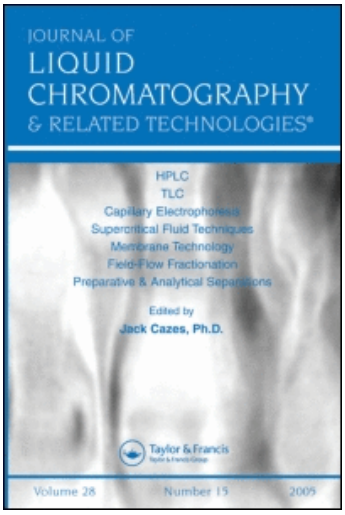

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HPLC ANALYSIS OF CEFMETAZOLE AND NOCARDICINS A AND E IN HUMAN SERUM AND URINE USING SOLID-PHASE EXTRACTION

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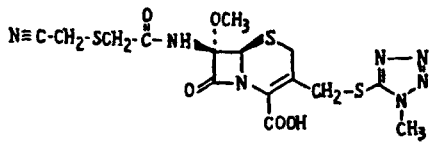
ABSTRACT

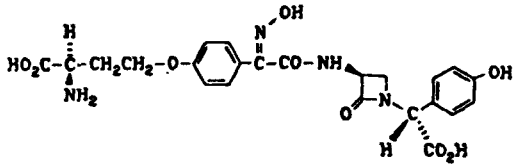
A method for extraction and quantification of cefmetazole and nocardicins A and E in serum and urine samples is described in this paper. Sample pretreatment is carried out using solid-phase extraction cartridges, resulting in very high extraction recoveries of these β-lactam antibiotics. The procedure, which prepares biological fluids for reversed-phase high-performance liquid chromatographic analysis is convenient, rapid and reproducible. An water-methanol-acetic acid mobile phase was used with benzotriazole as an internal standard. The detection limit was 0.2 μg/ml at 280 nm.

INTRODUCTION

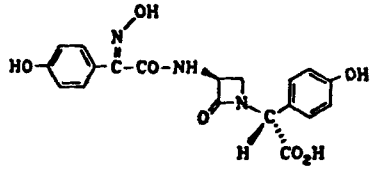
Cefmetazole is a new 7α-methoxycephalosporin that is highly resistant to hydrolysis by β-lactamases (1,2). Nocardicins A (3,4) and E (5) are novel monocyclic β-lactam antibiotics. Figure 1 shows the structures of these β-lactam antibiotics.



Cefmetazole



Nocardicin A



Nocardicin E

FIGURE 1. Structures of cefmetazole and nocardicins A and E

The determination of β-lactam antibiotics in biological fluids is often performed by microbiological assays (6-9). Recently, because of its rapidity, specificity and sensitivity, HPLC has proved to be a powerful analytical method for quantitating the β-lactam antibiotics in biological fluids and pharmaceutical preparations (10,11).

HPLC assays for the determination of β-lactam antibiotics in biological fluids using a rapid bonded-phase extraction technique have been reported (12,13). This report describes a simple, sesitive,rapid and specific HPLC technique for the determination of cefmetazole and nocardicins A and E in small volume samples of serum and urine, using

benzotriazole as an internal standard. The procedure includes the use of Sep-Pak disposable C_{18} cartidges for the extraction of antibiotics.

The bonded-phase extraction method involves the activation of the bonded-phase extraction material, application of the serum and urine samples onto the bonded-phase resin, clean up and selective elution.

MATERIALS AND METHODS

Materials and Reagents

Standard laboratory antibiotics powders were used in these studies.

All the water used was purified by the Milli-Q-reagent-grade Water System (Millipore Bedford, MA, U.S.A.). Benzotriazole of reagent grade (internal standard) was purchased from Aldrich Chemical Co. (Milwankee, WI). Internal standard was dissolved in mobile phase. HPLC-grade water and methanol (Carlo Erba, Milán, Italy) and reagent-grade acetic acid and hydrochloric acid (Merck, Darmstadt, G.F.R.) were used in these studies.

The bonded-phase extraction cartridges containing C_{18} reversed-phase packing (Sep-Pak C_{18} cartridges, catalogue No. 51910) were obtained from Waters Assoc. (Milford, MA).

Instruments

The HPLC system consisted of a Constametric pump II G (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne Model 7210 loop injector (volume 50 μl) and a Model 441 UV detector manufactured by Waters.

Quantitation was based on integration of peak areas using a Varian computing integrator (Model 4290, Palo Alto, CA).

The chromatograph was equipped with a μBondapak C_{18} column (10 μm particle size; 30 cm x 3.9 mm I.D.).

Chromatographic procedure

The mobile phase consisted of a mixture of water-methanol-acetic acid (163.5:35:1.5) for cefmetazole and (153:45:2) for nocardicins A and E. A pre-column (3 cm x 4.6 mm I.D.) packed with the same packing

materials was used to guard the main column. The flow-rate was set at 1.5 ml/min and 50 l samples were injected. The detector was set at 280 nm. All chromatographic operations were carried out under ambient conditions.

Preparation of standards

The serum standards containing 2 - 100 µg/ml of antibiotic were prepared by adding a working solution to pooled, drug-free blood blank serum. Urine standards containing 5 - 200 µg/ml of antibiotic were prepared by adding working solutions to fresh urine.

A solution of benzotriazole (200 µg/ml) prepared in the mobile phase was used as an internal standard for measuring antibiotic concentration in serum and urine samples.

Extraction procedure and analysis

Extraction procedure : Transfer 1 ml of serum into a 5-ml glass test-tube. Add 100 µl of I.S. solution and 0.5 ml of 0.5 M hydrochloric acid and mix. The sample was then applied to a Sep-Pak cartridge that had been conditioned with 4 ml of methanol followed by 4 ml of distilled deionized water at a flow-rate of 1 - 2 ml/min using a water aspirator as a vacuum source connected to the cartridge rack. After aspiration of the sample, the cartridge was washed with 2 ml of 0.1 M hydrochloric acid and dried under vacuum for 1 min. The antibiotic was then eluted from the cartridge with 1 ml of methanol in opposite direction. Figure 2 is a schematic representation of extraction procedure.

A aliquot of the methanol eluent was injected into the HPLC system.

Quantitation : Calibration lines were calculated using the linear least-squares method for data acquired from HPLC analysis of the serum and urine standards. The X-coordinate values were represented by the ratio :peak area of antibiotic /peak area of benzotriazole. The Y coordinates were represented by the known concentrations.

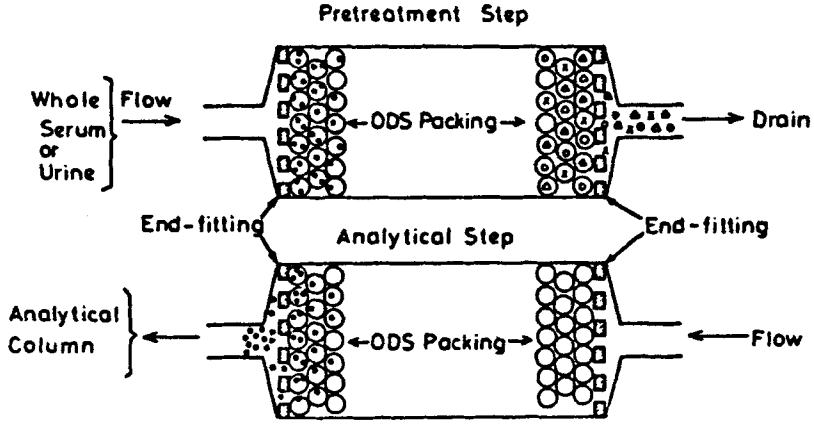


FIGURE 2. Schematic representation of the pretreatment step for extraction of antibiotics and benzotriazole (I.S.) of serum and urine samples.
● antibiotics and benzotriazole
△ ○ ▲ ✗ other serum and urine compounds

RESULTS

Extraction of antibiotic and benzotriazole

Extraction with C_{18} disposable Sep-Pak cartridges resulted in greater than 95 % recovery of antibiotic from serum and urine standards. Analysis of the eluent, after application of the sample to the C_{18} Sep-Pak cartridges, revealed no loss of whatsoever antibiotic from the cartridges; also, analysis of the acid wash eluent revealed loss of β-lactam antibiotics in

Chromatographic separation

Chromatograms of blank serum and urine containing benzotriazole as internal standard, and serum and urine samples spiked with β-lactam antibiotics are showed in figures 3 and 4 The substances are well separated without interference.

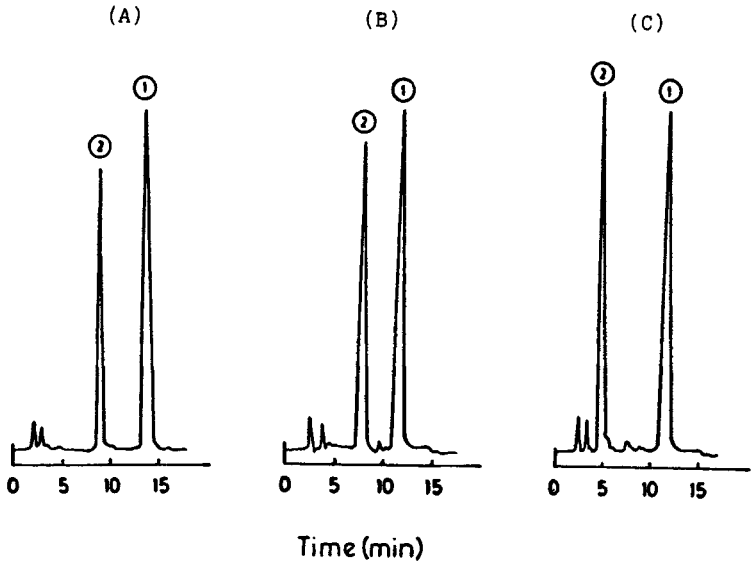


FIGURE 3. Typical chromatograms of blank serum containing benzotriazole (I.S.), peak 1: (A) spiked with cefmetazole, peak 2; (B) spiked with nocardicin A, peak 2; (C) spiked with nocardicin E, peak 2.

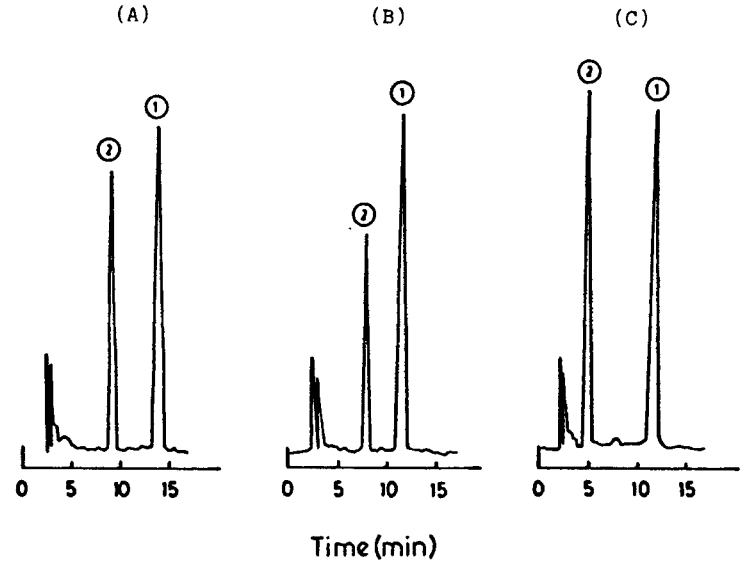


FIGURE 4. Typical chromatograms of blank urine containing benzotriazole (I.S.), peak 1: (A) spiked with cefmetazole, peak 2; (B) spiked with nocardicin A, peak 2; (C) spiked with nocardicin E, peak 2.

Recovery

Table 1 shows the total recoveries of the antibiotics and benzotriazole. Detector responses of serum and urine samples spiked with the antibiotic and benzotriazole were compared with detector responses of directly injected aqueous solutions that had identical concentrations of compounds investigated.

Linearity

Linearity was checked by measuring six different concentrations in the range of 2 - 100 μg/ml for serum assay and 5 - 200 μg/ml for urine assay. There was a good linear relationship between the ratio : peak area of antibiotic /peak area of I.S. and the concentration of antibiotic in serum and urine samples, with regression analysis of the data revealing a correlation coefficient of 0.99 (or more).

Sensitivity

We estimated that the limit of accurate determination was 0.2 μg/ml with a 50 μl injection. Levels below 0.2 μg/ml of antibiotic can be measured, if desired, by increasing the injection volume or decreasing the methanol volume in the extraction procedure.

Accuracy and precision

Accuracy and precision data were obtained by analyzing serum and urine samples containing different concentrations of antibiotic The results are listed in tables 2-4

TABLE 1

Extraction recoveries from serum and urine.

Compound	Concentration added (μg/ml)	Recovery (%)	
		Serum (n=5)	Urine (n=5)
Cefmetazole	20	98.4 ± 2.8	97.3 ± 4.1
Nocardicin A	10	98.7 ± 2.3	96.9 ± 3.7
Nocardicin E	10	97.2 ± 3.6	95.7 ± 2.9
Benzotriazole (I.S.)	20	96.6 ± 3.1	94.2 ± 3.7

TABLE 2

Accuracy and precision of the cefmetazole assay in serum using benzotriazole as I. S.

Concentration added μg/ml	n	Concentration found μg/ml	C.V. (%)
Within run			
1	5	0.97 ± 0.04	4.1
5	5	5.04 ± 0.13	2.6
10	5	9.91 ± 0.18	1.8
20	5	19.79 ± 0.41	2.1
Within day			
5	3	4.91 ± 0.18	3.7
10	3	9.84 ± 0.39	4.0

TABLE 3

Accuracy and precision of the cefmetazole assay in urine using benzotriazole as I. S.

Concentration added μg/ml	n	Concentration found μg/ml	C.V. (%)
Within run			
1	5	0.96 ± 0.05	5.2
5	5	4.97 ± 0.16	3.2
10	5	10.08 ± 0.26	2.6
20	5	19.82 ± 0.58	2.9
Within day			
5	3	4.96 ± 0.16	3.2
10	3	9.81 ± 0.37	3.7

TABLE 4

Accuracy and precision of the nocardicins assays in urine and serum using benzotriazole as I.S.

	Concentration added μg/ml	n	Concentration found μg/ml	C.V. (%)
URINE	Within run			
	Nocardicin A			
	5	5	5.03 ± 0.16	3.2
	10	5	10.02 ± 0.23	2.3
	Nocardicin E			
	5	5	4.95 ± 0.17	3.4
	10	5	9.99 ± 0.24	2.4
	Within day			
	Nocardicin A			
	10	3	9.91 ± 0.36	3.6
	Nocardicin E			
	10	3	10.03 ± 0.51	5.1
SERUM	Within run			
	Nocardicin A			
	5	5	4.96 ± 0.12	2.4
	10	5	10.04 ± 0.19	1.9
	Nocardicin E			
	5	5	4.92 ± 0.14	2.8
	10	5	9.94 ± 0.21	2.1
	Within day			
	Nocardicin A			
	10	3	9.96 ± 0.42	4.2
	Nocardicin E			
	10	3	10.01 ± 0.37	3.7

DISCUSSION

The HPLC method has many advantages over previously reported assays of β-lactams antibiotics which rely on antimicrobial activity to estimate concentration in biological fluids (14-17)

The antimicrobial assays have disadvantages, according to Aravind et al. (18) : long turn-around time, a propensity for random error due to unavoidable biological variables inherent in the assay system, possible decomposition of the antibiotic during the 37°C incubation, and interference by concurrently administered antibiotics. In constrast, in the HPLC method developed, antibiotic concentrations can be determined in approximately 30 min and it can be measured in the presence of other commonly used antibiotics.

The main advantage of the method is that combines the assets of Sep-Pak cartridges with a versatile analytical method for quantitating antibiotics using an internal standard. The method is also quicker than earlier HPLC methods based on liquid extraction.

In conclusion, a practical RP-HPLC method to determine antibiotics in serum and urine is described. The proposed method is rapid, requires small sample volume, and can be used for quantitating the antibiotic concentration in serum and urine.

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