Journal of Chromatography, 223 (1981) 393—399
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 819

DETERMINATION OF CEPHALOSPORINS IN BIOLOGICAL MATERIAL BY REVERSED-PHASE LIQUID COLUMN CHROMATOGRAPHY

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(First received October 28th, 1980; revised manuscript received December 17th, 1980)

SUMMARY

Seven cephalosporins (β-lactam antibiotics), viz. cefazolin, cephalotin, cefoxitin, cefotaxime, cefamandole, cefuroxime and cefoperazone (T 1551) were determined in biological material. The compounds were extracted from acid-treated body fluids into chloroform—1-pentanol (3 : 1) and re-extracted into a small volume of an aqueous phase at pH 7, which was injected into the chromatographic column. The chromatographic support was μBondapak C_{18} (10 μm) and the mobile phase was a mixture of 0.01 *M* acetate buffer (pH 4.8) and methanol or acetonitrile. Detection limits are about 50 ng/ml for extractions from 1 ml of serum and have permitted pharmacokinetic studies of the seven cephalosporins.

INTRODUCTION

Pharmacokinetic studies of cephalosporins are usually performed by conventional microbiological assay procedures [1—6]. However, high-performance liquid chromatography is specific, rapid and sensitive, and is to be preferred when analysing samples of biological fluids, for separating the drug from interfering substances or for dosing eventual metabolites. A number of liquid chromatographic methods have been reported for the determination of cephalosporins in blood serum and urine [7—10]. Although some of these methods used anion-exchange columns for the separation of the drugs [11], some recent methods [12—14] recommended the reversed-phase technique with octadecyl-bonded packings. The major differences between these methods were in the serum sample preparation procedure. We have previously used serum protein precipitation techniques for sample preparations [15, 16] but with a low sensitivity (500 ng/ml). Although rapid and convenient, this method is not readily adaptable to trace amounts of drugs.

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This paper describes a method for the preparation and determination of seven cephalosporins, cefazolin, cefoxitin, cephalotin, cefuroxime, cefotaxime, cefamandole and cefoperazone (T 1551) and metabolites, using reversed-phase HPLC after extraction.

These semi-synthetic cephalosporins have similar structures (Fig. 1), based on the 7-aminocephalosporanic nucleus. The extraction depends on the carboxylic group at the C_3 position. Cephalosporins are isolated from the biological material in an organic phase at pH 3 and brought into a small volume of aqueous phase at pH 7. The drugs are then separated from co-extractants by reversed-phase chromatography and quantified by UV absorbance measurement at an appropriate wavelength. Detection limits are about 50 ng/ml for extraction from 1 ml of serum.

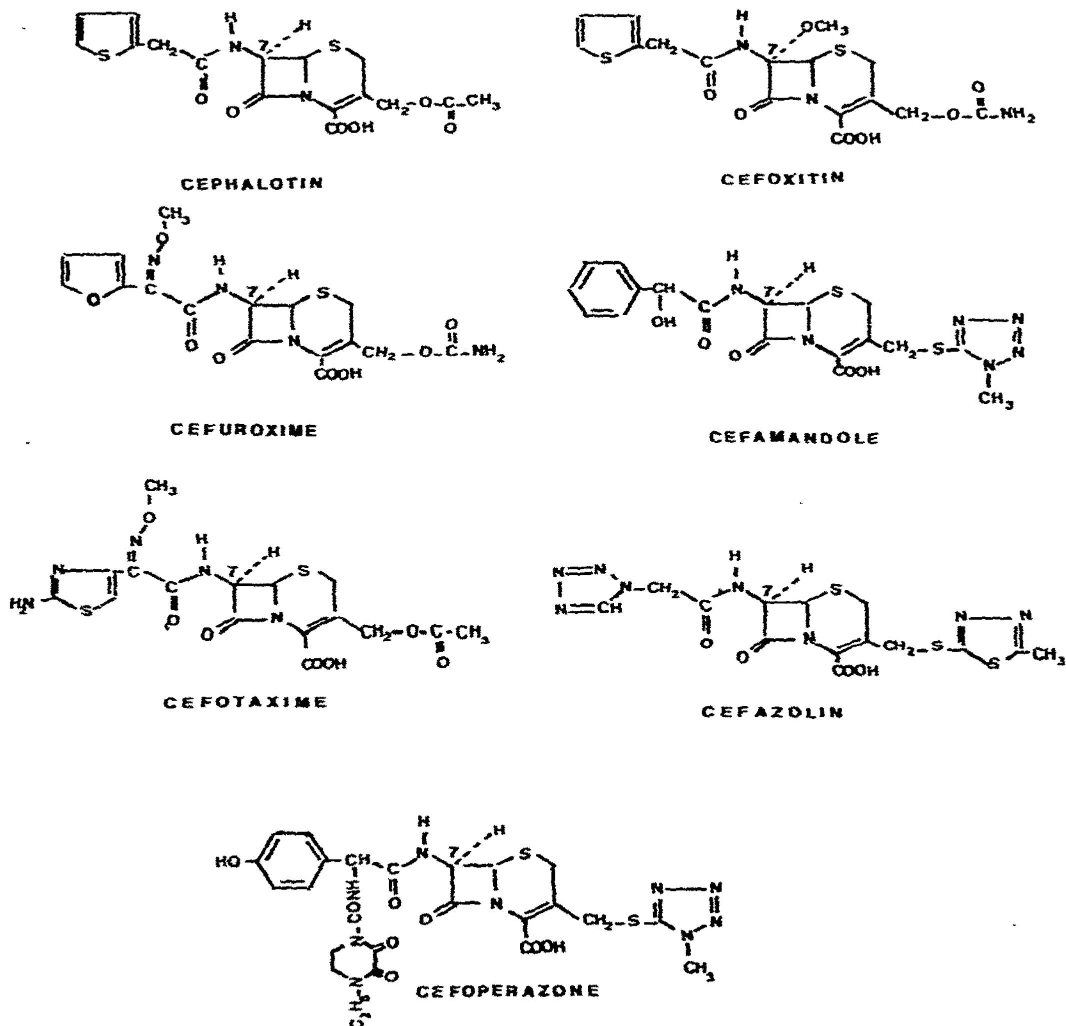

Fig. 1. Structures of cephalosporins.

EXPERIMENTAL

Drug standards

Pure drug samples were obtained as follows: cefazolin from Allard (Paris, France), cefoxitin from Merck, Sharp & Dohme (Paris, France), cephalotin and cefamandole from E. Lilly (Saint-Cloud, France), cefotaxime from Roussel (Paris, France), cefuroxime from Glaxo (Paris, France) and cefoperazone (T 1551) from Pfizer (Orsay, France).

Chemicals

Reagent-grade acetic acid, sodium acetate, hydrochloric acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, chloroform, 1-pentanol (R.P. Normapur, Prolabo, Paris, France), acetonitrile and methanol (Uvasol grade, E. Merck, Darmstadt, G.F.R.) were used without further purification.

Plasma and urine samples

Blood samples (5 ml) were collected in 10-ml glass test-tubes (Vacutainer) containing heparin, immediately before and at appropriate times after the start of drug administration. The samples were rapidly centrifuged at 1000 *g* for 15 min. The plasma fraction was carefully separated using a sera-clear and frozen at −20°C until taken for assay. Urine samples were stored at −20°C until taken for assay.

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used, which included a dual 6000A solvent delivery system, a WISP 710A sample processor and an M450 variable-wavelength UV detector. An Omniscribe B5000 recorder (Houston Instruments, Austin, TX, U.S.A.) was used. A reversed-phase μBondapak C_{18} (10 μm; 30 cm × 3.9 mm I.D.) column was employed (Waters Assoc.). Filtration of solvents was carried out using a Pyrex filter holder (Millipore Corp., Bedford, MA, U.S.A.). The curves were plotted by a Tektronix 4052 computer with an interactive graphic package for pharmacokinetic analysis [17].

Procedure for plasma samples

Extraction. The plasma sample (1 ml) was mixed with 0.50 ml of 0.4 *M* hydrochloric acid in a 10-ml centrifuge tube. The mixture was extracted with 7 ml of chloroform—1-pentanol (3 : 1), the extraction time being 10 min. After centrifugation at 1000 *g*, 5 ml of the organic phase were transferred into another centrifuge tube and cephalosporins were back-extracted (10 min) into 350 μl of phosphate buffer (pH 7). After centrifugation at 1000 *g*, part (10—50 μl) of the (upper) aqueous phase was injected into the chromatographic column.

Chromatography. The mobile phase was a mixture of 0.01 *M* acetate buffer (pH 4.8) and methanol or acetonitrile. The flow-rate was 1.5 ml/min at a pressure of about 150 bar. The separations were carried out at room temperature.

Quantitation. The chromatograms were quantitated by measuring the peak heights manually with a ruler. Calculations were performed by using a calibra-

tion graph of peak height versus concentration obtained by analysing known amounts of the compounds added to plasma samples obtained before administration of the drugs.

Procedure for urine samples

After centrifugation, urine samples were diluted with doubly distilled water and injected into the liquid chromatograph without extraction. To avoid interfering peaks, a mobile phase containing a lower percentage of acetonitrile or methanol was generally used. Calculations were performed by using a calibration graph obtained by analysing known amounts of the cephalosporins added to urine samples obtained before administration of the drugs.

RESULTS AND DISCUSSION

Extraction

Results for extraction from serum using various solvents are given in Table I.

TABLE I

PERCENTAGE EXTRACTION OF CEFAZOLIN WITH DIFFERENT SOLVENTS

Solvent	Extraction (%)	Solvent	Extraction (%)
Benzene	6.5	Amyl acetate	12
Chloroform	3	Ethyl acetate	83.5
Cyclohexane	5	Chloroform—1-butanol (3 : 1)	84.5
Methylene chloride	9	Chloroform—1-propanol (3 : 1)	84.5
Diethyl ether	7	Chloroform—1-pentanol (3 : 1)	85
n-Heptane	4		
n-Hexane	5		

Chloroform—1-pentanol (3 : 1) gave the best quantitative extractions. A theoretical elucidation of the extraction mechanism is probably possible based on the pK_a of the cephalosporins given by the carboxylic group at the C_3 position on the cephem nucleus: this pK_a is about 4—5. When the pH is lower than the pK_a, cephalosporins are in non-ionized form and therefore soluble in organic phase, whereas when the pH is about 7 (higher than the pK_a) they are extracted by an aqueous phase. The ratio of the volume of the organic to that of the aqueous phase is 20. This extraction cannot be used for orally absorbed cephalosporins, which contain an amino group that is ionized at pH 3.

Liquid column chromatography

Retention. We first performed the chromatographic analysis of cephalosporins using anion-exchange techniques, but a continuously decreasing retention was obtained.

The present method, using reversed-phase chromatography, can be used for all cephalosporins, even the orally absorbed cephalosporins cefadroxil, cephradine, cefalexin and cefaclor [18]. The retention times of the seven cephalosporins are given in Table II for a mobile phase consisting of 0.01 *M* acetate buffer (pH 4.8)—15% methanol.

TABLE II

ELUTION TIMES OF CEPHALOSPORINS

Column, μBondapak C_{18} (10 μm, 30 cm × 3.9 mm I.D.); eluent, methanol—0.01 *M* acetate buffer (pH 4.8) (15 : 85); flow-rate, 1.5 ml/min.

Cephalosporin	λ_{max}	Elution time (min)
Cefuroxime	254	3.1
Cefoxitin	245	3.3
Cefotaxime	234	3.45
Cefazolin	275	4.20
Cefamandole	270	6.15
Cephalotin	240	8
Deacetylcephalotin	240	2.7
Cefoperazone (T 1551)	240	8.35

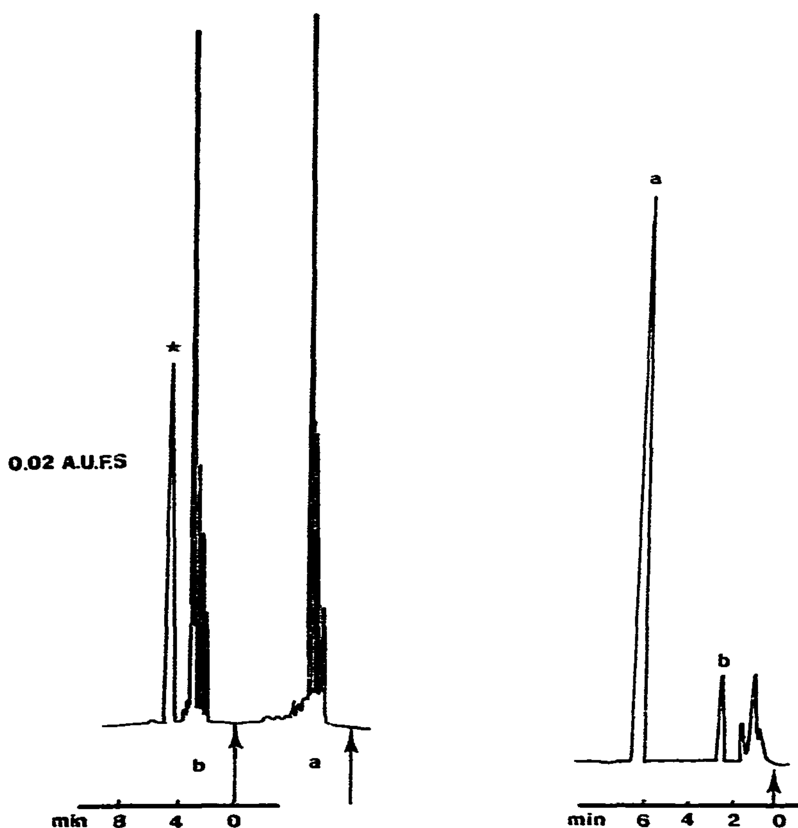

Fig. 2. (a) Chromatogram of extract from blank plasma (1 ml). (b) Chromatogram of extract from 1 ml of plasma containing 2.5 μg of cefoperazone (*) (λ = 240 nm; 0.02 a.u.f.s.; injection volume 40 μl).

Fig. 3. Chromatogram of extract from 1 ml of plasma after administration of cephalotin: a, cephalotin; b, deacetylcephalotin.

Detection and linearity. The detection wavelengths (Table II) were chosen so as to maximize the sensitivity. For instance, as shown in Fig. 2, cefoperazone (T 1551) was monitored at a UV wavelength of 240 nm with a detector sensitivity of 0.02 a.u.f.s. The detection limit under these conditions is about 50 ng/ml. The linearity of the method was determined for all cephalosporins and is illustrated in Table III.

TABLE III

LINEAR REGRESSION ANALYSIS OF STANDARD CURVES

Cephalosporin	Concentration range (µg/ml)	Correlation coefficient	Slope	Intercept
Cefazolin	0.075—1.25	0.9979	40.16	0.213
Cephalotin	0.15—5	0.9999	28.71	−0.055
Cefamandole	0.15—5	1.0000	26.92	0.189
Cefoxitin	0.15—5	0.9999	37.47	1.8
Cefuroxime	0.075—5	0.9999	82.09	−0.088
Cefotaxime	0.15—2.5	0.9999	57.16	0.924
Cefoperazone	0.15—2.5	0.9995	46.07	0.975

Selectivity

Serum protein precipitation techniques have not permitted the separation of cephalosporins from their metabolites. The method proposed here permits the quantitative determination of cephalotin and deacetylcephalotin (Fig. 3), cefotaxime and deacetylcefotaxime after administration of cephalotin or cefotaxime to a patient.

Precision

The precision of the method was studied, for each cephalosporin, by repeated analysis of serum solutions containing the drug. The data in Table IV show that the peak height for eight samples ranged from 106 to 108 mm [mean = 106.5 ± 0.47 mm (S.E.M.) for 2.5 µg/ml of cefoperazone].

TABLE IV

REPEATABILITY OF ANALYSIS OF CEFOPERAZONE

Analysis of 8 blood serum samples containing 2.5 µg/ml of cefoperazone

Sample No.	Peak height (mm)	Sample No.	Peak height (mm)
1	106	5	109
2	106	6	106.5
3	105.5	7	107
4	105	8	108

Plasma samples from patients

Chromatograms of plasma sample from a patient treated with cefoperazone are shown in Fig. 2. The plasma levels after intravenous administration of cefoperazone (1 g, bolus) are presented in Fig. 4.

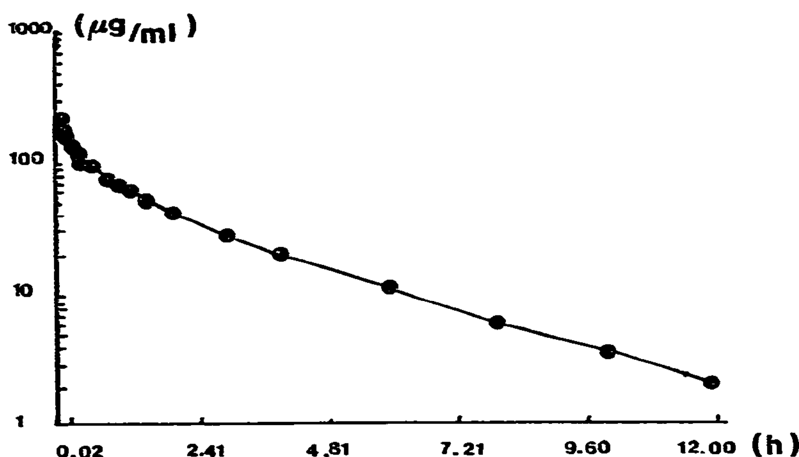

Fig. 4. Decrease of plasma levels of cefoperazone with time after intravenous administration (1 g, bolus).

This method permitted us to determine and compare the pharmacokinetic properties of the seven cephalosporins cefazolin, cephalotin, cefoxitin, cefamandole, cefotaxime, cefuroxime and cefoperazone (T 1551) under the same conditions [19].

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