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Assay methodology for the quantitation of unbound ertapenem, a new carbapenem antibiotic, in human plasma

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Abstract

Ertapenem is a new once-a-day antibiotic with excellent coverage of common community gram negative and gram positive aerobes and anaerobes. It demonstrates nonlinear protein binding in human plasma (about 94% bound). An assay for unbound drug was developed to study the pharmacokinetics of unbound ertapenem in plasma. Unbound drug is separated from plasma samples (1.0 ml) by ultrafiltration using a Centrifree® centrifugal filter device. Ertapenem (vulnerable to hydrolysis of the beta-lactam moiety) is stabilized in the filtrate by adding an equal volume of 0.1 *M* MES buffer, pH 6.5 and then is analyzed by reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection (300 nm). Non-specific binding to the Centrifree® device is <3%. A suitable internal standard is not available. The assay is specific and linear over the concentration range of 0.25 to 100 μg/ml in plasma filtrate. The lower limit of quantitation (LLOQ) is 0.25 μg/ml. Intra-day precision is C.V.<10% and accuracy ranges from 97 to 101% of nominal concentration. Inter-day precision and accuracy were determined using quality control samples (QCs) prepared in plasma ultrafiltrate at 0.5, 12 and 80 μg/ml and stored at −70 °C with stabilizer. Inter-day assay accuracy and precision ranged from 100 to 111% of nominal concentration and 1.8 to 5.3% C.V. (n=40), respectively. The assay has been used to analyze plasma samples from subjects receiving 500 and 2000 mg i.v. doses of ertapenem (30 min infusion).

Keywords: Ertapenem; Carbapenem

1. Introduction

Ertapenem is a new beta-lactam antibiotic (Fig. 1) [1,2]. It is a structurally unique member of the carbapenem class of antibiotics currently under development for once-a-day dosing in community acquired and mixed infections. The drug possesses a β-lactam group sensitive to acid and base catalysis (λ_{max} 300 nm→end absorption) and is resistant to β-lactamases. Unlike imipenem, it has a unique anionic side-chain and a 1-β-methyl group. The 1-β-methyl is believed to provide stability against human renal dehydropeptidase-I (DHP-I). The anionic side-chain is presumably responsible for the once-a-day dosing and the unique spectrum of coverage. In particular, ertapenem has excellent coverage of common community acquired gram positive and gram negative aerobes and anaerobes, and minimal coverage of pseudomonas, a common nosocomial patho-

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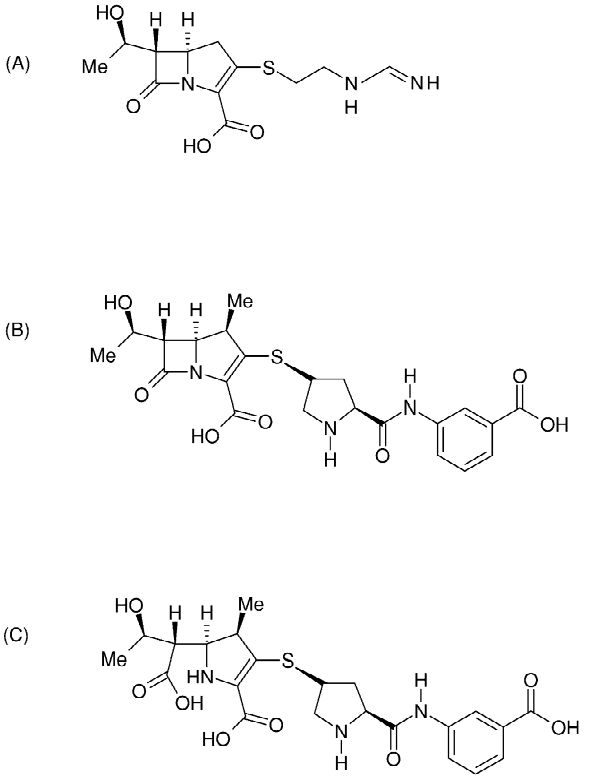

Fig. 1. Structures of imipenem (A), ertapenem (B) and its open-lactam form (C).

gen. Its major metabolite is the open-lactam form of the carbapenem. Also, ertapenem is highly protein bound (about 94% at total drug concentrations <100 μg/ml). It demonstrates non-linear binding in human plasma and slightly non-linear pharmacokinetics for total drug in healthy male subjects given single i.v. doses of 250 to 3000 mg [3].

An assay for unbound ertapenem was necessary to study its pharmacokinetics. Total plasma concentrations are determined via a direct-injection HPLC assay using absorbance detection at 300 nm [4]. An assay for unbound drug was developed using ultrafiltration of plasma samples and HPLC analysis of the stabilized filtrate. The standards and QCs were prepared in control plasma ultrafiltrate. An additional set of quality controls was prepared in control plasma to evaluate the ultrafiltration step. An internal standard was not used in the assay.

The method has been applied to several clinical studies in which the pharmacokinetics of total drug and unbound drug were compared.

2. Experimental

2.1. Chemicals and reagents

Ertapenem, (4*R*,5*S*,6*S*,8*R*,2′*S*,4′*S*)-3-[[2-[[3-carboxyphenylamino]carbonyl]-pyrrolidin-4-yl]thio]-4-methyl-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-en-2-carboxylic acid monosodium salt [5] and its open-lactam metabolite were obtained from Merck Research Laboratories (Rahway, NJ, USA). 2-[*N*-Morpholino]ethanesulfonic acid (MES acid) and its respective sodium salt (MES sodium salt) were purchased from Sigma (St. Louis, MO, USA). ACS grade sodium phosphate dibasic anhydrous, *o*-phosphoric acid, 85%, and optima methanol, were obtained from Fisher Scientific (Pittsburgh, PA, USA). All chemicals were used as received.

2.2. Equipment

The HPLC system consisted of equipment from Waters (Milford, MA, USA) and Applied Biosystems (Foster City, CA, USA): Model 717plus refrigerated autosampler, a 600E system controller, and 600P pump, and a Spectroflow 783A ultraviolet absorbance detector. The detector was set at 300 nm and its signal was acquired and processed by a Perkin-Elmer Nelson Turbochrom Client/Server data acquisition system (Cupertino, CA, USA).

2.3. Preparation of buffers

A 1.0 *M* MES buffer, pH 6.5 was prepared by mixing 15.5 g MES sodium salt and 5.58 g MES acid in a 100-ml flask and diluting with Milli-Q filtered water (18.2 Meq ohm/cm, Millipore, Bedford, MA, USA) to the fiducial mark. The 1.0 *M* MES buffer was further diluted to 0.1 *M* MES buffer, pH 6.5 (stabilizer) with Milli-Q water (1:10 v/v).

2.4. Ultrafiltration

Clinical or quality control samples, stored at −70 °C, were thawed to room temperature, mixed and centrifuged at 1200 *g* at room temperature for 5 min. A 1-ml aliquot was transferred to a Centrifree® Centrifugal Filter Device (Amicon Bioseparations, Millipore). At ~1.25 h after thaw, the device was centrifuged at 1500 *g* for 15 min at room temperature in a Beckman Model J-68 centrifuge (Beckman Instruments, Fullerton, CA, USA). The filtrate (about 80 μl) was then transferred using a micropipette to a 12×75 mm polypropylene conical centrifuge tube and mixed with an equal volume of 0.1 *M* MES buffer, pH 6.5. The sample was diluted further with control plasma filtrate-stabilizer (1:1, v/v), if necessary. The sample was then placed in an autosampler vial and refrigerated at 5 °C. All the procedures from thawing to placement in the autosampler were performed at room temperature.

2.5. Chromatographic conditions

The filtrate sample was chromatographed on a Keystone Scientific BDS Hypersil C_{18} 5 μm (100× 4.6 mm) column with an inline filter of 0.5 μm (Upchurch Scientific, Oak Harbor, WA, USA) at ambient temperature. The mobile phase was 10% methanol in 25 m*M* phosphate buffer, apparent pH 6.5. The autosampler was set to 5 °C and an injection volume of 50 μl. The pump flow was 2 ml/min and the absorbance detector was set at 300 nm.

2.6. Non-specific binding

The non-specific binding of ertapenem to the Centrifree® device during ultrafiltration was assessed (n=5) in 0.1 *M* sodium phosphate buffer, pH 7.4 at 1.0, 5.0, 50, and 100 μg/ml. Ertapenem was analyzed before and after the ultrafiltration process to obtain recovery data using the Centrifree® device.

2.7. Stability

The stability of ertapenem in plasma ultrafiltrate was conducted at room temperature and 5 °C with and without a buffer, 0.1 *M* MES, pH 6.5 (1:1, v/v). Plasma ultrafiltrate samples were prepared at 5 and 40 μg/ml. The samples were placed in the autosampler for HPLC analysis at room temperature over 24 h. A similar set of samples were tested at 5 °C for 24 h.

2.8. Effect of heparin on ertapenem protein binding

The effect of heparin on the protein binding of ertapenem was assessed by comparing ertapenem in heparinized plasma and serum at 10 and 75 μg/ml. Fresh heparinized plasma and fresh serum samples were prepared at each concentration (n=3) and analyzed on day 0. The samples were then frozen overnight, thawed and analyzed again on day 1.

2.9. Preparation of calibration standards

Stock solutions of ertapenem in the carboxylic acid form were prepared at 10, 100, and 500 μg/ml in 0.1 *M* MES buffer, pH 6.5 (stable for 14 days at −20 °C). Control plasma filtrate was prepared by centrifuging control human plasma (Sera Tec, New Brunswick, NJ, USA) in a Filtron Macrosep 30K (PALL Filtron, PALL Corporation, Northborogh, MA, USA) at 2500 *g* at room temperature. Calibration standards were prepared by mixing appropriate volumes of the stock solutions and control plasma filtrate in 12×75 mm conical polypropylene test tubes to achieve nine different concentrations from 0.25 to 100 μg/ml. The standards were then mixed with an equal volume of 0.1 *M* MES buffer, pH 6.5, transferred to autosampler vials and placed in a refrigerated autosampler at 5 °C.

2.10. Preparation of plasma filtrate QCs to evaluate accuracy and precision of the HPLC assay method

Stock solutions of ertapenem were prepared at 0.1, 1.0, and 10 mg/ml in 0.1 *M* MES buffer, pH 6.5. Appropriate volumes from the stock solutions were transferred to 25-ml flasks and the flasks were diluted to the fiducial mark with control human plasma filtrate and then mixed by vortex. The concentrations of the plasma filtrate QC solutions were initially prepared at 0.5 and 40 μg/ml. Later, the number of QC concentrations was expanded to a

low, middle, and high concentration at 0.5, 12.0, and 80 μg/ml.

The QC solutions were then mixed with an equal volume of 0.1 *M* MES buffer, pH 6.5. The stabilized solutions were then divided into 600-μl aliquots in conical polypropylene tubes with caps and stored at

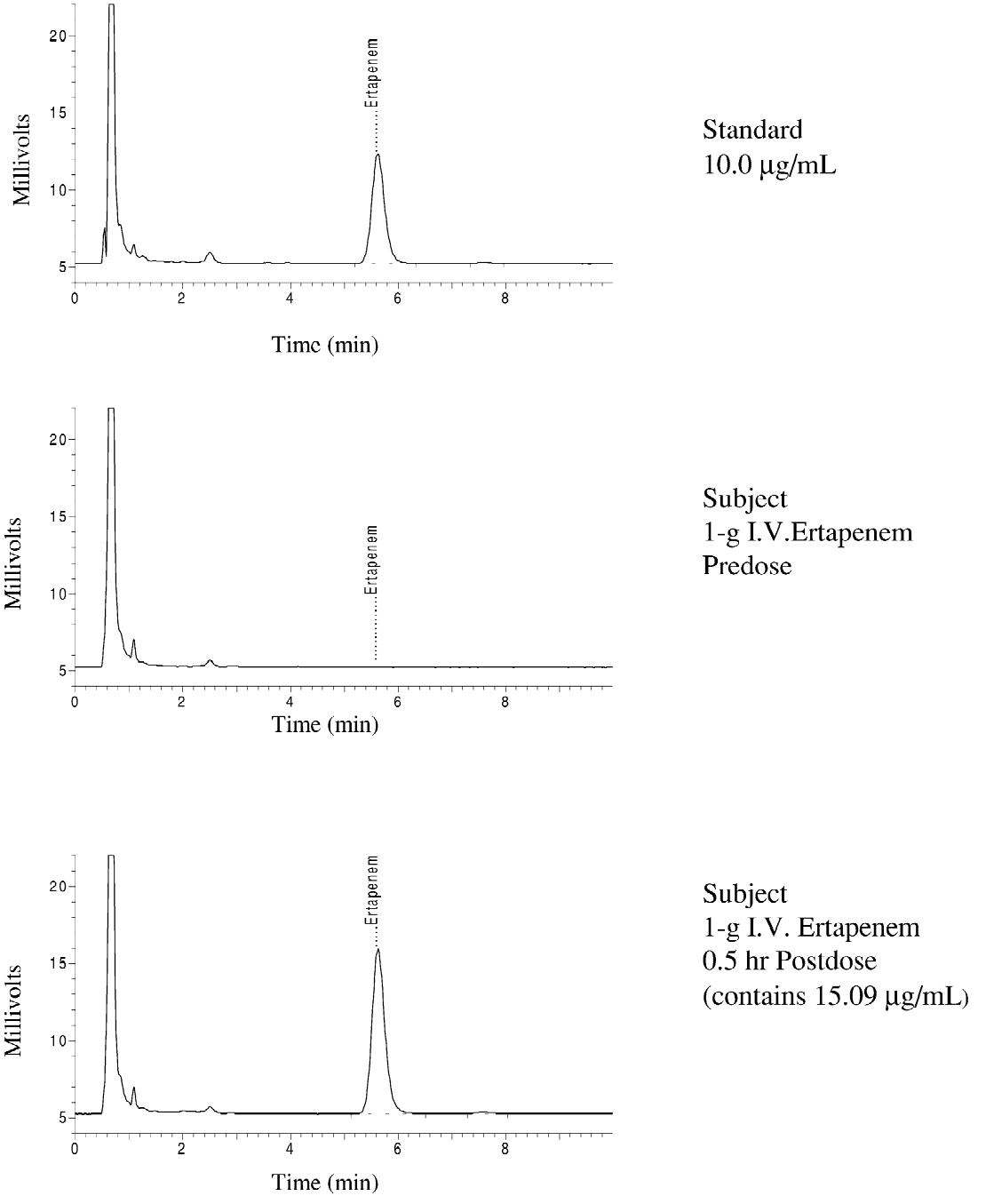

Fig. 2. Representative chromatograms of unbound (free) ertapenem in human plasma.

−70 °C. The QCs were removed from storage at a specific time for analysis to determine assay inter-day accuracy and precision.

2.11. Preparation of plasma QCs to evaluate ultrafiltration, long-term and freeze–thaw stability

QC samples of ertapenem were prepared in commercial control plasma using a similar procedure described for the plasma filtrate QCs. Appropriate volumes from the 0.1 mg/ml stock solution of ertapenem were transferred to 100-ml volumetric flasks and diluted to the fiducial mark with plasma. The flasks were gently mixed by inversion (to avoid emulsions) and incubated for 30 min at 37 °C in a water bath (TurboVap LV, Zymark, Hopkinton, MA, USA). Ertapenem is stable (>96%) for about 1.0 h under these conditions. The flasks were mixed again by inversion and divided into 1.2-ml aliquots in 12×75 mm polypropylene tubes with caps and stored at −70 °C. The QCs were prepared at 10, 75 and 180 μg/ml.

2.12. Collection and storage of plasma samples

The blood samples were collected in heparinized tubes and placed on wet ice. The samples were then centrifuged (1200 *g*) at 0–5 °C within 1 h of collection for plasma. The plasma samples were stored long-term in polypropylene cryotubes at −70 °C until analysis.

3. Results and discussion

3.1. HPLC assay

The chromatography and detection for unbound ertapenem is very similar to the assay methodology previously reported for determination of total ertapenem in plasma [4], except that column-switching used for on-line extraction was not necessary for this assay. A 50-μl aliquot from a plasma filtrate sample, buffered with 0.1 *M* MES, pH 6.5, is injected directly onto a reversed-phase column and chromatographed using mobile phase containing 10% methanol in a 25 m*M* phosphate buffer, apparent pH 6.5. The UV absorbance detector was set at 300 nm, a λ_{max} for ertapenem. The chromatography is specific for separation of ertapenem from endogenous plasma material and its major metabolite, the open β-lactam (Fig. 2). The LLOQ is 0.25 μg/ml and the lower limit of detection is 0.06 μg/ml (*S*/*N*: 3:1). The assay method is linear from 0.25 to 100 μg/ml and a representative regression curve $y = mx + b$ with a weighting of $1/y$ is characterized where $m = 12248$, $b = -147$ and $r^2 = 1.0000$. Initial attempts at a lower limit of quantitation (LLOQ) of 0.1 μg/ml were unsuccessful because of poor integration precision.

3.2. Ultrafiltration

A plasma sample is thawed at room temperature, mixed, centrifuged (1200 *g*) for 5 min at room temperature and a 1.0-ml aliquot is transferred to the Centrifree® device. Approximately 1.25 h after thawing, the ultrafiltration of the sample by centrifugation in the Centrifree® device is conducted at room temperature for 15 min at 1500 *g*. The filtrate sample is then stabilized with 0.1 *M* MES buffer, pH 6.5 (1:1, v/v). Performing the above procedures within a specified time and stabilization of the filtrate as soon as possible minimized losses of the ertapenem to hydrolysis. This procedure has been semi-automated using the Packard MultiPROBE® IIEX Robotic Liquid Handling System (four fixed probes) and custom designed racks to handle the transfer of the plasma aliquots to the Centrifree® devices [6]. The automation improved the assay turn-around time and reproducibility by providing a quicker and more

Table 1
Summary of non-specific binding of ertapenem to Centrifree® device after ultrafiltration (*N* = 5)

Concentration (μg/ml)	Recovery after ultrafiltration[a] (%)	Non-specific binding[b] (%)
1	98.97	1.03
5	94.13	5.87
50	97.88	2.12
100	98.23	1.77
Mean	97.30	2.70
SD	2.163	2.163
% C.V.	2.22	80.19

[a] % Recovery is determined by [mean conc. after filtration/mean conc. before filtration]×100%.

[b] % Non-specific binding is determined by (100%−% Recovery).

consistent method for transfer of samples to the Centrifree® devices.

Non-specific binding of ertapenem during the ultrafiltration process to the inner walls, filter membrane or receiving cup of the Centrifree® device was determined to be <3% (Table 1).

The effect of heparin on the protein binding of ertapenem was evaluated by comparing unbound concentrations (and % unbound of total) of ertapenem determined from human heparinized plasma and from human serum. The unbound concentrations in Table 2 do not indicate a significant effect of heparin on the equilibrium of drug bound to protein. The unbound drug concentrations after freezing the samples (from day 0 to day 1) showed similar results.

3.3. Short-term stability

Ertapenem in plasma filtrate is stable (>95%) for more than 25 h at room temperature when buffered with 0.1 *M* MES, pH 6.5 (1:1, v/v). Without the buffer, the analyte in plasma filtrate degrades to 95% at 4.5 h and to decrease, thereafter (Fig. 3).

Ertapenem is stable (>98%) for at least 23 h at 5 °C in plasma filtrate and plasma filtrate with 0.1 *M* MES buffer, pH 6.5.

3.4. Accuracy and precision

Within-day accuracy and reproducibility at each calibration standard prepared in plasma filtrate are listed in Table 3 and show precision with <6.2% C.V. ($n=5$) and accuracy of 100.1±1.3% based on nominal concentrations (each concentration is calculated from an average regression curve). Intra-day evaluation of plasma filtrate QCs ($n=5$) gave similar results with precision <2.1% C.V. and accuracy of 102.8+7.9%.

Inter-day precision and accuracy for plasma filtrate QCs were used to evaluate the assay methodology (Table 4). Average precision ranged from 1.8 to 5.3% C.V. over 5 months ($n=40$); accuracy ranged from 100 to 111%. Similarly, plasma QCs were used to assess the reproducibility of the ultrafiltration step and the average precision ranged from 9.3 to 16.2% C.V. Plasma filtrate QCs were analyzed with the plasma QCs.

3.5. Long-term stability

The long-term storage and freeze–thaw stability of ertapenem in plasma was assessed for chemical/metabolic loss and for protein-binding equilibrium. For drug degradation, total drug was measured using a previously reported assay [4] and for protein binding, unbound drug was quantitated using the method described herein. Plasma QC samples without stabilizer were used to measure long-term storage at −70 °C and freeze–thaw stability.

The chemical and metabolic stability of ertapenem in plasma samples stored long-term at −70 °C was evaluated at 0.25 and 40 μg/ml. Total drug was analyzed in these QCs over 15 months and the results showed no significant degradation. Freeze–thaw stability of these plasma QCs was assessed over four cycles. Samples were frozen, thawed, and analyzed, and then the cycle was repeated. The concentration values suggest that there was no loss of ertapenem through the four cycles.

The protein binding stability of ertapenem was evaluated by measuring unbound drug in plasma at 10, 75, and 180 μg/ml stored at −70 °C. Plots of unbound concentrations of ertapenem in low,

Table 2
Protein binding stability for unbound ertapenem in fresh plasma (heparin) and serum from day 0 to day 1 after one freeze–thaw cycle

Sample ($n=3$)	10.0–Heparin[a]		75.0–Heparin[a]		10.0–Serum[a]		75.0–Serum[a]	
	Conc. (μg/ml)	Unbound[b] (%)	Conc. (μg/ml)	Unbound[b] (%)	Conc. (μg/ml)	Unbound[b] (%)	Conc. (μg/ml)	Unbound[b] (%)
Mean day 0	0.43	4.33	4.48	5.98	0.44	4.40	4.29	5.72
Mean day 1	0.55	5.50	4.82	6.43	0.47	4.70	4.35	5.80

[a] 10 and 75 μg/ml of total ertapenem (nominal) in heparinized plasma and in serum.
[b] Value based on nominal concentration for total ertapenem.

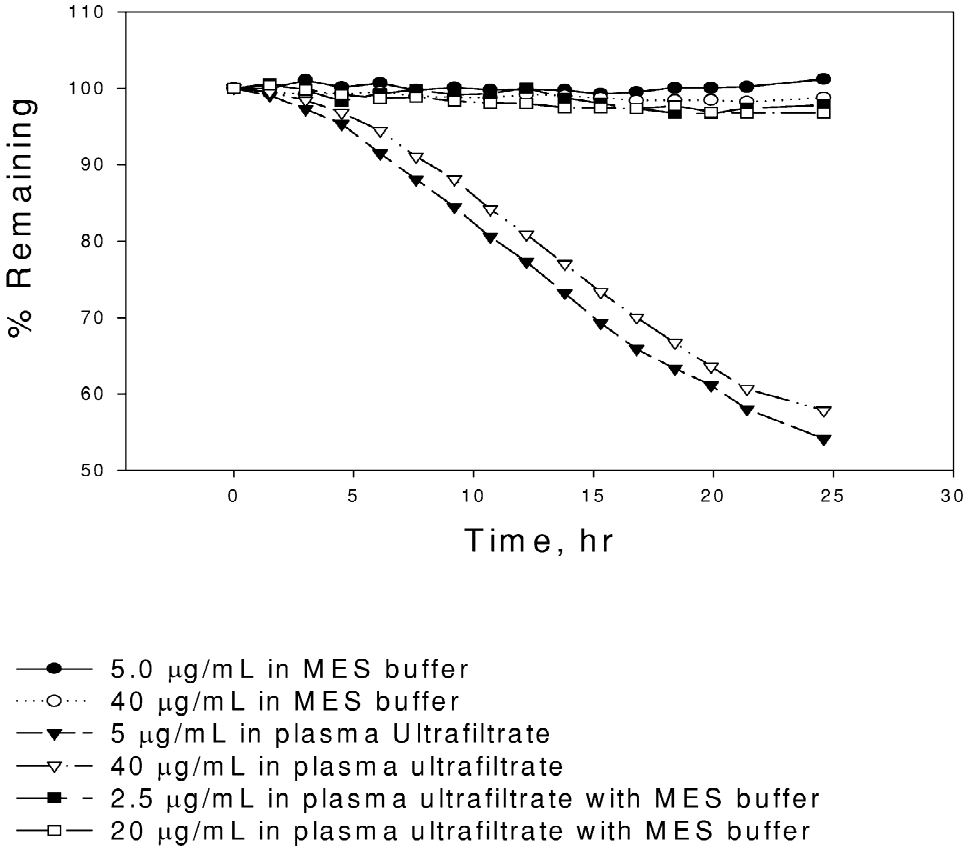

Fig. 3. Stability of ertapenem in 0.1 *M* MES buffer, pH 6.5 and in plasma ultrafiltrate with and without stabilizer at room temperature (MES buffer = 0.1 *M* MES buffer, pH 6.5).

Table 3
Intra-day precision and accuracy[a]

Nominal concentration (μg/ml)	Mean (μg/ml)	Precision (% C.V.)	Accuracy (% of nominal)
Standards[b]			
0.25	0.24	6.19	96.8
0.50	0.50	3.06	100.8
1.0	1.01	1.43	101.2
2.5	2.52	1.08	100.6
5.0	5.04	0.53	100.8
10.0	10.05	0.66	100.5
25.0	25.11	0.91	100.4
50.0	49.87	0.62	99.7
100.0	99.92	0.60	99.9
QCs[b]			
0.5	0.468	2.05	93.5
12	12.8	0.18	106.7
80	86.6	0.13	108.3

[a] $n = 5$.
[b] Ultrafiltrate plasma.

Table 4
Inter-day precision and accuracy ($n=40$)

QC matrix	Nominal concentration (μg/ml)	Mean (μg/ml)	C.V. (%)
Plasma ultrafiltrate	0.5	0.5	5.3
	12	13.1	1.9
	80	89.0	1.8
Plasma	10	0.86	16.2
	75	8.01	10.3
	180	28.1	9.3

medium, and high QCs show stability over 500 days (Figs. 4 and 5). For comparison, the concentrations of unbound ertapenem from plasma QCs were plotted with concentrations of ertapenem measured in plasma filtrate QC samples (stabilized), stored and analyzed with the plasma QCs. The trends for both sets of data suggest similar stability for total drug and unbound drug (separated from protein bound drug) over long-term storage.

Freeze–thaw analysis of unbound ertapenem concentrations in plasma QCs was tested over three cycles at 10, 75, and 180 μg/ml (Table 5). Unbound concentrations measured from the QCs after one cycle showed a very modest rise.

3.6. Application

The plasma assay method was used to quantitate unbound ertapenem concentration in phase 1 pharmacokinetic studies. For a single ascending dose study, plasma profiles of unbound ertapenem from a representative healthy male subject, administered single i.v. doses (30 min infusion) of ertapenem at 500 mg and 2000 mg, are shown in Fig. 6. A plot of percent unbound drug versus total drug from the same subject is shown in Fig. 7. The first plot shows higher unbound plasma concentrations with the 2000 mg dose as compared to the 500 mg dose. The second plot demonstrates an increase in percent unbound with an increase in total drug concentration.

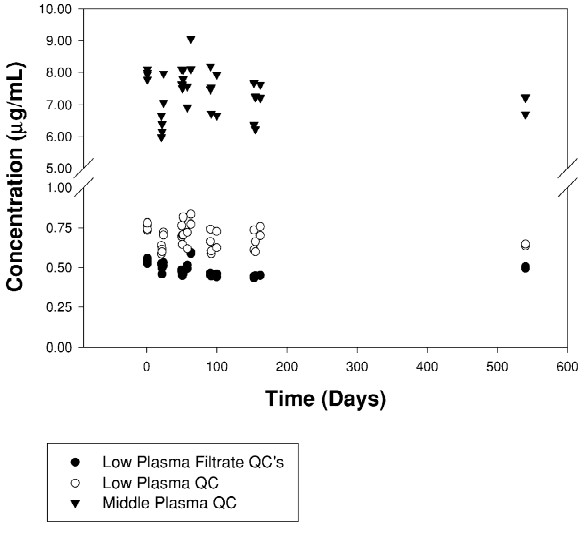

Fig. 4. Long-term stability of unbound ertapenem in low and middle plasma QC samples (10 and 75 μg/ml) and low plasma ultrafiltrate QC samples (0.54 μg/ml) stabilized with 0.1 *M* MES buffer, pH 6.5 stored at −70 °C.

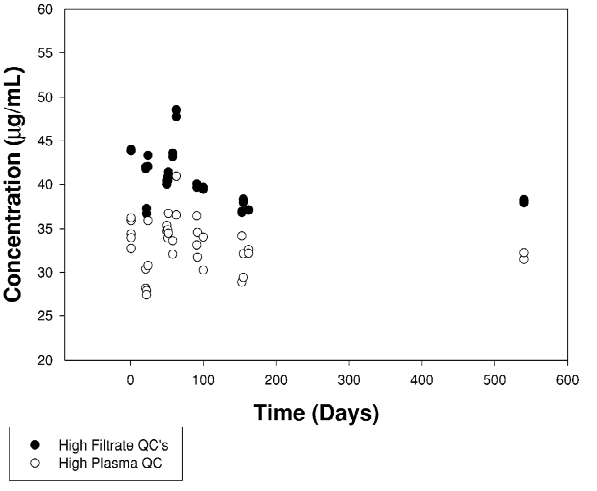

Fig. 5. Long-term stability of unbound ertapenem in high plasma QC samples (180 μg/ml) and in high plasma ultrafiltrate QC samples stabilized with 0.1 *M* MES buffer, pH 6.5 (40 μg/ml) stored at −70 °C.

Table 5
Freeze–thaw cycles of quality control samples for unbound ertapenem in human plasma[a]

Cycle	N	Low QC		Middle QC		High QC	
		Mean conc. (μg/ml)	Standard deviation	Mean conc. (μg/ml)	Standard deviation	Mean conc. (μg/ml)	Standard deviation
Nominal		10		75		180	
1	4	0.85	0.04	9.39	0.46	32.27	0.30
2	4	0.84	0.06	9.86	0.16	34.53	1.19
3	3	0.90	0.01	11.46	0.14	36.76	0.59

[a] Commercial plasma from Sera Tec (previously frozen).

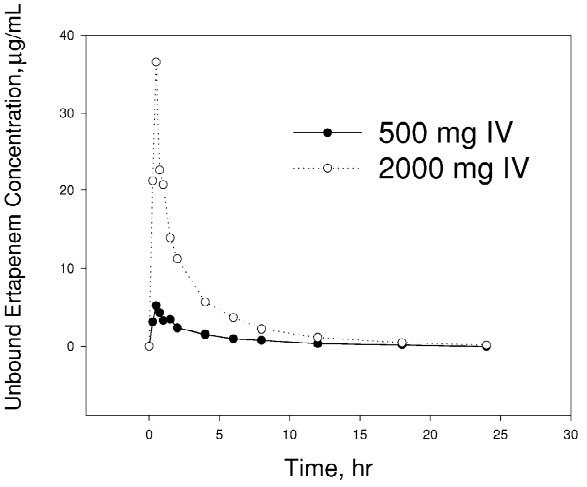

Fig. 6. Plasma profiles of unbound ertapenem from a healthy male subject receiving single intravenous infusions (30 min) of ertapenem at 500 mg and 2000 mg.

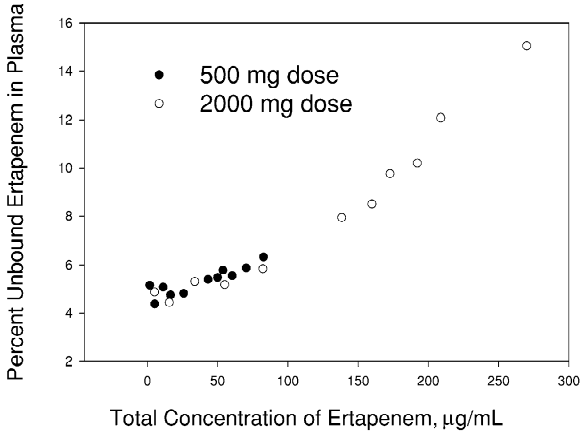

Fig. 7. Percent unbound ertapenem concentrations in plasma from healthy male subject plotted against corresponding total concentrations of ertapenem.

4. Conclusions

A methodology to separate unbound drug from plasma proteins and to assay ertapenem in human plasma filtrate has been developed and implemented for clinical pharmacokinetic studies. Stability studies show that total drug and the unbound concentration of drug in plasma are stable during short term periods for sample preparation and over long-term storage at −70 °C.

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