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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF A NOVEL CARBAPENEM ANTIBIOTIC IN HUMAN PLASMA AND URINE

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SUMMARY

High-performance liquid chromatographic methods for quantification of a novel carbapenem anti-infective agent, I, in plasma and urine have been developed, validated, and applied to clinical samples. The carbapenem is stabilized in the matrix by the addition of a non-nucleophilic buffer, rapid freezing, and storage at −70°C. After addition of another carbapenem, II, as internal standard, plasma proteins are precipitated with acetonitrile, which is subsequently extracted from the sample with methylene chloride. A portion of the aqeuous phase is injected onto a reversed-phase phenyl column that is eluted with 4% (v/v) acetonitrile in 15 m*M* ammonium phosphate (pH 7.4). The urine assay entails addition of the internal standard II to buffered urine, which is subsequently extracted with methylene chloride prior to injection of the aqueous phase onto a cation-exchange column. The urine assay mobile phase is 5% v/v tetrahydrofuran in 100 m*M* sodium acetate (pH 5.4). The detector response at 313 nm is a linear ($r > 0.99$) function of concentration over the ranges 0.50–100 μg/ml and 2.0–200 μg/ml for the plasma and urine assays, respectively. Thermal degradation products do not interfere with either assay. These assays have proven to be accurate, precise, reproducible, and rugged during clinical sample analyses.

INTRODUCTION

The carbapenem I (BMY-25174, Fig. 1), (5*R*,6*S*)-3-[(N-methylpyridinium)-2-yl-methylthio]-6α-[1-(*R*)-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate, is a semi-synthetic antibiotic that exhibits significant activity against important aerobic and anaerobic pathogens. Like the prototypic carbapenem imipenem [1], I is resistant to hydrolysis by bacterial β-lactamases; however, I is more stable to degradation by renal dipeptidase, an enzyme localized to the proximal kidney tubules of animals and humans [2]. This resistance to enzymatic degradation obviates the need for a renal dipeptidase inhibitor, such as cilastatin, that is coadministered with imipenem for clinical use [3, 4]. These

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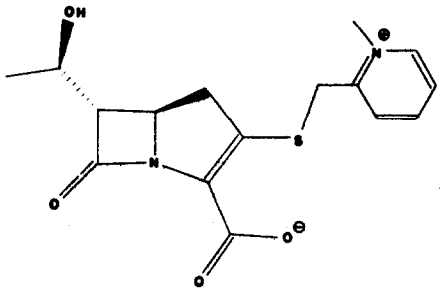
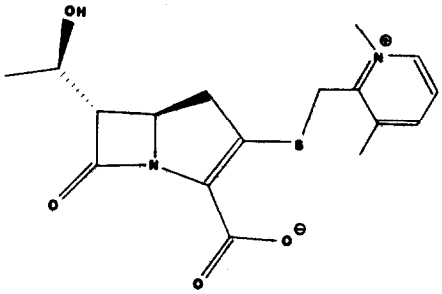
CARBAPENEM ANTIBIOTIC, I





INTERNAL STANDARD, II

Fig. 1. Structures of the carbapenem antibiotic (I) and the internal standard (II).

carbapenems are chemically labile owing to steric strain in the ring system, a fact of practical importance in analytical applications.

This report describes rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assays for I that have been applied to clinical pharmacokinetic studies [5].

EXPERIMENTAL

Chemicals

The carbapenem I and the internal standard (II) were obtained from Bristol-Myers, Pharmaceutical Research and Development Division (Syracuse, NY, U.S.A.) with assigned purity greater than 95%. The structure of II, which is (5*R*,6*S*)3-[(1,3-dimethylpyridinium)-2-yl-methylthio]-6α-[1-(*R*)-hydroxyethyl]-7-oxo-1-aza-bicyclo[3.2.0]hept-2-ene-2-carboxylate, is shown in Fig. 1. Compound I has a water solubility of greater than 100 mg/ml, a pH stability optimum of ca. 6.4, and UV absorption maxima at 270 nm ($\epsilon \simeq 11\,000$) and 292

($\epsilon \simeq 9000$). 3-(N-Morpholino)propanesulfonic acid (MOPS: pK_a 7.2; Calbiochem-Behring), HPLC-grade solvents (Burdick & Jackson, or Fisher), reagent-grade chemicals (Fisher), and control human serum (Interstate Blood Bank, Philadelphia, PA, U.S.A.) were used as received. Heparinized blood was obtained from volunteers, and plasma prepared by centrifugation. House-distilled water was purified with an ion-exchange filtration system (Milli-Q; Millipore).

Instrumentation

The chromatographic equipment used for the assays was obtained from Waters Assoc. A Model 440 dual-channel absorbance detector was fitted with a 313-nm wavelength filter. Either a Model M-45 or a Model 6000A reciprocating pump was used in conjunction with a microprocessor-controlled sample injector (WISP Model 710B). The detector's 0–10 mV analog signal was recorded with a Model 1200 chart recorder (Linear Instruments) and the 0–2 V signal was digitized and recorded with a Hewlett-Packard Model 3357 laboratory data automation system.

Sample collection and processing

Heparinized blood samples were collected and held on ice until plasma was prepared by centrifugation (1000 *g* for 10 min at 4°C). Three parts plasma were buffered with one part of 400 m*M* MOPS (pH 6.5), and frozen as rapidly as possible in a dry ice–methanol bath prior to storage (−70°C). During sample processing, thawed samples were held on ice and, if necessary, diluted into the range of the standards with buffered serum (400 m*M* MOPS–control serum, 1:3). Subsequently, 0.40 ml were transferred to a glass tube, to which 50 μl of 40 m*M* MOPS containing 7.5 μg of II were added. Thereafter, processing was done at room temperature (water and acetonitrile are practically immiscible at 0–4°C). After the addition of 0.60 ml of acetonitrile and centrifugation (1000 *g* for 5 min), the supernatant was poured into a clean tube, to which 2.0 ml of methylene chloride were added, with vortexing, and the tube was recentrifuged. A portion of the upper, aqueous phase was transferred to an injection vial insert in preparation for HPLC.

Urine was collected in containers held on ice, and a sample (seven parts) was buffered with 400 m*M* MOPS (one part) prior to freezing and storage as described above. At the time of sample analysis, a 0.50-ml thawed sample was held on ice and, if necessary, diluted with 50 m*M* MOPS buffer. After the addition, with vortexing, of 100 μl of 50 m*M* MOPS containing 12 μg of II, and then 1.5 ml of methylene chloride, each tube was centrifuged (1000 *g* for 5 min). A portion of the upper, aqueous phase was taken for HPLC.

Standards and solutions

The stock solution of 300 μg/ml I, in 400 m*M* MOPS buffer, was stored at −70°C, and used within one week of preparation. On each assay day, analytical standards containing 0.5, 1.0, 4.0, 10, 40, 70 and 100 μg/ml were prepared, in duplicate, by spiking 0.30-ml serum portions with the requisite amount of stock solution and 400 m*M* MOPS, such that the final sample volume was 0.40 ml. Each stated standard concentration denoted the drug concentration for the plasma

component alone. Thus, concentrations predicted from the standard curve did not require a correction factor for buffer dilution.

For the urine assay, a stock solution of 500 μg/ml I, in 125 m*M* MOPS, was stored at −70°C, and used within one week of preparation. Analytical standards containing 2.0, 5.0, 20, 50, 100 and 200 μg/ml were prepared, in duplicate, by spiking 0.30-ml portions of pooled control human urine with 0.20 ml of 125 m*M* MOPS containing the requisite amount of I. All urine concentrations denote the amount of drug in buffered urine.

Blank and spiked quality control (QC) samples were prepared in the biological matrices at the time of study sample collection, and were stored, thawed, and analyzed with the samples to verify sample stability and the accuracy and precision of every assay sequence. The buffer concentrations of QC samples were the same as that of study samples.

HPLC conditions

All chromatographic procedures were performed at ambient temperature. For the plasma assay, 20-μl injections were made onto a guard column (2.3×0.4 cm I.D) packed with C_{18} pellicular media (30–38 μm particles; Whatman) in series with a μBondapak phenyl analytical column (30×0.39 cm I.D.; 10-μm particles; Waters), which was eluted (1.2 ml/min) with 4% v/v acetonitrile in 15 m*M* ammonium phosphate (pH 7.4). For urine, 12-μl injections were made onto a guard column packed with pellicular cation-exchange media (30–38 μm particles; Whatman) in series with a Partisil-10 SCX analytical column (25×0.46 cm I.D.; 10-μm particles; Whatman). The mobile phase consisted of 5% (v/v) tetrahydrofuran in 0.1 *M* sodium acetate (pH 5.4), and the flow-rate was 2.0 ml/min. Mobile phases were recycled (column eluate returned to solvent reservoir) during typical analytical sequences. After ca. 300 injections the mobile phase was replaced with a fresh solution.

Data processing

Retention times for I and the internal standard II and the peak-height ratios (PHR) were recorded for each chromatogram with the computer system. The least-squares linear regression of PHR on concentration for each standard, weighted by the inverse of the standard's nominal concentration, was computed. Outlier rejection of assay standards was done by the procedure of Prescott [6]. Unknown sample concentrations were calculated by inverse prediction from the linear regression line. The management of the assay sequence data with the Hewlett-Packard 3357 system has been described by Farmen et al. [7].

Assay validation

Specificity was assessed with regard to interference by endogenous matrix constituents, degradation products, and common drugs. Heparinized plasma from ten individuals and urine from five different people were processed, both as blank samples and as samples spiked at the lower limit of quantitation (LLQ), defined as 0.50 and 2.0 μg/ml for plasma and urine, respectively. The degreee of interference by plasma and urine constituents with the peaks of interest was evaluated

by inspection of chromatograms, and by determining the accuracy and precision with which the spiked samples were assayed.

Possible interference by degradation products of I was investigated by correlating UV and proton NMR results. A 2.0 mg/ml solution of I in deuterium oxide was scanned (WM360 WB spectrometer; 360 MHz; Brucker Instruments) prior to and immediately after a 5.5-h incubation period at 53°C. Aliquots of the same sample were taken before and after incubation, diluted with MOPS buffer, and held on ice pending direct injection for HPLC. Peak heights at both 280 nm and 313 nm were monitored with the dual-channel detector. Some common drugs were checked for chromatographic interference by directly injecting 0.5–2 μg and recording retention times and peak heights.

Recovery was assessed by comparison of peak heights for processed standards with peak heights for standards prepared with water rather than the matrix, and for direct injections of standard solutions. The fact that 50 m*M* MOPS was suitable for diluting urine samples into the standard curve range was verified by sequential two-fold dilutions of control samples with this buffer.

Accuracy and intra-assay precision were assessed by assaying ten replicate samples of spiked plasma or urine pools in a blinded manner. The deviation of the mean predicted concentration from nominal was taken as an index of accuracy, and precision was expressed as relative standard deviation (% R.S.D.). Additional portions of each pool were frozen, and on subsequent days, ten replicates of each were assayed to assess inter-assay precision. The results for each assay sequence (treatment) were evaluated by ANOVA to obtained the treatment mean square (TrMS), error mean square (EMS), and grand mean (GM). Inter-assay precision was calculated as: $100[\mathrm{TrMS}-\mathrm{EMS})/n]^{0.5}/\mathrm{GM}$. If EMS > TrMS, then the inter-assay precision was estimated as the standard deviation (% R.S.D.) of the individual assay sequence means about the GM.

The stability of the analyte in fresh pooled human plasma or urine was assessed at 37°C. The plasma samples were held under an atmosphere of 95:5 oxygen–carbon dioxide. Periodically during incubation in a Dubnoff shaker, aliquots were abstracted, buffered, and held on ice until assayed. Stability during storage at −20°C and −70°C was evaluated with spiked plasma and urine samples that were divided into aliquots, buffered, and frozen as described above. Some storage aliquots were thawed, held on ice for ca. 2 h, and refrozen to assess the effect of additional freeze–thaw cycles on storage stability. Stability of I and II in the final aqueous phase prior to injection was evaluated by repeatedly injecting an assay standard, and determining peak height and PHR as a function of time after processing.

Pharmacokinetic application

Serial plasma samples were collected from a normal male volunteer who received 500 mg of I as a 25-min intravenous infusion. The total urine output of the subject was collected over the intervals 0–1, 1–2, 2–3, 3–4, and 4–6 h after dosing. Plasma concentration, (C) versus time (t) data were fit to a biexponential equation of the form $C=A'\mathrm{e}^{-\alpha t}+B'\mathrm{e}^{-\beta t}$, by means of NONLIN84, an iterative, non-linear

least-squares regression program [8]. Optimized coefficients for infusion administration were converted into their bolus dosing equivalents, A and B, as described by Loo and Riegelman [9]. These coefficients, and the exponential terms, were used to derive the following kinetic parameters by standard equations [10, 11]. The elimination half-life was calculated as $t_{1/2} = (\ln 2)/\beta$. The area under the C versus t curve (AUC) and the area under the first moment curve (AUMC) were calculated as $A/\alpha + B/\beta$ and $A/\alpha^2 + B/\beta^2$, respectively. Mean residence time (MRT) was obtained as MRT = AUMC/AUC − (T/2), where the infusion time (T) was 0.42 h. Total body clearance, Cl = dose/AUC, and the steady-state distribution volume, $V_{ss} = \text{MRT} \times Cl$, were also calculated.

The amount of intact I remaining to be excreted (A_{re}) at the end of each collection interval was obtained by subtracting the cumulative amount excreted by the end of that interval from the total amount excreted over all intervals. An estimate of $t_{1/2}$ was obtained as $(\ln 0.5)/b$, where b was the slope of the least-squares line for the regression of $\ln (A_{re})$ on time [12].

RESULTS AND DISCUSSION

The sample processing and HPLC procedures yielded chromatograms (Fig. 2) that were devoid of significant interference in the regions of the I peak and the peak for internal standard II. Plasma, but not serum, from some individuals contained an endogenous component that produced a peak equivalent to less than 2 μg/ml in the vicinity of the I peak. Although human serum proved chromatographically distinguishable from plasma, serum was used for assay standards, because this matrix was stocked in quantity. The absence of endogenous interference was verified further, by evaluating accuracy and precision at the assay's LLQ. The mean (% R.S.D.) observed concentrations for ten plasma samples spiked to contain 0.50 μg/ml and for five urine samples nominally containing 2.0 μg/ml were 0.50 μg/ml (2) and 2.0 μg/ml (5.8), respectively. Thus, the range of reliable response included the LLQ.

Evidence that degradation products of I do not interfere with the assay was obtained by correlating peak-height and proton NMR results. Heat degradation of a test sample caused a 24% decrease in peak height on the cation-exchange column. NMR analysis of this sample revealed a 24% decomposition of the β-lactam bond and a 12% loss of the aromatic moiety. Since loss of the two characteristic proton signals could have occurred independently, 24–36% of the carbapenem could have degraded during heating. Similarly, for the plasma assay evaluation, a 24% decrease in peak height was accompanied by a 24% loss in the aromatic signal and a 16% loss of the β-lactam. Thus, the HPLC and NMR results for the extent of degradation are consistent and help support the conclusion that the assays are specific for intact I. The 313 nm/280 nm ratios for the I peak were the same (0.67) on both HPLC columns and were invariant with respect to heat degradation.

Of the compounds tested, only aspirin and salicylate absorbed appreciably at 313 nm and were eluted within 30 min. However, normal serum levels of these two drugs would not be expected to cause a substantive problem. Other tested

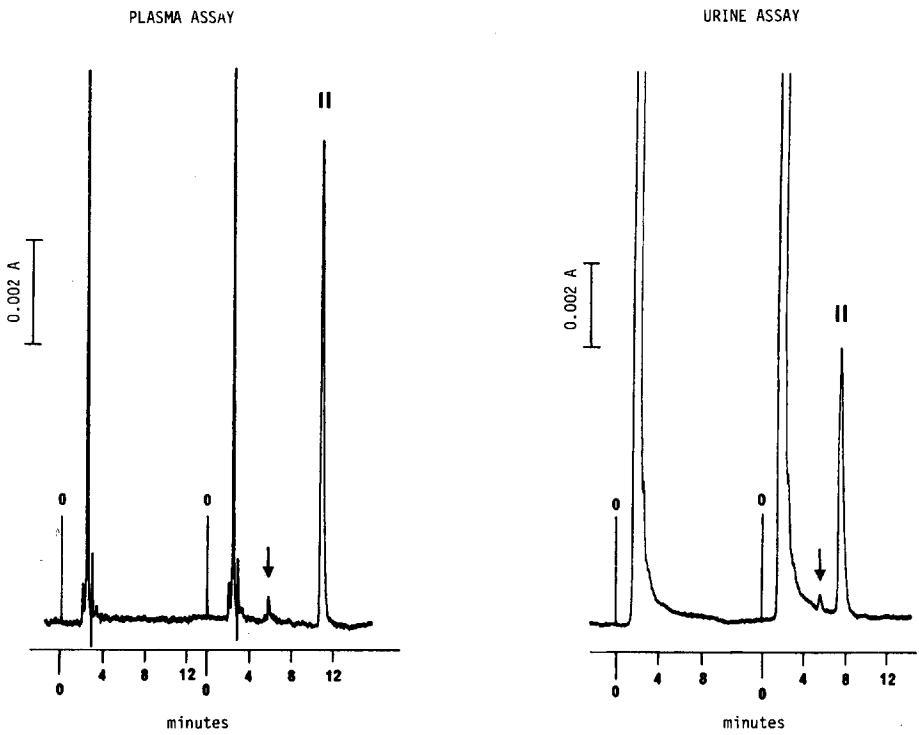


Fig. 2. Representative HPLC profiles of human plasma and urine. The left panel contains a chromatogram of blank plasma followed by a recording for plasma spiked at the lowest limit of quantification with carbapenem (I; arrow) and the internal standard (II). An analogous pair of chromatograms for blank and spiked urine is shown in the panel on the right.

drugs were penicillin G, ampicillin, carbenicillin, cephalothin, cefazolin, cimetidine, acetaminophen, and caffeine.

The plasma and urine assays were evaluated with respect to recovery, linearity of response, accuracy, and precision. Recovery of I was assessed despite the fact that drug extraction was not involved for either assay. Recovery was essentially 100% relative to direct injections of standard solutions or processed standards prepared in buffer alone. The UV response was a linear ($r > 0.99$) function of concentration over the ranges 0.5–100 and 2.0–200 μg/ml for plasma and urine, respectively. Representative assay results for accuracy and intra-assay precision assessments are summarized in Table I. For the plasma assay, inter-assay (between-day) precision values were 1.5% and 1.2% at 5.7 μg/ml and 89 μg/ml, respectively. Values of 6.6% at 13.2 μg/ml and 4.9% at 173 μg/ml were obtained for the urine assay.

The new carbapenem is unstable in both human urine and plasma; a decomposition rate of 6–9 %/h, at 37°C, was measured. The addition of a non-nucleophilic buffer, such as MOPS, maintenance of samples on ice, rapid freezing in dry ice–methanol, and storage at −70°C have been used to achieve sample stability for at least one month (Tables I and II). Spiked samples were unstable at −20°C,

TABLE I

REPRESENTATIVE ANALYTICAL RESULTS FOR ACCURACY, PRECISION, AND STORAGE STABILITY ASSESSMENT OF PLASMA AND URINE CARBAPENEM ASSAYS

Either five or ten replicate samples were assayed in each case.

Matrix	Nominal concentration (μg/ml)	Storage period (days)	Mean observed concentration (μg/ml)	Intra-assay precision (% R.S.D.)	Deviation from nominal (% nominal)
Plasma	5.7	0	5.6	2.3	−1.8
		1	6.0	6.9	+5.3
		98	5.3	5.4	−7.0
	8.0	210	8.3	3.5	+3.8
	80	210	80	1.3	0.0
	89	0	89	2.3	0.0
		1	93	1.5	+4.5
		98	85	1.5	−4.5
Urine	13.3	0	12.7	3.8	−4.4
		1	12.3	1.5	−7.9
		8	13.7	6.3	+3.1
		22	14.5	1.9	+8.7
		37	12.1	1.0	−9.2
	173	0	153	8.3	−11.5
		1	159	3.9	−8.2
		8	170	1.3	−1.5
		22	179	1.0	+3.6
		37	158	0.8	−8.8

TABLE II

SUMMARY OF QUALITY CONTROL SAMPLE RESULTS OBTAINED DURING SINGLE-DOSE CLINICAL SAMPLE ANALYSES

Samples were assayed in triplicate.

Matrix	Total number of assays	Nominal concentration (μg/ml)	Mean observed concentration (μg/ml)	Intra-assay precision (% R.S.D.)	Inter-assay precision (% R.S.D.)
Plasma	16	8.0	8.0	4	2
	17	80	80	3	4
	18	160	160	4	6
Urine	8	15	15	7	3
	8	150	152	2	3
	8	400	394	4	4

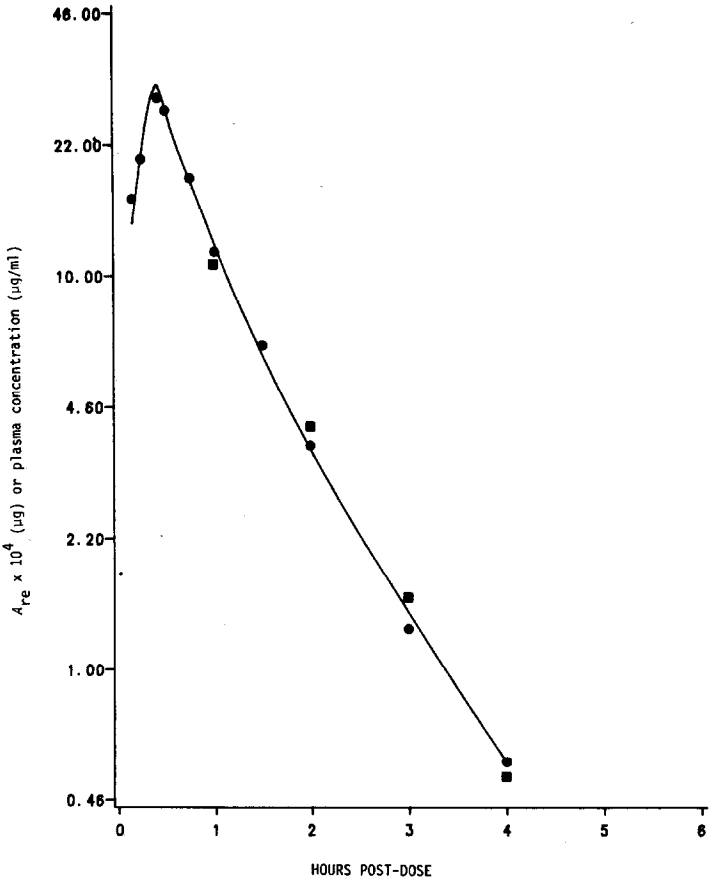


Fig. 3. Application of the plasma and urine assays for clinical studies. A male subject received 500 mg of carbapenem I as a 25-min intravenous infusion. Plasma concentration data (●) were fit to a biexponential function, shown as a continuous curve, which was used to derive pharmacokinetic parameters. Superimposed on the graph are urinary recovery data (■) expressed as the amount of intact I remaining to be excreted (A_{re}) at the end of each interval.

losing 30% of the compound within one week. It may be possible to enhance the stability of samples stored at −20°C by the addition of ethylene glycol [13] to prevent freezing, and the concomitant formation of foci of high drug concentration. However, this solvent led to substantive chromatographic interference, a problem noted by Myers and Blumer [14] for their imipenem assay. At least two freeze–thaw cycles can be tolerated without substantial (more than 10%) loss of activity. The stability of the processed samples at room temperature was adequate for use with an automatic injector. Although peak heights for I and the internal standard both decreased by ca. 0.7%/h, the PHR was essentially invariant over a 20-h period. Thus, the use of carbapenem analogue II as an internal standard compensates for decomposition of I prior to injection.

These methods have been used extensively for the analysis of plasma and urine samples from clinical pharmacokinetic studies. Routinely, 50–100 injections have

been made in each assay sequence, with predictable results, and no indication of interfering chromatographic peaks. When applied in other laboratories, changes in sample handling and instrumentation, e.g. make and model of detector, were almost inevitable. It was impractical to precisely determine the consequences of each and every alteration on sample integrity and assay accuracy. Therefore, QC samples were prepared, stored, thawed, and assayed with each set of study samples. Verification of sample stability under actual study conditions, and of analysis validity, was based on the accuracy and precision of QC results as well as standard curve statistics. Representative QC results accrued during a clinical, safety, and pharmacokinetic study [5] are compiled in Table II. These data are clearly indicative of assay accuracy and precision adequate for kinetic evaluation.

Representative analytical data from the clinical study are illustrated in Fig. 3. Serial plasma samples and the total urine output of a male subject, who received 500 mg of I, were collected, and all samples were assayed as described. The plasma data were fit to a biexponential function and the urine data were evaluated to determine the amount of intact carbapenem remaining to be excreted (A_{re}) at the end of each collection interval. The following kinetic parameters were obtained from the characteristic plasma function: $t_{1/2}=0.81$ h; AUC=29 μg h/ml; MRT=0.65 h; $Cl_T=285$ ml/min; and $V_{ss}=11$ l. Approximately 42% of the dose administered to this subject was recovered in the urine as intact I. The $t_{1/2}$ of 0.69 h, based on the A_{re} evaluation, was reasonably consistent with the plasma data. The plasma and urine assays have thus proven reliable and rugged, despite the inherent instability of the carbapenem.

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