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Journal of Pharmaceutical and Biomedical Analysis
27 (2002) 755–770

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Direct-injection HPLC assay for the determination of a new carbapenem antibiotic in human plasma and urine

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Received 31 January 2001; received in revised form 5 June 2001; accepted 26 June 2001

Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) assays using ultraviolet (UV) absorbance detection have been developed for the determination of a new carbapenem antibiotic **I** in human plasma and urine. A column-switching technique is employed in the HPLC methods to perform on-line extraction and separation for each sample. Each plasma sample is thawed, centrifuged, stabilized, and then injected onto an in-line reversed-phase extraction column using a methanol (8%)/phosphate buffer, pH 6.5. After 3 min, the analytes are back-flushed off the extraction column with a mixture of acetonitrile (5.5%) and methanol (10%)/phosphate buffer (pH 6.5) for 3 min onto a BDS Hypersil 3 μm C18 (100 × 4.6 mm i.d.) analytical column. The sample preparation and HPLC conditions for the urine assay are similar to the plasma assay, except that a CN extraction column is used. Both assays are specific with respect to endogenous material and the major metabolite **II**, and both are linear over the concentration range of 0.25–50, and 2–200 μg/ml, respectively. The assays were successfully applied to a clinical dose-ranging study. One limitation of the on-line extraction method is that the extraction column needs to be replaced regularly every 100–150 plasma samples and every 200–300 urine samples. Subsequently, the urine method was modified to an ion-pair HPLC assay for the simultaneous determination of both the antibiotic **I** and its metabolite **II**.

Keywords: Carbapenem antibiotic; Direct-injection HPLC; Column-switching; Plasma; Urine; Ion-pair

1. Introduction

The new carbapenem antibiotic **I** with broad gram-positive activity (Fig. 1) was designed for intravenous use for the specific and empiric treatment of gram-positive infections. It shows notable activity against methicillin-resistant and vancomycin-intermediate *Staphylococci*, as well as against vancomycin-and ampicillin-resistant *Enterococci* [1]. Enzymatic hydrolysis via dehydropeptidase-I of the β-lactam moiety in **I** produces the metabolite **II**, which is the 1,8-naphthalene sultam side group of the drug. Chemical hydrolysis of **I** generates the same product. An accurate and precise plasma and urine assay was needed to

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0731-7085/02/$ - see front matter
PII: S0731-7085(01)00500-3

support Phase I pharmacokinetic study. The assay should minimize the possible degradation of **I** during sample analysis and enable the evaluation of **I** stability in biological fluid.

An assay methodology using sample ultrafiltration and high performance liquid chromatography (HPLC) with UV detection at 300 nm was developed for the first carbapenem, imipenem [2]. Column-switching HPLC assays have been reported for carbapenems in rat and monkey plasma, and in human plasma and urine [3,4]. The column-switching technique allows for direct injection of a sample aliquot; this involves on-line sample extraction followed by chromatographic separation. Since the extraction and separation for the analytes were performed in a closed system, drug loss was minimized compared with off-line sample preparation procedures (e.g. plasma deproteination or analyte extraction), which might also enhance drug degradation prior to HPLC analysis. This approach eliminated a need for an internal standard with similar stability. Also, column-switching HPLC assays are more reproducible, require less total analysis time with minimal sample preparation, and provide higher sample throughput. The cost of the extraction column ($< \$100$) used in the assay is much less than the cost of labor for off-line extraction.

Considering the vulnerability of the beta-lactam moiety in **I** to hydrolysis, on-line sample extraction and reversed-phase HPLC with UV absorbance detection were developed and validated for **I** in human plasma and urine to support human pharmacokinetic studies. Furthermore, the urine method was subsequently modified using an ion-pairing reagent for the simultaneous determination of both the antibiotic **I** and its major metabolite **II**.

2. Experimental

2.1. Apparatus

The HPLC system consisted of equipment from Waters (Milford, MA, USA) and Applied Biosystems (Foster City, CA, USA): Model 717 plus autosampler, a 600E system controller and pump, a Perkin Elmer Series 200 pump and a Spectroflow 783A absorbance detector. The detector signal was acquired and processed using a Perkin Elmer Nelson Access*Chrom data acquisition system (Cupertino, CA, USA). The column switching was performed using an Autochrom M10 column switching valve (10-port) (Valco Instruments, Houston, TX, USA).

2.2. Chemicals and reagents

I, which is 2-(naphthosultamyl)methyl-carbapenem analog, and its metabolite **II**, which is 1,8-naphthalene sultam group of **I** (Fig. 1), were obtained from Merck (Rahway, NJ, USA). 2-[*N*-Morpholino]ethane-sulfonic acid (MES acid) and its respective sodium salt (MES sodium salt) and ethylene glycol were purchased from Sigma (St. Louis, MO, USA). Sodium phosphate dibasic anhydrous, ACS grade, *o*-phosphoric acid 85%, acetonitrile, isopropyanol and methanol optima were purchased from Fisher (Fair Lawn, NJ, USA).

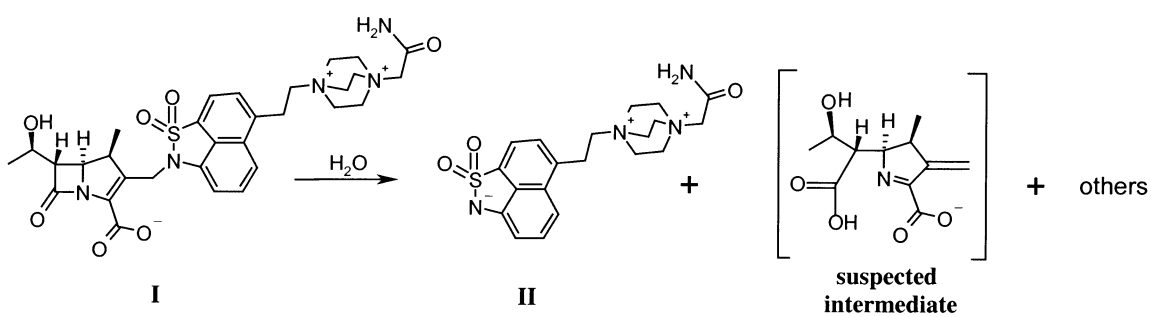

Fig. 1. Chemical structures of the antibiotic **I** and the metabolite **II**.

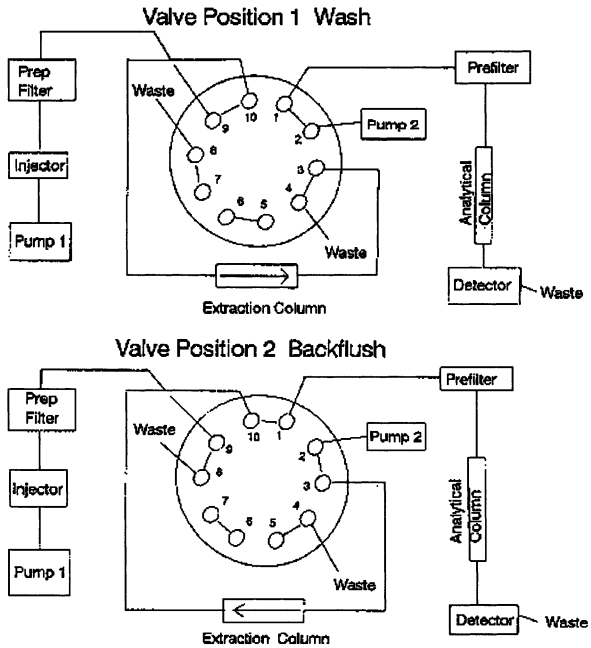

Fig. 2. HPLC system configuration.

1-Hexane sulfonate sodium salt (HS) was purchased from Alltech (Deerfield, IL, USA). All chemicals were used as received.

2.3. Buffers, needle wash solution and stabilizer

Buffer, A: 0.1 M MES buffer, pH 6.5; needle wash solution: acetonitrile/isopropyanol/water 1:1:1 v/v/v; stabilizers, A: 1:1 (v/v) 0.1 M MES buffer, pH 6.5:ethylene glycol, B: 0.1 M MES buffer, pH 6.5.

2.4. Chromatographic conditions

2.4.1. Plasma assay

An extraction column Maxsil C18 10 μm (30 × 4.6 mm i.d.) from Phenomenex (Torrance, CA, USA) was placed in-line after Pump 1 (Fig. 2), injector and the prep inline filter with a 2 μm frit, connected to a 10-port valve. The BDS Hypersil C18, 3 μm (100 × 4.6 mm i.d.) analytical column and BDS Hypersil C18 (20 × 4 mm i.d.) guard column from Keystone Scientific were placed in-line after Pump 2, prefilter with a 0.5 μm frit, and before the detector. Pumps 1 and 2 flow-rates were set at 1.0 and 0.8 ml/min, respectively.

The autosampler was programmed with an injection volume of 50 μl, a carousel temperature of 5 °C, and a run time of 17 min. The absorbance wavelength on the detector was set at 248 nm. The pneumatic switching valve was activated by contact closures on the PE Access*Chrom A/D box. The valve was in Position 1 (Fig. 2) for 3 min after sample injection onto the extraction column and wash of the column with the phosphate buffer, pH 6.5. The valve was then switched to Position 2 for 3 min during backflush of the analyte onto the analytical column. After 6 min from injection, the valve moved back to position 1, waiting for the next injection as summarized in Table 1.

The extraction mobile phase for Pump 1 was methanol (8%) in sodium phosphate buffer (25 mM), pH 6.5. The analytical mobile phase for Pump 2 was a mixture of acetonitrile (5.5%) and methanol (10%) in phosphate buffer (25 mM), pH 6.5. The mobile phases were degassed under vacuum by ultrafiltration using Nylon-66 (0.2 μm) filters (Rainin Instrument Co Inc, Woburn, MA, USA).

2.4.2. Urine assay for *I*

Chromatographic conditions for the urine assay were similar to those for the plasma assay except for the extraction column, valve-switching time on Position 2, and the content of acetonitrile in the analytical mobile phase 2. The injection volume of the autosampler was 10 μl. A Maxsil 10 μm CN column (50 × 4.6 mm i.d.) (Phenomenex, Torrance, CA, USA) was selected as the extraction column to extract the analyte from the endogenous urine background. There was no guard column needed for the analytical column. The backflush time period was 2 min, and the analytical mobile phase for Pump 2 was optimized with a mixture of acetonitrile (7%) and methanol (10%) in a phosphate buffer (25 mM), pH 6.5 (Table 1).

2.4.3. Ion-pair HPLC urine assay for *I* and *II*

Based on the original urine assay for **I**, the chromatographic conditions were modified using an ion-pair reagent for an ion-pair HPLC urine

Table 1
The switching-valve configuration for three assays

On-line analysis stage	Valve configuration (Fig. 2)	Plasma assay			Urine assay			Ion-pair urine assay		
		Time (min)	Flow rate (ml/min)		Time (min)	Flow rate (ml/min)		Time (min)	Flow rate (ml/min)	
			Pump 1	Pump 2		Pump 1	Pump 2		Pump 1	Pump 2
Extraction and cleanup	Position 1	0.0–3.0	1.0	0.8	0.0–3.0	1.0	0.8	0.0–3.5	1.0	1.5
Backflush and elution	Position 2	3.0–6.0	1.0	0.8	3.0–5.0	1.0	0.8	3.5–6.5	1.0	1.5
Analyte analysis and equilibration of extraction column	Position 1	6.0–17.0	1.0	0.8	5.0–17.0	1.0	0.8	6.5–26.0	1.0	1.5

Pump 1 (mobile phase 1): 8% MeOH/25 mM Na_2HPO_4 buffer pH 6.5 used for all three assays in Table 1. Pump 2 (mobile phase 2): 5.5% ACN + 10% MeOH/25 mM Na_2HPO_4 buffer pH 6.5 for plasma assay. 7% ACN + 10% MeOH/25 mM Na_2HPO_4 buffer pH 6.5 for urine assay. 7% ACN + 10% MeOH/25 mM Na_2HPO_4 buffer pH 6.5 with 10 mM 1-hexane sulfonate sodium salt for ion-pair urine assay.

assay for the simultaneous determination of both the antibiotic **I** and its metabolite **II**.

The modifications for the ion-pair HPLC urine assay included the following: (a) using an Prism RP, 5 μm (100 × 4.6 mm i.d.) as the analytical column; (b) adding HS (10 mM) into the original analytical mobile phase (7% acetonitrile and 10% methanol in 25 mM phosphate buffer, pH 6.5) with a flow-rate set at 1.5 ml/min; (c) changing the timing of the switching-valve at Position 1 from 3.0 to 3.5 min, and at Position 2 from 6.0 to 6.5 min as shown in Table 1.

2.4.4. Carry-over assessment

Carry-over was evaluated in the method development stage to eliminate a possible source of a carry-over from residue analyte backflushed from the extraction column. The column-switching time window for backflush of the analyte onto the analytical column was optimized for each assay. Additionally, the needle wash solution acetonitrile/isopropyanol/water in 1:1:1 (v/v/v) was used after each injection to clean up the needle of the autosampler system. There was no obvious carry-over peaks observed around the retention times of the analytes in plasma, urine and ion-pair HPLC urine assays.

2.5. Standard and quality control (QC) samples preparation

2.5.1. Plasma

A stock solution at 0.5 mg/ml and working standard solutions at 2.5, 5, 10, 25, 50, 100, 250 and 500 μg/ml of **I** (stored at approximately −70 °C with drierite) were prepared as a free quaternary ammonium ion form in MES (0.1 M) buffer, pH 6.5. Aliquots (20 μl) of the working standards were spiked into aliquots (180 μl) of control human plasma to obtain a plasma calibration standards covering the range of 0.25–50 μg/ml. Stabilizer A was added to each plasma standard in a 1:1 (v/v) ratio. Three levels of pooled plasma QC samples were prepared at 0.5, 5.0 and 40.0 μg/ml from separate stock and working solutions. Aliquots of the QC samples were transferred into polypropylene microcentrifuge tubes and stored at approximately −70 °C until analysis.

2.5.2. Urine

Urine calibration standards for **I** were prepared in a manner similar to that described for plasma standards. A stock solution at 0.50 mg/ml and working standard solutions covering the range of 10–1000 μg/ml in MES (0.1 M) buffer, pH 6.5 were prepared and aliquots were mixed with human control urine to obtain concentrations covering the range of 2–200 μg/ml. Stabilizer B was added to all urine standards in a 1:1 (v/v) ratio. Three levels of urine QC samples pooled at 5, 20, and 150 μg/ml in control urine were prepared and stabilized with Stabilizer B in a 1:1 (v/v) ratio. Urine QC sample aliquots were stored at approximately −70 °C until analysis.

2.5.3. Ion-pair urine assay for *I* and *II*

Two individual sets of urine standards for **I** and **II** were prepared separately. The same urine **I** standards, including the stock and working standard solutions, were prepared as described in the original urine assay. Similarly, the urine **II** standards covering the same concentration range of 2–200 μg/ml were prepared as described for urine **I** standards. Stabilizer B was added to each urine standard for both **I** and **II** in a 1:1 (v/v) ratio prior to analysis. QC samples were not prepared for metabolite **II**.

2.6. Sample preparation for direct-injection HPLC

Frozen plasma QC and clinical samples were thawed at room temperature, mixed by vortex and centrifuged at 2500 rpm at 5 °C for 5 min. Aliquots of the supernatants were transferred to autosampler tubes which contained an equal volume of Stabilizer A. Urine samples were stabilized at the clinical site after collection, with Stabilizer B in a 1:1 (v/v) ratio. Frozen clinical urine samples and QC samples were prepared in a similar way as described for the plasma samples, with no further stabilization step. Stabilized samples were transferred to the autosampler refrigerated at 5 °C, prior to analysis. Samples with measured concentrations greater than the upper calibration standard were diluted with control matrix and then reanalyzed. Sample dilution was evaluated

by analyzing high QC samples ($n = 6$) that were diluted as samples at 10-fold. The mean deviation was less than 10% of the nominal concentration.

2.7. pH stability

A stock solution (1.8 ml) containing 10 μg/ml **I** and 5.0 μg/ml **II** was prepared in MES (0.1 M) buffer pH 6.5. The effect of pH 6.5 on **I** was evaluated by analyzing an aliquot of the stock solution. The adjustment of pHs greater or less than 6.5 was accomplished by dividing the stock solution into two portions and adjusting the pH of one portion to 3 with orthophosphoric acid (1 M) and then to pH 5 with sodium hydroxide (1 M) to assess the effect of acid catalyzed hydrolysis. To evaluate base hydrolysis, the pH of the other portion was adjusted sequentially from 6.5 to 7, 8, 9, and 11 using sodium hydroxide (1 M). For each pH tested, the solution was mixed by vortex after pH adjustment, and allowed to stay at room temperature for 12 min prior to injection. The solutions were considered not to be buffered outside the buffering capacity of MES (useful pH range 5.5–6.7).

2.8. Assay validation procedure

Intra-day accuracy and precision of the plasma and urine assay was determined by analyzing replicate calibration curves ($n = 5$). The peak areas generated for drug and metabolite were used to determine a response at each standard concentration for drug and metabolite. The calibration curves were constructed by weighted ($1/y$) least-squares linear regression analysis of peak-area versus nominal concentrations.

A set of QC samples at low, medium, and high concentrations ($n = 2$) was analyzed with a standard curve prepared daily with clinical samples. Acceptance of sample concentration data was based on QC results in each sample analysis run. QC sample results at three concentrations were used to determine inter-day assay variability.

Freeze-thaw stability was evaluated on repeat analysis of QC samples ($n = 3$) at three concentrations. Three sets of QC samples at each level were initially quantitated prior to freezing; they were then frozen and thawed up to three cycles prior to quantitative analysis.

3. Results and discussion

3.1. Detection

A standard solution containing 10 μg/ml of the antibiotic **I** and 5.0 μg/ml of the major metabolite **II** was prepared in MES (0.1 M) buffer pH 6.5, and the UV spectra for **I** and **II** was obtained at room temperature as shown in Fig. 3. The assay methods for earlier carbapenems [2–4] used the wavelength maximum (λ_{max}) at 300 nm. The spectrum for the antibiotic **I** shows a major λ_{max} at 248 nm and a minor λ_{max} at 340 nm. The by-product of **I** generated after hydrolysis is **II**, the major metabolite observed in urine, with a major λ_{max} at 260 nm. During assay development, the wavelength chosen for detection was 248 nm, which provided optimized assay sensitivity for **I** and allowed for detection of **II**.

3.2. Chromatography

A reversed-phase C18 Maxsil extraction column and a reversed phase BDS Hypersil C18 analytical column were chosen for assay sensitivity and specificity in plasma (Fig. 4). The HPLC of **I** in plasma involves three analysis stages: sample on-line extraction and clean up, analyte elution and analysis, and system equilibration. In the extraction stage, protein and biomolecules in the plasma sample are washed out of the extraction column, and initial retention of the analyte on the extraction column for 3 min is achieved using methanol (8%)/phosphate buffer, pH 6.5 mobile phase. At the elution stage, the analyte partitioned on the front portion of the extraction column is then back-flushed off the column with acetonitrile (5.5%)/methanol (10%)/phosphate buffer into the analytical column. The equilibration stage, with the valve switched back to the extraction column, allows the extraction column to equilibrate with the methanol/phosphate buffer mobile phase, prior to the next injection. The chromatography was shown to be specific for the analyte in human

plasma (Fig. 4), which included different lots of control plasma obtained commercially, and many plasma pre-dose samples from different clinical studies. The metabolite **II** elutes before the drug. The lower limit of detection is 0.1 μg/ml (S/N ratio: 8:1).

Other stationary phases evaluated for the extraction column (e.g. Maxsil CN) did not provide enough chromatography or retention of the analyte in plasma. On-line extraction was modulated using methanol in phosphate buffer mobile phase during sample injection and wash on the Maxsil C18 column. The methanol content in the extraction mobile phase was critical to assay specificity, sensitivity, and column back-pressure. For optimum selectivity, the methanol and acetonitrile in phosphate buffer organic modifiers were used to adjust chromatography on the BDS Hypersil C18 column. Other conditions considered were flow rates, timing for the column-switching valve, and injection volume. For the Maxsil C18, routine use eventually resulted in peak broadening, which was observed after the analysis of about 100–150 samples and was remedied by replacement. For similar reasons, the analytical column was changed about every 200–250 samples.

It is worth noting that the reagent ethylene glycol used in Stabilizer A played an important role in maintaining the extraction column back-pressure at an acceptable level (about 300 psi) during plasma sample analysis. The extraction column back-pressure remained stable with the number of sample injections ≥ 120 using Stabilizer A (with ethylene glycol), but only about 30

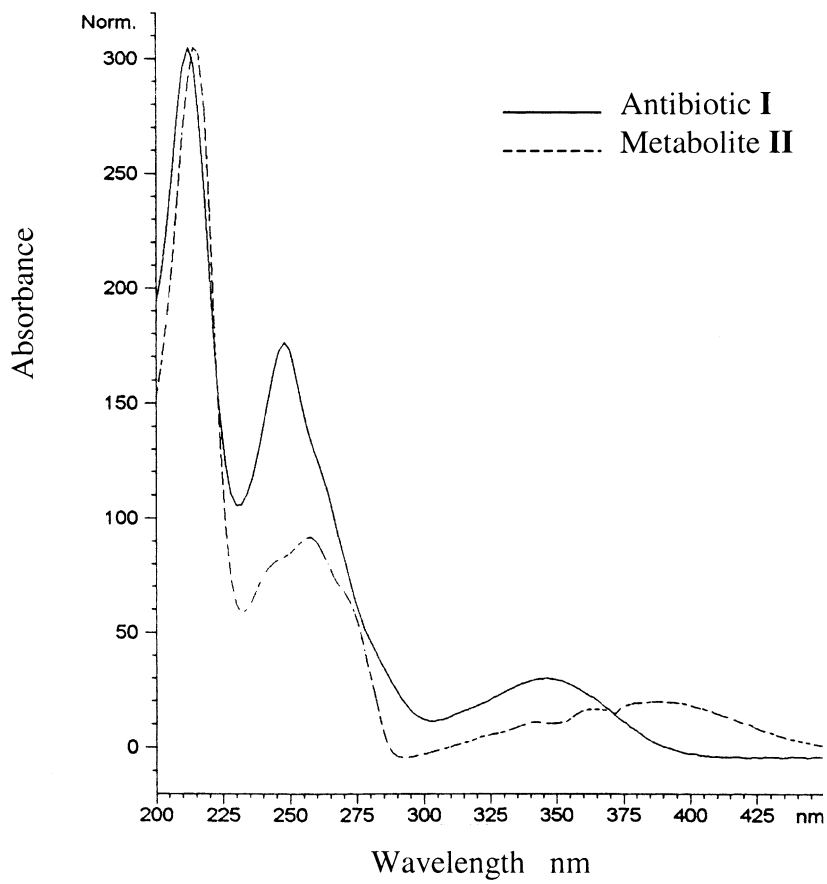

Fig. 3. UV absorbance spectrum of the antibiotic **I** and the metabolite **II**.

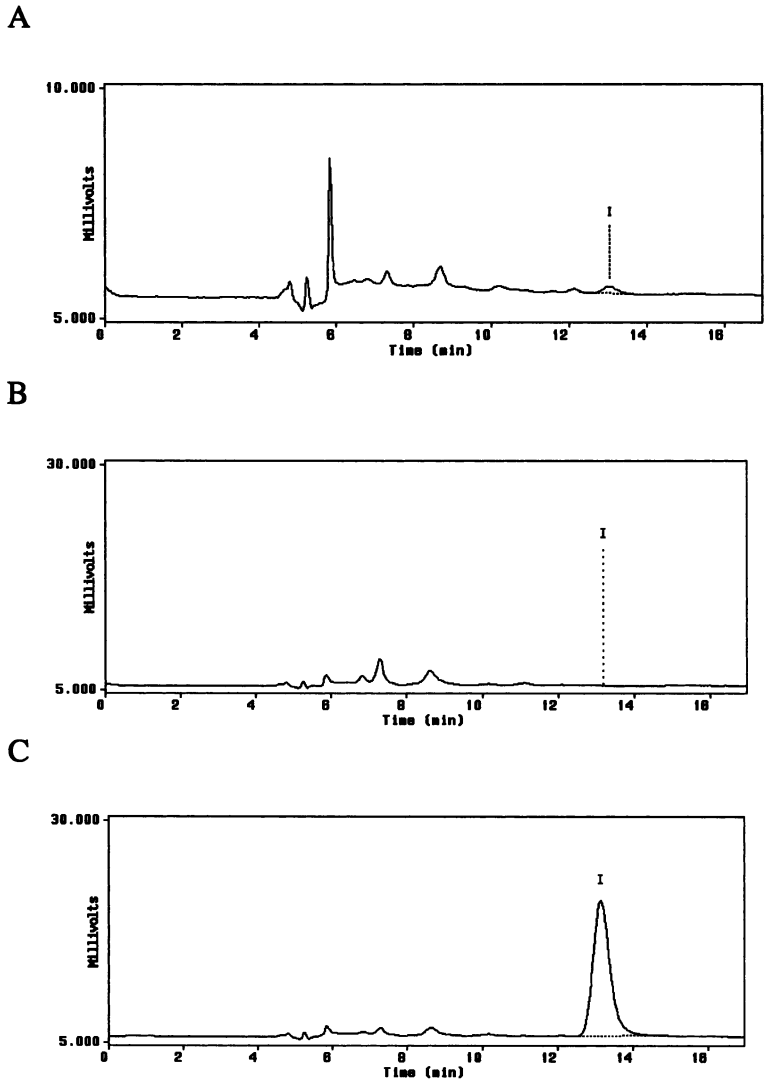

Fig. 4. Representative chromatograms of the antibiotic **I** in human plasma. (A) 0.25 μg/ml standard. (B) Predose subject sample. (C) Postdose (30 min, 500 mg dose) sample with 1:5 dilution, contains 57.1 μg/ml **I**.

injections using Stabilizer B (without ethylene glycol) before back-pressure rose to unacceptable levels. This observation suggests that ethylene glycol contained in the MES buffer may lessen protein denaturation in the extraction step and slow the build-up of denatured proteins on the extraction column. A possible explanation for the effects of Stabilizer A on plasma protein under the chromatographic condition may be that ethylene glycol is solvating protein molecules or acting as a surfactant to maintain protein solubility in the mobile phase.

Chromatography of **I** in urine initially involved HPLC conditions similar to those for the plasma assay. A Maxsil 10 μm C18 column, used as the extraction column in the plasma assay, did not provide enough on-line isolation of **I** in urine, and thus, many interfering endogenous peaks co-

eluted with the analyte, even though a sharper peak was obtained. On-line extraction of the analyte in urine was successful using a Maxsil CN 10 μm guard column to achieve specificity. The injection volume was optimized at 10 μl to minimize the effect of endogenous urine background and provide enough sensitivity. The extraction column in the urine assay did last longer than in the plasma assay, and needed to be replaced regularly every 200–300 urine samples. Specificity of the assay is demonstrated in Fig. 5.

3.3. Linearity, reproducibility and accuracy

Intra-day accuracy for each plasma standard concentration ranged from 93.0 to 113.6% of the nominal concentrations, and the precision of five replicates was $< 2.8\%$ coefficient of variation (CV) (Table 2). A representative plasma standard curve is shown in Fig. 6A. Intra-day validation of plasma QC samples at three concentrations ($n = 6$) is also indicated in Table 2; the accuracy ranged from 95.14 to 98.52%, and the precision

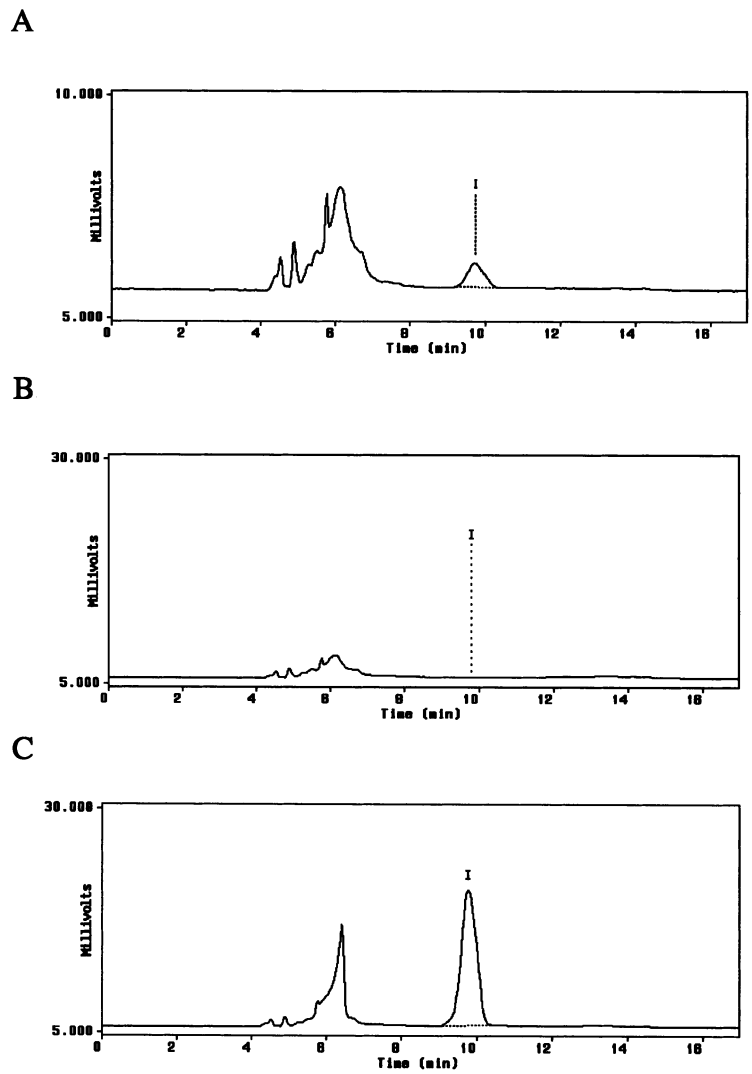

Fig. 5. Representative chromatograms of the antibiotic **I** in human urine. (A) 2.0 μg/ml standard. (B) Predose subject sample. (C) Postdose (4–6 h, 500 mg dose) sample, contains 48.2 μg/ml **I**.

Table 2
Intra-day accuracy and precision of the antibiotic **I** in human plasma

Nominal concentration (μg/ml)	Number of replicates	Mean calculated concentration (μg/ml)	Mean accuracy[a] (Calc/Nom, %)	Precision[b] (CV, %)
Standard				
0.25	5	0.28	113.6	1.92
0.5	5	0.49	98.8	2.76
1.0	5	0.96	95.8	2.51
2.5	5	2.37	94.7	2.27
5.0	5	4.65	93.0	0.95
10.0	5	9.94	99.4	2.09
25.0	5	24.79	99.2	1.62
50.0	5	50.84	101.7	1.58
QC				
0.5	6	0.49	98.52	5.85
5	6	4.77	95.48	0.33
40	6	38.06	95.14	0.12

[a] Mean accuracy for each standard concentration was calculated using the nominal concentration.
[b] CVs for standard replicates are based on peak area; CVs for QC replicates are based on calculated concentrations.

was $\leq 5.85\%$. Standard plasma curves ($n = 14$) were run daily over the calibration range of 0.25–50 μg/ml and gave representative r^2 and slopes of 0.9995 ± 0.0004 and 5.54 ± 0.60 (E4), respectively. Inter-day precision of QC samples over 14 runs ($n = 14$) were $< 6.9\%$ CV and accuracy ranged from 93.3 to 96.7% (Table 4A).

Similarly, for the urine assay, standard curves over the concentration range 2.0–200 μg/ml gave representative r^2 and slopes of 0.99996 ± 0.00001 and 9.23 ± 0.43 (E3), respectively. A representative urine standard curve is shown in Fig. 6B. Intra-day precision and accuracy for each urine standard and QC sample ($n = 5$) are listed in Table 3. Over the concentration range, for urine standards, the accuracy ranged from 98.1 to 104.0% with the precision at $\leq 5.6\%$ CV; for QC samples, the accuracy ranged from 97.9 to 107.1% with the precision at $\leq 6.41\%$. Inter-day QC samples prepared at 5, 20 and 150 μg/ml over day-to-day measurements ($n = 5$) gave precision at $< 6.9\%$ CV and accuracy ranged from 93.7 to 97.4% (Table 4B).

3.4. Ion-pair HPLC urine assay for simultaneous determination of *I* and *II*

Chromatographic conditions of the validated urine method for **I** were inadequate for the simultaneous determination of **I** and **II**. Metabolite **II** eluted before **I**, with endogenous urine material. The urine method was modified using HS, an ion-pairing agent, to enhance the retention of **II**. The on-line extraction column was not changed, but the analytical column was changed from BDS Hypersil C18 to Prism RP, which provided special selectivity for multi-ionic and quaternary groups. Adjusting the column-switching valve timing and increasing the flow-rate for the analytical column to 1.5 ml/min, the eluting window for **I** and **II** was optimized with the HS concentration at 10 mM in the analytical mobile phase. The assay is specific for **I** and **II** in human urine over the concentration range of 2–200 μg/ml (Fig. 7). The retention time is about 9 and 21 min for **I** and **II**, respectively. A run time of 26 min was used for the sample analysis. Validation of this metabolite assay involved determining assay specificity and linearity. Linear regression analysis for **I** over the concentration range 2.0–200 μg/ml gave r^2 at 0.9997; for **II**, r^2 was at 0.9995.

3.5. Stability

3.5.1. Solution stability at different pH conditions

The β-lactam moiety in a carbapenem is chemically and enzymatically vulnerable to hydrolysis

to the open-lactam form [3], suspected intermediate and other adducts (Fig. 1). Thus, the solution stability of **I** and **II** was evaluated from pHs 3 to 11, using the direct-injection HPLC. As shown in Fig. 8, the peak area of the antibiotic **I**, the metabolite **II**, and unknown peak **X** were measured at pH 3, 5, 6.5, 7, 8, 9, and 11, and peak areas were plotted against pH. Above pH 8, peak area of **I** dramatically decreased with increasing pH, while the other two degradation products increased accordingly. In acidic conditions at pHs below 6.5, **I** gradually increased with increasing pH while **II** decreased. It appeared that the loss of **I** occurred with a gain of **II** and **X**. The trend of the three curves reflects the degradation of **I** and the formation of **II** and **X**. The optimal pH for solution stability of **I** is 6.5.

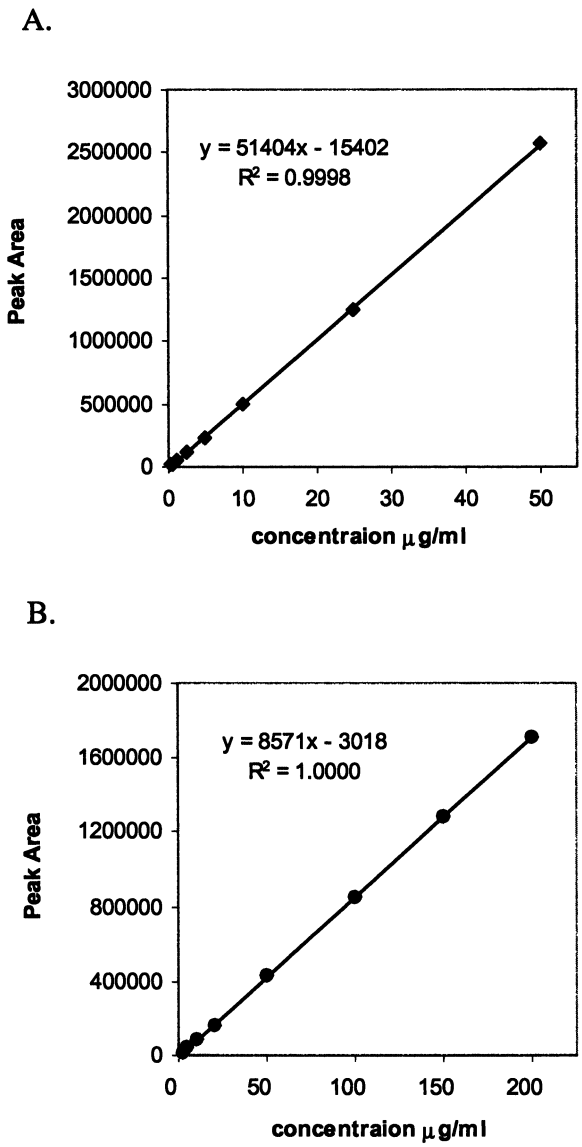

Fig. 6. Representative standard curves of the antibiotic **I** in human plasma and urine. (A) Plasma assay. (B) Urine assay.

*3.5.2. Short-term stability of **I** in plasma and urine at 5 and 25 °C*

For the stability evaluation of **I**, the test samples were placed in an autosampler set at 5 °C or room temperature (25 °C) and periodically, over a period of time, directly analyzed using the column-switching HPLC procedure. The changes in analyte concentrations with time were measured by comparing percent peak areas, starting with 0-time.

The drug was stable ($>95\%$ remaining) in MES (0.1 M) buffer, pH 6.5 at room temperature for at least 18 h. The stability of **I** in plasma without stabilizer was 95% at room temperature for at least 8 h. The stability of **I** in urine with and without stabilizer B was monitored at 5 °C and at room temperature. The antibiotic **I** in both urine matrices was stable ($>95\%$ remaining) at 5 °C for 28 h and at room temperature for 10 h.

*3.5.3. Long-term storage stability of **I** in plasma and urine at −20 and at −70 °C*

The long-term storage stability of the antibiotic **I** in plasma without stabilizer was tested at −20 and at −70 °C. At −20 °C, plasma concentrations dropped to an unacceptable level ($<90\%$) over 87 days, however, remained acceptable at −70 °C for 200 days (Table 6). The stability data for the antibiotic **I** in urine with stabilizer at −70 °C is shown in Table 6. After 82 days, the percent of drug remaining in urine QC samples was about 90% of the initial.

*3.5.4. Freeze-thaw stability of the antibiotic **I** in plasma and urine*

Freeze-thaw stability studies for **I** indicated no effect on the antibiotic **I** concentrations in plasma

Table 3
Intraday accuracy and precision of the antibiotic **I** in human urine

Nominal concentration (μg/ml)	Number of replicates	Mean calculated concentration (μg/ml)	Mean accuracy[a] (Calc/Nom, %)	Precision[b] (CV, %)
Standard				
2.0	5	2.08	104.0	5.6
5.0	5	4.99	99.8	2.3
10.0	5	9.82	98.2	2.0
20.0	5	19.62	98.1	0.8
50.0	5	50.10	100.2	0.3
100.0	5	99.60	99.6	0.2
150.0	5	150.14	100.1	0.5
200.0	5	200.68	100.3	0.1
QC				
5	5	5.35	107.1	6.41
20	5	19.58	97.9	1.86
150	5	151.15	100.8	0.56

[a] Mean accuracy for each standard concentration was calculated using the nominal concentration.
[b] CVs for standard replicates are based on peak area; CVs for QC replicates are based on calculated concentrations.

Table 4
Inter-day accuracy and precision of the antibiotic **I** assays

(A) Inter-day accuracy and precision in plasma QC samples				(B) Inter-day accuracy and precision in urine QC samples			
	LQC 0.5 μg/ml	MQC 5 μg/ml	HQC 40 μg/ml		LQC 5 μg/ml	MQC 20 μg/ml	HQC 150 μg/ml
Mean concentration[a]	0.48	4.46	36.00	Mean concentration[c]	5.19	19.07	141.61
S.D.	0.03	0.15	0.95	S.D.	0.36	1.25	7.64
CV (%)	6.82	3.39	2.64	CV (%)	6.87	6.55	5.40
% Accuracy[b]	96.67	93.33	94.59	% Accuracy[d]	96.87	97.44	93.69

[a] $n = 14$, representing 14 different days of analysis.
[b] Based on the initial mean of QC samples.
[c] $n = 5$, representing 5 different days of analysis.
[d] Based on the initial mean of QC samples.

QC samples objected to three freeze-thaw cycles. There was a slight decrease in urine QC sample concentration (Table 5), supporting the apparent loss observed at -70 °C long-term storage.

4. Application

Clinical plasma samples generated from a Phase I, single rising intravenous dose study in healthy subjects were analyzed using the described assay methods. Fig. 9 shows mean plasma levels with time of six subjects dosed intravenously at 50–1500 mg. Urinary concentrations of the analyte for a representative subject receiving 500 mg i.v. dose ranged from 260.2 μg/ml at 0–2 h to 3.6 μg/ml at 12–24 h collection intervals. Percent urinary excretion was calculated at 21.0%. A mean percent urinary excretion of six subjects was $16.8 \pm 5.3\%$.

Selected urine samples from subjects receiving 250 mg multiple doses were analyzed simulta-

neously for **I** and **II**. Postdose concentrations of **I** and **II** for a representative subject ranged from 120.8 and 184.4 μg/ml at the 0–2 h to 19.0 and 59.2 μg/ml at the 12–24 h, respectively. For this subject, the calculated ratio of the metabolite **II** to the drug **I** excreted was 1.3; the percent urinary excretion of **I** was 18.7%.

5. Conclusion

Precise and accurate HPLC assays have been developed for the high throughput analysis of **I** in plasma and urine using column-switching for direct-injection of a sample aliquot. This technique precludes the need for sample preparation proce-

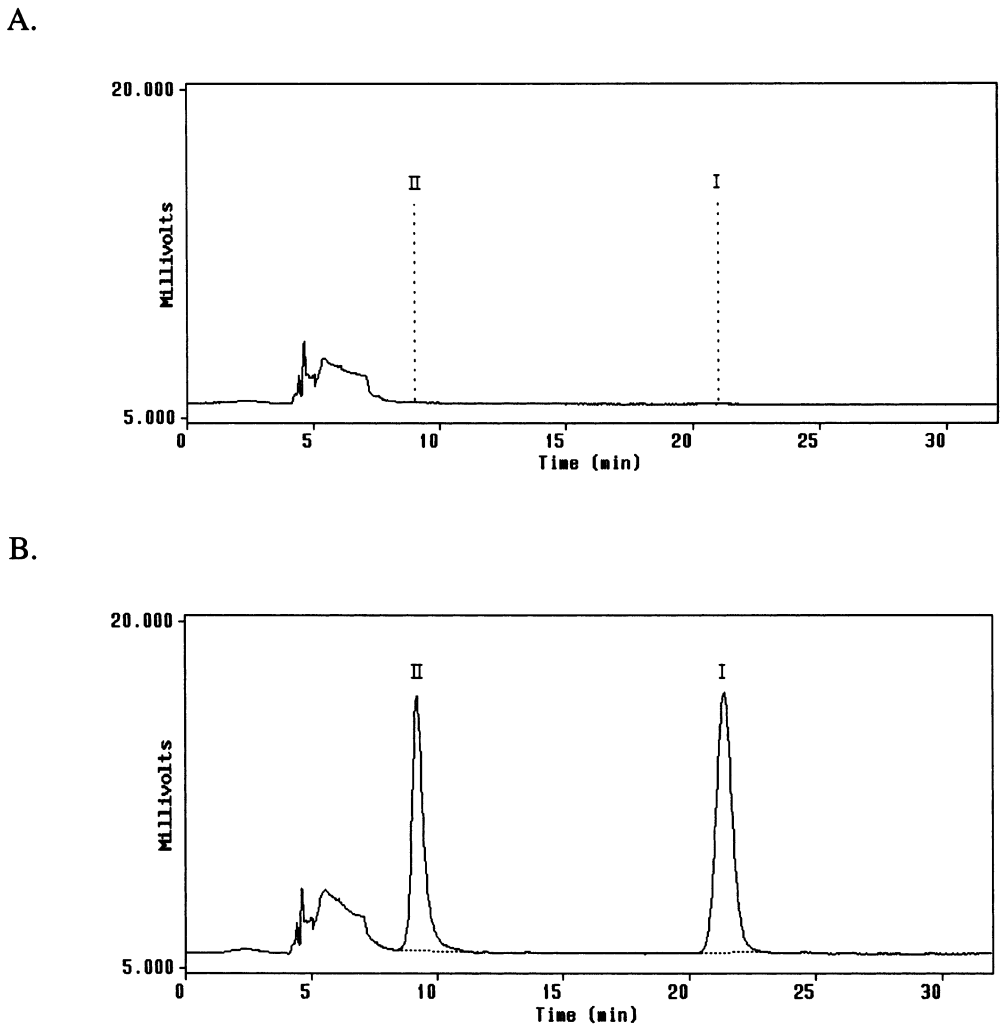

Fig. 7. Representative chromatograms of the antibiotic **I** and the metabolite **II** in human urine. (A) Predose subject sample. (B) Postdose (2–4 h, 250 mg dose) containing the metabolite **II**—182.8 μg/ml (R_t **II** = 9.2 min) and the antibiotic **I**—184.2 μg/ml (R_t **I** = 21.4 min) (dilution factor = 3).

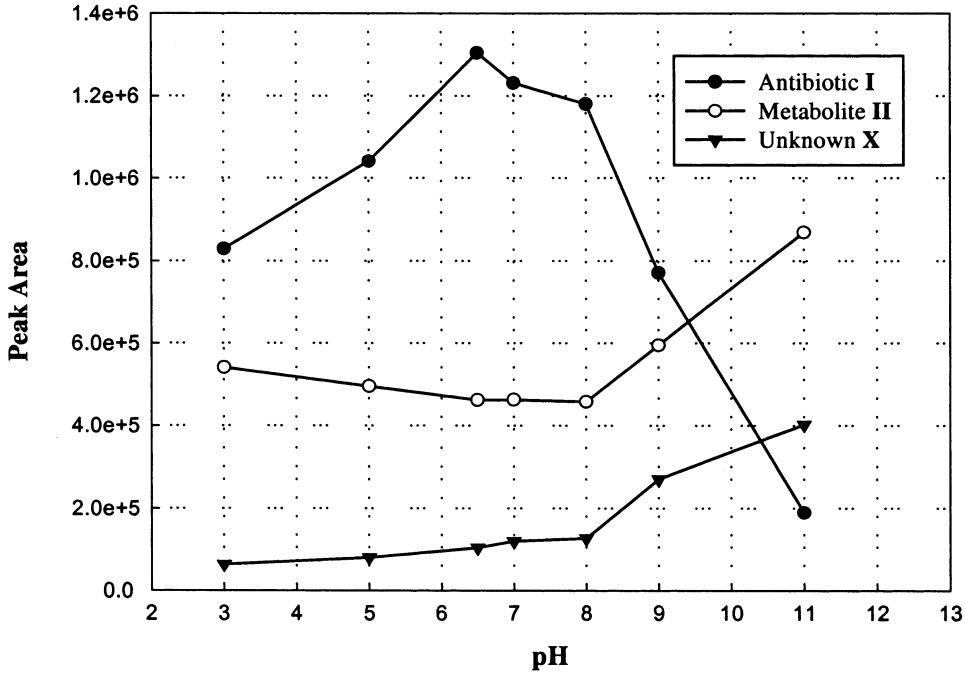

Fig. 8. Effect of pH on degradation of antibiotic **I** and formation of metabolite **II** and unknown **X**.

Table 5
Freeze-thaw stability (−70 °C) of the antibiotic **I** in human plasma and urine

Cycle	*n*	Mean concentration of **I** (μg/ml)		
(A) Plasma		F/T LQC 0.5	F/T MQC 5	F/T HQC 40
1	3	0.50	4.38	35.81
2	3	0.50	4.35	35.67
3	3	0.51	4.28	35.10
Mean		0.50	4.34	35.53
SD		0.01	0.05	0.38
CV (%)		1.15	1.18	1.06
% of nominal concentration		96.24	98.23	98.68
(B) Urine		F/T LQC 5	F/T MQC 20	F/T HQC 150
1	3	5.56	19.70	151.16
2	3	4.88	18.37	135.67
3	3	4.90	17.91	134.22
Mean		5.12	18.66	140.35
SD		0.38	0.93	9.39
CV (%)		7.53	4.99	6.69
% of nominal concentration		94.38	91.79	92.89

Table 6
Stability of the antibiotic **I** at −70 °C

% Remaining of **I** in human plasma QC samples without the stabilizer				% Remaining of **I** in human urine QC samples with the stabilizer			
Days ($n = 3$)	LQC 0.5 μg/ml	MQC 5 μg/ml	HQC 40 μg/ml	Days ($n = 3$)	LQC 0.5 μg/ml	MQC 20 μg/ml	HQC 150 μg/ml
1	100.0	100.0	100.0	0	100.0	100.0	100.0
9	86.7	90.9	91.7	7	98.8	96.3	100.0
32	94.5	97.8	98.1	18	106.4	102.3	101.5
51	101.8	98.4	98.2	79	90.9	85.9	86.5
58	97.8	104.9	104.1	82	89.2	87.9	90.3
63	94.7	92.0	92.2				
70	97.3	94.6	94.6				
87	104.3	95.9	93.2				
144	93.1	94.4	101.3				
200	95.6	105.5	103.2				

dures that might enhance drug degradation and allows for direct evaluation of the carbapenem stability in biological fluids. One disadvantage of the methods was the needs to replace the extraction column approximately every 100–150 plasma samples and 200–300 urine samples. Application of the assays on samples from subjects administered 50–1500 mg intravenously showed adequate assay sensitivity for clinical studies. The employment of an ion-pairing reagent in the column-switching HPLC method was successfully applied for the separation and the quantitation of both **I** and **II** in urine simultaneously.

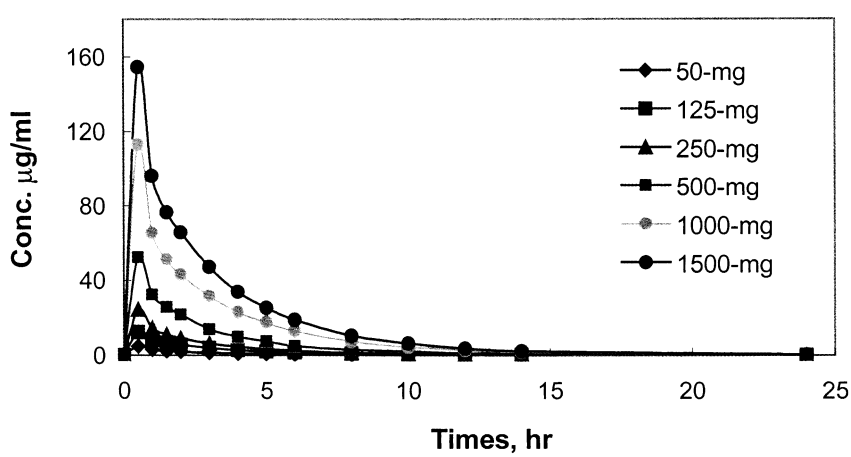

Fig. 9. Mean plasma profiles of the antibiotic **I** of six healthy subjects receiving different i.v. doses of drug (clinical study).

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