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Column-switching technique for the sensitive determination of ertapenem in human cerebrospinal fluid using liquid chromatography and ultraviolet absorbance detection

Chester J. Kitchen*, Donald G. Musson, Alison L. Fisher

Merck Research Laboratories, WP75A-303, P.O. Box 4, West Point, PA 19486, USA

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Abstract

A sensitive reversed-phase high-performance liquid chromatographic (RP-HPLC) assay with on-line extraction was developed for quantifying ertapenem in human cerebrospinal fluid (CSF). This assay is at least five times more sensitive than previously published ertapenem methods with a lower limit of quantitation at 0.025 μg/ml. In this assay, a CSF sample is extracted on-line using a RP extraction column and an aqueous acidic mobile phase (0.1% formic acid) to wash away polar endogenous materials, while ertapenem is retained on the column. Ertapenem is then back-flushed off the extraction column and directed to a RP analytical column using an acidic mobile phase with an organic modifier (acetonitrile/0.1% formic acid, 15:85 (v/v)) and detected using UV absorbance. The acidic mobile phase provided a sharper chromatographic peak and on-line extraction allowed large injection volumes (≥150 μl) of buffered CSF to be injected without compromising column integrity. These assay conditions were necessary to quantify ertapenem at levels expected to be found in human CSF (<0.05 μg/ml). The method was successfully validated and implemented for a clinical study: intraday precision and accuracy of the CSF assay for calibration standards (0.025–10 μg/ml) and quality control samples (0.1, 0.5, and 2.5 μg/ml) were <6.2% coefficient of variation and 96.8–104.0% of nominal concentration, respectively.

Keyword: Ertapenem

1. Introduction

Ertapenem (INVANZ™, Fig. 1) is a new once-a-day parenteral, broad-spectrum antibiotic approved for a variety of community-acquired and mixed infections. Ertapenem is a β-lactam that is structurally unique from imipenem in that it contains a 1-β-methyl group that provides stability against human dehydropeptidase-1 (DHP-1), and a benzoate anionic side chain that contributes to high protein-binding (∼94%) and prolongs the $t_{1/2}$, thereby allowing once daily dosing [1].

Assays for total ertapenem in plasma and urine [2] have been reported and employ column-switching for on-line extraction at a neutral pH followed by reversed-phase high-performance liquid chromatography (RP-HPLC). An assay has also been reported for unbound ertapenem in plasma that uses ultrafiltration followed by direct injection on to an RP-HPLC system without on-line extraction [3]. Lower limits of quantitation (LLOQs) for the plasma, urine, and plasma filtrate assays are 0.125, 2.5, and 0.25 μg/ml, respectively.

On-line extraction using an acidic mobile phase and a large injection volume was necessary to develop a sensitive assay for ertapenem in human CSF. This assay is at least five times more sensitive than any other reported ertapenem assay [2] with a LLOQ of 0.025 μg/ml. Greater sensitivity was attained using a combination of an acidic mobile phase (0.1% formic acid, pH 3) to sharpen the chromatographic peak and on-line extraction using column-switching to allow injection of a large sample volume (≥150 μl) without compromising chromatography. Using column-switching, ertapenem is back-flushed off of the extraction column by increasing the solvent strength of the mobile phase and is then loaded onto the analytical column before UV detection.

* Corresponding author. Fax: +1-215-652-4524.
E-mail address: chet_kitchen@merck.com (C.J. Kitchen).

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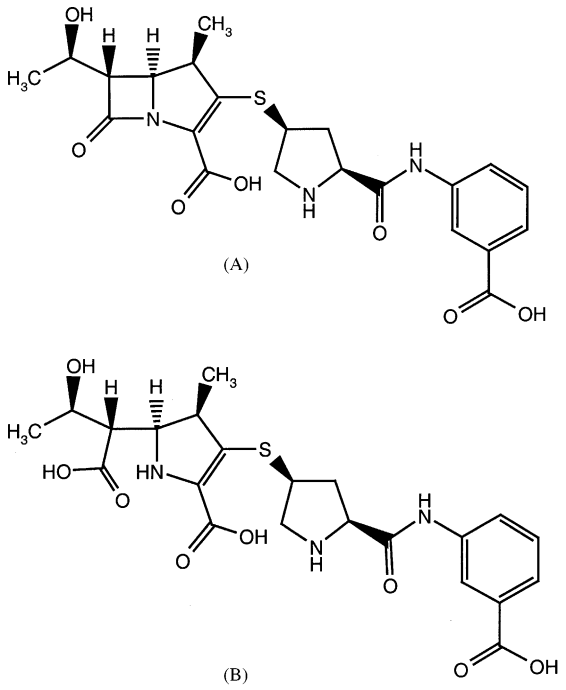

Fig. 1. Structure of ertapenem (A) and the open-lactam metabolite of ertapenem (B).

2. Experimental

2.1. Chemicals

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-carboxylic acid and its open-lactam metabolite were obtained from Merck Research Laboratories (Rahway, NJ, USA) and their structures are shown in Fig. 1 [4]. Ampicillin, cefazolin, cefotaxime, ceftriaxone, oxacillin, and piperacillin (all sodium salts), clindamycin HCl, gentamicin sulphate, 2-[*N*-morpholino]ethane-sulfonic acid (MES acid) and its sodium salt (MES sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ceftazidime pentahydrate was purchased from US Pharmacopeia (Rockville, MD, USA). ACS grade formic acid was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Optima grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Purified, filtered water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). Assayed liquid human CSF control (Level 1) was purchased from VWR Scientific (Bridgeport, NJ, USA). All chemicals were used as received.

2.2. Instrumentation and chromatography

The HPLC autosampler (7l7plus) and loading pump for column-switching (pump 1, 600E) were from Waters (Milford, MA, USA). The elution pump (pump 2, Series 200 LC Micropump) and the UV-Vis detector (785A) were from Perkin-Elmer (Cupertino, CA, USA). Column-switching was performed by an Autochrom M10 column-switching valve (10-port) purchased from Valco Instruments Company (Houston, TX, USA). On-line extraction was performed using a Maxsil C18 (50 mm × 4.6 mm, 10 μm) column from Phenomenex (Torrance, CA, USA) and chromatography was performed using a BDS Hypersil C18 (100 mm × 4.6 mm, 5 μm) HPLC column from Keystone Scientific (Bellefonte, PA, USA). Data were collected, stored, and analyzed using Turbochrom Navigator Client/Server version 6.1 (Perkin-Elmer, Cupertino, CA, USA). An Eppendorf EDOS 5222 (Brinkmann Instruments, Westbury, NY, USA) pipettor was used for sample and standard aliquots.

Mobile phase 1 (MP1, 0.1% formic acid) and mobile phase 2 (MP2, ACN/0.1% formic acid, 15:85 (v/v)) were filtered and degassed through a 0.22 μm Magna-R nylon filter (Whatman International, Maidstone, UK). The flow rates for pump 1 and pump 2 were 1.5 and 2.0 ml/min, respectively. A diagram of the different column-switching positions in the HPLC system is shown in Fig. 2.

2.3. Preparation of standards and quality control (QC)

Stock standard solutions of ertapenem were prepared at a concentration of 500 μg/ml free acid in 0.1 M MES buffer, pH 6.5. The stock standard of ertapenem was diluted again to yield a series of working standards. CSF calibration standards were prepared by diluting aliquots of the working standards with control human CSF to obtain concentrations of 0.025, 0.05, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, and 10 μg/ml.

A separate weighing of ertapenem was used to prepare a 500 μg/ml stock solution for preparing QC samples. Batches of QC samples were prepared from this stock solution at 0.1, 0.5, and 2.5 μg/ml in human CSF, separated into individual polypropylene tubes, and stored at −70 °C until used.

A volume of 0.1 M MES buffer, pH 6.5, was added to each CSF standard, QC sample, and clinical sample at the time of analysis to increase the total sample volume by 25% in the autosampler vial. This was required since the volume of CSF available from the clinical samples was often not much more than the maximum residual volume of the autosampler vials and might result in an incomplete injection without adding buffer to increase the volume. MES buffer (0.1 M, pH 6.5) was chosen because it is in the most stable pH range for ertapenem [2].

2.4. Method validation

Intraday accuracy and precision were determined by analyzing replicate calibration curves ($n = 5$) prepared in different lots of control human CSF. The calibration curves were constructed by performing weighted ($1/y$) linear regression analysis of peak areas versus nominal concentrations.

QC samples were used to determine intraday and interday assay variability and accuracy. A set of QC samples at low, medium, and high concentrations ($n = 2$) were analyzed

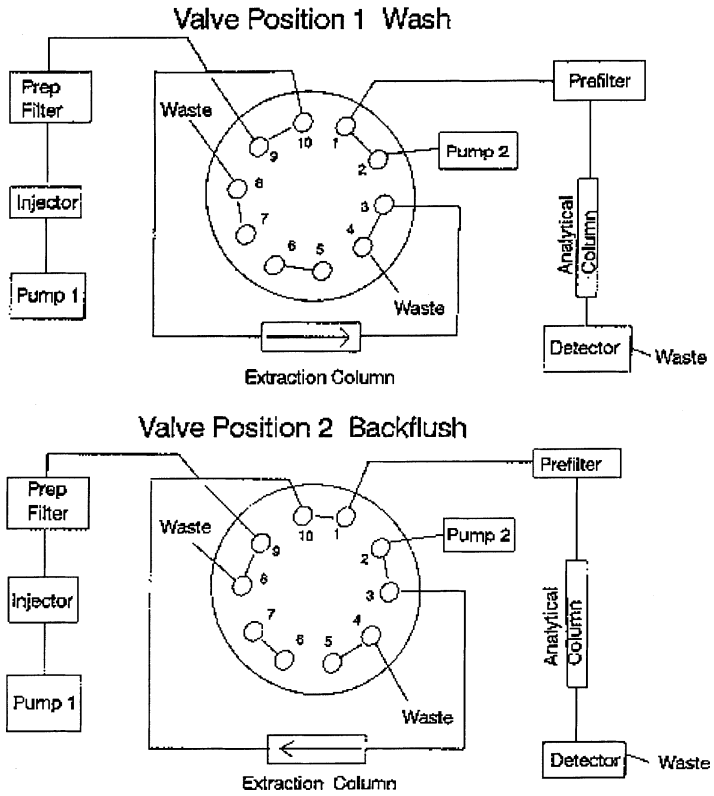

Fig. 2. Diagram of the different column-switching positions of the HPLC system.

with the daily standard curve and clinical samples. Acceptance of the daily analysis run was based upon acceptable QC results for that batch.

The absolute recovery of ertapenem from CSF standards using on-line extraction was evaluated by comparing peak areas of standards of ertapenem assayed with and without the extraction column. The standards were prepared in triplicate at 0.1, 1.0, and 10 μg/ml.

Relative ertapenem recoveries from diluted plasma (1:10) and control CSF were assessed. This was done to determine if a matrix with protein content slightly higher than the levels found in control CSF (1–4 g/l versus 0.3–0.6 g/l) or in CSF of patients with meningitis (≥1 g/l) would adversely affect the recovery of drug from the respective matrices [5]. This was accomplished by preparing ertapenem solutions to 0.1, 2.5, and 10 μg/ml ($n = 3$ at each concentration) in 0.1 M MES buffer (pH 6.5), 1:10 diluted plasma/saline (v/v), and human control CSF. The mean relative recovery of ertapenem from CSF and 1:10 plasma was calculated by comparing the analyte peak area in CSF or 1:10 plasma to the analyte peak area in 0.1 M MES buffer.

Stability studies of ertapenem in CSF were conducted at 5 °C and approximately 22 °C. For each study, test samples were placed in the autosampler that was set to 5 °C or turned off (~22 °C) and analyzed over time. Standards ($n = 2$) at 0.1 and 1 μg/ml in control human CSF were used for the 5 °C stability study over 23 h and standards at 0.5 and 10 μg/ml were used for the 5 and 22 °C stability study over 6.4 h. Freeze–thaw stability studies were also assessed by thawing and refreezing QC samples for 24 h ($n = 2$ or 5 at three concentrations).

2.5. Sample preparation

All CSF samples (including CSF standards and quality control standards) were thawed and then vortexed. Sample aliquots of 200 μl were mixed with 50 μl of buffer (0.1 M MES buffer, pH 6.5) and transferred to autosampler vials. The vials were capped, vortexed, and stored in a 5 °C autosampler until 150 μl of each sample was injected onto the HPLC.

2.6. Sample analysis

The sample was loaded onto the extraction column for 3.5 min at a flow rate of 1.5 ml/min with MP 1. During this time, ertapenem was concentrated on the head of the column while endogenous constituents were passed through the column to waste. After 3.5 min, the analyte was back-flushed off of the extraction column and directed to the analytical column using MP 2, at a flow rate of 2.0 ml/min. At 5 min after injection, the switching valve returned MP 1 through the extraction column for equilibration prior to the next injection. The total run time was 10 min. ACN/H_2O (50:50 (v/v)) was used as a needle wash on the autosampler.

3. Results and discussion

Preliminary CSF experiments using a neutral mobile phase on reversed phase chromatography [2] resulted in broad ertapenem peaks giving inadequate sensitivity. Low concentrations of drug were expected in CSF samples at <0.05 μg/ml. Subsequent development work used an acidic mobile phase (ACN/0.1% formic acid 15:85 (v/v)) to improve assay sensitivity: the acidic mobile phase quickened the elution of the analyte, sharpened the peak, and enhanced sensitivity. Large injection volumes were also necessary to attain the required sensitivity. However, initial results, without column-switching, from repeated injections of large volumes (100–200 μl) of buffered CSF standards containing ertapenem showed progressive deterioration of its chromatographic peak shape. Fig. 3 shows representative chromatograms from replicate 200 μl injections of a 1 μg/ml ertapenem standard without using column-switching. The peaks appeared to be splitting, which suggested degradation of the column packing. When the large injection volume was extracted on-line using column-switching and back-flushed to the analytical column, the peak remained sharp with repeated injections (at least 57 injections). Increasing the injection volumes from 50 to 200 μl increased the peak area response proportionally four-fold.

On-line extraction was accomplished by combining a two-pump HPLC system with a column-switching valve, extraction column, and an analytical column. A large volume (150 μl) of buffered CSF sample at pH 6.5 was injected into an acidic mobile phase (MP1, 0.1% formic acid) and the analyte was extracted on a Maxsil C18 column, 4.6 mm × 50 mm, 10 μM. At 3.5 min after injection, the column-switching valve was activated and an organic mobile phase (MP2, 15% ACN:85% of 0.1% formic acid (v/v)) was directed through the extraction column in the reverse direction. The focused analyte was then chromatographed on the analytical column (Keystone Scientific BDS Hypersil C18, 4.6 mm × 100 mm, 5 μm) and detected on the UV absorbance detector. The column-switching valve was activated again at 5 min post injection to condition the extraction column and await the next injection.

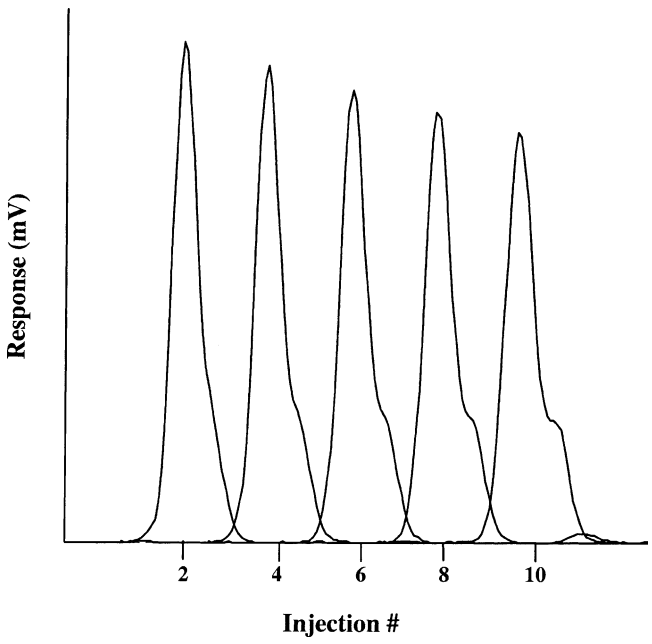

Fig. 3. Representative ertapenem chromatographic peak deterioration as a consequence of replicate large CSF sample injections without using column-switching.

Ertapenem and other β-lactams are unstable in an acidic environment and will tend to convert to the open-ring form [2,6]. However, no conversion of ertapenem to the open-ring form was detected using this assay, possibly because of the short time the drug was exposed to the acidic mobile phase: ertapenem eluted in approximately 7 min and was probably protected from the acid by partitioning into the oily C18 stationary phase. Human clinical samples containing ertapenem also required storage at a neutral pH in the autosampler tray due to the susceptibility of the β-lactam moiety of ertapenem to hydrolysis [6]. This column-switching approach allowed the CSF samples to be stored on the autosampler carousel at 5 °C at a neutral pH until injection, thus minimizing the possibility of compound degradation in the sample or on the system.

Representative chromatograms of a known standard, predose sample, and a post-dose sample are shown in Fig. 4. The open-lactam metabolite elutes with the solvent front immediately after column-switching occurs. No interfering peaks have been detected in an ongoing clinical study. The assay has been validated over the concentration range of 0.025–10 μg/ml. The calculated concentrations were determined using linear regression of the equation $y = mx + b$ where y is the response concentration of ertapenem with $1/y$ weighting and x is the concentration of ertapenem. Intraday accuracy and precision of ertapenem standards in CSF ranged from 98 to 104% of nominal and from 0.86 to 6.22% (% CV), respectively (Table 1). The mean ($n = 5$) standard curve had a slope, y-intercept, and correlation coefficient of 59243, 44.0, and 0.9998, respectively. Intraday accuracy and precision ($n = 5$) for low, medium, and high concentration QCs ranged from 96.8 to 98.3% of nominal and from 0.38 to 0.90% (% CV), respectively (Table 2).

At 5 °C and room temperature (range: 21–25 °C), >95% of ertapenem was found remaining in control human CSF at 6.4 h (Table 3). At 5 °C, ertapenem at 0.1 and 1.0 μg/ml was stable (>96%) for at least 23 h. Ertapenem was found to be stable for at least 13 months in control human CSF stored at −70 °C without buffer addition. Three freeze–thaw cycles showed no apparent degradation of the compound (Table 4). The geometric mean of duplicate injections at three concentrations for two and three freeze–thaw cycles was 101.5 and 96.6%, respectively.

This assay has applicability to compounds where a large injection volume may be needed to attain a very low LLOQ. The assay is also specific for cefotaxime and would allow

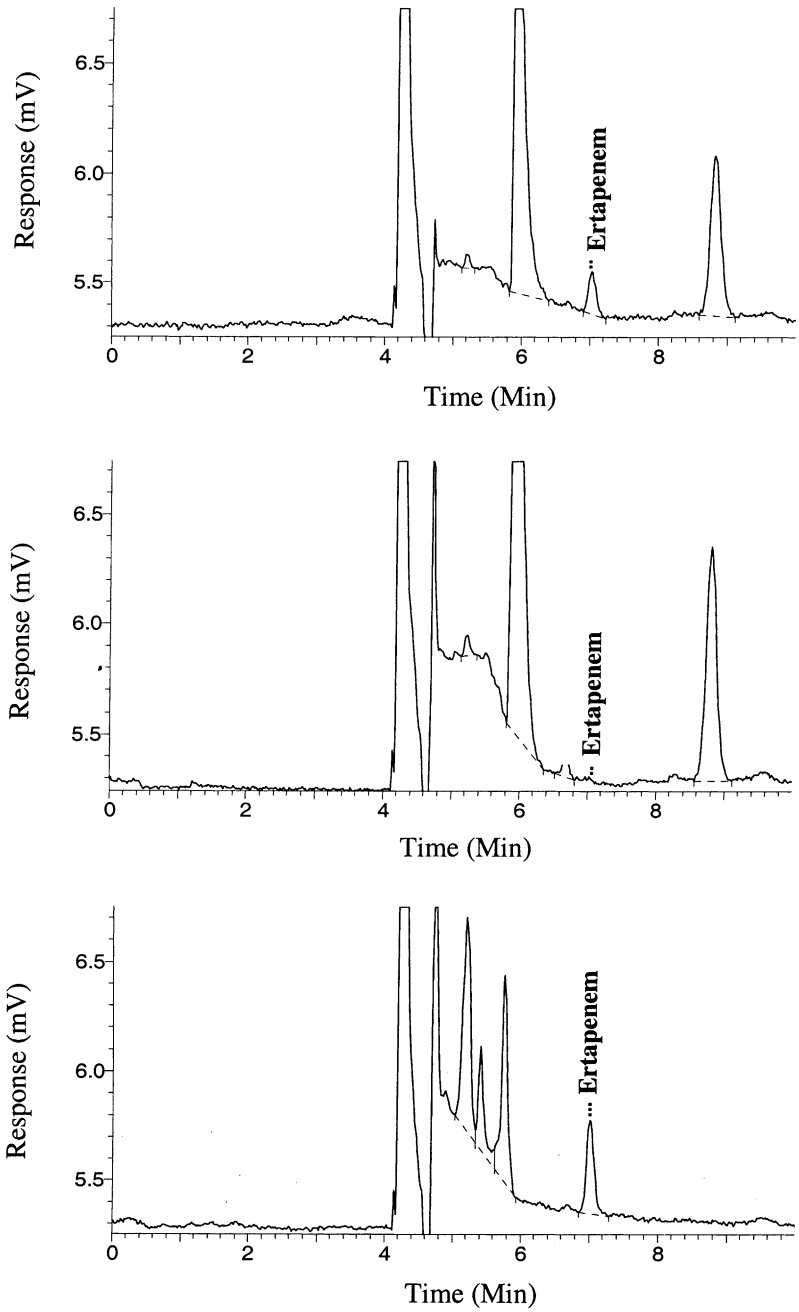

Fig. 4. Representative chromatograms of a 0.025 μg/ml ertapenem standard in human cerebrospinal fluid (top), blank CSF sample (middle), and clinical CSF sample 12 h after a single 20 mg/kg IV injection (0.0574 μg/ml) (bottom).

simultaneous quantification of ertapenem and cefotaxime, with cefotaxime eluting before ertapenem with baseline separation. Ceftriaxone elutes near the solvent front and may be assayed using this assay if less organic modifier is used in the mobile phase. Oxacillin, vancomycin, clindamycin, gentamicin, piperacillin, and ampicillin have also been tested using this assay, and do not co-elute with the ertapenem peak.

Ertapenem is approximately 94% protein bound in plasma and concentrations of ertapenem in the plasma are measured as total and free ertapenem [1]. Protein levels are considerably lower in CSF than in plasma (0.1–1.0 g/l versus ~70 g/l). The amount of protein in CSF is age dependent where protein is highest in the CSF of children <6 months of age (0.4–1.0 g/l) [5]. CSF protein levels may also be higher (>1 g/l) in patients with diseases such as meningitis [7]. Considering the similarity of protein composition between plasma and CSF and the very limited availability of CSF from patients with meningitis, human control plasma diluted 1:10 with saline solution was chosen as a test matrix for the preparation of ertapenem calibration standards [8]. Plasma diluted 1:10 has slightly higher levels of protein (7 g/l) than would be anticipated in sick patients with

Table 1
Representative intraday precision and accuracy data for the determination of ertapenem in human cerebrospinal fluid

Nominal concentration (μg/ml)	Mean[a] concentration (μg/ml)	Precision[b] CV (%)	Accuracy[c] (%)
0.025	0.026	6.22	104.0
0.05	0.049	3.76	98.0
0.1	0.100	3.07	100.4
0.25	0.245	2.63	98.1
0.5	0.499	3.94	99.8
1	1.00	2.32	99.8
2.5	2.50	2.24	99.9
5	5.03	1.64	100.5
10	9.99	0.86	99.9

[a] Mean concentrations calculated from the weighted ($1/y$) linear least-squares regression curve using all five replicates at each concentration.
[b] Percent coefficient of variation (CV) of peak areas ($n = 5$), expressed as [(standard deviation/mean) × 100].
[c] Expressed as [(mean observed concentration/nominal concentration) × 100] ($n = 5$).

Table 2
Initial intraday analysis of quality control samples ($n = 5$) for the determination of ertapenem in human cerebrospinal fluid

Nominal concentration (μg/ml)	Accuracy[a] (%)	Precision[b] CV (%)
0.1	96.8	0.90
0.5	98.3	0.38
2.5	97.2	0.51

[a] Expressed as [(mean observed concentration/nominal concentration) × 100] ($n = 5$).
[b] Percent coefficient of variation (CV) of peak areas ($n = 5$), expressed as [(standard deviation/mean) × 100].

Table 3
Mean ($n = 2$) stability of ertapenem in human CSF at 5 and 22 °C

Time (h)	% remaining			
	5 °C		22 °C	
	0.5 μg/ml	10 μg/ml	0.5 μg/ml	10 μg/ml
0	100.0	100.0	100.0	100.0
2.4	100.2	99.2	98.1	98.2
4.5	98.0	99.1	96.5	97.3
6.4	97.3	98.6	95.1	95.8

Table 4
Freeze–thaw stability of ertapenem in human CSF

Freeze–thaw (FT) cycles	Concentration (μg/ml)		
	0.1	0.5	2.5
1 ($n = 5$)	0.097	0.492	2.43
2 ($n = 2$)	0.102	0.493	2.41
3 ($n = 2$)	0.093	0.477	2.35
2FT/1FT peak area ratio[a]	105.37	100.18	99.16
3FT/1FT peak area ratio[a]	96.07	96.93	96.75

[a] Peak area ratio = (2FT or 3FT area/1FT area) × 100.

Table 5
Recovery[a] of ertapenem from CSF and 1:10 diluted plasma

	Concentration (μg/ml)		
	0.1	2.5	10
0.1 MES (pH 6.5)	5.97	149	599
Cerebrospinal fluid (CSF)	6.07	147	594
1:10 diluted plasma/saline (v/v)	6.18	147	596
CSF/MES peak area ratio[b]	101.59	98.52	99.10
Diluted PL/MES peak area ratio[b]	103.56	98.69	99.52

[a] Mean ($n = 3$) area counts × 1000 (mV).
[b] Peak area ratio = (CSF or diluted PL area/MES area) × 100.

meningitis (≥1 g/l). The mean ($n = 3$) percent relative recovery of ertapenem from 1:10 diluted plasma was determined to be 100.6% with a range from 98.7 to 103.6%. The mean recovery from control human CSF was determined to be 99.7% with a range from 98.5 to 101.6% (Table 5). Considering nearly identical recoveries of ertapenem standards from control CSF and diluted plasma, control CSF was used for the calibration standards and QC preparations rather than diluted plasma. The mean ($n = 3$ at 3 concentrations) percent absolute recovery of ertapenem from CSF was 104.1% with a range from 97.2 to 110.1%.

4. Conclusions

An assay for quantification of ertapenem to 0.025 μg/ml was developed, validated, and implemented for a clinical study. The use of column-switching for on-line extraction with an acidic mobile phase enables large sample volumes to be injected giving sharp chromatographic peaks. This improves assay sensitivity for ertapenem at least five times lower than previously published methods. Also, chromatographic conditions and volatile mobile phase buffers used for this assay are compatible with tandem mass spectrometry (HPLC–MS–MS).

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