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High-performance liquid chromatographic methods for the determination of the penems SCH 29482 and FCE 22101 in human serum and urine

R. Méndez, A. Negro and J. Martín-Villacorta

Departamento de Bioquímica y Biología Molecular, Facultades de Biología y Veterinaria, Universidad de León, 24071 León (Spain)

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ABSTRACT

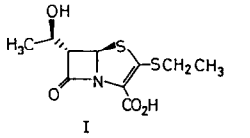
High-performance liquid chromatographic methods have been developed for the determination of two 6-(1-hydroxyethyl)penems, SCH 29482 (I) and FCE 22101 (II), in serum and urine. Serum samples were combined with an equal volume of methanol to remove proteins and, after centrifugation, an aliquot of the supernatant was analysed by ion-pair chromatography on a reversed-phase C_{18} column with hexadecyltrimethylammonium bromide as the ion-pairing agent. The compounds were detected by their ultraviolet absorbance at 305 nm for II and 322 nm for I. Urine samples were diluted, filtered and analysed by the same chromatographic procedure. At concentrations of 1–500 μg/ml of each compound, the within- and between-day precisions were 1.8–3.6 and 2.6–5.1%, respectively. The detection limit was 0.2 μg/ml for I and 0.3 μg/ml for II.

INTRODUCTION

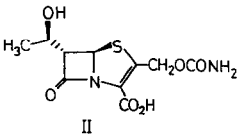
Penems are synthetic β-lactams [1,2] characterized by their broad antibacterial spectrum and low susceptibility to hydrolysis by β-lactamases. Several agents of this class have undergone preclinical investigation. These include SCH 29482 (I), SCH 34343, FCE 22101 (II) and FCE 22891 [3–7].

The determination of β-lactam antibiotics in biological fluids is often performed by microbiological assays [8,9]. Recently, high-performance liquid chromatography (HPLC) has come into widespread use for the analytical determination of β-lactam antibiotics in biological fluids [10,11]. The speed, sensitivity and specificity of these techniques justify their use for the quantification of antimicrobial agents.

This paper presents an HPLC assay for the determination of 5*R*,6*S*,8*R*-6-(1-hydroxyethyl)-2-ethylthiopenem-3-carboxylic acid (I) and 5*R*,6*S*,8*R*-6-(1-hydroxyethyl)-2-carbamoyloxymethylpenem-3-carboxylic acid (II, Fig. 1) in human serum and urine. It involves ion-pair reversed-



I



II

Fig. 1. Structures of penems: SCH 29482 (I) and FCE 22101 (II).

Correspondence to: Dr. J. Martín-Villacorta, Departamento de Bioquímica y Biología Molecular, Facultades de Biología y Veterinaria, Universidad de León, 24071 León, Spain.

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phase chromatography with UV detection at 322 and 305 nm for I and II, respectively. The sample preparation procedure is simple and rapid, requiring only precipitation of protein with methanol.

EXPERIMENTAL

Reagents and materials

Compounds I and II were provided by Schering (Bloomfield, NJ, USA) and by Farmitalia Carlo Erba (Milan, Italy), respectively. Chloramphenicol was obtained from Fluka (Buchs, Switzerland). Methanol and 2-propanol were purchased from Carlo Erba. Potassium phosphate was obtained from Merck (Darmstadt, Germany), and hexadecyltrimethylammonium bromide was from Sigma (St. Louis, MO, USA). All water used in this study was purified with a Milli-Q water purification system (18 MΩ resistance) (Millipore, Bedford, MA, USA).

Ultrafiltration tubes (Ultrafree C3LGC, low binding cellulose, molecular mass cut-off 10 000) were from Nihon Millipore (Yonezawa, Japan).

Instruments

The HPLC system (Spectra-Physics SP 8800) was equipped with a variable-wavelength UV–visible detector (SP 84500) and a computing integrator (SP 4290). Samples were loaded onto the column via a Rheodyne 7125 loop injector (volume 50 μl). The column (15 cm × 3.9 mm I.D.) was of stainless steel, prepacked with 4-μm Nova-Pak C_{18} packing (Waters Chromatography Division, Millipore, Milford, MA, USA) and protected with a pre-column (30 mm × 4 mm I.D.) fitted with New Guard Cartridge RP-8 (Brownlee Labs., Santa Clara, CA, USA).

Penem standard solutions

Stock solutions of penems are prepared by dissolving 20 mg of the compounds in 10 ml of 50 m*M* phosphate buffer (pH 6.0). These stock solutions were stored at 5°C for up to two days. The 2.0 mg/ml stock solutions were further diluted 1:20 with phosphate buffer to prepare additional standards at a concentration of 100 μg/ml.

Serum and urine calibration standards

Serum and urine calibration standards were prepared by adding microlitre amounts of the 100 μg/ml or 2.0 mg/ml stock solutions to the appropriate volume of drug-free biological fluids, to produce concentrations in the range 0.5–100 μg/ml for serum and 5–500 μg/ml for urine.

Internal standard solution

Working standard solutions for the serum assay were prepared fresh on each day of analysis by dissolving amounts of chloramphenicol (internal standard) in methanol to produce concentrations of 15 and 30 μg/ml for II and I, respectively. For the urine assay the working standard solutions were prepared by dissolving the chloramphenicol in appropriate volumes of the mobile phases to produce concentrations of 10 and 15 μg/ml for II and I, respectively.

Chromatographic procedure

The chromatographic conditions are summarized in Table I. The mobile phases were prepared fresh on the day of analysis and were filtered and degassed by vacuum. All chromatographic operations were carried out at 20°C. The column was conditioned by passing the mobile phase through it for 2 h at a flow-rate of 0.5 ml/min. The detection wavelength was set at 305 nm for II and 322 nm for I.

The apparent pH values of the mobile phases used were within the pH interval where these antibiotics are most stable [12].

Sample preparation and analysis

For both antibiotics the conditions for preparation and analysis of samples were established as follows.

For serum samples, 0.5 ml of the serum calibration standard or unknown sample was pipetted into a glass tube, and 0.5 ml of working standard solution containing the internal standard was added. The tube was then vortex-mixed for 15 s and centrifuged for 15 min at 10 000 *g*. A 50-μl aliquot of the clear supernatant was loaded into the HPLC column.

For urine samples, a 0.5-ml aliquot of the urine

TABLE I

HPLC CONDITIONS FOR THE ANALYSIS OF I AND II

Parameter	Compound I	Compound II
Column type	Nova-Pak C_{18} (4 μm) (15 cm × 3.9 mm I.D.) for both antibiotics	
Flow-rate (ml/min)	0.5	0.5
Temperature (°C)	20	20
Mobile phase		
0.1 *M* Potassium phosphate (%)	75	60
Methanol (%)	–	40
2-Propanol (%)	25	–
Hexadecyltrimethylammonium bromide (ion pair) concentration (m*M*)	0.1	1.5
Apparent pH	6.20	5.75
Detection wavelength (nm)	322	305
Injection volume (μl)	50	50
Internal standard	Chloramphenicol	Chloramphenicol
Calculation	Peak area	Peak area

calibration standard or unknown sample was transferred to a 10-ml glass tube, to which 4.5 ml of working standard solution for the urine assay were added. The tubes were mixed vigorously for 15 s; 1 ml of this solution was filtered through an assembly consisting of a 0.45-μm filter (Millex-HA, Millipore) attached to a 5-ml syringe. A 50-μl aliquot of the filtrate was injected into the column.

Quantification

The serum and urine calibration standard solutions of the antibiotics were prepared at seven different concentrations between 0.5 and 100 μg/ml for serum and between 5 and 500 μg/ml for urine, and treated in the manner described above. Calibration graphs of the chromatographic peak-area ratios (antibiotic/internal standard) *versus* antibiotic concentration were constructed. Antibiotic concentrations in the unknown serum and urine samples were calculated by interpolation from the calibration graphs by a least-squares regression line treatment.

RESULTS AND DISCUSSION

Selectivity

Chromatograms from the isocratic ion-pair HPLC analysis of I and II in human serum and urine samples are shown in Figs. 2–5. Under the conditions used, I and II were well resolved from endogenous serum or urine compounds. Minor changes to the organic modifier content and/or the ion pair of the mobile phase were occasionally required to accommodate column efficiency loss, or interference from atypical serum and urine samples.

Recovery

The total recoveries of the penems were measured on blank human serum and urine spiked

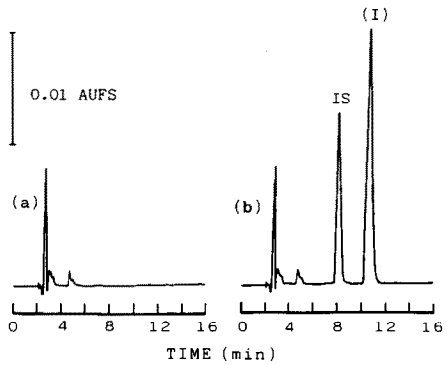

Fig. 2. Chromatograms of (a) drug-free human serum and (b) human serum spiked with 15 μg/ml SCH 29482 (I). IS = internal standard (30 μg/ml).

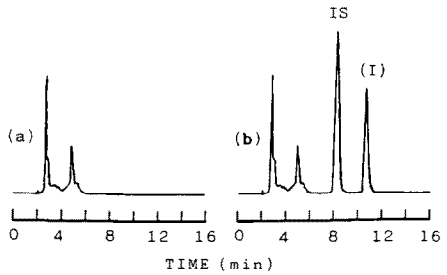

Fig. 3. Chromatograms of (a) drug-free human urine and (b) human urine spiked with 30 μg/ml SCH 29482 (I) IS = internal standard (15 μg/ml).

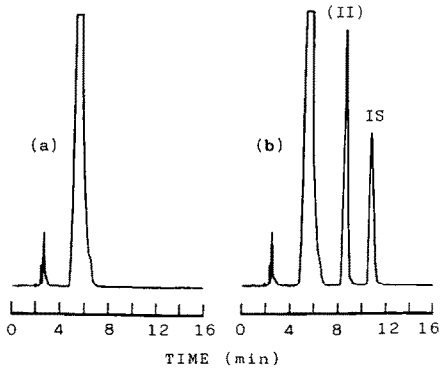

Fig. 4. Chromatograms of (a) drug-free human serum and (b) human serum spiked with 20 μg/ml FCE 22101 (II). IS = internal standard (15 μg/ml).

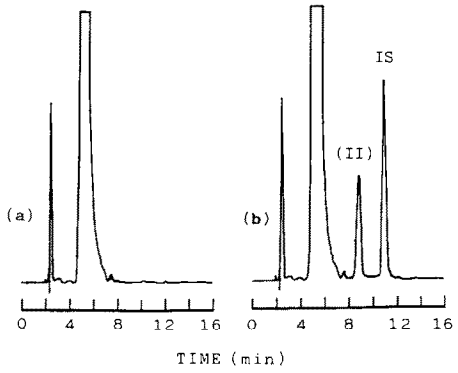

Fig. 5. Chromatograms of (a) drug-free human urine and (b) human urine spiked with 40 μg/ml FCE 22101 (II). IS = internal standard (10 μg/ml).

with these antibiotics at different concentrations. The detector responses to spiked samples were compared with those to 50 m*M* phosphate buffer solutions (pH 6.0) with identical concentrations of the compounds under study. The results (Table II) show that the recoveries were *ca.* 100%, and therefore protein binding of penems has no effect on the recoveries in serum when methanol is used to precipitate the serum proteins.

We also studied the efficacy of the ultrafiltration method for removing proteins from serum. The results showed that the main drawbacks are that *ca.* 10% of the antibiotics are absorbed in the ultrafiltration membrane, and that the method is slower and more expensive than methanol precipitation. Furthermore, the chromatograms obtained by the two methods are practically identical. When the ultrafiltration method is used, however, it is not necessary to change the precolumn as often as with methanol precipitation.

Linearity and sensitivity

For both penems we found a good linear relationship between the peak-area ratios and the penem concentrations in serum and urine samples in the range studied, with regression analysis of the data revealing a correlation coefficient of $\leqslant 0.997$ for both penems. We estimated that the limits of determination were 0.2 μg/ml for I and 0.3 μg/ml for II, with a signal-to-noise ratio of approximately 3.

TABLE II

EXTRACTION RECOVERIES OF I AND II FROM SERUM AND URINE

Sample	Concentration added (μg/ml)	Recovery (mean ± S.D.)(%)	
		Compound I	Compound II
Serum	5	99.4 ± 1.9	96.8 ± 3.3
	20	98.6 ± 3.2	99.3 ± 1.5
	50	103 ± 2.8	102 ± 3.7
Urine	5	102 ± 4.2	99.0 ± 3.1
	100	97.8 ± 2.3	97.3 ± 4.1
	500	99.3 ± 1.2	98.2 ± 2.8

Accuracy and precision

The accuracy and precision of the assays developed for I and II in serum and urine were determined by adding known amounts of these penems to blank serum and urine. The within-day reproducibility was studied at three concentrations of I and II. To evaluate the between-day reproducibility, two concentrations of I and II were used. For both cases six serum and urine samples at each concentration were analysed by the HPLC procedure described. The results are shown in Tables III and IV, from which one can see that the coefficients of variation (C.V.) ranged from 1.8 to 5.1%, and the accuracy, defined as (amount found/amount added) × 100 (%), was *ca.* 100% for all samples assayed.

Storage stability at different temperatures

The *in vitro* storage stability in serum and urine of I and II was evaluated at −70°C, −30°C and 5°C. Freshly collected normal human serum and urine samples were supplemented with the antibiotics at concentrations of 10 and 100 μg/ml, respectively. These were assayed initially, and then stored in the dark at the stated temperatures. When aliquots of these standards were periodically analysed, I and II in serum and urine samples were stable at −70°C over the 60-day study period; however, at 5°C and −35°C, loss of both penems from serum samples was evident. The results of the analysis of these samples, given in Table V, indicate that the penems studied are especially degraded in serum samples stored at 5°C.

These results indicate that serum samples may be safely stored for up to 60 days at −70°C, and urine samples may be stored for up to 30 days at −30°C.

TABLE III

ACCURACY AND PRECISION RESULTS FOR SERUM AND URINE SAMPLES SPIKED WITH I

Concentration (mean ± S.D.) (μg/ml)		Accuracy[a] (%)	Precision[b] (%)
Added	Found		
Serum			
Within-day (n = 6)			
1	0.97 ± 0.03	97	3.3
25	24.2 ± 0.47	96.8	2.0
50	50.9 ± 1.43	102	2.8
Between-day (n = 6)			
5	4.97 ± 0.17	99.4	3.5
100	101 ± 2.63	101	2.6
Urine			
Within-day (n = 6)			
5	5.3 ± 0.17	106	3.3
250	246 ± 6.80	98.4	2.8
500	508 ± 14.7	101.6	2.9
Between-day (n = 6)			
5	5.1 ± 0.21	102	4.2
300	294 ± 11.2	98	3.8

[a] (Found/added) × 100.
[b] Coefficient of variation.

TABLE IV

ACCURACY AND PRECISION RESULTS FOR SERUM AND URINE SAMPLES SPIKED WITH II

Concentration (mean ± S.D.) (μg/ml)		Accuracy[a] (%)	Precision[b] (%)
Added	Found		
Serum			
Within-day (n = 6)			
1	1.03 ± 0.03	103	2.8
25	25.4 ± 0.59	101.6	2.3
50	50.1 ± 0.90	100.2	1.8
Between-day (n = 6)			
5	5.2 ± 0.16	104	3.0
100	100 ± 4.20	100	4.2
Urine			
Within-day (n = 6)			
5	4.8 ± 0.17	96	3.6
250	255 ± 6.88	102	2.7
500	491 ± 15.2	98.2	3.1
Between-day (n = 6)			
5	5.03 ± 0.21	100.6	4.3
300	294 ± 15.0	98	5.1

[a] (Found/added) × 100.
[b] Coefficient of variation.

TABLE V

STORAGE STABILITY DATA FOR I AND II IN HUMAN SERUM AND URINE AT 5°C AND −30°C

Storage time (days)	Found concentration (μg/ml)		C.V. (n = 3) (%)		Percentage of day zero concentration	
	5°C	−30°C	5°C	−30°C	5°C	−30°C
Compound I						
Serum						
Day zero	10.2	10.2	1.2	2.8	100	100
5	7.0	10.1	4.1	3.1	68.6	99
10	5.5	9.5	2.3	1.6	53.9	93.1
15	3.6	9.4	3.5	2.4	35.3	92.1
20	2.9	9.1	2.8	3.7	28.4	89.2
Urine						
Day zero	98	98	5.4	4.2	100	100
10	88	96	4.2	2.4	89.8	97.9
20	82	101	2.6	4.0	83.7	103
30	79	98	3.9	5.1	80.6	100
Compound II						
Serum						
Day zero	10.3	10.3	2.9	3.0	100	100
5	5.4	9.8	1.3	0.7	52.4	95.1
10	3.1	8.3	2.6	2.1	30	80.6
15	2.1	8.4	4.3	3.2	20.4	81.5
20	1.2	7.4	2.7	4.1	11.6	71.8
Urine						
Day zero	101	101	1.6	4.7	100	100
10	91	93	5.3	3.8	90	92
20	83	102	2.9	5.8	82.2	101
30	80	96	4.3	1.7	79.2	95

CONCLUSION

It is interesting to observe that in the penem system, in contrast with penicillins and cephalosporins, a sulphur atom is conjugated directly through a carbon–carbon double bond to the carboxylic acid and, thus, the UV spectra of penems display a long-wavelength maximum at *ca.* 310 nm. This suggests that the methods developed may be applicable to other 6-(1-hydroxyethyl)penems, with slight modifications in the mobile phase and detection at *ca.* 310 nm.

The HPLC methods developed for the quantification of I and II in human serum and urine are quick, sensitive, accurate and precise, and may be easily applied to pharmacokinetic studies of the penems in humans.

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