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A new simple HPLC assay for the quantification of ertapenem in human plasma, lung tissue, and broncho-alveolar lavage fluid

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Abstract

Ertapenem is an important newer broad-spectrum carbapenem antibiotic covering various infections caused by common gram-positive and -negative aerobes and anaerobes. Due to its physicochemical peculiarities, pharmacokinetic data of other carbapenems are of limited value in predicting ertapenem distribution into particular compartments of the body. This raises demand for detailed pharmacokinetic studies and, as a consequence, rapid and specific ways of analysis. The HPLC assays for the quantification of ertapenem in biological matrices reported so far are based on columns of 4.6 mm I.D. and involve pre-concentration by use of column-switching. However, automated column-switching technique is not standard equipment with all analytical laboratories. Furthermore, signal-to-noise ratios are likely not to be sufficient for quantification of specimens of low concentration. Therefore, a new HPLC/UV method based on narrow-bore column design using sample pre-cleaning by liquid–liquid extraction has been developed. The assay is rapid for specimen concentrations ≥1 mg/l and is easily tuned to achieve low quantification limits at high chromatographic resolution for lower concentrated samples. The method has been successfully applied to plasma, serum, lung tissue or cell homogenates, and broncho-alveolar lavage fluid with lower limits of quantification of 40 and 20 μg/l, respectively. It was also used for the pharmacokinetic monitoring of ertapenem in humans.

Keywords: Cell; Ertapenem; HPLC; Lavage fluid; LLE; Plasma; Serum; Tissue; UV

1. Introduction

Ertapenem is a newer β-lactam antibacterial agent which covers various infections by gram-positive and -negative aerobes and anaerobes [1]. It has been approved for intra-abdominal and acute gynaecological infections, as well as community-acquired pneumonia. Ertapenem shows structural peculiarities which make it unique among the members of the carbapenem class of antibiotics and markedly influence the physicochemical properties (Fig. 1). One characteristic is the 1-β-methyl residue that is believed to lower the vulnerability of the aza-ring towards hydrolytic cleavage by both acid or base and enzymatic catalysis through β-lactamases and renal dehydropeptidase-I [2]. Another feature is an anionic side chain which is thought to be the reason for the broad spectrum activity and the protein binding properties. Ertapenem binding to human albumin is generally high while decreasing from ∼95% at blood concentrations <50 mg/l to ∼85% at a level of 280 mg/l [3]. This contributes to the observed long plasma half-life of ∼4 h that declines slightly with increasing concentration, i.e. the unbound fraction of drug.

Due to the structural differences compared to other carbapenems, the pharmacokinetics of ertapenem, in particular its distribution into the various compartments of pharmacological interest, are unlikely to be predicted based on data of related analogues. The capabilities of ertapenem regarding the penetration into tissues or lumina, and the permeation of cell membranes into the inner compartment are expected to differ markedly from imipenem or meropenem and need to be addressed in separate studies which require a broad array of analytical methods.

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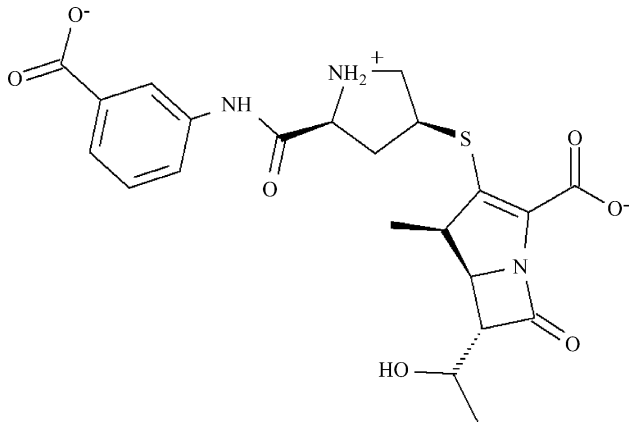

Fig. 1. Chemical structure of ertapenem.

However, only few HPLC/UV methods for the determination of ertapenem in biological matrices are published [3–8]. All are based on standard 4.6 mm I.D. columns and all except one assay for ultrafiltrate of plasma [2] involve a pre-concentrating step using reversed-phase extraction by column-switching prior to separation of the retained compound mixture on an analytical column. These methods show overall run times varying from 7 to 26 min.

On the other hand, the multi-port switching valves required for these methods are not available with all laboratories. Furthermore, compounds of similar to lower polarity are bound to the collecting cartridge and back-flushed onto the separation column, too. This may compromise the signal-to-noise ratio of detection and demand subsequent separation steps.

For a broad applicability, methods for the determination of ertapenem dispensing with automated switching techniques are of particular interest. Such assays need to cope with restrictions by a poor signal-to-noise ratio in alternative ways of sample pre-cleaning and/or using high chromatographic resolution. Analyte separation should be adjustable to avoid interferences of the respective matrix by simple eluent control. With respect to mass spectrometry the eluent should preferably be free of non-volatile components and support high yields in target ion generation.

2. Experimental

2.1. Chemicals, solvents, and solutions of ertapenem

Ertapenem [(4*R*,5*S*,6*S*,8*R*,2′*S*,4′*S*)-3-[[2-[[3-carboxyphenyl) amino]carbonyl]-pyrrolidin-4-yl]thio]-4-methyl-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-en-2-carboxyclic acid monosodium salt, purity 98.2% (LC)] was kindly provided by MSD Sharp & Dohme GmbH (Haar, Germany), 2-[*N*-morpholino]ethanesulfonic acid (MES) and 3-[*N*-morpholino] propanesulfonic acid (MOPS) were obtained from Sigma (St. Louis, USA). All other reagents were purchased in analytical quality from Merck (Darmstadt, Germany); water, methanol, acetonitrile and dichloromethane were of HPLC grade.

Stock solutions and dilutions of ertapenem (1.00 mg/ml) were prepared with MES buffer (0.1 M, pH 6.5) and stored at −80 °C for no longer than one month. MOPS buffer (0.1 M, pH 6.5) was used for the homogenisation of lung tissue.

2.2. Instrumentation

The HPLC system consisted of Shimadzu (Duisburg, Germany) series 10A VP components (CV-AL four way valve with degasser, LC-AD pump, SIL-AD auto-injector, CTO-AS column oven, and a SPD-AV spectrophotometric detector) interfaced via a SCL-A controller module to a PC operating by CLASS VP software (version 6.12 SP5). For mass spectrometry experiments a finnigan TSQ7000 was used with an ESI I ion source.

2.3. Sample preparation

Specimens of (heparinised) blood and lung tissue were taken from patients undergoing therapy by broncho-alveolar lavage (BAL) and/or surgical resection. Blood and lavage fluid were centrifuged (1500 × *g*, 10 min) to yield serum (plasma) or pellets of lung cells, respectively. All samples were stored at −80 °C until analysis. Written informed consent of the subjects and approval of the local ethics committee has been granted prior to collection.

Serum (plasma) aliquots of 200 μl were mixed with an equal volume of MES buffer prior to protein precipitation with 600 μl of acetonitrile. After centrifugation (1500 × *g*, 5 min), the supernatant was poured into a volume of dichloromethane equal to that of acetonitrile (600 μl), vortexed for 5 min and centrifuged again to separate the phases (1500 × *g*, 5 min). The upper aqueous layer (∼400 μl) was separated and subjected to HPLC analysis.

Lung tissues were kept deep frozen with liquid nitrogen and triturated using a swing-mill. Portions of 50–60 mg mush were homogenised with an equal amount of MOPS buffer (w/v), and aliquots of 100 μl homogenate further diluted 1:1 with MES buffer. Protein precipitation was achieved by vortexing (5 min) with 300 μl acetonitrile and subsequent centrifugation (1500 × *g*, 5 min) and extraction with dichloromethane as done with serum (plasma). Pellets of lung cells (2–20 mg) were mixed with 200 μl of MES buffer, then 600 ml acetonitrile and treated with ultrasound for 15 min in an ice bath prior to centrifugation and extraction as with tissue homogenates.

Aliquots of 200 μl BAL liquid were diluted 1:1 with MES buffer and analysed by HPLC without further treatment.

Blanks, quality controls, and calibrators were prepared in the same manner, except that both of the latter were spiked prior to preparation with authentic pure ertapenem to result in final concentrations covering the range of interest of the respective biological matrix (Table 1).

2.4. High pressure liquid chromatography and UV-spectrophotometry (HPLC/UV)

A volume of 10 μl prepared sample was injected for analysis. HPLC was carried out at 15 °C using a guarded (8 mm × 3 mm) column of Hypersil® ODS 125 × 3, 120 Å particle size, 3 μm pore diameter (Macherey & Nagel, Düren, Germany) and a

Table 1

Matrix	Controls (mg/l)	CV (%)		RE (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Plasma	0.040[a]	5.6	17.3	−8.3	3.0
	1.4	5.3	1.5	−9.8	−2.6
	27.7	3.4	3.3	4.1	4.0
	53.5	6.9	6.6	3.6	3.5
Lung tissue homogenate	0.040[a]	1.6	n.d.	−2.7	n.d.
	2.3	1.9	2.9	−1.3	−0.6
	7.7	4.1	2.6	0.1	−0.0
	10.0	0.6	3.4	7.3	3.2
BAL fluid	0.020[a]	3.9	3.6	6.0	7.4
	0.050	3.1	5.3	−9.3	−6.2
	0.080	0.6	2.6	−1.9	−0.1
	0.100	1.1	2.5	−0.4	1.2

Precisions (coefficients of variation, CV) and accuracies (relative errors, RE) for the quantification of ertapenem in selected matrices ($n = 5$–10, n.d. not determined).
[a] Denotes the lower limit of quantification (LLoQ) for the respective matrix.

gradient eluent at a flow rate of 80 μl/min. The mobile phase comprised initially of 95:5 water/methanol (v/v, 25 mM ammonium acetate) and was then ramped to 50:50 within 5 min for samples of all matrices ≥1 mg/l ertapenem. For samples of BAL fluid the same mixing ratio was adjusted within 30 min, for samples of all other matrices containing <1 mg/l ertapenem a double column was used and a final ratio of 52.5:47.5 attained within 50 min. Absorbance of the eluate was recorded at a wavelength of 300 nm.

2.5. Calibration and validation of the assays

Calibrations were calculated by linear least-squares regression analyses of the signal area of ertapenem versus the corresponding concentrations. Calibrators contained ertapenem at final concentrations from as low as 40 μg/l up to 100.0 or 16.0 mg/l for serum and plasma or tissue, respectively, and from 20 μg/l to 1.0 mg/l for BAL fluid. Each calibration function was based on at least eight calibrators prepared and measured in duplicate. The validation of the method was performed according to the guidelines of the FDA [9,10] involving blanks and quality controls. The intra-day repeatability of the method was tested by multiple analyses of individual samples within the same day ($n \geq 5$). Inter-day reproducibility was tested on 3 or more consecutive days. The recoveries of ertapenem were assessed at 0.1 and 20.0 mg/l by comparing the peak areas obtained from spiked and prepared biological matrices to those determined from aqueous standards.

2.6. Medical application of the assays

Blood, lung tissue, and BAL liquid of patients having received a single dose of 1 g ertapenem (Invanz®) by intravenous infusion over 30 min were collected and analysed. Plasma concentration–time profiles were established withdrawing blood samples before and 0.5, 1, 2, 4, 6, 8, 12, 16, 18, and 24 h after the start of administration, and the data evaluated using the software Kinetica® (version 4.2; InnaPhase Corp., Philadelphia, USA). The apparent half-life was calculated as $\ln(2)/\lambda z$, where λz denotes the time constant of the terminal slope. The area under the plasma concentration–time curve (AUC) was calculated using the log–linear trapezoidal rule as covered by data points (AUC_{last}) or extrapolated to infinity (AUC_{total}). The concentrations in lung tissue, BAL fluid and alveolar cells from BAL fluid were assessed in order to prove the method's suitability under a clinical therapeutic regimen.

3. Results and discussion

A new HPLC-method for the determination of ertapenem in biological matrices was developed which focusses on improving the signal-to-noise ratio by sample pre-cleaning and modifications of the chromatographic system rather than applying a pre-concentrating step by column-switching. The LC-system was set up with widespread standard constituents and a simple gradient eluent composed of water and methanol containing ammonium acetate in order to stabilise the pH and reduce the influence of endogenous ions. The mobile phase is also suitable for electrospray mass spectrometry.

3.1. Improvement of the signal-to-noise ratio

In a general modification of the methods described before [3–8] an HPLC column of 3.0 mm I.D. with an appropriate lining (0.13 mm I.D.) was used instead of a 4.6 mm I.D. system which corresponds to a theoretical increase in analyte peak concentration at the column outlet up to a factor of 2.35 [11]. This narrow-bore HPLC system was useful for the rapid determination of ertapenem exceeding concentrations of 1 mg/l by steep gradients (from 5% or 10 to 50% MeOH in 5 min) at retention times between 10 and 15 min (Fig. 2).

In contrast, for samples of low ertapenem concentration (<1 mg/l) signal-to-noise ratios of fast elutions were not sufficient for quantification. Such unfavourable condition was offset earlier by pre-concentrating ertapenem via reversed-phase extraction using column-switching [4,5,7]. However, this tech-

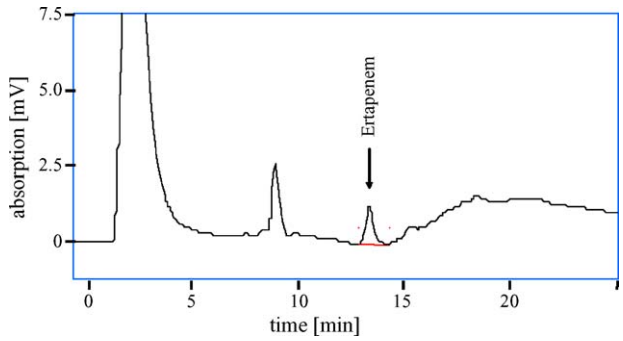

Fig. 2. Representative chromatogram of ertapenem from human plasma at a concentration of 1.4 mg/l using an initial eluent of 95:5 water/methanol (v/v, 25 mM ammonium acetate) ramped to 50:50 within 5 min (UV absorption at 300 nm).

nique is not available at all laboratories. Furthermore, preconcentrating may not only accumulate the analyte but also matrix components of similar polarity and, thus, selective elution procedures flushing compounds of higher and retaining compounds of lower hydrophilicity might be required. Similar circumstances occur when liquid–liquid extraction is applied in order to isolate and concentrate the target analyte; another approach to sample processing by LLE is to remove concomitant material. Since ertapenem shows particular high solubility in water the latter alternative was chosen using dichloromethane as the organic medium. The resulting aqueous phase showed a significantly diminished chromatographic background and was considered applicable for HPLC analysis. Optimisation of HPLC was directed toward shifting the ertapenem signal into an interference-free range of the chromatogram which was subsequently managed for sample concentrations $\geq$1 mg/l ertapenem. Lower concentrated samples were resolved further by duplicating the column length. Finally, excellent signal-to-noise ratios with low quantification limits (cf. Table 1) were accomplished even for diluted samples of each matrix without involving a concentrating step (e.g., Figs. 3 and 4, insets I). However, the benefit was achieved at the expense of longer run times (total 50–65 min). Typical retention times of ertapenem were

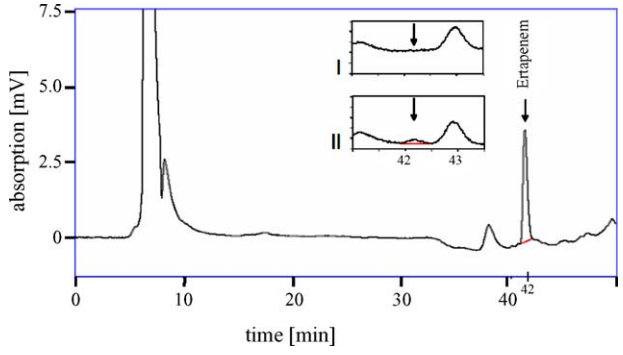

Fig. 3. Representative chromatograms of ertapenem from human plasma at a concentration of 1.1 mg/l and the LLoQ (40 μg/l, inset II) using a duplicate column and an initial eluent of 95:5 water/methanol (v/v, 25 mM ammonium acetate) changed to 52.5:47.5 within 50 min (absorbance at 300 nm). Inset I indicates the low and smooth background of plasma blanks in the relevant part of the chromatogram.

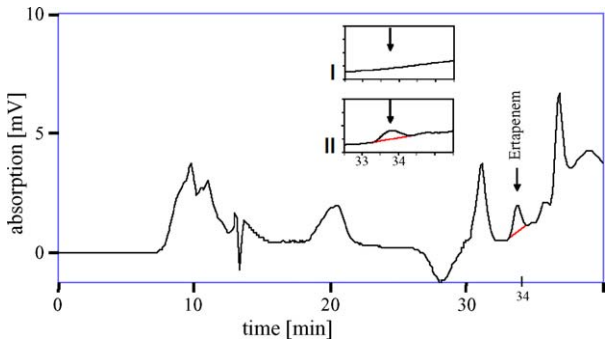

Fig. 4. Representative chromatograms of ertapenem at level of 0.3 μg/g in alveolar cells precipitated from BAL fluid and at the respective LLoQ (20 μg/l, inset II) registered using a duplicate column and an initial eluent of 95:5 water/methanol (v/v, 25 mM ammonium acetate) adjusted to 52.5:47.5 within 30 min (absorbance at 300 nm); inset I shows a blank recording.

42–43 min for plasma or serum and lung tissue, and 34–36 min for BAL liquid and alveolar cell homogenates.

3.2. Validation of the assays

The recoveries of ertapenem ranged 87–93%. The calibration functions of all matrices were linear within the calibration ranges with correlation coefficients $r^2 \geq 0.998$.

The lower limits of quantification (LLoQs) were determined in order to comply with established state-of-the-art criteria [9,10] and set to 20 μg/l for BAL fluid and alveolar cell homogenates, and 40 μg/l for plasma, serum and tissue homogenates. The intra- and inter-day precisions for all controls as expressed by the coefficient of variation (CV) were <6% except day-to-day variation at the LLoQ for plasma (17.3%); the intra- and inter-day accuracies expressed as the relative errors (RE) ranged from −9.8 to +7.4%. The data are summarised in Table 1. Typical chromatograms at the LLoQ for plasma and alveolar cell are depicted in the insets II of Figs. 3 and 4, respectively.

The specificity of the assay was tested for each matrix using blanks from 25 different healthy donors and from 15 patients receiving co-medication for thoracotomy [12]. Furthermore, blank samples of each matrix spiked with the other β-lactams meropenem, imipenem or piperacillin were checked for interferences with the ertapenem signal. Neither chromatogram revealed any limitations for the assay.

Long-term stability of ertapenem was monitored at the concentrations 0.02, 0.2 and 4.0 mg/l. No significant loss was observed at −80 °C for at least one year and at +6 °C for at least 7 days.

3.3. Medical implications of the assays

These data demonstrate that the described method is valid for monitoring ertapenem concentrations in body liquids and tissues. The quantification limits are sufficiently low to assess the level of the minimum inhibitory concentrations of clinically relevant germs (MIC_{90}: inhibition of 90% of isolates) such as Streptococcus pneumoniae (0.03–2.0 mg/l) Haemophilus influenzae (0.06 mg/l), Enterobacteriaceae (0.008–0.5 mg/l) or methicillin-susceptible Staphylococci and Anaerobes ($\leq$0.5 mg/l) [13].

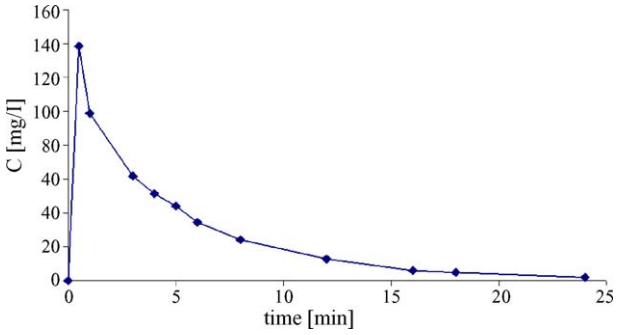

Fig. 5. Example of a concentration vs. time profile of ertapenem in human plasma after i.v. administration of a single dose 1 g ertapenem (Invanz®, infusion time 30 min). The maximum concentration observed after the start of infusion was 138.5 mg/l, the total area under the curve (AUC_{total}) was extrapolated 563.3 mg l^{-1} h, the clearance and the terminal half-life were calculated 29.6 ml min^{-1} and 4.2 h, respectively.

The method has been used to study the pharmacokinetics of ertapenem in plasma or serum, tissue compartments, and BAL liquid after intravenous administration of a single dose of 1 g ertapenem (Invanz®, Figs. 2–4) [12]. A representative concentration–time curve for plasma is shown in Fig. 5; the maximum concentration (C_{max} = 138.5 mg/l), and the calculated values as the areas under the curve (AUC_{total} = 563.3 mg l^{-1} h, AUC_{last} = 551.2 mg l^{-1} h), the Clearance (29.6 ml min^{-1}), and the terminal half-life (4.2 h) are in well accordance with published data [3,8,14].

4. Conclusions and summary

A method has been developed to assess ertapenem concentrations in various matrices such as plasma, serum, tissue homogenates, and BAL fluid without a pre-concentrating step like column-switching.

For higher ertapenem concentrations (≥1 mg/l) the method performs very well with steep gradient ramps at retention times from 10 to 15 min. These rapid assays meet the requirements for most pharmacokinetic studies in humans like monitoring concentration–time curves in body liquids or tissue homogenates after administration of therapeutic standard doses of Invanz™.

For low concentrated specimens (≤1 mg/l) and/or samples of very low amount as, for instance, poorly penetrated tissue or exiguous cell pellets, the signal-to-noise ratio was significantly enhanced by removing noise, i.e., interfering compounds from the matrix and improving the chromatographic resolution, the latter, however, at the expense of longer retention time (≥33 min).

In summary, the presented analytical method supplements the array of established procedures using column-switching technique [4,5,7] thereby broadening the scale of analytical approaches to the quantification of ertapenem in biological matrices. The method is suitable for rapid measurements at moderate sensitivity as well as for highly selective and sensitive determinations. The assays have been applied for pharmacokinetic profiling of ertapenem in humans [12] and provide useful tools for further pharmacokinetic studies.

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