simultaneous quantification of meropenem and its main degradation product, the open-ring metabolite (ORM).
Methods: The method involves a protein precipitation followed by chromatographic separation using a formic acid-ammonium formate methanol gradient on a pentafluorophenyl column. Multiple reaction monitoring in the positive ion mode and stable isotope labeled internal standards were used for quantification. Validation was performed according to the European Medicines Agency guideline.
Results: Validation was successful performed within the linear drug concentration range of 1.0–100.0 mg/l for meropenem and 0.62–62.30 mg/l for the ORM. Investigation of selectivity, accuracy and precision showed good results and potential matrix effects were successfully compensated by the internal standards. The suitability of the method was shown by the comparison of 35 anonymized leftover serum samples from intensive care patients with routine analyses.
Conclusion: For the first time, we herein describe a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantification of meropenem and its ORM in human serum. The ratio of active to inactive compound provides valuable pharmaceutical and pharmacokinetic information, which may contribute to therapeutic efficacy.

1. Introduction

Therapeutic drug monitoring (TDM) of meropenem (MER), one of the most commonly used broad spectrum beta-lactam antibiotics [1], is an increasingly important task in clinical diagnostics [2]. The rising demand is a result of frequent sub-therapeutic levels and alternative dosing strategies such as continuous infusion [3,4]. This is caused by unpredictable pharmacokinetics, especially in severely ill patients due to different volumes of distribution, renal and/or hepatic impairment or organ replacement procedures. To maximize antimicrobial activity and efficacy, recent guidelines for beta-lactam antibiotics recommend a target free drug concentration between four and eight times the (minimum inhibitory concentration) MIC of a causative pathogen for the entire dosing interval, ($fT \geq 4-8x$ MIC = 100%) [5]. Without monitoring, therapy failure, resistances and potential side effects can be the result of under-or overdosing [6–8].

The instability of MER with conversion to its inactive open-ring metabolite (ORM) (Fig. 1a-c) is an issue for both, therapeutic application, and TDM of MER. Consequently, MER is stored as a crystalline substance and not dissolved until the point of care. The stability of MER has been the subject of many studies, both in neat solutions, especially in terms of infusion solutions, and matrix-based conditions [9,10]. Zander et al. reported a concentration-dependent degradation of more than 15% when stored for more than 12 or 24 h at 4 °C [11].

In former studies, pharmacokinetic of MER and its ORM were investigated using HPLC-UV and radioimmunoassay [12–14]. MER is mostly (~70%) renal eliminated unchanged with a half-life of about 1 h. Depending on the health condition, this may vary. These studies assumed that the ORM is the main degradation product, which results e.g. by hydrolysis [15]. This mechanism was confirmed by a recently published study that qualitatively investigated the degradation products in non-matrix-based samples under different storage conditions by direct infusion of ESI-MS/MS [16].

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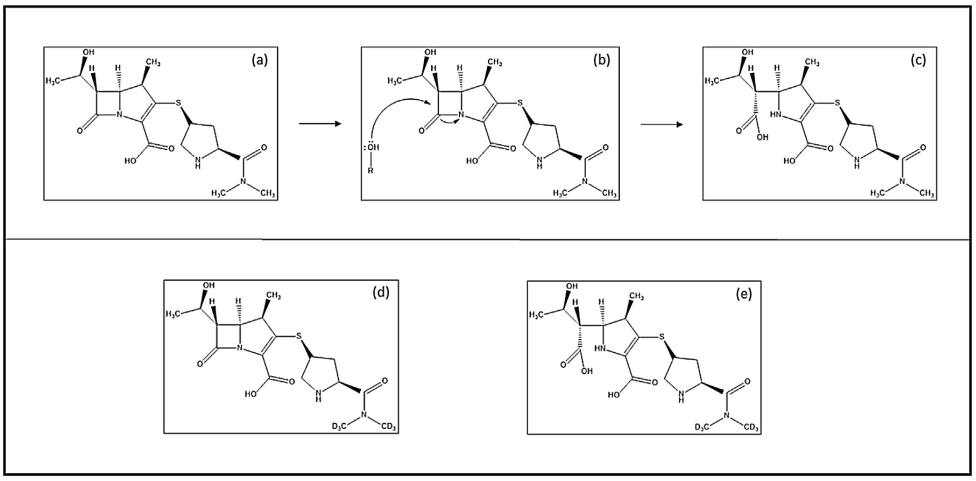

Fig. 1. Degradation pathway: meropenem (a) is attack at the beta-lactam ring by a nucleophilic substance (b) resulting in the open-ring metabolite (c). Corresponding internal standards are meropenem-d6 (d) and open-ring metabolite-d6 (e).-.

Degradation of MER may occur at several stages between administration of the drug and the measurement of blood concentrations. For instance, prolonged infusion of aqueous MER solutions may lead to an insufficient therapeutic effect, given that MER may be converted to in-active ORM in relevant amounts already *ex vivo*. From the diagnostic perspective, the instability of MER can lead to misleading results of MER measurement: Increased degradation is particularly likely if an adequate refrigeration chain of samples shipped for TDM of MER is not maintained in individual samples. Substantially higher ORM concentrations than usually found can be a hint to pre-analytical issues in a specific hospital setting. Consequently, reporting of the ORM concentrations along with MER can be an added value for TDM, with unusual ratios being indicative of both, pre-administration and / or pre-analytical issues. For external quality control it would be a benefit in the same respect. The verification of how much analyte has been degraded during transport or storage, is an aspect that would possibly improve the results of inter-laboratory comparisons. Additionally, the analysis of the ORM can be an advantage for a more comprehensive pharmacokinetic monitoring.

Therefore, our aim was to develop a method for the simultaneous quantification of MER and its ORM in human serum. We applied isotope-dilution LC–MS/MS as the highest metrological standard for TDM purposes.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, water, acetonitrile, isopropanol and formic acid were obtained in UHPLC quality from Biosolve (Valkenswaars, the Netherlands) and ammonium formate from Sigma Aldrich (St. Louis, Missouri, USA). Sodium chloride was from Merck (Darmstadt, Germany). Antibiotic MER (certified reference material) was from Supelco (Sigma Aldrich, St. Louis, Missouri, USA) and the isotope-labelled MER-d_6 (Dimethylamino-d_6) from Toronto research chemicals (Toronto, Ontario, Canada). Both, the trifluoroacetate salt of the open-ring metabolite and the isotope labelled analogue the trifluoroacetate salt of the open-ring metabolite - d_6 (Dimethylamino-d_6) were obtained from AlsaChim (Illkirch, France). MER, ORM and the corresponding internal standards are shown in Fig. 1. A drug free serum pool was purchased from the blood donation center of the Bavarian Red Cross (Munich, Germany).

2.2. Calibrator samples, quality control samples and internal standards

Stock solutions of MER and ORM were weighed individually for calibrators and the quality control (QC) samples and dissolved in a 0.9% NaCl solution. Spiking solutions were prepared by combining both stock solutions and subsequently by dilution. Finally, drug free serum was mixed with respective spiking solutions (9:1; v/v) to obtain seven calibrator and four QC levels. The concentrations of the calibration standards and QC samples are listed in Table 1. All prepared samples, stock and spike solutions were aliquoted and stored at −80 °C.

Both internal standards, MER-d_6 and the ORM-d_6, were dissolved in a 0.9% NaCl solution to yield a concentration of 1 mg/mL. An internal standard working solution was prepared by mixing both stock solutions with a 0.9% NaCl solution to achieve final concentrations of 10 μg/mL each. Stock solutions and the internal standard working solution were stored at −80 °C.

2.3. Sample preparation

A 50 μL aliquot of each sample (calibrator, quality controls or patient serum samples) were mixed with 25 μL of the internal standard working solution, vortexed and shaken for 5 min at room temperature with 1400 rpm (Eppendorf, Germany). The blank sample was treated similar, but with 25 μL of the 0.9% NaCl solution. Protein precipitation was performed by adding 325 μL methanol as precipitation solution, followed by vortexing and 5 min shaking. After centrifugation (10 min, 5 °C, 14,000 x g), 30 μL supernatant were diluted with 120 μL water in a vial with micro-insert and placed in an autosampler (4 °C sample cooling) for analysis.

2.4. HPLC conditions

Chromatography was performed on an Agilent 1290 Infinity I LC system (Santa Clara, California, USA) coupled to an AB Sciex TripleQuad 6500+ mass spectrometer (Framingham, Mas-

Table 1
Concentrations of calibration standards and quality control samples for meropenem and its open-ring metabolite.

Analyte	Calibration standards [mg/l]							Quality controls [mg/l]			
	1	2	3	4	5	6	7	A	B	C	D
Meropenem	1.0	2.5	5.0	10.0	25.0	50.0	100.0	1.0	3.0	30.0	75.0
Open-ring metabolite	0.62	1.56	3.12	6.23	15.57	31.15	62.30	0.62	1.78	18.69	46.72

sachusetts, USA). Instrument control and data acquisition were done with the Analyst software 1.6.3 (Sciex), data processing, and evaluation with the MultiQuant software 3.0.3 (Sciex). An XSelect HSS PFP column (100 × 2.1 mm, 2.5 μm) with a preceding XSelect HSS PFP Van Guard Cartridge from Waters (Milford, Massachusetts, USA) was used for chromatographic separation. The column oven was kept at 45 °C and the injection volume was set to 2 μL. The total run time was 5.5 min at a flow rate of 0.5 mL/min using mobile phases A 10 mM ammonium formate in water-formic acid (99.8/0.2, v/v) and B methanol. The gradient was run with the following conditions for mobile phase B: 0.00–1.00 min → 7%; 1.00–1.01 min → 80%; 1.01–1.80 min → 100%; 1.80–2.50 min →100%; 2.50–2.51 min → 20%; 2.51–4.00 min→100%; 4.00–5.00 min → 100%; 5.00–5.25 min →7%; 5.25–5.50 min →7%.

A switching valve allowed the elution to enter the mass spectrometer between 0.9–2.4 min.

2.5. Mass spectrometry conditions

MER and ORM were analyzed in the positive ion mode (ESI+) with the following ion source settings: curtain gas 50 psi, collision gas 8 (medium), source temperature 300 °C, ion spray voltage 5000 V, ion source gas 1 50 psi (atomizing gas) and ion source gas 2 40 psi (heating gas), using nitrogen as gas. Mass transitions and their specific parameters were manually tuned with neat solutions (Table 2).

2.6. Method validation

Method validation was performed according to the guideline of bioanalytical method validation from the European Medicines Agency (EMA) [17]. The following characteristics of a bioanalytical method were studied: selectivity, carry-over, lower limit of quantification, calibration curve, accuracy and precision, dilution integrity, matrix effects, recovery and stability.

2.6.1. Selectivity

To determine selectivity, different MER-free serum samples from poly-pharmacized non-intensive care (n = 10) and intensive care (n = 5) patients were analyzed. In addition, MER and its metabolite were spiked to native samples to achieve final concentrations of 3.00 and 1.87 μg/mL, respectively. According to EMA, the absence of interfering compounds is accepted if their response is ≤20% of the lower limit of quantification (LLOQ) for the analyte and ≤5% for the internal standard at analytical retention time. The mean value of the ion ratios (quantifier/qualifier) of the calibration standards from the intra-assay experiment was calculated for both compounds. These values were compared to the ion ratios of all calibration standards, quality control samples and clinical samples and should not differ by more than 20% [18].

2.6.2. Carry-over

Blank samples were analyzed after the highest calibration samples (ULOQ) to define the carry-over. In accordance with the EMA guideline, the area of the blank samples should be ≤20% of the area of the LLOQ and ≤5% of the internal standard.

2.6.3. Limits of quantification

The LLOQ indicates the lowest concentration of an analyte in a sample that can be reliably quantified and provides an inter-day imprecision and inaccuracy of ≤20% and a signal of at least 5 times the signal of a blank sample. In contrary, the calibrator with the highest analyte concentration represents the upper limit of quantification (ULOQ).

2.6.4. Calibration curve

Selected concentration range for MER with 1−100 mg/L was based on our experience from routine MER TDM and the recommended therapeutic target for beta-lactam antibiotics (fT ≥ 4−8x MIC = 100%) [5]. The calibration range for the ORM was considered to be approximately half that of MER assuming equimolar degradation. A blank (without analyte and internal standard) and a zero sample (including the internal standard only) were processed and analyzed additionally in each run. Meeting the EMA guidelines, the recalculated concentrations of 75% of at least 6 calibration standards, should be within ±15% (for the LLOQ ± 20%) of the nominal value. A 1/x weighting function was used for linear regression (included origin).

2.6.5. Accuracy and precision

Accuracy (indicated as inaccuracy by the relative bias d_r) and precision (indicated as imprecision by the coefficient of variation CV) were investigated as intra-assay (n = 5) and inter-assay (n = 15) on three separate days covering four quality control samples. All results should be within deviations of ±15% (for the LLOQ ± 20%) as defined by the EMA. In addition, according to the guidelines of the German Federal Medicine Council (Rilibäk) [19], the total error can be calculated as the relative root-mean-square-error (%RMSE), which should also not exceed 15%:

$$\%\mathrm{RMSE} = \frac{\sqrt{\sum_{i=1}^{n}(\mu_i - \mu)^2}}{\mu}$$

where μ_i is the measured value of a sample and μ the target nominal value.

2.6.6. Dilution integrity

Dilution experiments were performed for samples with concentrations exceeding the calibration range. Drug-free serum was spiked with 75% and twice the ULOQ of MER and its ORM (n = 5), diluted with water (1:2, 1:3, 1:5), processed, analyzed and concentrations back calculated to undiluted concentration levels. As defined by the EMA, inaccuracy and imprecision should not exceed 15%.

2.6.7. Matrix effects and recovery

A post-column infusion experiment was used to study matrix effects [20]. For this purpose a neat solution of calibrator 7 in methanol-water (20:80, v/v) with a flow rate of 10 μL/min was applied. In addition, matrix effects and recoveries were assessed by using three different sample sets, each with the identical final analyte and internal standard concentration. Antibiotic-free patient serum (n = 6) was spiked with a low (3x LLOQ) and a high (75% ULOQ) concentration of both analytes and the corresponding internal standards before (set B) and after (set C) sample preparation. In

Table 2
Multiple reaction monitoring transitions with precursor and product ions and specific mass spectrometry parameters for quantifier and qualifier and the corresponding internal standards.

Analyte	RT (min)		Quantifier				Qualifier			
		Precurson ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)
Meropenem	1.98	384.2	114.0	40	36	18	254.3	40	22	25
Open-ring metabolite	1.85	402.3	140.2	20	19	16	358.2	20	10	24
Meropenem-d6	1.98	390.2	114.0	40	31	15	–	–	–	–
Open-ring metabolite-d6	1.85	408.1	140.1	26	21	16	–	–	–	–

addition, the identical final concentrations were analyzed in a neat solution (set A).

According to EMA matrix effects are described by the matrix factor (MF), calculated from the ratio of areas of set C to the areas of set A. Additionally, the internal standard MF was calculated as the ratio of the MF of the analyte to the MF of the corresponding internal standard, where the CV should be ≤15%. The recovery is defined by the CLSI-IFCC [21] as the ratio of set B and set C, where the deviations of the mean value should be ≤15%.

2.6.8. Stability experiments

Stability experiments in serum were performed for 24 h at 4 °C, 7 days at −20 °C and 11 weeks at −80 °C storage with 3 replicates of two quality control samples (QC B and QC D). Stability of two freeze-thaw (−80 °C ≥ 24 h and RT for about half an hour) cycles and 24 h autosampler stability of processed samples were investigated. According to EMA, the mean values for each concentration level may not decrease more than 15% of the nominal concentration as determined by fresh counterparts.

2.7. Further investigations

Degradation of MER can already occur during the preparation of the standard solutions, which would result in an incorrect measurement. To verify this, fresh prepared quality control samples containing MER and ORM in 0.9% NaCl solution were investigated individually and in combination to evaluate possible degradation.

In addition, the presence of a possible methyl ester product of MER was tested, which could result from a nucleophilic attack at the beta-lactam ring by methanol. For this purpose, the *m/z* of the precursor and product ion were calculated (414.2 > 173.1) and implemented in the method. This product was measured in the negative ion mode using the fast polarity switching of the mass spectrometer with following settings: ion spray voltage was set to −4500 V, the declustering potential to −40 V; the collision energy to −38 V and the collision cell exit potential to −14 V. The remaining LC–MS/MS method parameters were adopted. During validation, random samples were analyzed using this method.

2.8. Application to clinical samples

We measured 35 leftover serum samples derived from routinely TDM from intensive care patients receiving MER according to clinical guidelines. Results for MER were compared with those from the routine laboratory using a linear regression analysis. Linear relationship between the results of both methods were determined with the Pearson's correlation coefficient (r). In addition, ratios of ORM-to-MER were calculated.

3. Results

3.1. Method development

Method development was initiated based on a fast routine HPLC-MS/MS method for antibiotics TDM in our laboratory [22]. The protein precipitation procedure was adopted and reproducible extraction yields were obtained with methanol.

Several reverse phase C8 and C18 columns were tested for chromatographic separation, with none being suitable for the analysis of the ORM. The best results were obtained with a 2.5 µm XSelect HSS PFP column (2.1 mm x 100 mm) (Milford, Massachusetts, USA). The addition of 0.2% of formic acid in mobile Phase A is necessary to prevent strong tailing of the ORM. Typical MRM chromatograms are depicted in Fig. 2.

To reduce carry over for MER and the ORM, a second gradient was added to the chromatography method and the washing program for the injection needle was improved by using different wash solutions.

3.2. Method validation

3.2.1. Selectivity

No interfering signal was visible in the analyzed intensive care and non-intensive care patient samples. The quantification of the spiked samples was not disturbed by native sample matrix or co-medication: determined accuracies were 91.7–98.9% for MER and 92.0–108.3% for the ORM. The ion ratio of all analyzed samples were comparable (within ± 10%) to the calculated ion ratio obtained from calibrator samples for both analytes.

3.2.2. Carry over

The mean carry over was 3% for MER and ≤ 20% for its ORM. For the corresponding internal standards, both mean values were 0.16%.

3.2.3. Limits of quantification

Intra- and inter-assay imprecision, inaccuracy and RMSE were ≤11.1% for the LLOQ (details are shown in Table 3). The analyte signal for the LLOQ was at least ≥25 x the signal of the blank samples at the respective retention times.

3.2.4. Calibration curve

Seven calibration levels were used for quantification. A linear regression model by applying a weighting factor of 1/x and an $R^2 \geq 0.999$ was obtained for both analytes.

3.2.5. Accuracy and precision

All limits required by the EMA guideline were achieved by maximum values of ≤ 6.5% for the coefficient of variation, ≤ 9.3% (LLOQ) for the relative bias and ≤ 11.1% (LLOQ) for the relative RMSE for both intra- and inter-day studies (Table 3).

3.2.6. Dilution integrity

With deviations from the nominal values between 0.5% and 3.6%, the dilution integrity for both analytes could be demonstrated for all three dilution steps and both concentrations.

3.2.7. Matrix effects and recovery

The post-column infusion experiment did not indicate ion suppression or enhancement for both analytes at the predicted

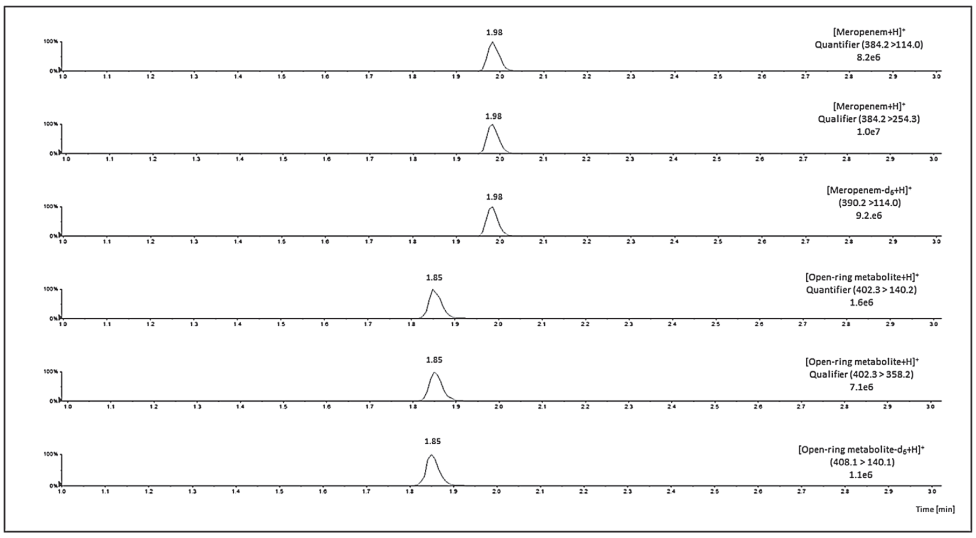

Fig. 2. Multiple reaction monitoring (MRM) chromatograms of all transitions for both analytes and the corresponding internal standards.

Table 3
Intra-day (n = 5) and inter-day (n = 15) validation results for the assessment of accuracy, precision and the root-mean-square-error of quality control samples (QC A, B, C and D).

Intra-day and inter-day assay									
Analyte		QC A		QC B		QC C		QC D	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Meropenem									
	$\bar{x}$ (mg/l)	0.93	0.93	2.87	2.88	29.22	29.0	71.06	73.85
	CV (%)	2.4	3.8	1.0	4.5	2.1	2.4	1.5	4.4
	d_T (%)	6.9	6.6	4.3	3.9	2.6	3.3	5.3	1.5
	RMSE (%)	7.2	7.5	4.3	5.8	3.1	4.1	5.4	4.6
Open-ring metabolite									
	$\bar{x}$ (mg/l)	0.65	0.68	1.94	1.98	18.62	19.01	47.57	49.67
	CV (%)	2.7	5.4	4.5	6.5	3.8	3.6	2.5	5.2
	d_T (%)	5.3	9.3	3.8	5.9	0.4	1.7	1.8	6.3
	RMSE (%)	6.0	11.1	6.0	9.1	3.8	4.0	3.1	8.4

retention times. A mean normalized matrix factor of 100% with a coefficient of variation of $\leq$ 0.9% for MER and of 99.5% with a coefficient of variation of $\leq$ 2.7% for ORM was achieved. Results of recovery rates were found to be between 81.8% and 96.2% for both concentration levels.

3.2.8. Stability experiments

The stability was proven to be sufficient for 24 h at 4 °C, 1 week at −20 °C and 11 weeks at −80 °C. Furthermore up to two freeze-thaw cycles showed to be stable. The processed samples stored in the autosampler at 4 °C did not show relevant degradation. Detailed stability data can be seen in Table 4.

3.3. Further investigations

Accuracies between 98.8% and 104.7% were found for the ORM solely as well as in combination with MER within the degradation experiment.

In addition, the methyl ester product showed an area below 1% compared to the area of MER.

3.4. Application to clinical samples

Linear regression analysis for method comparison with the results of the established routine method for the analyses of MER in the averaged range of 1.79 μg/mL and 49.05 μg/mL yielded a slope of 0.85, an intercept of 0.06 mg/l, and a Pearson's correlation coefficient (r) of 0.99. The ORM-to-MER ratio ranged from 19.7% to 186.7%, with a median of 69.1%. Detailed results are shown in Fig. 3.

4. Discussion

In this article, a validated isotope dilution LC-tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantification of meropenem (MER) and its main metabolite, the open-ring metabolite (ORM), in human serum is described for the first time.

There are several publications regarding therapeutic drug monitoring of MER by LC–MS/MS [9,22–24] and routine therapeutic monitoring of MER is performed in a rapidly increasing number of clinical laboratories worldwide [25]. However, none of these methods included a quantitative measurement of ORM. HPLC-UV and radioimmunoassay techniques were designed for the analysis of the ORM alone, but none of these methods simultaneously

Table 4
Results of the stability experiment for 4 °C (24 h), −20 °C (1 week), −80 °C (11 weeks), 4 °C autosampler (24 h), freeze-thaw and twice freeze-thaw stability tested with quality control samples B and D.

Analyte		4 °C (24 h)		−20 °C (1 week)		−80 °C (11 weeks)		4 °C autosampler (24 h)		2x freeze-thaw	
		QC B	QC D	QC B	QC D	QC B	QC D	QC B	QC D	QC B	QC D
Meropenem											
	x (mg/l)	3.00	75.0	3.00	75.0	3.00	75.0	3.00	75.0	3.00	75.0
	Recovery (%)	86.6	88.9	92.5	86.5	93.6	92.1	94.3	96.5	93.7	91.2
Open-ring metabolite											
	x (mg/l)	1.87	46.7	1.87	46.7	1.87	46.7	1.87	46.7	1.87	46.7
	Recovery (%)	110.7	107.3	100.0	102.8	105.9	107.2	103.0	104.9	102.7	99.1

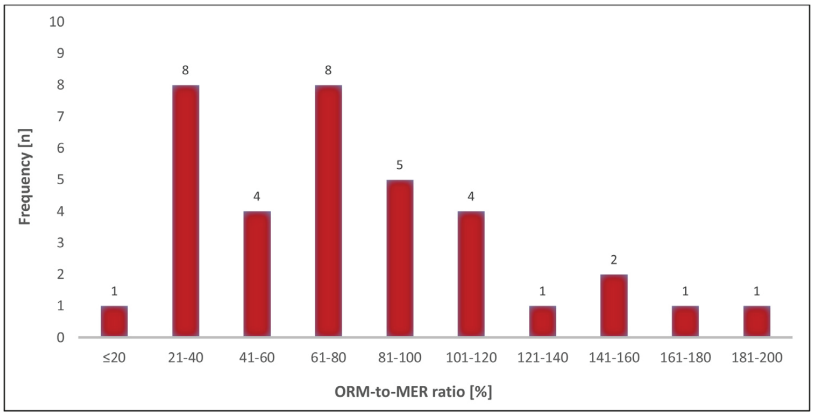

Fig. 3. Distribution of measurement results for the open-ring metabolite (ORM)-to-meropenem (MER) ratio for 35 anonymized leftover routine serum samples.

quantified intact MER too [26,27]. Although ORM has no antimicrobial activity itself, simultaneous quantification of MER and ORM is of clinical interest: an unusually high proportion of ORM found in a sample in relation to MER can result both, from pre-application issues (extended time interval between dissolvation of the compound and administration), and from pre-analytical issues (extended time interval between sampling and reception in the laboratory for centrifugation and freezing until analysis). A high ratio potentially may also be the result of the enzymatic activity of resistant pathogens as well as non-renal clearance. In our preliminary assessment of anonymized clinical samples we indeed observed a substantial degree of between-patient variation (Fig. 3). This is in accordance with previous reports [27,28]. Clinical studies are necessary to elucidate these findings; the method described herein represents an essential tool for addressing these questions.

MER and its ORM were dissolved in 0.9% NaCl solution according to Patel and Cook [29] to minimize potential stability issues of MER in aqueous solution. This was confirmed, as no relevant degradation (< 1%) of MER was observed in the stock solution.

An important detail for ORM measurement in the method described herein is the addition of 0.2% of formic acid in mobile phase A. The addition of only 0.1% of formic acid, as routinely used, was not sufficient and resulted in an unseparated double peak. However, this had no effect on MER. Two possible causes are proposed: first, the standard of the ORM is the salt of the trifluoroacetic acid, which acts as an ion pair reagent in the positive ion mode. This can be cleaved by the addition of formic acid. Secondly, the ring opening increases the basicity and a higher acid concentration is needed for optimal retention.

For method validation, all criteria proposed by the EMA were met [15]. The coefficient of variation and the relative bias were ≤ 9.3% for all quality control samples (both intra- and inter-assay). Matrix effects were negligible due to high dilution of samples, low injection volume, and the use stable isotope dilution internal standardization. The carryover of 20% for the ORM when compared to the LLOQ is adequate for analysis of clinical samples.

Sample stability was given for all tested conditions. However, the degradation of MER was close to 15% for 24 h at 4 °C and for 7 days at −20 °C. As advised in the literature samples should be processed quickly – applying a refrigeration chain for clinical diagnostic samples - and stored at −80 °C until analysis [11]. According to autosampler stability data samples may be re-injected up to 24 h after sample cleanup.

The additional information of the concentration of the ORM along with MER can also help to assess stability issues with respect to external quality assessment of MER. The ORM transition could be used to verify whether degradation of MER has occurred in proficiency testing samples during shipment. In addition, the in vitro stability of MER can now be studied in further detail, both in terms of handling the dissolved compound prior to administration (e.g. as continuous infusion) and in terms of preparing diagnostic samples for antibiotic TDM.

In conclusion, we herein present a novel convenient and robust method for the simultaneous quantification of MER and its main metabolite ORM using isotope-dilution standardization LC–MS/MS. This method provides the option for a far more comprehensive MER TDM in routine diagnostics, and is applicable for further research concerning the stability of MER.

Author contributions

Sophie Rakete developed and validated the method and wrote the manuscript. Carina Schuster and Michael Paal contributed to methodological aspects and reviewed and edited the manuscript.

Michael Vogeser supervised the project. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

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Declaration of Competing Interest

The authors report no declarations of interest.

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The Role of Non-Enzymatic Degradation of Meropenem—Insights from the Bottle to the Body

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Abstract: Several studies have addressed the poor stability of meropenem in aqueous solutions, though not considering the main degradation product, the open-ring metabolite (ORM) form. In the present work, we elucidate the metabolic fate of meropenem and ORM from continuous infusion to the human bloodstream. We performed in vitro infusate stability tests at ambient temperature with 2% meropenem reconstituted in 0.9% normal saline, and body temperature warmed buffered human serum with 2, 10, and 50 mg/L meropenem, covering the therapeutic range. We also examined meropenem and ORM levels over several days in six critically ill patients receiving continuous infusions. Meropenem exhibited a constant degradation rate of 0.006/h and 0.025/h in normal saline at 22 °C and serum at 37 °C, respectively. Given that 2% meropenem remains stable for 17.5 h in normal saline (≥90% of the initial concentration), we recommend replacement of the infusate every 12 h. Our patients showed inter-individually highly variable, but intra-individually constant molar ORM/(meropenem + ORM) ratios of 0.21–0.52. Applying a population pharmacokinetic approach using the degradation rate in serum, spontaneous degradation accounted for only 6% of the total clearance.

Keywords: meropenem; open-ring metabolite (ORM); continuous infusion; stability; pharmacokinetic; isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS)

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1. Introduction

Meropenem is a broad spectrum antibiotic with excellent activity against many pathogens that is used to treat a variety of bacterial infections [1]. As a beta-lactam antibiotic, meropenem exhibits a time-dependent antibacterial effect, i.e., the free concentration should be maintained above the minimum inhibitory concentration (MIC) of a target pathogen for the entire dosing interval (expressed as $fT_{>MIC}$ = 100%) [2]. For complicated infections, some guidelines even recommend target trough levels with $fT_{>4\text{-}8x\ MIC}$ = 100% to optimize clinical efficacy [3]. Recently, increasing concerns have been raised that meropenem exposure might be inadequate, especially in critically ill patients, with the necessity for therapeutic drug monitoring (TDM)-guided individualized treatment [4–6].

In order to maximize antimicrobial target attainment and clinical cure in severe illness, meropenem is typically administered with continuous infusion regimens after application of a loading dose [7–9]. The administration of meropenem in outpatient parenteral antimicrobial therapy (OPAT), representing the administration of IV antibiotics without hospitalization, is also becoming increasingly popular in clinical practice [10].

However, continuous infusion of meropenem has been limited owing to concerns with instability, including prolonged storage time before administration.

According to the European and United States Pharmacopoeia, stability of an infusate is only given when the drug concentration remains above 90% of the initial concentration during the entire infusion interval [11,12]. Numerous studies have investigated the stability of meropenem in aqueous solutions at various conditions, including antibiotic concentration, temperatures and pH values [13–23]. All these studies focused solely on in vitro stability data. However, they do not examine meropenem degradation in vivo and they also do not investigate the formation of the open-ring metabolite (ORM) of meropenem, the main degradation product formed by hydrolysis of the beta-lactam ring [24]. In vivo, approximately 70% of meropenem is excreted by the kidneys, while approximately 30% is non-renally excreted. The high proportion of renal excretion necessitates dose adjustments based on the kidney function [25]. In addition, reporting of the ORM concentration is of added value for TDM purposes, given that unusually high ORM levels may be indicative of pre-analytical issues (e.g., improper sample handling with in vitro meropenem degradation).

The aim of the present study was thus the comprehensive assessment of meropenem stability, continuously tracking both meropenem and the ORM from the infusate to the in vivo condition by isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS). For this purpose, extensive in vitro stability testing was performed and supplemented by the investigation of serum samples from patients who received meropenem as a continuous infusion. A population pharmacokinetic approach was employed to investigate the proportion of spontaneous decay to the total elimination.

2. Results

2.1. Infusate Stability at Room Temperature

Meropenem dissolved with 2% in normal saline solution decreased continuously with a degradation rate of 0.006/h (=percentage decay per hour) at 22 °C, with a recovery of 86.6% (% of the initial concentrations, t = 0 h) after 24 h. After 17.5 h, the stability limit of 90% as proposed by the European and United States Pharmacopoeia was crossed. The ORM increased at the same time, but was not quantitatively recovered as a metabolite (see Figure 1). The concentration of meropenem in mg/L and the ORM in normal saline at each time point is visualized in Figure 1.

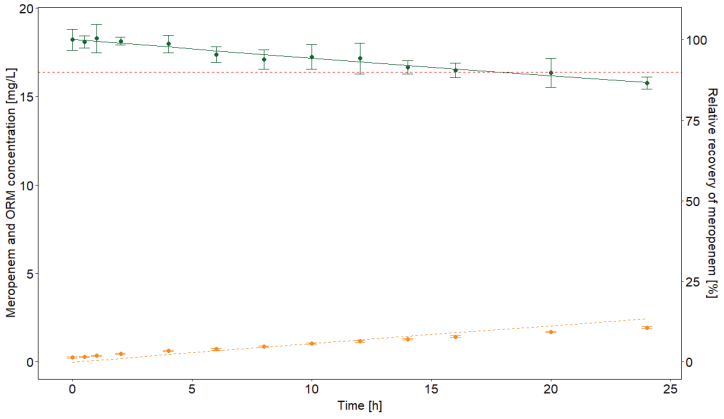

Figure 1. Degradation of 2% meropenem in vitro in normal saline infusate at 22 °C. Red dashed line: 90% relative recovery of meropenem. Green dots: mean measured concentrations of meropenem. Orange dots: mean measured concentrations of the open-ring metabolite (ORM). Standard deviations are depicted with error bars. The non-linear degradation is shown by the green line, while the orange dotted line indicates the theoretical increase in ORM, which is not entirely achieved.

2.2. Stability in Serum

No concentration-dependent degradation effect was observed in buffered serum heated to 37 °C. Instead, uniform degradation rates of 0.025/h were obtained for 2, 10, and 50 mg/L total meropenem with a recovery of 54.9% after 24 h. As observed in normal saline, the ORM increased at the same time, but was not quantitatively recovered as a metabolite (see Figure 2). The degradation of meropenem and the increase of ORM in 37 °C warmed buffered serum are illustrated in Figure 2.

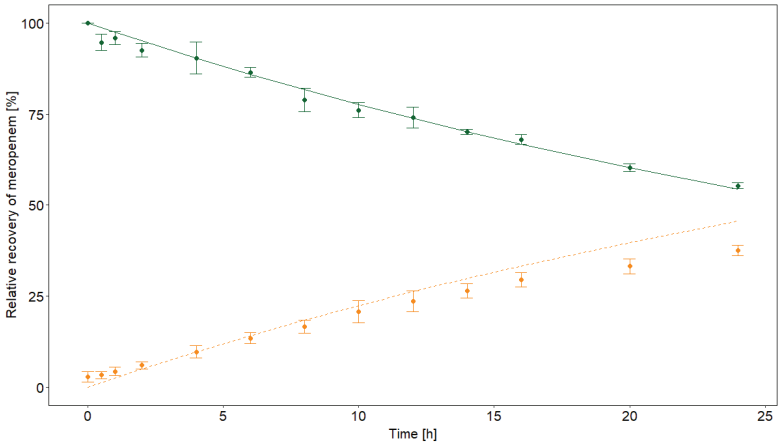

Figure 2. Combined degradation profiles of 2, 10, and 50 mg/L meropenem in vitro in buffered serum at 37 °C. Green dots: mean measured concentrations of meropenem. Orange dots: mean measured concentrations of the open-ring metabolite (ORM). Standard deviations are depicted with error bars. The non-linear degradation is shown by the green line, while the orange dotted line indicates the theoretical increase in ORM.

2.3. Pharmacokinetics in Patients

A total of 24 serum samples from 6 patients (4 male, 2 female) were included in the study (see Table 1). The median age was 46 years (range: 35–72 years), the median body weight was 86 kg (range: 47–170 kg), and the median meropenem infusion rate was 6 g/24 h (range: 3–6 g/24h). The glomerular filtration rates were 28–307 mL/min (median 139 mL/min). None of the patients received extracorporeal renal replacement therapy.

Table 1. Overview of patient characteristics.

Patient Characteristic [Unit]	Number/Median (Range)
No. of patients	6
No. of male patients	4
No. of samples	24
Meropenem concentration [mg/L]	19.74 (7.25–31.25)
Open-ring metabolite (ORM) concentration [mg/L]	7.73 (2.71–23.37)
Meropenem daily dose [g/24 h]	6 (3–6)
Age [years]	46 (35–72)
Weight [kg]	86 (47–170)
Glomerular filtration rate [mL/min]	139 (28–307)

Over several days of continuous meropenem administration, all patients showed stable intra-individual meropenem and ORM steady state concentrations, but highly variable

inter-individual ORM/(meropenem + ORM) metabolic ratios of 0.21–0.52 (median 0.28); see Figure 3. The ORM/(meropenem + ORM) ratio was correlated with the GFR (R^2 0.46, $p < 0.001$). Patient no. 2, with the highest renal impairment (median GFR 42 mL/min), also had the highest metabolic ratio. The median meropenem concentration was 19.74 mg/L (range: 7.25–31.25 mg/L) and the median ORM concentration was 7.73 mg/L (range: 2.71–23.37 mg/L).

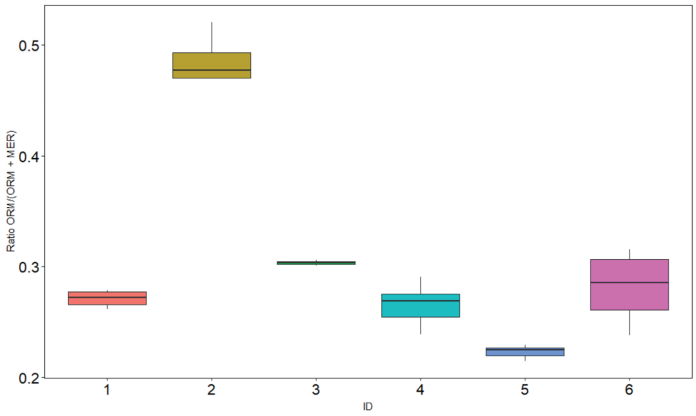

Figure 3. Intra- and interindividual variability of the ratio of the open-ring metabolite (ORM) to the total meropenem concentration in six critically ill patients.

The one-compartment model with first-order elimination, inter-individual variability on the total clearance, and a proportional residual variability adequately described the meropenem concentrations (see Table 2 and Figures 4 and 5). The total clearance was 11.4 L/h (range: 5.2–25.3 L/h). The glomerular filtration rate clearance (CL_{GFR}) represented the largest clearance fraction with 7.1 L/h (62% of total clearance). The unexplained residual clearance (CL_{nonGFR}) was 3.6 L/h (32%) and spontaneous decay (CL_{decay}) accounted for about 0.7 L/h clearance (6% of total clearance).

Table 2. Parameter estimates of the population pharmacokinetic model.

Parameter Estimates (RSE, %) [Shrinkage, %]	
	Meropenem
Parameter [unit]	**1-CMT Model**
OFV	121.8
Fixed-effects Parameter	
CL_{GFR} [L/h]	7.1
CL_{nonGFR}	3.6 (28)
CL_{decay}	0.66
V [L]	26.2
Interindividual variability	
ω CL (CV %)	14.9 (18) [2]
Residual variability	
σ Prop. (CV %)	13.5% (20) [11]

Abbreviations: RSE: relative standard error, CMT: compartment, OFV: objective function value, CL: total clearance, CL_{GFR}: clearance attributable to the glomerular filtration rate, CL_{nonGFR}: clearance not attributable to the glomerular filtration rate, CL_{decay}: clearance attributable to spontaneous decay in plasma at 37 °C (fixed at the experimentally determined value). V: volume of central compartment (fixed at a literature value [26]), ω: random-effects parameters for interindividual variability, CV: coefficient of variation, σ: random-effects parameters for residual variability, Prop.: proportional. Total clearance (CL) was calculated according to the following equation: $CL = CL_{GFR} + CL_{nonGFR} + CL_{decay}$.

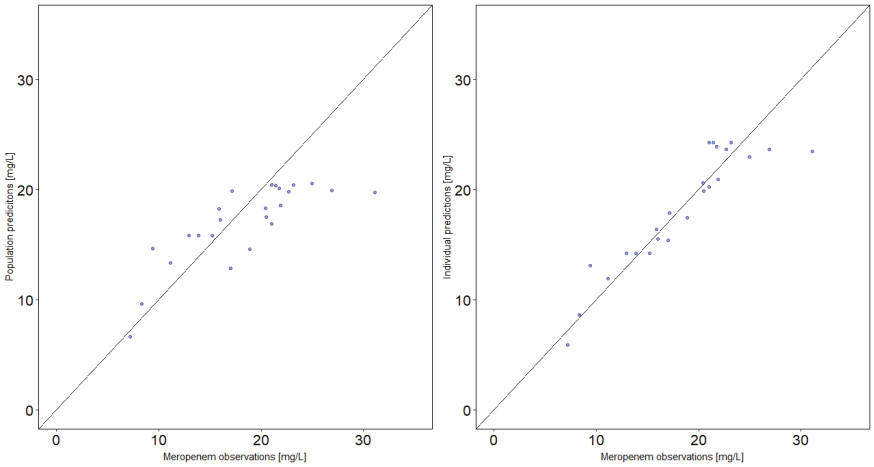

Figure 4. Goodness-of-fit plots for the final population pharmacokinetic model of meropenem in six critically ill patients. Left figure: Population-predicted concentrations versus observed concentrations. Right figure: Individual-predicted concentrations versus observed concentrations. Lines: Line of unity.

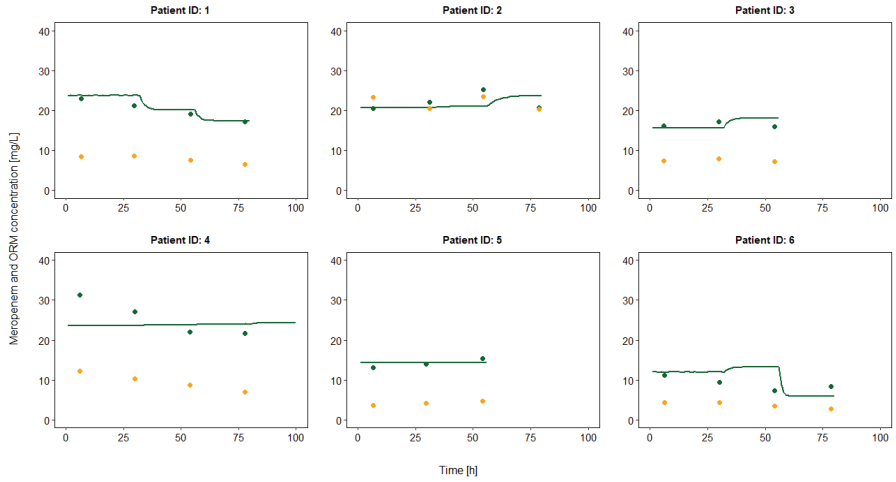

Figure 5. Observed meropenem and open-ring metabolite (ORM) concentrations and meropenem concentration–time profile predicted based on a one-compartment pharmacokinetic model. Green line: median predictions, green points: meropenem concentrations, orange points: ORM concentrations.

3. Discussion

In the present study, we investigated the degradation of meropenem both in vitro and in vivo, applying a high precision isotope dilution LC-MS/MS method designed to quantify both meropenem and its main degradation product, the open-ring metabolite (ORM) form, which is microbiologically inactive. The inaccuracy and imprecision of the quantitative assay were consistently $\leq$8% (in most cases < 5%), which allows reliable conclusions to be drawn.

Several studies have shown that meropenem is relatively unstable after reconstitution in aqueous solution. Accordingly, delivery by continuous infusion over 24 h is generally considered unacceptable. Consistent with previous findings by Manning et al. [27], our results show that normal saline solutions with the standard concentration of 2% meropenem

in normal saline retain >90% of their initial concentration for 17.5 h and 86.6% for 24 h at 22 °C, respectively. The ORM concentration is not increasing equimolarly with the degradation of meropenem, indicative of further conversion of the ORM (e.g., by decarboxylation) [24]. For clinical practice and in agreement with previous stability studies, we recommend the administration of meropenem prepared in two separate continuous 12 h infusions, respectively [27–30].

Meropenem stability has also been shown to be influenced by the drug concentration. Degradation rates of reconstituted meropenem increase with higher concentrations in infusates (mg/mL range) [22,27,31], which can be attributed to intermolecular aminolysis by a nucleophilic attack of one meropenem molecule opening the beta-lactam ring of a second molecule of meropenem. Concentration-dependent degradation in the mg/L scale is also evident in frozen human plasma samples [32]. To the best of our knowledge, there are no studies that simultaneously investigate the degradation of meropenem and its conversion to ORM in vivo using a single analytic method. In the present study, we used meropenem-fortified human serum at body temperature as an approximate in vitro model for the human bloodstream. To avoid sample alkalization and enhanced meropenem degradation due to loss of dissolved carbon dioxide, the serum pH was stabilized with phosphate buffer. The three tested concentrations of 2, 10, and 50 mg/L that are representative of the therapeutic range produced identical meropenem degradation rates of 0.025/h during the 24 h interval tested with a non-equimolar increase of the ORM. This degradation rate yielded a half-life of about 27.7 h, which is significantly longer than previously described by Harrison et al. with approximately 11 h [33]. The longer in vitro half-life of meropenem in our experiments might be explained by the fact that we stabilized the pH value by buffer addition. Our test conditions are closest to physiological in vivo conditions and should thus be considered the most valid.

We also investigated the relationship of meropenem and the ORM in vivo in patients receiving continuous infusion of meropenem. The formation of the ORM reflects the non-renal elimination in vivo and can either be caused by spontaneous, non-enzymatic beta-lactam opening or enzymatic hydrolysis by renal dehydropeptidase-1 (DHP-1), although meropenem appears to be very stable against human DHP-1 [33,34]. In healthy individuals, meropenem is rapidly excreted unchanged in the urine (approximately 70% of the dose, $t_{1/2} \approx 1$ h) and the remainder mainly by conversion to the ORM (approximately 30% of the dose, $t_{1/2} \approx 2$–3 h) [35]. In subjects with impaired renal function, non-renal meropenem clearance via the ORM formation increases up to 50% [36,37]. In line with these findings, continuous infusion samples from our patients exhibited highly variable inter-individual metabolic ratios of ORM/(ORM + meropenem), with the highest ratio for patient number 2 with the most compromised renal function (median GFR: 42 mL/min). With timewise stable renal function, all patients presented with almost constant metabolic ratios over several days. Consequently, a single determination of ORM in routine clinical practice could be informative about the proportion of non-renal elimination in an individual patient and helpful for personalized dose adjustments. Total clearance in our population was slightly increased compared with previously published meropenem models in critically ill patients (7.7–9.4 L/h), which can be attributed to an overall hyperdynamic renal function (median GFR 139 mL/min) [26,38,39]. Our population pharmacokinetic model indicated a low impact of spontaneous degradation on the total clearance (6%), while glomerular filtration rate accounted for about 62%. Consequently, the remaining 32% of the clearance can be attributed to elimination by the renal DHP-1 and net tubular secretion, as mentioned earlier [33]. However, the mean percentage of 28% ORM indicates metabolization by renal DHP-1 rather than tubular net secretion, as the latter would not yield further ORM.

Our study has several limitations. The analysis of spontaneous degradation was an in vitro analysis and the implementation in a population pharmacokinetic model only represents an approximation of the in vivo condition. Our study would also have benefited considerably from the analysis of urine data. Unfortunately, these samples were not available for analysis. Still, our approach provides interesting and novel clues about the

in vivo metabolism of meropenem. Furthermore, the determined elimination rates can be implemented in future model-based dose optimizations. In particular, physiologically-based pharmacokinetic models could benefit from integrating the determined elimination rates. Our population pharmacokinetic model was based on a reduced number of patients with sparse sampling and included fixed parameters. We would thus like to explicitly point out that this model should not be used for dose optimization strategies without restrictions.

In summary, the present study provides a deeper understanding about the stability of meropenem both in vitro and in vivo. Spontaneous degradation in serum accounts for only a small fraction of the non-renal elimination. Meropenem reconstituted at 2% in normal saline is reasonably stable at room temperature and, accordingly, requires only two separate 12 h infusions as a part of a 24 h continuous infusion regimen. Concomitant quantification of the open-ring metabolite (ORM) form could be helpful in dose adjustments in individual patients receiving meropenem therapeutic drug monitoring (TDM).

4. Materials and Methods

4.1. Chemicals and Reagents

Commercial powder for injection with one vial delivering 1 g of meropenem and 208 mg of sodium carbonate was from Hikma (London, United Kingdom). Certified reference material of meropenem as the trihydrate, di-potassium hydrogen phosphate trihydrate ($K_2HPO_4 \times 3\ H_2O$), and hydrochlorid acid (HCl) were obtained from Supelco (Bellafonte, United States). Commercially available quality controls (QCs) for the therapeutic drug monitoring (TDM) of meropenem in serum were obtained from Chromsystems (Gräfelfing, Germany). Normal saline for intravenous infusion was from B. Braun (Melsungen, Germany) and polypropylene syringes were from H-Medical (Bargteheide, Germany). Drug-free serum was purchased from the blood donation center of the Bavarian Red Cross (Wiesentheid, Germany). The 691 pH meter (Metrohm, Switzerland) was calibrated with standard buffers and used to record pH values of test solutions.

4.2. Infusate Stability at Room Temperature

Stability testing in 0.9% normal saline was performed in duplicate. We used only one specific meropenem brand given that generic brands of meropenem were shown to be equivalently stable in normal saline [17]. The antibiotic powder was dissolved in isotonic solutions giving a currently approved dose of 1.0 g meropenem in 50 mL normal saline, yielding a concentration of 2% (corresponding 20 mg/mL). The concentration chosen reflects the standard operating procedure for continuous infusion at the University Hospital, LMU Munich.

In accordance with clinical practice, meropenem continuous infusion solutions were transferred in polypropylene syringes and locked in an infusion pump, delivering with a flow rate of 2 mL/h at 22 °C (±1 °C). At t = 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, and 24 h, 200 µL infusates were sampled in polypropylene tubes, immediately frozen in liquid nitrogen, and stored at −80 °C for up to four weeks until ID-LC-MS/MS analysis.

4.3. Stability in Serum

Stability testing in serum was performed in duplicate. Drug-free serum was buffered with 3 M phosphate buffer pH 7.4 ($K_2HPO_4 \times 3\ H_2O$, titrated with 1 mol/L HCl) (30/1, *v*:*v*) as described by Kratzer et al. [40]. The buffered serum was then warmed to 37 °C (±1 °C) in a water bath and mixed with antibiotic fortified normal saline (95:5, *v*/*v*) prepared from meropenem certified reference material to obtain final concentrations of 2, 10, and 50 mg/L meropenem, respectively. These spiked sera were then aliquoted to 500 µL in polypropylene tubes, incubated at 37 °C (±1 °C) in a water bath. At t = 0, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, and 24 h, aliquots were frozen in liquid nitrogen and stored at −80 °C for up to four weeks until ID-LC-MS/MS analysis. The pH value was measured on replicates and proved to be stable with maximum deviations of +0.3 within these 24 h.

4.4. Laboratory Testing and ID-LC-MS/MS Analysis

General clinical chemical parameters were obtained with standard clinical chemical methods. Creatinine clearance was determined using urine collection ((CrCl = ($crea_{Urin}$ × $volume_{Urin}$)/(collection time × $crea_{Plasma}$)) and subsequently used as an estimate for glomerular filtration rate (GFR).

All samples were independently assayed in triplicate with an isotope dilution LC-MS/MS method designed to simultaneously quantify meropenem (molar mass, MM: 383.46 g/mol) and its open-ring metabolite (ORM) (MM: 401.16 g/mol) in a single analytic run [41] (see Figure 6).

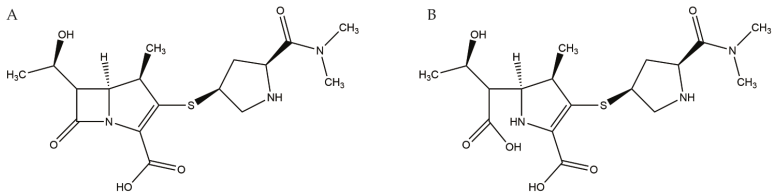

Figure 6. Molecular structure of meropenem (**A**) and its main degradation product, the open-ring metabolite (ORM) (**B**).

In LC-MS/MS analysis, small molecules can be quantified in various biological fluids by measuring the mass-to-charge ratios of target ionized molecular compounds, as well as analyte-specific fragments. Using neat analytes, calibrators and controls are prepared in a given matrix (e.g., serum, plasma) and used for quantitative analysis of matrix-matched samples. To obtain the highest metrological standard, LC-MS/MS is typically combined with isotope dilution standardization. During sample processing, a given concentration of stable isotope-labeled analogues of the analytes of interest is added to a sample (including calibrators and quality controls). These isotope-labeled standards can be selectively detected owing to different molecular weights and fragmentation patterns. Given that they have almost identical physicochemical behavior when compared with their unlabeled counterparts, these standards can be used to efficiently control analytical variations that are introduced from the sample matrix (termed matrix effects). Accordingly, isotope dilution LC-MS/MS has become the gold standard in quantitative small molecule analysis, including TDM [42].

Laboratory-developed calibrators and quality controls (QCs) in both normal saline and serum were obtained from 10x concentrated analyte stock solutions that were prepared by weighing in of meropenem and ORM from certified reference materials.

The linear ID-LC-MS/MS assay range was 1.0–100.0 mg/L for meropenem and 0.62–62.30 mg/L for ORM. Within- and between-run imprecision and inaccuracy were ≤8.0% for all quality controls tested (in most cases, <5%), including four laboratory-developed QCs (with meropenem and ORM) and two commercially available meropenem QC levels. Samples from the continuous infusion stability experiment in normal saline were diluted 1:250 into the linear ID-LC-MS/MS calibration range prior to cleanup. Briefly, 50 µL samples were admixed with internal standard working solution (including meropenem-D_6 and ORM-D_6) and precipitated with methanol, and the supernatants were diluted 1:4 in water and separated on a XSelect HSS PFP column (100 × 2.1 mm, 2.5 µm) with a preceding XSelect HSS PFP Van Guard Cartridge from Waters (Milford, MA, USA) with a 10 mM ammonium formiate in water-formic acid (99.8/0.2, *v*/*v*)/methanol gradient elution within 5.5 min. Analysis was done on an Agilent 1290 Infinity I LC system (Santa Clara, CA, USA) coupled to an AB Sciex TripleQuad 6500+ mass spectrometer (Framingham, MA, USA) with multiple reaction monitoring. Specimens from in vitro stability testing were considered stable if solutions retained >90% of the initial concentration at the timepoint t = 0 h [11,12,43].

4.5. Calculation of In Vitro Elimination Rate Constants

Graphical and statistical analysis was performed using R Version 4.0.2 (CRAN.R-project.org). A non-linear regression analysis was carried out to determine the first-order elimination rates in perfusor syringes and serum according to the following formula:

$$C(t) = C0 \times \exp(-t \times k)$$

where k is the elimination rate constant and t is the time.

4.6. Patient Serum Testing

Blood samples were collected in the pharmacokinetic steady state once daily in patients with infusion duration >48 h of continuous infusion. Dosing was at the discretion of the responsible physician with a median meropenem infusion rate of 250 mg/h. Patient sera were immediately obtained by centrifugation at 2000× *g* for 10 min at 20 °C and stably stored in polypropylene tubes at −80 °C for up to four weeks until ID-LC-MS/MS analysis. Owing to the negligible protein binding of meropenem, only total meropenem concentrations were considered [44]. Given that the ORM has similar physicochemical properties, it is also not assumed to have significant protein binding.

Only patients aged ≥18 years, admitted to the intensive care unit, treated with continuous infusion of meropenem, and subjected to antibiotic therapeutic drug monitoring (TDM) on at least three consecutive days were included in study. The percentage of ORM was calculated in steady state according to the following formula: Ratio = $C_{ORM}/(C_{ORM} + C_{Meropenem})$. Written informed consent was given by all subjects or their legal representatives and the local Ethics Committee of the Ludwig-Maximilians-Universität approved the study (registration number 18–578).

4.7. Population Pharmacokinetic Modelling

To compare the calculated elimination rates (see Sections 4.3 and 4.5) with the total body clearance, a pharmacokinetic model was employed for critically ill patients with continuous infusion. PK modelling was performed using NONMEM® 7.4 (ICON Development Solutions, Ellicott City, MD, USA) and Piraña version 2.9.9 (Certara USA, Inc., Princeton, NJ, USA). A one compartment model with zero-order input and first-order elimination, parameterized in terms of clearance (CL) and volume of distribution (V), was investigated. As all patients were in steady state and the volume of distribution could not be estimated, it was specified according to a published literature value of 26.2 L [26]. The calculated organ independent elimination rate constant in serum was integrated as a fixed value ($CL_{decay} = k*V$) into the model, and the renal clearance was fixed to the GFR. Inter-individual variability was incorporated using an exponential model, while residual unexplained variability was investigated using additive, proportional, and combined variability models.

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