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Quantitative determination of semisynthetic cephamycins in human serum and urine by ion-exchange, reversed-phase and ion-pair chromatography

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Abstract

High-performance liquid chromatographic methods have been developed for the determination of semisynthetic cephamycins: cefoxitin, cefmetazole and cefminox in human serum and urine samples. Serum samples spiked with each cephamycin were combined with an equal volume of methanol to remove proteins and, after centrifugation, an aliquot of the supernatant was analysed by ion-exchange, reversed-phase and ion-pair chromatography with hexadecyltrimethylammonium bromide as the ion-pairing agent. Urine samples were diluted, filtered and analysed by same chromatographic procedure. The cephamycins were detected by their ultraviolet absorbance (265–272 nm). It was possible to determine concentrations of cephamycins to 0.2 μg/ml in serum and 2 μg/ml in urine samples with a good level of reproducibility and accuracy.

Keywords: Cephamycins; Lactams; Antibiotics

1. Introduction

Cefoxitin, cefmetazole and cefminox (Fig. 1) are members of the cephamycins family. The cephamycins studied are β-lactam antibiotics with a molecular structure similar to that of cephalosporin, but including a methoxy group at position C-7α [1]. These compounds are much less sensitive to β-lactamase attack than cephalosporins suggesting an important role for the methoxy group in the mechanism of resistance against β-lactamase hydrolysis [2].

Several papers on high-performance liquid chromatography (HPLC) of cefoxitin [3–12] and cefmetazole [11,13,14] describes the determination of the these antibiotics in biological samples.

This paper describes and compares a set of HPLC methods using different techniques (ion-exchange, reversed-phase and ion-pair chromatography), for the determination of cephamycins studied in human serum and urine samples. The sample preparation procedure is simple and rapid, requiring only precipitation of proteins with methanol. These methods may be of use in the study of the pharmacokinetics of formulations of cephamycins.

2. Experimental

2.1. Reagents and materials

Cefoxitin and cefmetazole were obtained from Merck Sharp and Dohme Research Labs. (Rahway, NJ, USA) and cefminox was from Meiji Seika Kaisha Research Labs. (Japan). The structures of these antibiotics are shown in Fig. 1. Potassium

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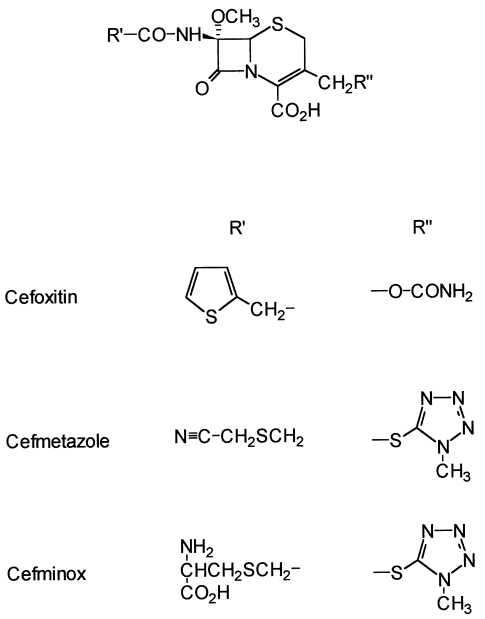

Fig. 1. Chemical structures of the investigated cephamycins.

phosphate (analytical-reagent grade), and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Hexadecyltrimethylammonium bromide (HTAB) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. 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, molecular mass cut-off 10 000) were purchased from Nihon Millipore (Yonezawa, Japan).

2.2. Instruments and chromatography

A HPLC System Gold liquid chromatograph from Beckman Instruments (Fullerton, CA, USA) equipped with a programmable solvent module 116 pump and a detector module 166 variable-wavelength UV–visible detector were used. For initial experiments the chromatograms of human serum and urine samples were recorded with a module 168 photodiode array detector system. Samples were loaded onto the column via a Rheodyne 7125 loop injector (volume 50 μl). A 150×4.6 mm I.D. Nucleosil-5 C_{18} column (5 μm particle size) (Macherey–Nagel, Düren, Germany) was used, protected with a pre-column (30×4.6 mm I.D.) packed with the same material. For ion-exchange chromatography a 250×4.6 mm I.D. Zorbax Sax column (5 μm particle size) was used.

2.3. Chromatographic conditions

Mobile-phase characteristics and other chromatographic parameters are given in Table 1. The mobile phases were prepared fresh on the day of analysis and were filtered through a Millipore filter (0.45-μm pore size) and degassed under vacuum before use. Chromatography was performed at 20°C. When ion-pair chromatography was used the column was conditioned by passing the mobile phase through it for at least 2 h at a flow-rate of 1 ml/min.

2.4. Cephamycins standard solutions

Stock solutions of cephamycins are prepared by dissolving appropriate amounts of cephamycins in 50 m*M* phosphate buffer (pH 6.00) to produce a concentration of 2 mg/ml. The 2 mg/ml stock solutions were further diluted 1:20 with phosphate buffer to prepare additional standards at a concentration of 0.1 mg/ml. These stock solutions were stored at 5°C for up to two days.

2.5. Serum and urine calibration standards

Serum and urine standards were prepared by adding μl amounts of the 0.1 mg/ml or 2 mg/ml stock solutions to the appropriate volume of drug-free biological fluids, to produce concentrations in the range of 0.5–100 μg/ml for serum and 5–500 μg/ml for urine.

2.6. Sample pretreatment

For all cephamycins studied the conditions for preparation 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 methanol was then added and the glass tube was shaken on a vortex mixer for about 30 s. After centrifugation at 10 000 *g* for 15 min, 50 μl of the clear supernatant was injected onto the chromatographic system.

Table 1
HPLC conditions for the analysis of studied cephamycins

HPLC method	Parameter	Antibiotic		
		Cefoxitin	Cefmetazole	Cefminox
	Column type	Nucleosil-5 C_{18} 5 μm (150×4.6 mm I.D)		
	Flow-rate	1 ml/min		
	Temperature	20°C		
	Mobile phase			
Reversed-phase	0.1 *M* potassium phosphate (pH 6.00) (%)	87	87	97
	Acetonitrile (%)	13	13	3
	λ (nm)	265	272	272
	Injection volume (μl)	50		
	Column type	Nucleosil-5 C_{18} 5 μm (150×4.6 mm I.D.)		
	Flow-rate	1 ml/min		
	Temperature	20°C		
	Mobile phase			
Ion-pair	0.1 *M* Potassium phosphate (pH 6.00) (%)	80	80	93
	Acetonitrile (%)	20	20	7
	Ion-pair	$1 \cdot 10^{-4}$ *M* Hexadecyltrimethylammonium		
	λ (nm)	265	272	272
	Injection volume (μl)	50		
	Column type	Zorbax Sax (250×4.6 mm I.D.)		
	Flow-rate	1 ml/min		
	Temperature	20°C		
Ion-exchange	Mobile phase			
	0.1 *M* Potassium phosphate (pH 6.00) (%)	100	100	100
	λ (nm)	265	272	272
	Injection volume (μl)	50		

For urine samples, a 0.5-ml aliquot of the urine calibration standard or unknown sample was transferred to a 10-ml glass tube, to which 4.5 ml of phosphate buffer (pH 6.00) 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) atached to a 5-ml syringe. A 50-μl aliquot of the filtrate was injected onto the chromatographic system.

3. Results and discussion

3.1. Selectivity

We studied the elution profiles of serum and urine before proceeding to optimize the mobile phase conditions for separating cephamycins from them. Under the chromatographic conditions of the methods used (Table 1), detection at 270 nm showed high peaks due to endogenous serum or urine compounds appearing at void volume and tailing up to 4 and 5–7 min, for serum and urine samples, respectively.

It is therefore necessary to get the cephamycins in question to elute at between 6 and 10 min by choosing the appropriate chromatographic conditions. The retention times for the cephamycins are relatively low and it is difficult to increase them by altering the mobile phase to obtain a good separation of these compounds in human serum and urine samples. However, reversed-phase and ion-pair chromatography do not have these limitations, and the results are more satisfactory.

For the ion-pair method, the ion-pair used was HTAB, the presence of which in the mobile phase noticeably increased the retention times of cefoxitin and cefmetazole while delaying the elution of some minor components of serum and urine that could interfere in the elution of cefminox. For this drug, therefore, the reversed-phase method is more suit-

able. As was to be expected with reversed-phase chromatography, the elution of the cephamycins studied depends on the amount of acetonitrile in the mobile phase.

Reversed-phase and ion-pair chromatography showed cephamycins well resolved from endogenous serum or urine compounds. Typical reversed-phase chromatograms are shown in Figs. 2–7. Under the chromatographic peaks, the cephamycins have baseline resolution and are also well resolved from other peaks in the chromatogram. Minor changes in the organic modifier content of the mobile phase were occasionally required to compensate loss of column efficiency or interference from atypical serum and urine samples.

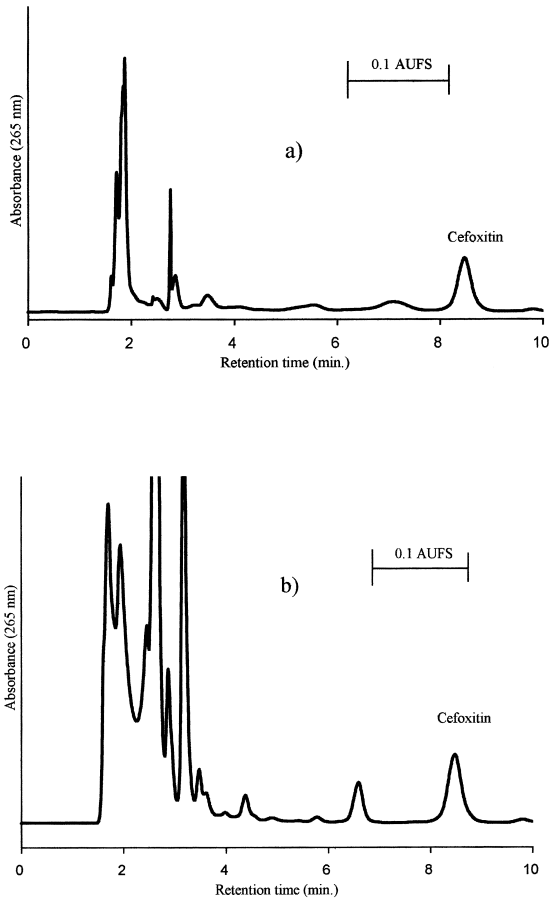

Fig. 2. Chromatograms of (a) human serum spiked with cefoxitin (20 μg/ml) and (b) human urine spiked with cefoxitin (20 μg/ml), chromatographed by RP-HPLC. See Section 2.3 for chromatographic conditions.

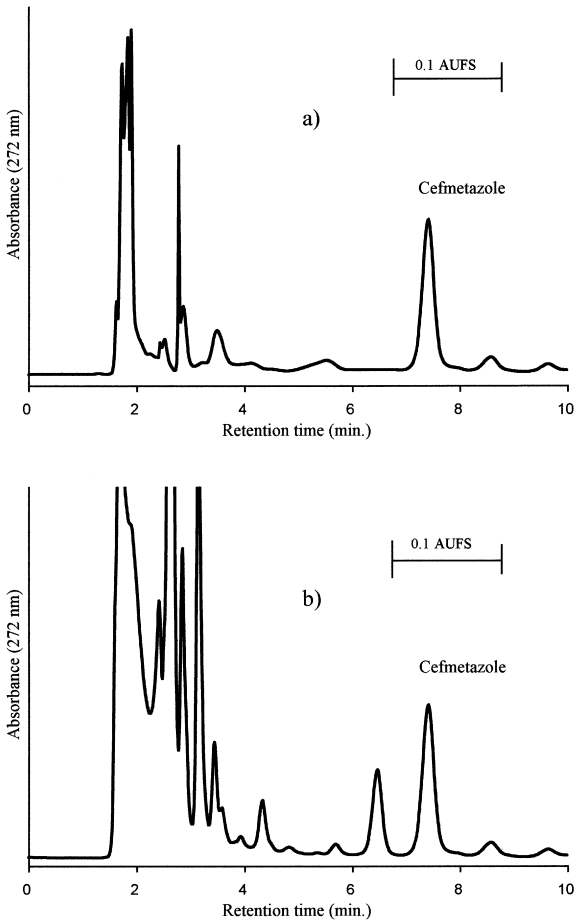

Fig. 3. Chromatograms of (a) human serum spiked with cefmetazole (20 μg/ml) and (b) human urine spiked with cefmetazole (20 μg/ml), chromatographed by RP-HPLC. See Section 2.3 for chromatographic conditions.

Generally speaking, reversed-phase and ion-pair chromatography may be considered very selective in the separation of the cephamycins studied from serum and urine samples.

3.2. Recovery

The total recoveries of the cephamycins were measured on blank human serum and urine spiked with these antibiotics at different concentrations. The detector responses to spiked samples were compared

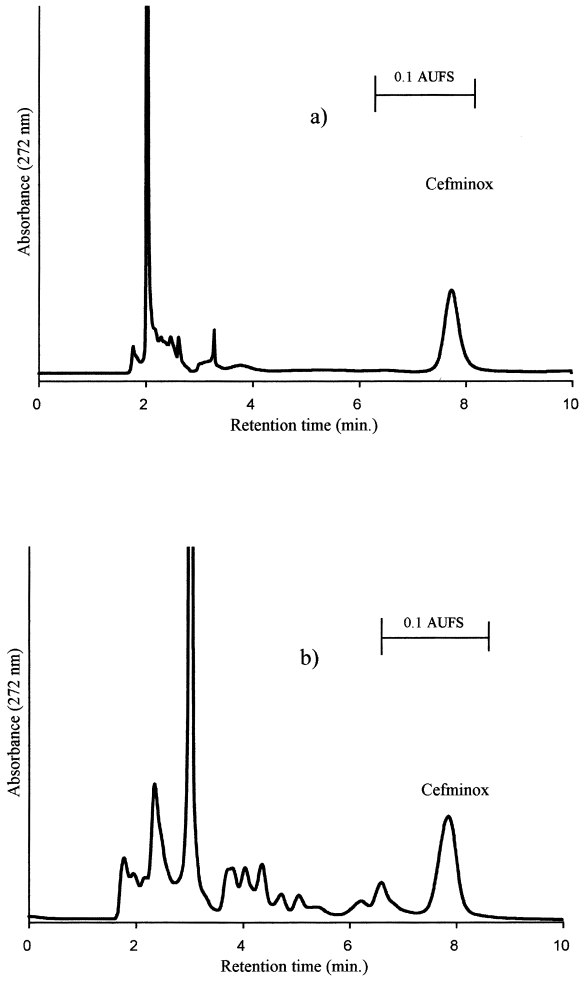

Fig. 4. Chromatograms of (a) human serum spiked with cefminox (40 μg/ml) and (b) human urine spiked with cefminox (40 μg/ml), chromatographed by RP-HPLC. See Section 2.3 for chromatographic conditions.

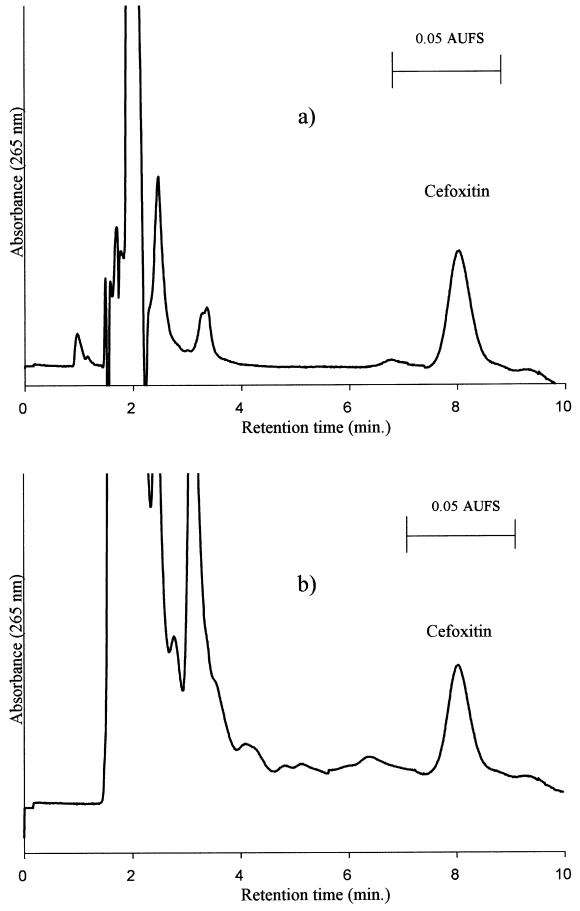

Fig. 5. Chromatograms of (a) human serum spiked with cefoxitin (20 μg/ml) and (b) human urine spiked with cefoxitin (20 μg/ml), chromatographed by ion-pair HPLC. See Section 2.3 for chromatographic conditions.

with those to 50 m*M* phosphate buffer solutions (pH 6.00) with identical concentrations of the cephamycins under study. Table 2 shows the recoveries of cephamycins from serum and urine when methanol is used to precipitate the serum proteins. In all cases the recoveries were greater than 95%.

We also studied the efficacy of the ultrafiltration method for removing proteins from serum. The results showed that ca. 5% 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 exchange the pre-column as often as with methanol precipitation.

3.3. Linearity and sensitivity

The serum and urine calibration standard solutions of the antibiotics were prepared at ten 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. Using the

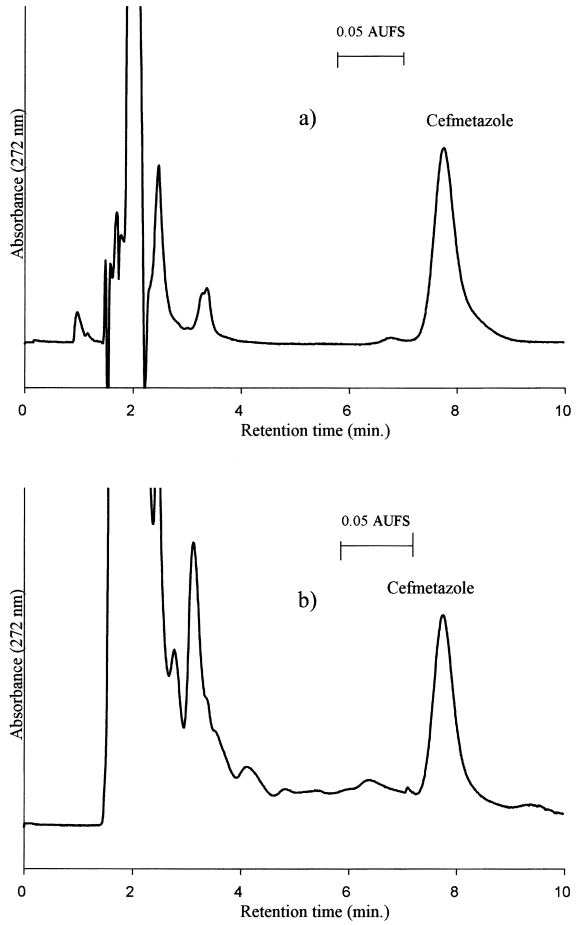

Fig. 6. Chromatograms of (a) human serum spiked with cefmetazole (20 μg/ml) and (b) human urine spiked with cefmetazole (20 μg/ml), chromatographed by ion-pair HPLC. See Section 2.3 for chromatographic conditions.

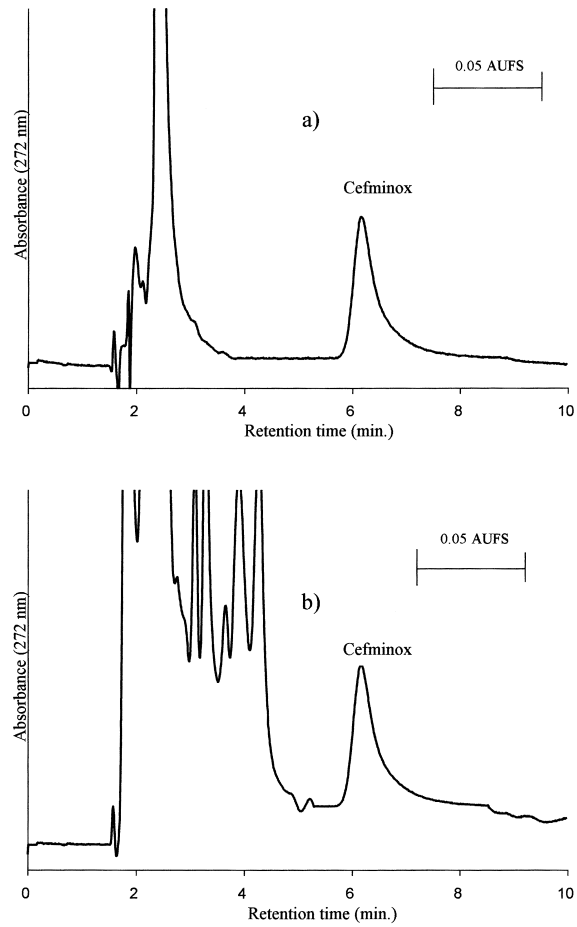

Fig. 7. Chromatograms of (a) human serum spiked with cefminox (40 μg/ml) and (b) human urine spiked with cefminox (40 μg/ml), chromatographed by ion-pair HPLC. See Section 2.3 for chromatographic conditions.

external standard method, a linear correlation was obtained between the amount of cephamycin chromatographed and the relative peak area in the range of 0.5–100 μg/ml for serum and 5–500 μg/ml for urine calibration standard solutions. Antibiotic concentrations in the unknown serum and urine samples were calculated by interpolation from the calibration graphs by a least-squares regression line treatment. The regression line obtained in most cases had a correlation coefficient higher than 0.998 ($n=8$). We estimated that the limits of determination were 0.2 μg/ml for all cephamycins studied, with a signal-to-noise ratio of approximately 3.

3.4. Accuracy and precision

The accuracy and precision of the assays developed for cephamycins in serum and urine were determined by adding known amounts of these cephamycins to blank serum and urine. The within-day and between-day reproducibilities were studied

Table 2
Recoveries of cefoxitin, cefmetazole and cefminox from serum and urine samples using reversed-phase chromatography

Sample	Concentration added (μg/ml)	Recovery (mean±S.D.) (%)		
		Cefoxitin	Cefmetazole	Cefminox
Serum (n=6)	5	102±2.7	101±1.9	103±3.4
	20	99.3±2.4	100±3.1	101±2.2
	50	103±1.7	96.6±1.6	97.1±1.9
Urine (n=6)	5	104±3.3	102±2.8	103±4.1
	100	98.1±2.6	96.9±1.6	97.8±2.8
	500	97.9±2.7	97.8±2.1	102±3.1

at three and two concentrations of the cephamycins respectively. For both cases six serum and urine samples at each concentration were analysed by the HPLC procedure described. The results obtained for cefoxitin and cefmetazole by RP-HPLC are shown in Tables 3 and 4, from which one can see that the relative standard deviations (R.S.D.s) ranged from 1.7 to 5.8%, and the accuracy, defined as (amount found/amount added)×100, was ca. 100% for all samples assayed. Similar values have been obtained for cefminox.

4. Conclusions

Of the three chromatographic methods developed for analysing cefoxitin, cefmetazol and cefminox (ion-exchange, reversed-phase and ion-pair), the

Table 3
Accuracy and precision results for serum and urine samples spiked with cefoxitin

Concentration (mean±S.D.) (μg/ml)		Accuracy[a] (%)	Precision[b] (%)
Added	Found		
Serum			
Within-day (n=6)			
1	1.04±0.06	104	5.8
25	24.1±0.62	96	2.6
50	50.3±0.85	101	1.7
Between-day (n=6)			
5	5.2±0.18	104	3.5
100	102±4.10	102	4.0
Urine			
Within-day (n=6)			
5	4.9±0.20	98	4.1
250	257±6.93	103	2.7
500	490±14.3	98	2.9
Between-day (n=6)			
5	5.13±0.23	103	4.5
300	298±11.3	99	3.8

[a] (Found/added)×100.
[b] R.S.D.

Table 4
Accuracy and precision results for serum and urine samples spiked with cefmetazole

Concentration (mean±S.D.) (μg/ml)		Accuracy[a] (%)	Precision[b] (%)
Added	Found		
Serum			
Within-day (n=6)			
1	1.05±0.05	105	4.8
25	24.4±0.48	98	2.0
50	51.3±1.33	103	2.6
Between-day (n=6)			
5	5.1±0.22	102	4.3
100	99.1±2.72	99	2.7
Urine			
Within-day (n=6)			
5	5.2±0.18	104	3.5
250	247±6.11	99	2.5
500	506±15.2	101	3.0
Between-day (n=6)			
5	4.9±0.26	98	5.3
300	290±11.3	97	3.9

[a] (Found/added)×100.
[b] R.S.D.

most selective are the last two, excepting the ion-pair method for cefminox, as some minor components of serum and urine samples may interfere in its elution. They are fast, sensitive and accurate, and are applicable to the determination of these cephamycins in clinical studies.

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