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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE PENEM ANTIBIOTIC (5*R*,6*S*)-2-AMINOMETHYL-6-[(1*R*)-HYDROXYETHYL]-2-PENEM-3-CARBOXYLIC ACID IN HUMAN PLASMA AND URINE

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SUMMARY

High-performance liquid chromatographic methods for the determination of (5*R*,6*S*)-2-aminomethyl-6-[(1*R*)-hydroxyethyl]-2-penem-3-carboxylic acid in plasma and urine have been developed, validated and applied to clinical samples. After addition of another penem, (5*R*,6*S*)-2-aminoethyl-6-[(1*R*)-hydroxyethyl]-2-penem-3-carboxylic acid, as an internal standard, plasma proteins are precipitated with a saturated solution of ammonium sulphate. A portion of the supernatant is injected on to a reversed-phase column (RP 8–10 μm) that is eluted with pH 6 phosphate buffer. The urine assay entails a 25-fold dilution with pH 6 buffer and addition of the internal standard prior to injection. The detector response at 320 nm is a linear function of concentration over the ranges 1.6–410 μmol/l (0.4–100 μg/ml) and 41–1025 μmol/l (10–250 μg/ml) for the plasma and urine assays, respectively. These methods have proved to be suitable for pharmacokinetic investigations in man.

INTRODUCTION

(5*R*,6*S*)-2-Aminomethyl-6-[(1*R*)-hydroxyethyl]-2-penem-3-carboxylic acid* (I) (Fig. 1) belongs to a new class of β-lactam antibiotics, the penems. It com-

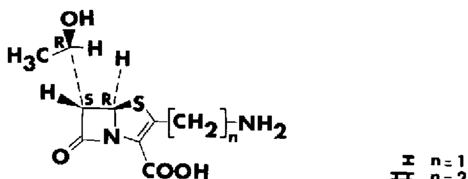

Fig. 1. Structures of the penem antibiotic (I) and the internal standard (II).

*Ciba-Geigy identification: CGP 31 608.

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bines in a single structure the essential elements to which penicillins and cephalosporins owe their biological activity. It represents a significant advance over third-generation cephalosporins and newer extended spectrum penicillins. The compound has a broad spectrum of activity in vitro that includes Gram-positive and Gram-negative aerobic and anaerobic bacteria. It is highly effective against *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*, the two most frequently encountered problem bacteria [1,2].

This paper describes high-performance liquid chromatographic (HPLC) methods for the determination of I in plasma and urine using the closely related aminoethyl derivative II (Fig. 1) as an internal standard. Their application to clinical pharmacokinetic studies is demonstrated.

EXPERIMENTAL

Chemicals

The penem I and the internal standard (5*R*,6*S*)-2-aminoethyl-6-[(1*R*)-hydroxyethyl]-2-penem-3-carboxylic acid (II) were supplied by Ciba-Geigy (Basle, Switzerland). Sodium hydrogenphosphate, potassium dihydrogenphosphate and ammonium sulphate were purchased from E. Merck (Darmstadt, F.R.G.). Water was purified and deionized using a Milli-Q ion-exchange filtration system (Millipore, Bedford, MA, U.S.A.).

Instrumentation

The chromatographic equipment was a Hewlett-Packard Model 1084B instrument, (Palo Alto, CA, U.S.A.) equipped with a variable-volume injector and a variable-wavelength detector set at 320 nm. The peak heights were measured manually and the peak areas were given by the integrator–recorder (79 850 A LC terminal). A Hewlett-Packard Model 1081B instrument, equipped with a variable-volume injector, a Model SF783 variable-wavelength detector (Kratos, Ramsey, NJ, U.S.A.) set at 320 nm and a Model CR3A integrator–recorder (Shimadzu, Tokyo, Japan) giving the heights or areas of peaks was also used.

HPLC conditions

The pre-packed column was a stainless-steel tube (20 cm$\times$4.6 mm I.D.) filled with LiChrosorb RP-8 (10 μm) (Hewlett-Packard, 79915 MO 174). A pre-column (1 cm$\times$4.6 mm I.D.) filled with Nucleosil C_{18} 30 μm was used.

The mobile phase consisted of pH 6 phosphate buffer ($8\cdot10^{-3}$ mol/l Na_2HPO_4–$5.9\cdot10^{-2}$ mol/l KH_2PO_4). It was filtered and degassed before use at a flow-rate of 1 ml/min. The mobile phase and the column were at room temperature.

Solutions

Solutions of internal standard (116.2 μmol/l, corresponding to 30 μg/ml for plasma; 387 μmol/l, corresponding to 100 μg/ml for urine) were prepared in pH 6 phosphate buffer.

Stock solutions of I (2.05 mmol/l, corresponding to 500 μg/ml for plasma; 410

μmol/l, corresponding to 100 μg/ml for urine) were prepared in pH 6 phosphate buffer. The reference solutions for calibration were prepared by dilution in the same buffer.

Calibration samples

Plasma calibration samples were prepared by adding 50 μl of reference solutions of I to 250 μl of plasma. The amounts added corresponded to concentrations ranging from 1.64 μmol/l (400 ng/ml) to 410 μmol/l (100 μg/ml).

Urine calibration samples were prepared by adding 100 μl of reference solutions of I to 1 ml of 25-fold diluted urine. The amounts added corresponded to concentrations ranging from 41 μmol/l (10 μg/ml) to 1.025 mmol/l (250 μg/ml).

Sample preparation

Plasma. A 50-μl volume of the internal standard solution, 50 μl of pH 6 phosphate buffer and 250 μl of a saturated solution of ammonium sulphate (53 g of ammonium sulphate in 72 ml of water) were added to 250 μl of plasma in a 10-ml conical glass tube. The mixture was shaken for 30 s on a vortex mixer and centrifuged at 1400 *g* for 10 min. The supernatant was transferred into a conical plastic vial and 100 μl were injected.

Urine. A 1-ml volume of urine was first diluted to 25 ml with pH 6 phosphate buffer, then 1 ml of the diluted urine, 100 μl of the internal standard solution and 100 μl of pH 6 phosphate buffer were introduced into a 10-ml glass tube. After shaking for 15 s on a vortex mixer, 30 μl of the mixture were injected using conical plastic vials.

RESULTS AND DISCUSSION

Plasma and urine interferences

Plasma from six volunteers and urine from nine volunteers were tested. I and the internal standard were well separated from plasma and urine components (Figs. 2 and 3).

For the assay in plasma, the analytical column and the pre-column must be replaced after 120–150 injections in order to prevent a decrease in separation efficiency.

Linearity, accuracy and precision

Calibration graphs for plasma were obtained by plotting the peak-area ratio (I/II) against the concentration of I in the range 1.6–410 μmol/l. Their equation was calculated using weighted linear regression with a weighting factor of $1/(\text{concentration})^2$. As shown in Table I, a calibration graph remained valid for one week. For urine, the peak-height ratios (I/II) were plotted against the concentration of I in the range 41–1025 μmol/l. Weighted linear regression was used. A calibration graph remained valid for one week.

The within-day precision for the plasma and urine assays was assessed by using five or six samples spiked with I at four different concentrations. These samples were prepared and analysed on the same day. The between day precision was

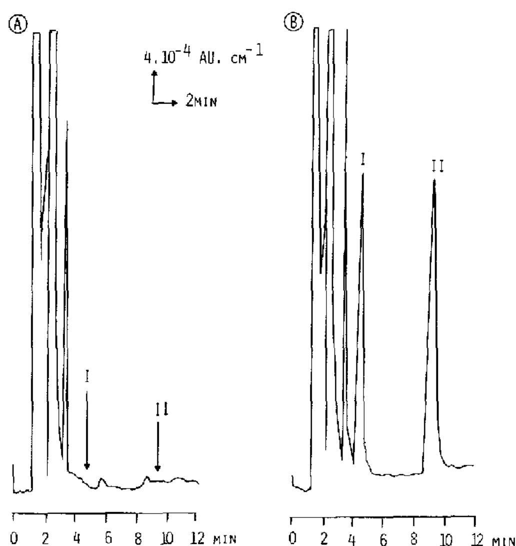

Fig. 2. Chromatograms of the supernatant of (A) a 250-μl sample of blank human plasma and (B) a 250-μl sample of human plasma spiked with 2.05 nmol (500 ng) of I and 5.81 nmol (1500 ng) of II (internal standard).

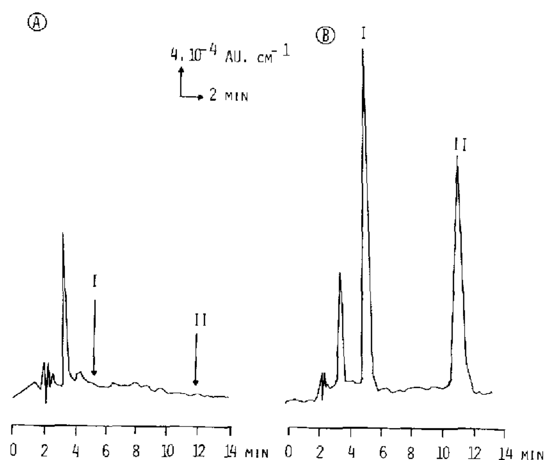

Fig. 3. Chromatograms corresponding to (A) a 1-ml sample of blank human urine (diluted 25-fold) and (B) a 1-ml sample (diluted 25-fold) of urine spiked with 16.4 nmol (4 μg) of I and 38.7 nmol (10 μg) of II (internal standard).

assessed by using six samples spiked with I at the same concentrations as mentioned above. Drug-free plasma and urine from several volunteers were used. These samples were analysed on six different days. The precision and accuracy were characterized by coefficients of variation (C.V.) lower than 10% and recoveries [(amount found $\times$ 100)/(amount introduced)] close to 100% (Tables II and III).

Concentrations down to 1.64 μmol/l (400 ng/ml) in plasma and 41 μmol/l (10 μg/ml) in urine can be accurately and precisely determined (Tables II and III).

TABLE I

ASSAY IN PLASMA: VALIDITY OF THE CALIBRATION GRAPH DURING ONE WEEK

Concentration values were calculated from the peak-area ratios on day 6 and the calibration graph was established with peak-area ratios on day 1.

Amount introduced (μmol/l)	Day 1	Day 6	
	Peak-area ratio	Peak-area ratio	Recovery* (%)
1.64	0.129	0.122	91
4.10	0.303	0.295	105
12.30	0.748	0.765	97
20.50	1.251	1.271	99
41.00	2.545	2.451	96
205.00	12.782	11.781	93
410.00	25.293	25.300	100

*Recovery = (amount found × 100) / (amount introduced).

TABLE II

WITHIN-DAY AND BETWEEN-DAY PRECISION AND ACCURACY OF THE ASSAY OF I IN PLASMA

Nominal concentration (μmol/l)*	Within-day ($n=6$)			Between-day ($n=6$)**		
	Mean concentration found (μmol/l)	C.V. (%)	Recovery*** (%)	Mean concentration found (μmol/l)	C.V. (%)	Recovery*** (%)
1.64	1.61 ($n=5$)	8.6	98	1.64	6.3	100
32.8	32.4	2.6	99	32.0	3.6	97
205.0	203.0	3.9	99	199.0	4.6	97
328.0	328.0	3.0	100	329.0	2.6	100

*To convert into μg/ml, multiply the data by 0.2443.
**Drug-free plasma from four volunteers was used.
***Recovery = (amount found × 100) / (amount introduced).

TABLE III

WITHIN-DAY AND BETWEEN-DAY PRECISION AND ACCURACY OF THE ASSAY OF I IN URINE

Nominal concentration (μmol/l)*	Within-day ($n=6$)			Between-day ($n=6$)**		
	Mean concentration found (μmol/l)	C.V. (%)	Recovery*** (%)	Mean concentration found (μmol/l)	C.V. (%)	Recovery*** (%)
41.0	42.8	7.9	104	41.7	2.3	102
82.0	84.9	5.0	103	81.4	3.5	99
410.0	397.0	4.0	96	412.0	3.0	100
1025.0	1014.0	5.5	99	1044.0	3.3	102

*To convert into μg/ml, multiply the data by 0.2443.
**Drug-free urine from six volunteers was used.
***Recovery = (amount found × 100) / (amount introduced).

The routine use of the method with plasma showed that concentrations down to 0.8 μmol/l (200 ng/ml) can be precisely and accurately determined (mean recovery ± standard deviation for six replicates = 107 ± 6%).

Selectivity

As the metabolism of I in man has not yet been elucidated, the selectivity remains to be checked.

Stability

The reference solutions of I in pH 6 phosphate buffer remained stable for only one week at 5°C. The stability was evaluated as follows: on day 1, a solution of I was prepared and an aliquot was injected; the corresponding peak height was taken as the reference. This solution was kept at 5°C for thirty days and the same aliquot as on day 1 was injected on several occasions during the storage period. The decrease from the initial concentration was 10% after eight days of storage and 40% after thirty days. For routine analysis, new solutions of I and internal standard were prepared every week.

The extracts in ammonium sulphate-saturated solution remained stable for only 2 h at room temperature, so it was not possible to prepare samples in advance and to inject them during the night. They must be injected as soon as possible after preparation.

Diluted urine samples in pH 6 phosphate buffer can be kept for one night at 5°C without any degradation of the compounds.

A decrease (about 15%) in the concentration of I was observed after storage of spiked plasma samples for two months at −20°C. With spiked urine samples, there was no decomposition after 49 days at −20°C. No decrease in concentration was observed in actual plasma and urine samples from animals submitted to two freezing–thawing cycles. Re-analysis of actual plasma samples after storage for 39 days at −20°C gave concentrations accounting for 94 ± 8% ($n = 5$) of the initial values. With urine, the second analysis (after 36 or 50 days at −20°C) accounted for 90 ± 6% ($n = 4$) of the initial values.

Pharmacokinetic applications

Serial plasma and urine samples were collected from healthy volunteers who had received a single dose of I (250, 500, 1000 or 2000 mg) as a 5-min intravenous (i.v.) injection. Mean plasma concentration–time curves and cumulative urinary excretion profiles are shown in Figs. 4 and 5.

I was rapidly cleared from plasma after i.v. administration. In most instances, up to 2 h after administration, two elimination phases with short half-lives (0.04–0.26 h for the α-phase, 0.12–1.0 h for the β-phase) were observed. Later, a longer half-life (0.7–4.0 h) was observed for a few subjects. The areas under the plasma concentration–time curve and the maximum concentrations at the end of the i.v. infusion were linearly correlated with the dose in the range 250–2000 mg. I appeared rapidly in urine after i.v. administration. The excretion was almost completed by 4 h after dosing; 71–84% of the dose was recovered in urine as the

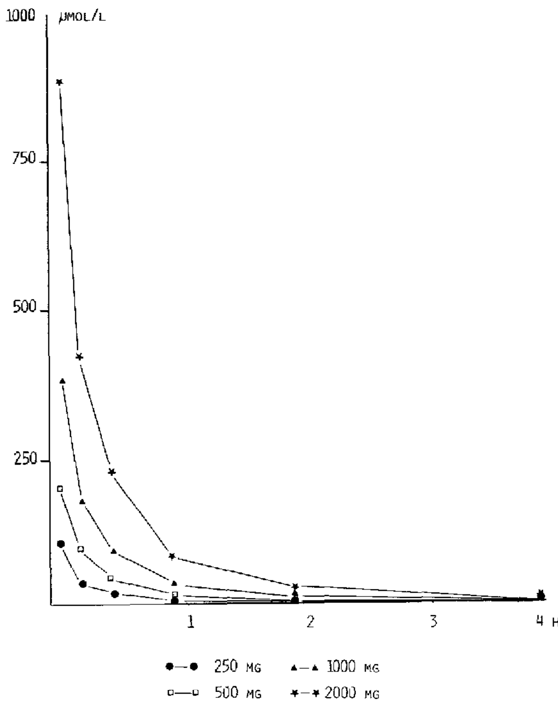

Fig. 4. Plasma concentrations of I following a 5-min i.v. injection of a single dose of 250, 500, 1000 or 2000 mg to healthy volunteers. Mean values of six subjects.

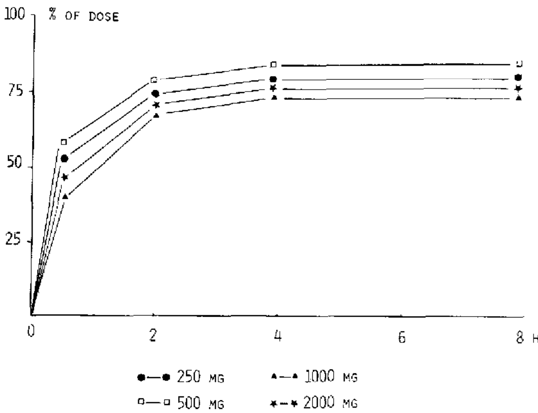

Fig. 5. Cumulative urinary excretion (% of dose) of I following a 5-min i.v. injection of a single dose of 250, 500, 1000 or 2000 mg to healthy volunteers. Mean values of six subjects.

unchanged drug. The urinary excretion appeared to be independent of the i.v. dose.

CONCLUSION

The methods described here appear to be suitable for determining I in plasma and urine samples from pharmacokinetic studies in man.

REFERENCES

1 R. Wise, J.M. Andrews and L.J.V. Piddock, Antimicrob. Agents Chemother., 31 (1987) 267.
2 H.C. Neu, N.-X. Chin and N.M. Neu, Antimicrob. Agents Chemother., 31 (1987) 558.