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RAPID CHROMATOGRAPHIC DETERMINATION OF CEFOTAXIME AND ITS METABOLITE IN BIOLOGICAL FLUIDS

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SUMMARY

A reversed-phase high-performance liquid chromatographic assay for the simultaneous determination of cefotaxime and its metabolite desacetylcefotaxime in plasma and urine was developed. Plasma was deproteinized with small amounts of acetonitrile. After separation of the proteins the supernatant was extracted with a mixture of chloroform and 1-butanol. A phase separation was obtained leaving the cephalosporin and its metabolite in the aqueous part and extracting most of the interfering endogenous material. The aqueous phase was injected directly into the chromatograph. As part of the plasma water was dissolved in the acetonitrile—1-butanol—chloroform layer, the concentration of the cephalosporin in the aqueous phase was significantly higher than in the original plasma sample. Therefore, the usual diluting effect of the deproteinization could be avoided. In a similar way the assay was applicable to measure cefotaxime and its metabolite in urine. Calibration curves were set up and were linear up to 25 μg/ml for desacetylcefotaxime and 250 μg/ml for cefotaxime. The assay was applied to study the pharmacokinetics of cefotaxime and its metabolite in a healthy volunteer. In a similar way this deproteinization and extraction method was also applied to assay for ceftazidime, cephalexin, cephazolin and cefoxitin.

INTRODUCTION

Pharmacokinetic studies of antimicrobial agents require sensitive assay methods that can be applied to detection of drug and metabolites in plasma and urine. Cefotaxime sodium is an intravenously administered third-generation cephalosporin antibiotic active against a wide variety of bacteria [1]. Studies of its pharmacokinetics have used microbiological and high-performance liquid chromatographic (HPLC) techniques for quantitation of the drug [2—6].

Microbiological assays frequently lack the sensitivity and specificity desired

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for pharmacokinetic studies. If the drug has an active metabolite, microbial assays may overestimate the concentration of the parent drug. Cefotaxime's metabolite, desacetylcefotaxime, has antimicrobial activity [1] and has been demonstrated to interfere with the determination of cefotaxime by microbiological assay techniques [5, 6]. Therefore, microbiological assays are not useful in the determination of the pharmacokinetic parameters for cefotaxime.

Difficulty in the HPLC analysis of cefotaxime and desacetylcefotaxime has been related to their highly polar nature and their instability. The primary difficulty in their HPLC analysis has been in obtaining suitable chromatographic separation of the metabolite from plasma components. Several methods have been described for precipitation of the plasma proteins that generally involve the use of an acid, e.g. trichloroacetic acid [5], perchloric acid [6] or phosphoric acid—methanol [7]. However, stability studies have shown that in strong acid a rapid hydrolysis of cefotaxime takes place and also a lactonization

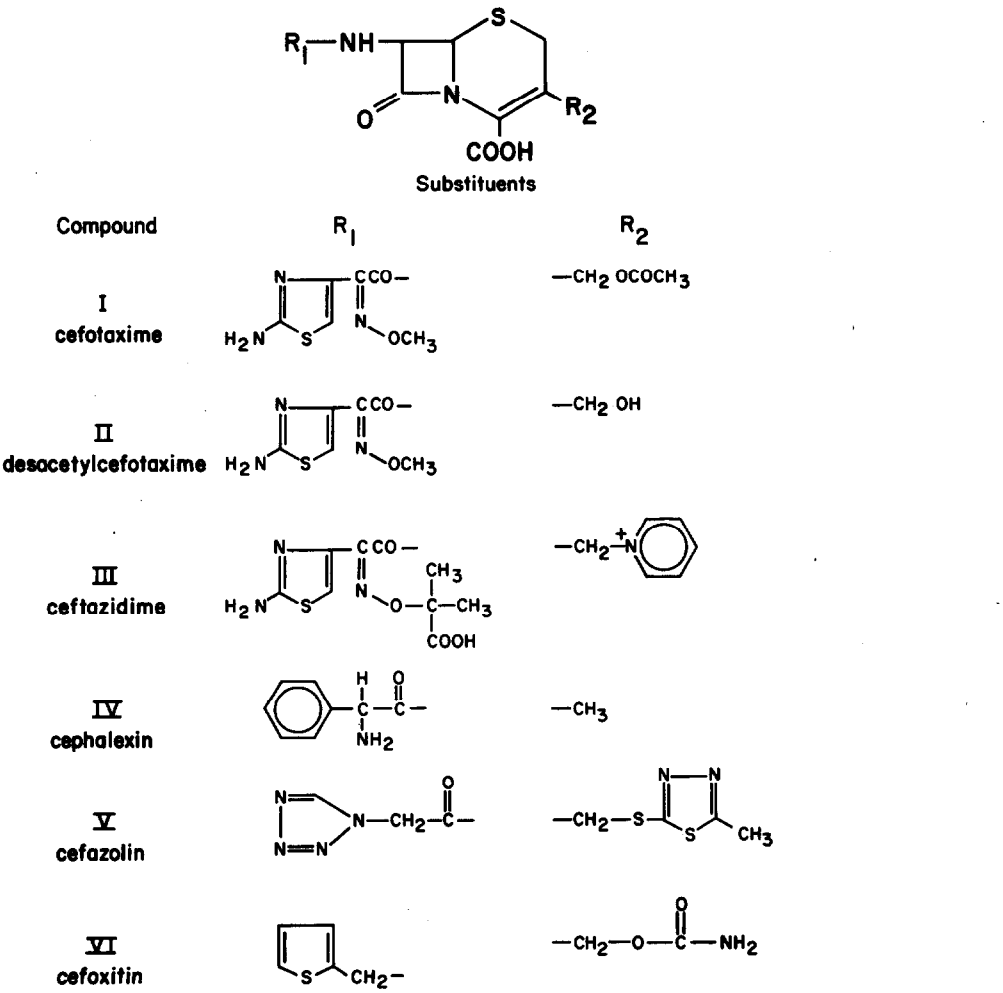

Fig. 1. Chemical structures of cefotaxime, desacetylcefotaxime, ceftazidime, cephalexin, cefazolin and cefotoxin.

of the desacetylcefotaxime occurs [8—10]. The optimum pH range of stability was reported to be 4.3—6.5 [8, 9]. Thus, acid deproteinization methods may convert significant amounts of cefotaxime to desacetylcefotaxime and to the lactone. One of the reported methods [6] even uses this conversion to the lactone to determine desacetylcefotaxime indirectly by measuring its degradation product. However, a direct determination of the unchanged compound would be a more desirable way to assay.

Another disadvantage of the reported deproteinization methods is the fact that the samples have to be diluted. Thus, sensitivity is decreased. For many drugs assay sensitivities can be increased by employing extraction techniques. However, neither cefotaxime nor its metabolite are extractable with organic solvents at neutral pH, and methods for extraction employing acid treatment and organic extraction [11] bear the danger of degradation and lactonization. A reported deproteinization method using chloroform—acetone to remove protein and freeze-drying to concentrate the sample [12] is feasible, but time-consuming and expensive.

This paper describes a simple assay method for cefotaxime and desacetylcefotaxime in plasma that does not degrade the parent drug or its metabolite, gives good separation, and is sensitive and linear over a wide range (0.5—250 μg/ml).

The assay procedure was also applied to ceftazidime, cephalexin, cefazolin and cefoxitin (Fig. 1).

EXPERIMENTAL

Materials

All chemicals were either USP, NF or ACS quality and were used without further purification. Cefotaxime sodium and desacetylcefotaxime were gifts from Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). Ceftazidime was a gift from Glaxo (Greenford, U.K.). Cephalexin, cefazolin and cefoxitin were gifts from Eli Lilly (Indianapolis, IN, U.S.A.). These compounds were used as supplied.

Apparatus

For the HPLC assay the following instruments were used: high-pressure pump, Constametric III, LDC/Milton Roy (Riviera Beach, FL, U.S.A.), variable-wavelength UV detector, SpectroMonitor D, LDC/Milton Roy, autosampler, Model ISS-100, Perkin-Elmer (Norwalk, CT, U.S.A.), integrator, Model 3390A Hewlett-Packard (Palo Alto, CA, U.S.A.), and an octadecylsilane column, C_{18} μBondapak 30 cm × 4.5 mm I.D., (10 μm), Waters Assoc. (Milford, MA, U.S.A.) with a guard column, octadecyl-silane 37—50 μm, 4 cm, Waters Assoc. A laboratory centrifuge from International Centrifuge Equipment (Needham, MA, U.S.A.) was used in the separation of organic extracts from aqueous phases.

Chromatographic conditions

The mobile phase consisted of 0.007 *M* phosphoric acid in water—acetonitrile (85:15). The flow-rate was 1.3 ml/min, sensitivity 0.001 a.u.f.s., and

the wavelength 254 nm. The chart speed was 0.3 cm/min and all assays were performed at ambient conditions.

Sample preparation

Stock solutions of cefotaxime and desacetylcefotaxime were prepared by dissolving 25 mg of drug in 10 ml of methanol. Plasma standards were prepared for a range of 0.5—250 μg/ml for cefotaxime and 0.5—25 μg/ml for desacetylcefotaxime by spiking blank plasma with the appropriate amounts of the two solutions. Blank plasma was obtained from a single human donor. Urine standards were prepared by spiking urine from a drug-free, caffeine-free volunteer with appropriate amounts of the stock solutions. Urine standards were prepared for a range of 5—500 μg/ml for cefotaxime and 5—1000 μg/ml for desacetylcefotaxime. Stock solutions of the other cephalosporins were prepared in an analogous way.

Plasma (1.0 ml) and acetonitrile (3.0 ml) were mixed and vortexed for 5 sec. The mixture was centrifuged for 20 min at 1500 *g*. Of the resulting supernatant 1 ml was extracted with 1 ml of a mixture of chloroform—1-butanol (3:1). The sample was vortexed for 10 sec and then centrifuged for 5 min at 1500 *g*. A 20-μl aliquot of the resulting aqueous supernatant was injected into the HPLC system.

Urine (0.1 ml) was diluted with water (5.0 ml), vortexed for 5 sec and then centrifuged for 5 min at 1500 *g*. A 20-μl aliquot of the resulting supernatant was injected into the HPLC system.

Subject

The subject, a 42-year-old white female, 52.3 kg and 165.7 cm, was administered 1000 mg of cefotaxime sodium intravenously, Claforan R from Hoechst-Roussel Pharmaceuticals (Somerville, NJ, U.S.A.). The drug was dissolved in 12 ml of sterile water for injection and administered via Harvard infusion pump over 5 min. Blood samples were collected in sodium heparin tubes (Venoject Lot No. 13024, Terumo Medical Co., Elkton, NV, U.S.A.). The plasma was immediately separated and frozen at −20°C. Samples were collected immediately before and at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 480 min after the start of the infusion. Urine was collected prior to drug administration, and in intervals of 0—2, 2—4, 4—8, and 8—24 h. Total urine volumes were measured, the pH was recorded, and an aliquot was immediately frozen.

RESULTS AND DISCUSSION

Chromatographic separation

With a mobile phase of 0.007 *M* phosphoric acid in water—acetonitrile (85:15) cefotaxime and desacetylcefotaxime are well separated and can be assayed simultaneously (Fig. 2A). The retention times of all investigated compounds for a flow-rate of 1.3 ml/min are listed in Table I.

Sample preparation

Plasma samples were deproteinized with acetonitrile. This was advantageous

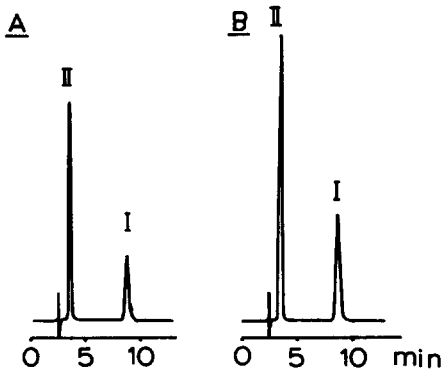

Fig. 2. Chromatograms of cefotaxime and desacetylcefotaxime in aqueous solution: (A) 5 μg/ml in water, injection volume 20 μl; (B) same sample as A after extraction of a mixture of 1 ml of the aqueous solution and 3 ml acetonitrile with the same volume of chloroform—1-butanol, injection volume 20 μl of the aqueous phase. Peaks: I = cefotaxime; II = desacetylcefotaxime.

TABLE I

RETENTION TIMES OF THE INVESTIGATED CEPHALOSPORINS

Chromatographic conditions: mobile phase 0.007 *M* phosphoric acid—acetonitrile (85:15), 10-μm octadecylsilane column, flow-rate 1.3 ml/min, UV detection at 254 nm.

Compound	Retention time (min)
Cefotaxime	8.7
Desacetylcefotaxime	3.5
Ceftazidime	4.2
Cephalexin	7.6
Cefazolin	12.0
Cefoxitin	16.3

as drastic pH changes as are obtained using trichloroacetic acid are avoided. However, two major problems remain using acetonitrile deproteinization. The sample is diluted to one fourth of its original concentration, thereby decreasing the sensitivity of the assay. Furthermore, it is very difficult to separate chromatographically the very polar desacetylcefotaxime from endogenous plasma components. Both of these problems can be overcome by a further sample preparation step. The supernatant—acetonitrile—plasma—water mixture is extracted with equal volumes of a mixture of chloroform—1-butanol (3:1). A phase separation is obtained with a small volume of an aqueous phase on top and a chloroform—1-butanol—acetonitrile—water mixture on the bottom. The cephalosporins concentrate in the aqueous phase whereas most of the interfering compounds are extracted into the organic. The final concentration of the cephalosporins in the supernatant is higher than the original plasma concentration (Fig. 2B), so the dilution step during the deproteinization is more than compensated. No evaporation of solvent is needed. The ratio 3:1 for the chloroform—1-butanol mixture was chosen as a further increase of 1-butanol prevents a good phase separation, whereas a decrease of the 1-butanol portion will lead to lower cephalosporin concentrations in the supernatant aqueous phase.

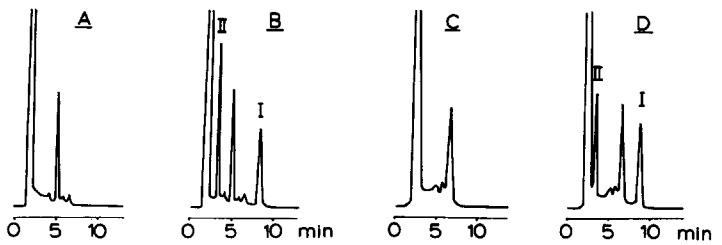

Fig. 3. Chromatograms of cefotaxime and desacetylcefotaxime in plasma and urine: (A) 1 ml of blank plasma, mixed with 3 ml acetonitrile, supernatant extracted with the same volume of chloroform—1-butanol, injection volume 20 μl of the aqueous phase; (B) 1 ml of plasma containing 5 μg/ml drug and metabolite, extracted and treated as in A; (C) 0.1 ml of blank urine, mixed with 5 ml water, injection volume 20 μl; (D) 0.1 ml of urine containing 20 μg of drug and metabolite, treated as in C. Peaks: I = cefotaxime; II = desacetylcefotaxime.

Typical chromatograms of cefotaxime and its metabolite in plasma after direct injection of the supernatant aqueous phase are shown in Fig. 3A and B. As the concentrations of the cephalosporins in urine are very high it is possible to assay urine by direct injection after dilution with water. Typical chromatograms of urine samples containing drug and metabolite are shown in Fig. 3C and D.

Simultaneous HPLC assay of cefotaxime and desacetylcefotaxime

With the described method calibration curves in plasma were set up over a range of 0.5—250 μg/ml for cefotaxime and 0.5—25 μg/ml for the metabolite. Under assay conditions no desacetylcefotaxime formed owing to degradation of parent drug. The calibration curves were linear over this wide range; their statistics are given in Table II. The limit of sensitivity was about 0.1 μg/ml for both cefotaxime and metabolite. The range for the urine calibration curves was 5—500 μg/ml for cefotaxime and 5—1000 μg/ml for its metabolite. The precision was determined by repetitive analysis of the same sample. The average relative standard deviation in the investigated concentration range was 1.8% for the parent drug and 1.1% for its metabolite.

Accuracy and linearity of the assay were challenged by measuring the concentrations of spiked plasma samples in the investigated range. Good agreements with an average error of 3.2% could be observed (Table III).

TABLE II

STATISTICS OF CALIBRATION CURVES

Concentrations (C) in μg/ml versus peak area (PA), $C \pm S_{x,y} = (m \pm s_m)PA + (b \pm s_b)$. Standard of error of estimate y on x, concentration μg/ml, on peak area.

Compound	Medium	Range	$m \pm s_m$	$b \pm s_b$	$S_{x,y}$
Cefotaxime	plasma	0.5—10	$1.5 \cdot 10^{-4} \pm 4.3 \cdot 10^{-6}$	0.20 ± 0.13	0.23
Cefotaxime	plasma	0.5—10	$1.7 \cdot 10^{-4} \pm 5.5 \cdot 10^{-6}$	0.37 ± 0.14	0.26
Cefotaxime	plasma	0.5—250	$1.2 \cdot 10^{-4} \pm 1.2 \cdot 10^{-6}$	1.17 ± 0.85	2.72
Cefotaxime	plasma	0.5—250	$1.7 \cdot 10^{-4} \pm 1.7 \cdot 10^{-6}$	2.11 ± 0.97	3.10
Desacetylcefotaxime	plasma	0.5—25	$9.0 \cdot 10^{-5} \pm 8.5 \cdot 10^{-7}$	−0.015 ± 0.10	0.21
Desacetylcefotaxime	plasma	0.5—25	$9.5 \cdot 10^{-5} \pm 1.0 \cdot 10^{-6}$	−0.077 ± 0.18	0.27
Cefotaxime	urine	5—500	2.55 ± 0.03	3.7 ± 2.1	4.8
Desacetylcefotaxime	urine	5—1000	1.93 ± 0.03	20.1 ± 15.6	28.9

TABLE III

ACCURACY OF THE ASSAY IN PLASMA

Compound	Concentration (μg/ml)	Assayed (μg/ml)	Error (%)
Cefotaxime	0.5	0.54	8.0
	2.0	1.94	3.0
	15.0	15.05	0.3
	100.0	96.67	3.3
	200.0	203.12	1.6
	250.0	253.04	1.2
Desacetylcefotaxime	0.5	0.53	6.0
	2.0	2.11	5.5
	15.0	14.81	1.3
	25.0	24.67	1.3

Application of the assay to pharmacokinetic studies

After intravenous administration a rapid decrease in the plasma level of cefotaxime can be observed (Fig. 4). Formation of the metabolite is fast, it reaches its maximum plasma concentration within a few minutes after administration of cefotaxime. The metabolite has a longer half-life than the parent compound. Analysis of the urine showed that about 35% of the given dose is

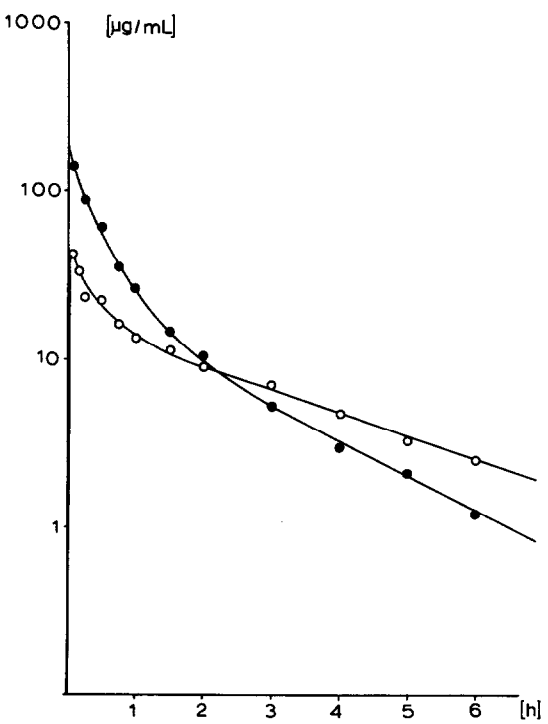

Fig. 4. Plasma levels for cefotaxime (•) and desacetylcefotaxime (○) after intravenous administration of 1 g of cefotaxime to a healthy volunteer.

TABLE IV

URINARY EXCRETION OF CEFOTAXIME AND DESACETYLCEFOTAXIME

Sample time (h)	Amount excreted (mg)	
	Cefotaxime	Desacetylcefotaxime
0—2	306.8	90.8
2—4	27.8	37.8
4—8	8.6	40.6
8—24	—	70.4
Total	343.2	239.6

excreted into the urine as unchanged cefotaxime and 25% as its metabolite (Table IV). As the metabolite also has microbiological activity [1], the results again underline the need for chromatographic assay methods to study the pharmacokinetics of cefotaxime, since microbiological assays will measure the antibacterial activities derived from the combination of drug and metabolite.

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