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Short communication

Modified high-performance liquid chromatographic method for the determination of ertapenem in human urine: enhanced selectivity and automation

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Abstract

A column-switching, reversed-phase high-performance liquid chromatographic (HPLC) assay for a new structurally unique carbapenem antibiotic, ertapenem, in urine has been improved for selectivity and automated using a Packard MultiPROBE® II EX pipetting station. The method uses column-switching for on-line extraction of the urine sample. The extraction column, analytical column, mobile phase, and timing of the column-switching valve have been changed to enhance selectivity for the analyte over endogenous background material. Sample transfer and dilution prior to direct-injection into the HPLC system have been accomplished using a Packard MultiPROBE® II EX robotic liquid handling system.

Keywords: Ertapenem

1. Introduction

Ertapenem (Fig. 1) is a new broad spectrum, once-a-day antibiotic currently under development for community-acquired and mixed infections. The antibiotic is a β-lactam with a unique anionic side chain and a methyl group on the azabicyclic ring (C_4). It is resistant to both β-lactamase and human renal dehydropeptidase-I (DHP-I).

Plasma and urine assay methodologies for ertapenem were previously reported [1] using reversed-phase HPLC and column-switching for on-line extraction. The urine method was validated for selectivity with respect to five different sources of human urine. Use of the method, however, on clinical samples would occasionally result in an endogenous interference. The method utilizes a reversed-phase guard column for on-line extraction and a reversed-phase analytical column with ultraviolet absorbance detection at 300 nm. The method has been modified to enhance selectivity for ertapenem by changing the on-line extraction column, analytical column, and wash-step timing for column-switching. In addition, the transfer and dilution steps during preparation of clinical and quality control samples have been automated using a Packard MultiPROBE® II EX Robotic Liquid Handling System.

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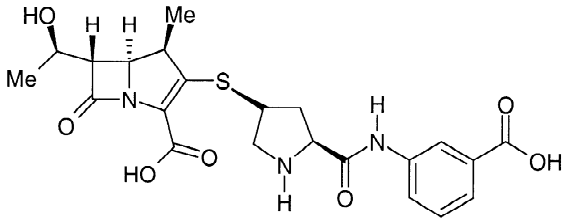
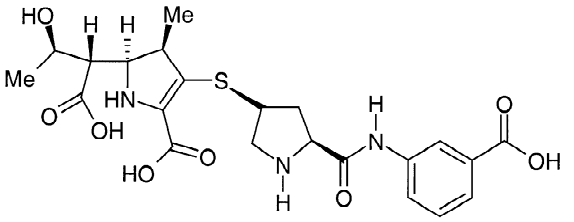


(A)



(B)

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

2. Experimental

2.1. Chemicals and reagents

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 monosodium salt [2], and its open-lactam metabolite [3] were obtained from Merck Research Laboratories (Rahway, NJ, USA). 2-[*N*-Morpholino]ethanesulfonic acid (MES acid), its respective sodium salt (MES sodium salt), and ethylene glycol were purchased from Sigma. ACS-grade sodium phosphate dibasic anhydrous, *ortho*-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): models 717plus autosampler, a 600E system controller and 600P pump, and a Spectroflow 783A ultraviolet absorbance detector. The column switching was performed by an Autochrom M10 column switching valve (10-port) purchased from Valco Instruments (Houston, TX, USA). The detector signal was acquired and processed by a Perkin-Elmer Nelson Turbochrom Client/Server data acquisition system (Cupertino, CA, USA). Automated sample transfers and dilutions were accomplished using a Packard MultiPROBE® II EX robotic liquid handling system (Downers Grove, IL, USA).

2.3. Chromatographic conditions

An extraction column Prism RP 5 μm (20×4.6 mm I.D.) from Keystone Scientific (Bellefonte, PA, USA) was installed in-line after pump 1, the injector, and a prep filter as diagramed in Fig. 2, using a 10-port valve. The analytical column, Aquasil C_{18} 5 μm (100×4.6 mm I.D.) from Keystone Scientific, was in-line after pump 2, protected by a prefilter, and followed by the detector. Pumps 1 and 2 provided mobile phases to the extraction column and analytical column at flow-rates set at 2.0 and 1.8 ml/min, respectively. The autosampler was programmed to inject 10 μl at 5 °C with a run time of 30 min. The

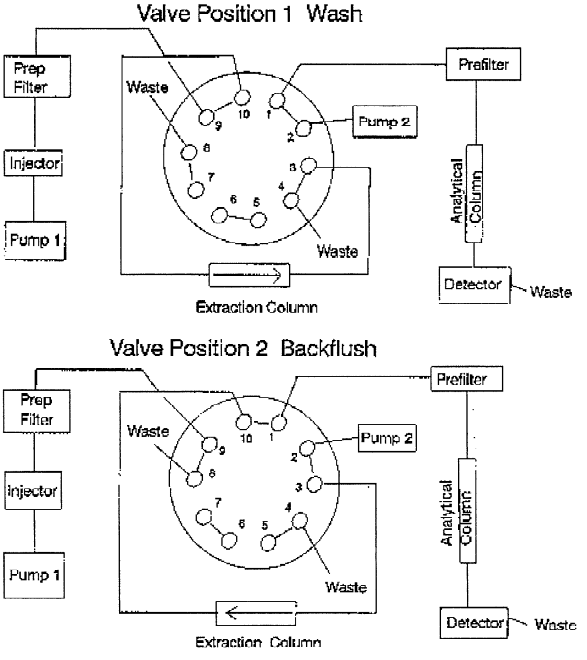


Fig. 2. Diagram of the different column-switching positions of the HPLC system.

absorbance detector was set at 300 nm. The electrically actuated switching valve was set in position 1 (Fig. 2) for 8.5 min, following sample injection of the analyte onto the extraction column and washing with a phosphate buffer, pH 6.5; the valve was then switched to position 2 during backflush of the analyte on to the analytical column for 3 min.

The valve switched back to position 1 from 11.5 min after injection to the end of the run while the chromatographic separation of analyte from endogenous components was achieved and detected. This cycle of events was repeated for subsequent injections.

The mobile phase for pump 1 was 25 m*M* sodium phosphate buffer, pH 6.5 (7.1 g sodium phosphate dibasic anhydrous in 2000 ml of water, pH adjusted to pH 6.5 with *ortho*-phosphoric acid, 85%); for pump 2, the mobile phase was 10% methanol in 25 m*M* phosphate buffer, apparent pH 6.5. The mobile phases were degassed under vacuum by ultrafiltration using 0.2 μm Nylon-66 filters (Rainin Instrument, Woburn, MA, USA).

2.4. Preparation of calibration standards

Stock solutions of ertapenem at 0.5 and 0.05 mg/ml were prepared as the carboxylic acid in 0.1 *M* MES buffer, pH 6.5. Appropriate volumes of the stock solutions were transferred using a micropipette to a 12×75 mm polypropylene test tube and mixed by vortex with control human urine to obtain concentrations of 2.5, 5.0, 12.5, 25, 50, and 100 μg/ml. The urine calibration standards were then mixed with an equal volume of stabilizer (0.1 *M* MES buffer, pH 6.5). Clinical urine samples were previously mixed with an equal volume of 0.1 *M* MES buffer, pH 6.5, at the clinical site to bring final pH to about 6.5 and then frozen [1].

2.5. Preparation of urine quality controls (QCs)

Stock solutions of 1.0 and 10 mg/ml of ertapenem were prepared as the carboxylic acid in 0.1 *M* MES buffer, pH 6.5. A 125-μl aliquot of the 1.0 mg/ml stock solution was transferred to a 25-ml volumetric flask and diluted to mark with control human urine giving a final concentration of 5 μg/ml. The urine QC solution was transferred to a 250-ml beaker and mixed with 25 ml of 0.1 *M* MES buffer, pH 6.5, using a stir bar. Aliquots of 1.25 ml from the stabilized QC urine solution were transferred to 12× 75-mm polypropylene tubes with caps (Sarstedt, Newton, NC, USA) and stored at −70 °C. Similar QC samples were prepared at 25, 80, and 500 μg/ml using the 1.0 and 10 mg/ml stock solutions.

2.6. Sample preparation and automation for HPLC analysis

Frozen QC and urine samples were thawed at room temperature, vortexed, and placed on the deck of the Packard MultiPROBE®II EX. A program was initiated for the transfer of 1000 μl of urine for those samples that did not need dilution. Single-step dilutions with 0.1 *M* MES buffer, pH 6.5, were programmed for samples requiring 2-fold (500 μl of urine with 500 μl of buffer), 5-fold (200 μl of urine with 800 μl of buffer), or 10-fold (100 μl of urine with 900 μl of buffer) dilutions. The urine and buffer were transferred directly to the HPLC autosampler vials. Two-step dilutions were performed for those samples where dilutions greater than 10-fold were required. For the two-step dilutions, the Packard MultiPROBE at first diluted the urine samples 10-fold by transferring sample and diluent to 12×75 mm polypropylene tube, and pausing when finished to allow the analyst to vortex the samples. Following intervention by the analyst to vortex the samples, the Packard MultiPROBE continued with the program. The Packard MultiPROBE then transferred the 10-fold diluted urine samples to the appropriate HPLC autosampler vials and diluted the samples two, five, or 10 more times in the vials. The vials were capped, vortexed, and placed in the autosampler rack for HPLC analysis. The autosampler was set at an injection volume of 10 μl and a temperature of 5 °C.

Verification of accurate and precise volume transfers by the Packard MultiPROBE involved weighing replicate ($n=5$) aliquots of Milli-Q water (Millipore, Bedford, MA, USA) for each syringe at each volume used to transfer and dilute.

2.7. Linear regression analysis

Batch analysis of clinical samples and QCs were performed with external standard calibration curves

constructed by plotting the peak areas of ertapenem against the nominal concentrations. Linear regression analysis of the calibration data was performed using the equation $y = mx + b$ and a curve weighting of $1/y$.

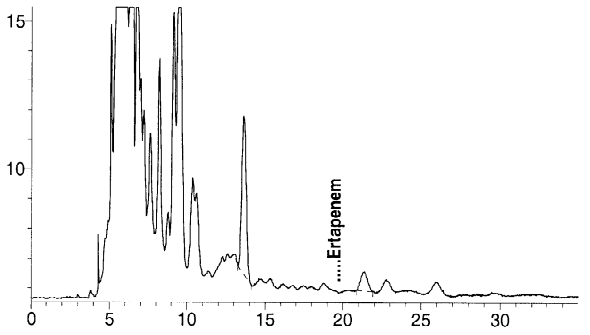
2.8. Collection, stabilization, and storage of urine samples

Urine fractions were temporarily stored on wet ice or in a refrigerator until all voids for each time period were collected and pooled. Each urine sample was mixed and a 10-ml aliquot was removed and stabilized by mixing with an equal volume of 0.1 *M* MES buffer, pH 6.5. The stabilized sample was then stored at −70 °C within 2 h of collection.

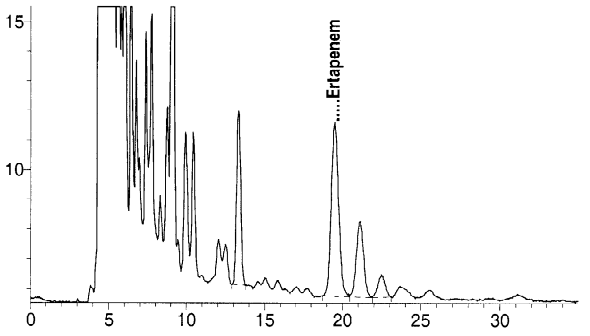
3. Results and discussion

3.1. Chromatography

Chromatography for ertapenem in urine by reversed-phase HPLC with UV absorbance detection has been reported previously [1] using column-switching for on-line extraction. The method was not always selective for the urine collections of some clinical subjects. Occasionally, the organic modifier methanol in the mobile phase had to be adjusted between 7 and 11% (v/v) to move the peak for ertapenem to a retention time free of endogenous interferences. The use of column-switching allowed several parameters to be modified for enhanced selectivity of ertapenem's chromatography. The extraction and analytical columns were replaced with columns providing more selectivity in a highly aqueous mobile phase. The mobile phase for the analytical column was set to contain a constant amount of organic modifier and the timing for the switching valve was optimized for the wash and elution steps. The chromatographic differences between the two methods are shown in Figs. 3 and 4. Most of the endogenous background observed in the chromatograms for the earlier assay were significantly reduced with the modified assay procedures. The injection volume was also reduced by 5-fold with comparable sensitivity. The lower limit of quantitation was changed from 1.25 to 2.5 μg/ml.



(a) Predose Clinical Sample

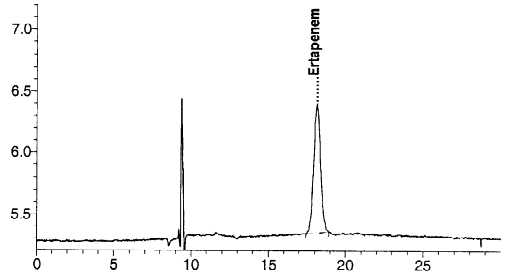


(b) Postdose Sample at 18-24 hr Collection (15.39 μg/mL)

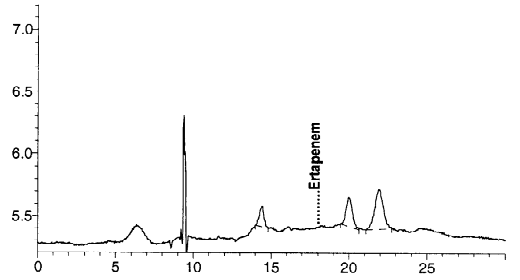
Fig. 3. Representative chromatograms of ertapenem in human urine using the original method (50 μl injection volume).

An adjustment for endogenous interferences was no longer needed.

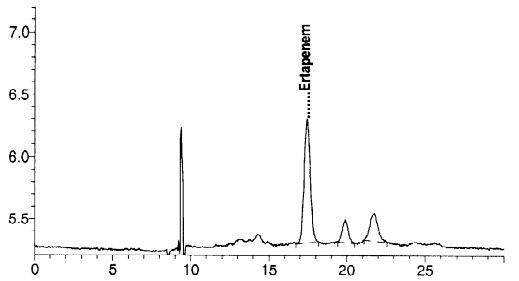
The objective of changing the on-line extraction column from Maxil ODS (10 μm, 50×4.6 mm) to Prism RP (5 μm, 20×4.6 mm) was to enhance the selectivity of the column for ertapenem over endogenous material by decreasing the secondary interactions of the polar acid using the extraction column. The Prism RP column generally shields the analyte from the secondary interactions due to impure silica and free silanols on the silica gel backbone of the column. The change of the on-line extraction column significantly reduced the retention of the analyte with less band broadening and allowed for a narrower peak back-flushed onto the analytical column, thus decreasing the endogenous material: the retention of the analyte changed from about 124 to 16 min; the



(a) Ertapenem Calibration Standard at 12.5 μg/mL



(b) Predose Clinical Sample



(c) Postdose Sample at 18-24 hr Collection (10.35 μg/mL)

Fig. 4. Representative chromatograms of ertapenem in human urine using the modified method (10 μl injection volume).

open-lactam metabolite changed from 21 to 5 min. The time from injection and wash of a sample aliquot on the extraction column with phosphate buffer was 8.5 min, before back-flushing with a stronger mobile phase. During this time the metabolite was eluted to waste. The back-flush was accomplished with 10% methanol in phosphate buffer for 3 min and the analyte peak was completely eluted onto the analytical column within 2 min.

The analytical column was also changed from Hypersil BDS to Aquasil C_{18} with enhanced selectivity of polar compounds in a highly aqueous mobile phase The selectivity factor for ertapenem and the few remaining endogenous peaks was much enhanced with the Aquasil C_{18} column. The objective, as before, with this change was to enhance the selectivity of the column for ertapenem over endogenous material.

3.2. Linearity, accuracy, and precision

The urine assay was linear from 2.5 to 100 μg/ml. A linear regression analysis of a representative calibration curve resulted in a slope of 2700, intercept of −455, and $r^2 = 0.9998$.

Within-day assay precision and accuracy were determined by analyzing replicate samples ($n = 5$) in a batch at each standard concentration. Precision varied from 1.5 to 3.1% C.V. and accuracy ranged from 98.6 to 101.6% of nominal (Table 1).

Interday precision and accuracy was evaluated from assay QC samples analyzed prior to and with clinical samples at 5, 25, and 80 μg/ml. Precision and accuracy ranged from 1.4 to 2.7% C.V. and 108 to 111% of nominal ($n = 14$), respectively (Tables 1 and 2).

Clinical urine samples were diluted as necessary depending on the dosage, route of administration, and collection time interval. Samples were diluted using the Packard MultiPROBE robotic liquid handling system. The accuracy and precision of the Packard MultiPROBE were established before the analyses of samples using water and during sample analyses using QCs. The precision and accuracy for the dilution QCs at 25 and 80 μg/ml are listed in Table 2 and show statistical data similar to the assay QCs described above. Dilution QC samples were also prepared at a concentration above the upper limit of the calibration curve at 500 μg/ml. The measured concentrations of this QC were always acceptable (within 20% of nominal) and statistics were similar to the QC data at lower concentrations.

The stock solutions of ertapenem were found to be stable at −20 °C for at least 14 days. Short- and long-term stability studies of ertapenem in urine

Table 1
Intraday precision and accuracy data for the determination of ertapenem in human urine as assessed by replicate analysis of standards ($n=5$)

Nominal	Mean analyzed[a]	Precision[b] (%)	Accuracy[c] (%)
Standard concentration (μg/ml):			
2.5	2.46	3.12	98.6
5.0	5.05	1.81	101.0
12.5	12.7	1.55	101.6
25.0	24.8	2.34	99.3
50.0	49.8	1.51	99.6
100.0	99.5	2.03	99.5
Assay QCs:			
5	4.93	4.76	98.6
25	24.4	4.87	97.6
80	78.7	4.58	98.4
Dilution QCs:			
25	25.4	4.67	101.6
80	84.9	1.41	106.1
500	519.3	1.99	103.9

[a] Analyzed concentrations calculated from the weighted ($1/y$) linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as the coefficient of variation (% C.V.); based on response factor (peak area).
[c] Expressed as [(mean calculated concentration)/(nominal concentration)×100%].

were previously conducted [1]. Briefly, the data showed that the stability (≥95%) of ertapenem in urine increased from 8 to about 12 h at room temperature with the MES buffer, pH 6.5; the stability of these samples at 5 °C was >24 h with and without the buffer; the long-term stability of ertapenem in urine with the buffer at −70 °C was >11 months.

3.3. Application

Healthy subjects received a single 1-g infusion of ertapenem over 30 min and urine samples were collected at predose, 0–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–18 and 18–24 h. Representative measured concentrations from a single subject were 0, 512, 665, 196, 235, 229, 166, 66, and 15.3 μg/ml, respectively.

4. Conclusion

The modified column-switching, reversed-phase HPLC assay for ertapenem in urine is much more selective than the previously reported method [1] and validated with comparable precision and accuracy. The transfer and dilution steps of the sample preparation procedure have been automated, and dilution QCs analyzed with clinical samples verified inter-day accuracy and precision of the Packard MultiPROBE.

References

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Table 2
Interday precision and accuracy for ertapenem in urine QCs ($n=14$)

Nominal:	QC concentration (μg/ml)					
	Assay QCs			Packard dilution QCs		
	5	25	80	25	80	500
Mean	5.14	25.0	81.3	25.7	82.3	526.3
C.V. (%)	8.92	2.97	2.22	5.16	7.96	6.80
Accuracy (% nominal)	102.8	100.0	101.6	102.8	102.9	105.3