Quantification of Imipenem's Primary Metabolite in Plasma by Postcolumn Chemical Rearrangement and UV Detection

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Imipenem (thienamycin formamidine) is an antibiotic active against a broad spectrum of bacteria. Its primary metabolite arises from cleavage of the lactam ring. The metabolite can be formed *in-vitro* by acid-catalyzed or enzymatic hydrolysis. In animals and man, this metabolite can be generated systemically as well as in the kidneys following the excretion of imipenem into the urine. In man, this dehydropeptidase-catalyzed renal metabolism is minimized by the coadministration of cilastatin, a competitive inhibitor. A specific HPLC assay has been developed to evaluate the disposition of this metabolite in humans having normal or end-stage renal function. The assay employs ion-pair, reversed-phase chromatography, and post-column acid treatment of the analyte for ultraviolet detection.

KEY WORDS: imipenem; metabolite; assay; stability.

INTRODUCTION

Imipenem (*N*-formimidoyl thienamycin; Fig. 1) is a potent carbapenem antibiotic active against a broad spectrum of bacteria (1–3). It is the first carbapenem antibiotic clinically available. Unlike penicillin analogues and many cephalosporins, imipenem is resistant to most beta-lactamases (4). The urinary recovery following intravenous administration in humans, however, is low and variable (5). Imipenem is highly metabolized in the kidneys by the renal dipeptidase, dehydropeptidase-I (DHP-I) (5,6). The renal metabolism occurs by cleavage of the beta-lactam, giving imipenemoic acid, the primary metabolite of imipenem (M1) (Fig. 1) (6). Coadministration of imipenem with the dehydropeptidase inhibitor, cilastatin sodium, increases the urinary recovery to 72% (7). An assay for M1 was needed to study its systemic formation and disposition in humans.

A biological assay for M1 is not available. M1 has been traditionally monitored by HPLC and radioactivity. The compound has no fluorescent, electrochemical, or ultraviolet physical properties to monitor. A study of its chemistry, spectrophotometrically, revealed the formation of a stable intermediate with an ultraviolet chromophore at a wavelength 283 nm in acid. An ion-pair, reversed-phase HPLC assay was developed using postcolumn, chemical rearrangement of M1 for ultraviolet detection. Spectral studies clarified the structural changes of imipenem and M1 in acid and in base. Stability studies also explained some of the observations made by other investigators concerning *in vitro* degradation products of imipenem (8).

EXPERIMENTAL

Reagents

Tetrabutylammonium HSO_4 was obtained from Regis Chemical Co. Phosphoric acid (85%), ethylene glycol, 4-morpholinoethanesulfonic acid (MES), 4-morpholinopropanesulfonic acid (MOPS), L-ascorbic acid, and ethylenediaminetetraacetic acid, disodium salt (EDTA, Na_2) was purchased from Fisher Scientific. Imipenemoic acid (6) and the internal standard, (4*R*, 5*S*, 6*S*)-3-((2-(dimethylamino)-2-iminoethyl)thio)-6-(1(R)-hydroxyethyl)-4-methyl-7-oxo-1-azabicycle(3.2.0)hept-2-ene-2-carboxylic acid (Fig. 1) (9), were synthesized at MSDRL. Mobile phase and stock solutions were prepared with Milli-Q water (supplied from the Millipore Reagent Water System). The reagents were used as they were received.

Apparatus

The isocratic HPLC system consists of a Perkin-Elmer ISS-100 autosampler with a temperature-control sample tray, two Perkin–Elmer Series 10 liquid chromatographs, two Kratos Spectroflow 783 (or 773) absorbance detectors, a Spectra Physics 4120 integrator, and a Waters 730 data module. The autosampler is set with a 30-μl injection volume and a 40- to 45-min run time; the sample tray is refrigerated at 0–5°C. One detector is set at 295 nm and the second at 320 nm; the integrators are set for peak height. The analyte is detected by a chemical treatment that occurs postcolumn. The eluent from the reversed-phase, analytical column is mixed with the reagent in a low-dead volume tee from Scientific Science, Inc., and flows to a reactor. The reactor is an empty stainless-steel column (4.6 mm × 25 cm in length) dry packed with 40-μm glass beads from Whatman. The temperature of the reactor is controlled by an Elelex temperature control unit Model III set at 29°C.

Chromatographic Conditions

The stationary phases consist of a guard column, RCSS Guard PAK C18, placed before the analytical column, a Resolve C18 Radial-PAK cartridge (8-mm ID × 10-cm length). Both columns were obtained from Waters Associates. The analytical column is conditioned with a concentrated solution of the mobile phase. The concentrated mobile phase is a solution of 350 mg of tetrabutyl ammonium HSO_4 and 4 ml of H_3PO_4 (85%) in 1800 ml of water. The solution is pH-adjusted with 1 *M* KOH to 6.85 and diluted to 2000 ml with water. The mobile phase is a further dilution of 400 ml of the solution with water to 2000 ml. The column is conditioned with methanol, water/methanol, and the concentrated mobile phase. The column is conditioned until the M1 peak

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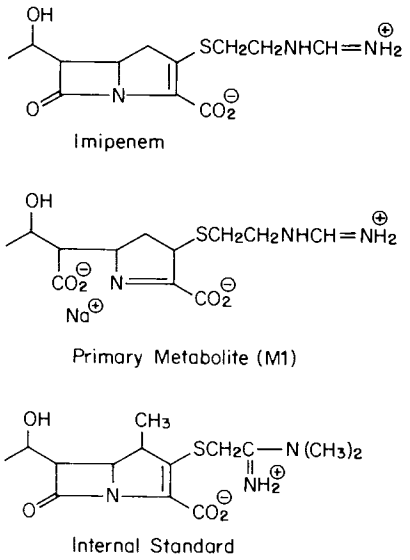

Fig. 1. Structures for imipenem, M1, and internal standard.

retention is about 9.0 ± 0.5 min. The mobile phase flow rate is 1.4 ml/min.

The postcolumn reagent is a solution of 98 g of H_3PO_4 (85%) in water diluted to 2000 ml (0.426 *M*). The reagent flow rate is set at 0.6 ml/min. The postreactor eluent flows to the first ultraviolet detector set at 295 nm and then to the second ultraviolet detector set at 320 nm. The range of the detectors was set at 0.002 aufs and the rise time was 1.0 sec. Stability studies have shown other M1 degradates to have similar chromophores and retention times. M1 absorbance is approximately threefold greater at 295 nm than at 320 nm, which is the wavelength chosen to monitor M1 degradates and imipenem. The response from the first detector should exceed that from the second detector if only M1 is present. Therefore, a comparison of the responses from both detectors qualitatively measures the homogeneity and authenticity of the M1 peak.

Stock Solutions

Imipenem and M1 Stabilizer for Plasma. Dissolve 0.744 g of L-ascorbic acid (10 m*M*) and 2.49 g of EDTA, Na_2 salt (10 m*M*), in 1 liter of MES buffer, pH 6.0 (1 *M*); mix the above with 1 liter of ethylene glycol. The stabilizer should be prepared routinely.

Imipenem and M1 Stabilizer for Urine and Dialysate. This is the same as discussed for plasma except the MOPS buffer, pH 6.8 (1 *M*), is used instead of the MES.

M1. A 1.0 mg/ml stock solution is prepared with dilute H_3PO_4 (0.212 *M* aq). A second stock solution for lower-concentration, calibration standards is prepared at 200 μg/ml with dilute H_3PO_4. The above solutions are kept at −70°C for long-term use. The solid, M1 material is hygroscopic and should be stored long-term in the presence of Drierite at −70°C.

Internal Standard. A 1.04 mg/ml solution is prepared with MES buffer/ethylene glycol (1:1). A second stock is prepared by dilution of the above to 104 μg/ml. These two solutions are kept at −70°C. A final stock solution is prepared by diluting 1 ml of the 104 μg/ml stock with 7 ml of MES buffer/ethylene glycol to 13.0 μg/ml. This stock is routinely drawn upon and kept at −20°C. It is prepared routinely from the above solution.

Sample Preparation Prior to HPLC

From each stabilized sample of plasma, a 250-μl aliquot is transferred to a Centrifree Micropartition System from Amicon. A 100-μl aliquot of the internal standard is added, the Centrifree System is capped, and the contents are mixed by vortex. The systems are centrifuged at 2000–2500 rpm for 25 min. The filtrate is analyzed. Urine and dialysate samples are prepared in the volume proportions described above, but ultrafiltration is not necessary.

Calibration Standards

Plasma and dialysate standards are prepared by mixing 1 ml of stabilizer with 1 ml of plasma or dialysate and an appropriate volume of the M1 stock solution. The concentrations mixed are 1, 2.5, 5, 10, 25, 50, 75, and 100 μg/ml. Urine standards are prepared similarly except for a different concentration range: 5, 10, 25, 50, 75, and 100 μg/ml.

NMR Analysis of Imipenem and M1

An NMR spectrum of imipenem was generated in D_2O + DCl (*in situ*); the same experiment was performed with M1 (generated using the enzyme DHP). The methyl protons (CH_3–CHOH-C-6) for imipenem gave the expected doublet splitting pattern before and after the addition of DCl (CH_3–, doublet, 1.37 to 1.22 ppm, respectively). The methyl protons for M1 in D_2O, however, gave a triplet pattern (CH_3–, triplet, 1.23 ppm). The addition of DCl resulted in a spectrum nearly identical to that of acidified imipenem (CH_3–, doublet, 1.21 ppm). There are also indications, from the NMR spectra, of other minor by-products resulting from the acid treatment of imipenem and M1.

Acid Hydrolysis of Imipenem

An analytical sample of imipenem was stirred in 1 *N* HCl for 1.0 hr at room temperature. The product was lyoph-

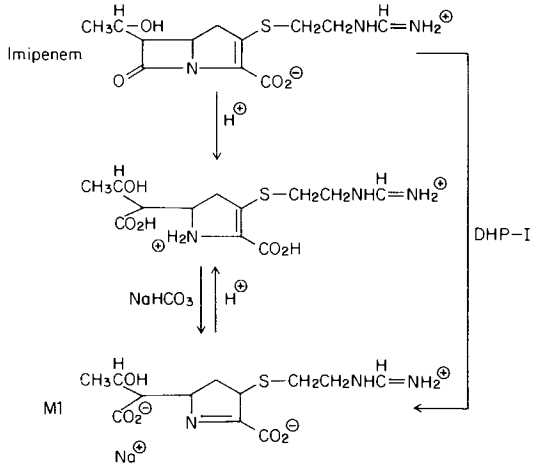

Fig. 2. Chemical transformations of imipenem during acid hydrolysis and of M1 during tautomeric rearrangement.

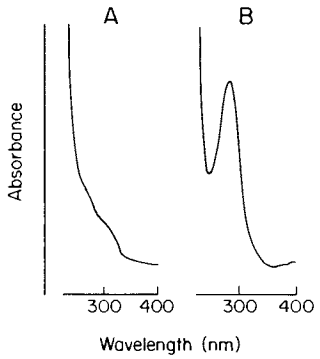

Fig. 3. UV spectra of M1 (enzyme-generated) in $KHCO_3$ (A) and in dilute H_3PO_4 (aqua.) (B).

ilized to an amorphous solid (glass-like). An (FAB-MS) analysis (MS analysis performed on a Varian MAT 731 mass spectrometer with an FAB ion source using a glycol matrix) gave a base peak at 318 z/e (MH+), the open lactam, and a peak at 635 z/e (2MH+), a dimer.

Clinical Study Protocol

Pharmacokinetic Study of Imipenem, Cilastatin, and M1 Following a Single 1000-mg iv Dose of Imipenem/Cilastatin Na (PRIMAXIN-MSD). Healthy volunteers received the PRIMAXIN iv dose by a 50-min constant-infusion rate. The subjects drank a glass of water hourly starting 1 hr before and up to 4 hr after administration. Blood samples were collected before infusion and after, starting at 0.42 hr and ending at 12 hr. Total urine voids were collected for 24 hr starting with hourly increments (−1 through 6) and ending with 2-, 4-, and 12-hr collections. The plasma specimens and urine aliquots were immediately mixed (1:1) with the appropriate stabilized and stored at −70°C.

RESULTS AND DISCUSSION

Chemistry

The metabolite can be formed from imipenem (6) (a) by acid hydrolysis and pH neutralization under argon or (b) by DHP-I hydrolysis of the beta-lactam. The UV absorption of the metabolite is negligible except for end-absorption at 230 nm (6). Previous studies with imipenem in acid, and neutral and alkaline buffers, showed extensive degradation. It is highly vulnerable to acid- and base-catalyzed hydrolysis of its beta-lactam and to subsequent degradation reactions (10–12). However, in acid, only one product was observed without further degradation.

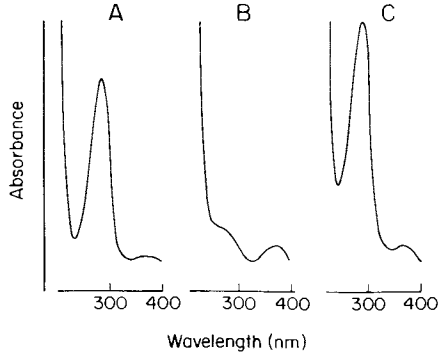

Fig. 4. UV spectra of M1 following (A) imipenem in H_2SO_4, (B) pH-neutralized with $KHCO_3$ under argon, and (C) pH-readjusted with H_3PO_4.

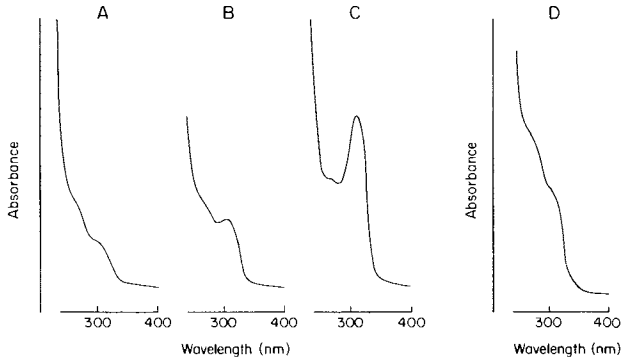

Fig. 5. UV spectra of M1 in $KHCO_3$ exposed to air at ambient temperature at (A) T = 0 hr, (B) T = 0.5 hr, and (C) T = 2.41 hr and M1 in $KHCO_3$ under an argon atmosphere at (D) T = 2.25 hr after incubation at ambient temperature.

Initial characterization of the physical properties of the metabolite revealed no inherent UV, fluorescent, or electrochemical properties. Thus, detection of the metabolite would have to be the result of a chemical change to the molecule. The reaction preferably would be postcolumn since any clinical sample analysis would involve imipenem.

The ultraviolet spectrum of M1 in a neutral aqueous matrix will change upon acidification to a strong band at 283 nm (Fig. 3). The chromophore at 283 nm can be formed from M1 whether M1 is generated by synthesis or by enzyme. Addition of $KHCO_3$ to the cuvette will result in a spectrum characteristic of M1, suggesting a reversible mechanism. The same chromophore at 283 nm can be observed from imipenem in an acid matrix. The synthesis of M1 from imipenem involves a bathochromic shift from 298 nm from imipenem to 283 nm upon addition of sulfuric acid. Neutralization of the acid under argon gas gives the uncharacteristic,

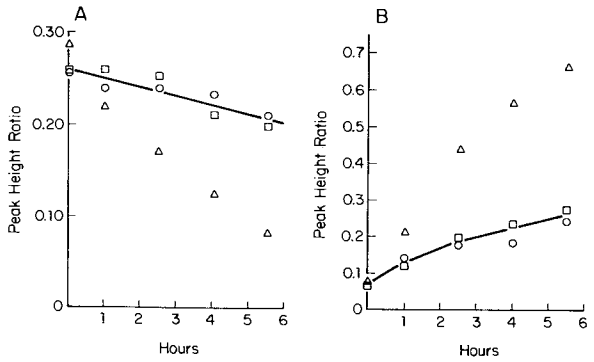

Fig. 6. (A) Degradation of M1 (10 μg/ml) in commercial human plasma with MES buffer/ethylene glycol (50:25:25) at ambient temperature ($T_{1/2}$ = 2.56 min) (△——△); (○——○) Same as above except the MES buffer was not present ($T_{1/2}$ = 16.23 min); (□——□) same as above except that EDTA, Na_2 was added (1 m*M*) ($T_{1/2}$ = 8.97 min). (B) Formation of the degradation product with a λ_{max} of 308 nm from M1 under the above conditions.

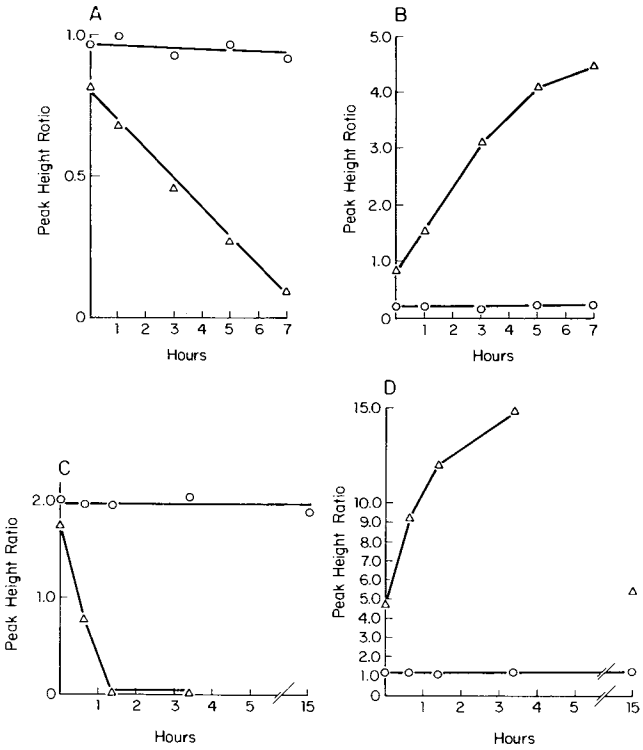

Fig. 7. (A) Degradation of M1 (20 μg/ml) in commercial human plasma with MES buffer/ethylene glycol (50:25:25) ($T_{1/2}$ = 3.217 min) (△——△); (○——○) Same as above except ascorbic acid and EDTA, Na_2 (1 m*M* each) are added. (B) Formation of the degradation product at λ_{max} 308 nm from M1 in commercial human plasma with MES buffer/ethylene glycol (△——△). (○——○) Same as above except ascorbic acid and EDTA, Na_2 (1 m*M* each) are added. (C) Degradation of M1 (40 μg/ml) in human plasma from subjects with renal dysfunction and containing MES buffer/ethylene glycol (50:25:25) ($T_{1/2}$ < 0.636 min) (△——△). (○——○) Same as above except ascorbic acid and EDTA, Na_2 (1 m*M* each) are added. (D) Formation of the degradation product at λ_{max} 308 nm from M1 in plasma under conditions described in C.

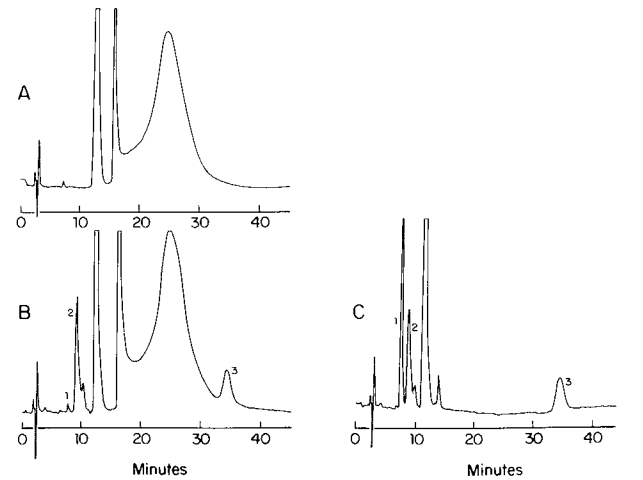

Fig. 8. Chromatogram A is a human plasma blank containing only stabilizers; B is human plasma containing M1 at 80 μg/ml (2), λ_{max} 308-nm degradation product (1), internal standard (3), and stabilizers; C is human plasma containing M1, degradation product, and internal standard (UV responses at 295 nm).

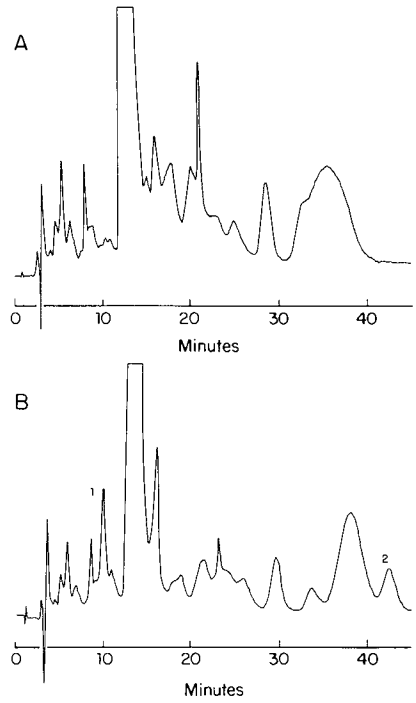

Fig. 9. Chromatogram A is a urine control blank; chromatogram B is a urine standard containing M1 (1) at 50 μg/ml and internal standard (2) (UV responses, at 295 nm).

ultraviolet spectrum of M1. Adding acid again (*in situ*) gives the 283-nm band (Fig. 4). The chromophore at 283 nm, once formed, appears stable under ambient conditions for at least 12 hr. Neutralization of the chromophore and exposure to air will give a multitude of side products.

FABS-MS of M1 in HCl gave a base molecular weight

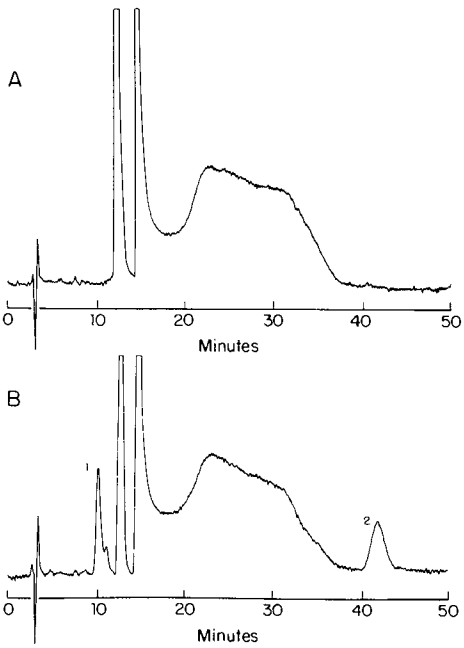

Fig. 10. Chromatogram A is a dialysate blank; chromatogram B is a dialysate standard containing M1 (1) at 50 μg/ml and internal standard (2) (UV responses at 295 nm).

Table I. Linear Regression Parameters for the Plasma, Urine, and Dialysate Assays for M1

Fluid	Y intercept	Slope	r^2	Concentration range (μg/ml)
Plasma	0.0098	0.0686	0.9991	1–100
Urine	0.3397	0.0797	0.9966	5–100
Dialysate	0.0019	0.0345	0.9978	1–100

ion peak of 318 z/e, the MH+ ion of M1. This value is consistent with the simple, lactam-opened, hydrolytic product. Loss of the *S*-alkyl side chain is not observed. The nearly identical NMR and UV spectra of acidified imipenem and acidified M1 along with the apparent splitting of the methyl doublet of M1 in neutral pH suggests the presence of two major isomers at neutral pH which convert to a single species upon acidification. These results are supported by recent work in our laboratories (13) in which the acidified species is shown to be the protonated 2-pyrroline product that isomerizes to the diasteriomeric 1-pyrroline mixture upon neutralization. This reversible reaction scheme is depicted in Fig. 2.

Stability

M1 undergoes oxidation in an aqueous matrix to an intermediate, degradation product that absorbs strongly at a wavelength 308 nm (Fig. 5). The intermediate degrades further to at least two products as determined by HPLC. The rate and extent of this oxidation of M1 are dependent on oxygen content (Fig. 5), metal catalyst (Fig. 6), temperature, pH, and type of aqueous matrix. The degradation product and other side products are formed during the synthesis of M1 from imipenem when an inert gas other than argon is used during pH neutralization. This particular product has been observed by other authors (8) during the incubation of imipenem with a microsomal kidney fraction. Isolation and characterization of this product have not been possible because of its own instability.

A MES buffer, pH 5.5–6.0, which is used to stabilize imipenem in plasma at a final pH of 6.8 appears to contain a catalyst that degrades M1 and forms the intermediate product with a chromophore at 308 nm. Adding EDTA, Na_2 to the MES buffer inhibits the degradation of M1. Adding EDTA, Na_2 to plasma containing the MES buffer and ethylene glycol slows the M1 degradation process and does not stop it (Fig. 6). In addition, M1 degrades at different rates in plasmas obtained from different sources. It degrades faster in plasma obtained from a human subject with renal dysfunction than in plasma from a normal subject or in commercially available plasma (Fig. 7). The difference in the degradation rates demonstrates the importance of a plasma stabilizer for M1 when collecting plasma samples for pharmacokinetic analysis.

Additional M1 stability studies with catalytic poisons (e.g., sodium azide, sodium cyanide), radical scavengers (e.g., 2,6-di-*t*-butyl-4-methoyl-phenol, vitamin E), and reducing agents (e.g., sodium metabisulfite, L-ascorbic acid) suggests a final combination of EDTA, Na_2, and L-ascorbic acid (10 m*M* each; Fig. 7). The final stabilizing solution contains MES buffer/ethylene glycol for imipenem and EDTA, Na_2/L-ascorbic acid for M1 in plasma; for urine and dialysate the above stabilization solution is modified by replacing the MES buffer with MOPS. The combination does not change the stabilizing effects of the MES buffer/ethylene glycol on imipenem in plasma or the MOPS buffer/ethylene glycol on imipenem in urine.

Assay Methodology

Postcolumn. The detection system for the assay used the chromophoric transformation of M1 in an acidic matrix to monitor the metabolite by UV. A postcolumn, dilute phosphoric acid reagent was pumped to a tee and mixed with the eluent of the analytical column. The mixture flowed to a packed-bed reactor set at 29°C (residence time of 0.75 min). The reaction time appears complete since increasing the reactor's temperature from 29 to 60 and 100°C did not improve the peak response. The detection wavelength which was set at 295 nm was chosen for specificity of M1 in an endogenous matrix. A second detector, set at 320 nm, was positioned in-line and after the first to monitor M1, the degradation product of M1 (described under Stability, with a wavelength maxima of 308 nm) and imipenem. The two UV responses gave information on the authenticity of the M1 peak and its degradation product(s) (i.e., ratio of absorbance responses at two different wavelength). The wavelength at 320 nm monitors about 80% of the degradation product and 5% of M1 in acid (based on their UV maximas). The peak for imipenem was better separated from the endogenous material at 320

Table II. Intraday Reproducibility of Replicate Standards (n = 5) of M1 in Plasma, Urine, and Dialysate

Concentration (μg/ml)	Mean[a] ± SD (Coefficient of variation)		
	Plasma	Urine	Dialysate
1.0	0.0720 ± 0.0022 (3.01%)	—	0.0321 ± 0.0037 (11.68%)
2.5	0.1699 ± 0.0071 (4.17%)	—	0.0788 ± 0.0045 (5.71%)
5	0.3096 ± 0.0143 (4.62%)	0.506 ± 0.021 (4.25%)	0.167 ± 0.15 (9.2%)
10	0.655 ± 0.0115 (3.43%)	1.063 ± 0.036 (8.05%)	0.361 ± 0.038 (10.6%)
25	1.750 ± 0.0447 (2.55%)	2.42 ± 0.187 (7.73%)	0.933 ± 0.033 (3.6%)
50	3.45 ± 0.106 (3.05%)	4.17 ± 0.215 (5.04%)	1.604 ± 0.119 (7.5%)
75	4.680 ± 0.259 (5.53%)	6.30 ± 0.329 (5.06%)	2.66 ± 0.27 (10.2%)
100	6.401 ± 0.3498 (5.45%)	7.726 ± 0.641 (8.29%)	3.44 ± 0.33 (9.62%)

[a] These values are averages of peak height ratios of M1 to internal standard at 295 nm.

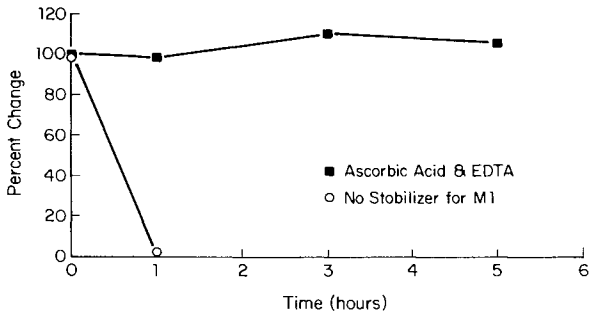

Fig. 11. Plot of M1 stability in human urine at ambient temperature over 5 hr: one curve (■——■) represents percentage M1 degradation in urine containing ascorbic and EDTA, Na_2; the second curve (○——○) represents M1 degradation in urine without stabilizers.

nm than at 295 nm. Imipenem elutes after M1 and is base lined resolved from M1.

Chromatography. The metabolite, imipenem, and degradation product (λ_{max}, 308 nm) can be separated by ion-pair, reversed-phase chromatography. M1 eluted as a broad peak on reversed phase without an ion-pairing agent. Optimal separation and peak shape for M1 and the degradation product were achieved using tetrabutylammonium HSO_4 on a C18 stationary phase that is not well end-capped, such as a μBondPAK ODS (10-μm) column or a Resolve ODS (10 μm, 8 mm × 10-cm length) radial-PAK cartridge for a RCM-100 (radial compression module). Initial studies with an Ultrasphere-IP (developed for ion-pairing by Beckman) did not separate M1 from the degradation product at λ_{max} 308 nm; an Ultrasil ODS (from Beckman) which is not well end-capped gave baseline separation as did the Resolve ODS cartridge. Thus, the exposed silanol groups of the silica backbone seem to be interacting with the analytes for baseline separation. The peak for M1 under the chromatographic conditions described for its quantification has a shoulder peak (Fig. 8B) that may represent one of two diastereoisomeric isomers. Imipenem is optically pure with three asymmetric centers.

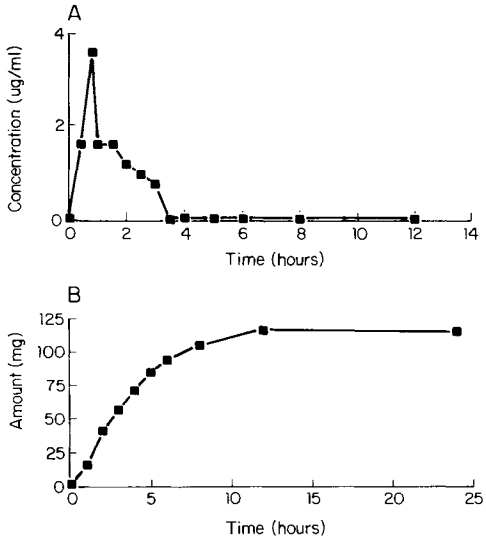

Fig. 12. The plots are (A) a plasma profile and (B) a cumulative urinary excretion of M1 from a healthy subject who received 1000 mg iv of PRIMAXIN by a 50-min constant infusion.

Formation of M1 gives an additional chiral carbon at C-3 and, thus, the formation of two diastereoisomers. For the above assay, only the larger peak is quantified by peak height. The ratio of the two peaks appears constant at 3.766 ± 0.147 across the concentration range 10–100 μg/ml.

Assay Parameters. The plasma assay for M1 is specific in the presence of endogenous material (Fig. 8), imipenem, cilastatin, stabilizers, and the degradation product of M1 that absorbs at the wavelength maxima of 308 nm. There are other degradation products that absorb around 300 nm and elute close to M1. The formation of these products is prevented with the proper stabilizers and can be identified in a chromatogram using the second UV detector set at 320 nm. A strong UV response from both detectors at or near the elution time of M1 suggests the presence of a degradation product. The dialysate and urine assays are specific for M1 with respect to the above except for a small endogenous interference present in the urine assay (Figs. 9 and 10). This is reflected in a positive *Y* intercept in the standard calibration curve.

The linear regression of representative calibration curves for plasma, urine, and dialysate are summarized in Table I. Intraday reproducibility of standard replicates (n = 5) of plasma and urine gave coefficients of variation (CV) which are <10% over the concentration ranges of 1 to 100 and 5 to 100 μg/ml, respectively. For dialysate the CVs varied from 3.6 to 11.7% (Table II). Interday reproducibility was determined with quality controls that were prepared, individualized, and stored at −70°C before the analysis of the clinical studies. The CVs were <10% for plasma, <11% for urine, and <5.6% for dialysate. Similar results were found with quality controls containing M1 and imipenem (at 1:2 and 1:5 molar ratios). Recovery of M1 from the ultrafiltration of a plasma standard at 10 μg/ml is 89.4 ± 2.7% (comparing with a standard prepared in MOPS buffer/ethylene glycol, not filtered). Recovery of M1 from urine at 100 μg/ml via ultrafiltration is 91.99 ± 1.00%. The dialysate samples are not filtered.

The degradation of M1 in plasma and urine at ambient temperature is negligible in the presence of the stabilizers discussed above (Figs. 7 and 11).

Human Studies. Healthy volunteers received 1000 mg iv of PRIMAXIN by a 50-min constant infusion. The plasma concentration profile and urinary excretion of M1 from one representative subject (No. 7) are shown in Fig. 12.

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