Journal of Chromatography, 419 (1987) 388–395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3708

Note

Micromethod for the determination of cefpiramide in human plasma and urine by high-performance liquid chromatography using automated column switching

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(First received November 28th, 1986; revised manuscript received March 13th, 1987)

The third-generation cephalosporins are characterized by their high stability to β-lactamases and their broad antibacterial spectrum especially against gram-negative bacteria. The properties are achieved by substitution at position 7 on the β-lactam ring [1]. Cefpiramide (Fig. 1), a new semisynthetic third-generation cephalosporin, has been shown to have a potent activity against *Pseudomona aeruginosa* [2].

High-performance liquid chromatography (HPLC) is in widespread use for analytical determination of cephalosporins in biological fluids. The rapidity, sensitivity and specificity of these techniques justify their use for the quantitation of antimicrobial agents. Microbiological assays for cefpiramide utilizing *Escherichia coli* NIHJ or Wy-001 as indicator organism require overnight incubation and lack sensitivity (detection limit in plasma 1.5 μg/ml) [3–5]. Since the aqueous solubility of cephalosporins is too high to allow their extraction by organic solvents, precipitation of serum proteins prior to injection onto a reversed-phase analytical column is generally used. We have previously reported a micromethod for the determination of cefotaxime in biological fluids utilizing a sample pretreatment prior to chromatography [6]. In this paper, we describe a fully automated HPLC method that does not involve this sample pretreatment step. This

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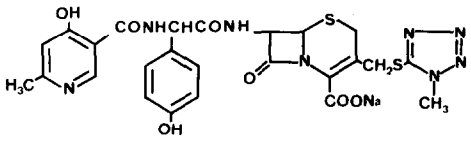

Fig. 1. Structure of cefpiramide.

results in a more rapid quantitation without loss of sensitivity or precision, which makes the method suitable for pharmacokinetic studies.

EXPERIMENTAL

Materials and reagents

Cefpiramide (SM-1652) was supplied by Clin Midy (Montpellier, France) and cefoperazone by Pfizer Labs (Orsay, France). Acetonitrile (HPLC grade) was from Rathburn (Walkerburn, U.K.). All other chemicals (ammonium acetate, acetic acid, orthophosphoric acid and triethylamine) were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). Water was deionized and doubly glass-distilled.

Apparatus and chromatographic procedure

A fully automated high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) was used. Its configuration was similar to one initially described by Roth [7]. The system is equipped with two solvent-delivery pumps, Model 501 (pump A) and Model 590 (pump B), a WISP Model 710 B automatic injector, two six-port switching valves, a Lambda-Max Model 480 ultraviolet detector operated at 270 nm and a 10-mV recorder Omniscribe (Houston Instruments).

Two pre-columns (20 mm×4 mm I.D.) filled with a reversed-phase material (C_{18} Corasil, 35–50 μm; Waters) are connected alternately with pump A and the injection system or with pump B and the analytical column. Compounds are chromatographed on a Radial-Pak μBondapak C_{18} column (100×8 mm I.D., 10 μm particle size; Waters).

The mobile phase A (wash-phase) consists of water–triethylamine (1000:4); the pH is adjusted to 3.0 with orthophosphoric acid. This phase is delivered by pump A at a flow-rate of 2 ml/min. The mobile phase B is water–acetonitrile–triethylamine (250:750:4); the pH is adjusted to 3.0 with orthophosphoric acid. The working pressure of pump B is 55 bar at a flow-rate of 3.8 ml/min. The apparatus is shown in Fig. 2.

Assay

To a 1.5-ml conical centrifuge tube containing 100 μl of plasma are added 100 μl of cefoperazone at 10 μg/ml in 0.1 *M* ammonium acetate buffer (pH 5), for low plasma concentrations (2–50 μg/ml). For high concentrations (50–600 μg/ml), 500 μl of acetate buffer containing cefoperazone at 20 μg/ml are added to 50 μl of plasma. The mixture is vortex-mixed for 15 s and centrifuged for 2 min at 8700 *g*

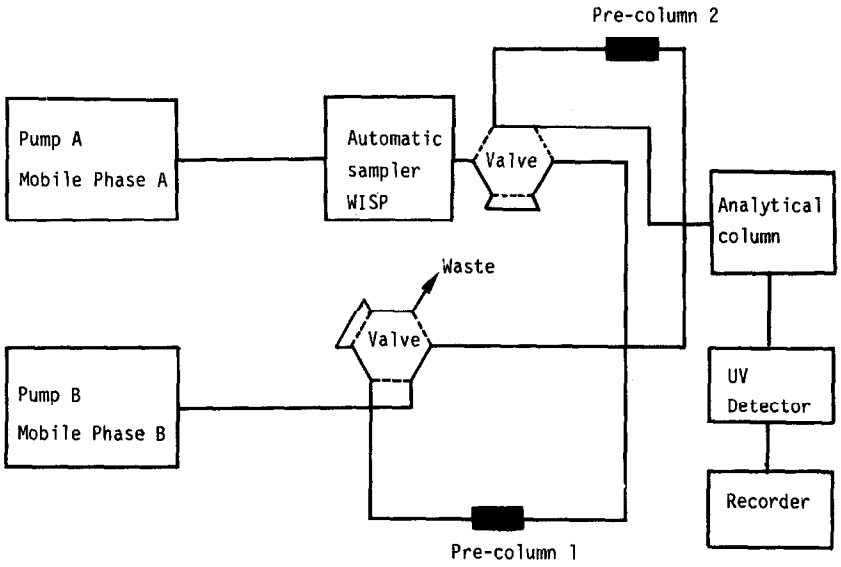

Fig. 2. Schematic diagram of the alternating precolumn enrichment system.

(Beckman Microfuge centrifuge). For urine, 500 μl of buffer containing cefoperazone at 10 μg/ml are added to 100 μl of diluted sample in physiological salt solution. An aliquot of 20–200 μl is injected into the chromatograph.

The time between two injections is 7 min (sample loading 1 min plus wash-period 2 min plus chromatography 4 min). After two quantitative analyses, the electronic timer restarts. Both valves are controlled by the programmable time function of pump B.

RESULTS

Selectivity

Typical chromatograms obtained from human plasma and urine samples are shown in Figs. 3 and 4. Under the chromatographic conditions described, cefpiramide and cefoperazone were well resolved from endogenous plasma or urine compounds (retention times 2.33 and 3.66 min, respectively). Potential interference by some other cephalosporins was investigated (Table I). Most of these drugs were eluted before cefpiramide.

Linearity and sensitivity

The HPLC response was found to be linear over the ranges 2–600 μg/ml in plasma and 1–50 μg/ml in diluted urine. A good correlation was obtained between concentrations and peak-height ratios (cefpiramide/cefoperazone): for plasma, $r=0.997$ (low concentrations, 2–50 μg/ml) and $r=0.998$ (high concentrations, 50–600 μg/ml); for urine $r=0.999$. Calibration lines were chosen from results of pharmacokinetic studies. The detection limit was 0.25 μg/ml in plasma and diluted urine (signal-to-noise ratio ≥ 2).

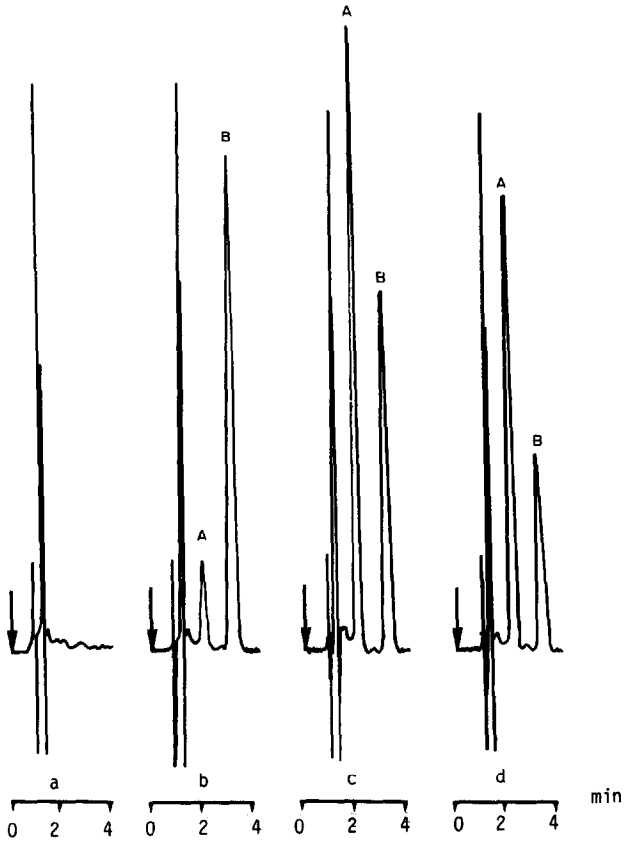

Fig. 3. Chromatograms of human drug-free plasma (a); human plasma spiked with 2 μg/ml (b) and 200 μg/ml (c) cefpiramide; and plasma sample from a subject 6 h after an intravenous dose of 1 g (d). Peaks: A = cefpiramide, B = cefoperazone.

Recovery

Recovery was measured on blank human plasma and urine spiked with cefpiramide at different concentrations. These spiked samples were compared with 0.1 *M* acetate buffer solutions directly injected onto the column. The values obtained were 105.7 ± 4% (plasma, 100 μg/ml, $n=5$) and 105.3 ± 0.3% (urine, 50 μg/ml, $n=5$). For the internal standard, cefoperazone, the mean recovery was 98.8 ± 8.8%.

These results show that there is no effect of protein binding of cefpiramide on the recovery in plasma. Cefpiramide is a cephalosporin which is strongly bonded to proteins (90% of binding [1]), but the drug affinity seems to be stronger towards the precolumn reversed-phase material than towards plasma protein.

Accuracy and precision

The accuracy and precision of the HPLC assay for cefpiramide in plasma and urine were determined by adding known amounts of cefpiramide to blank plasma and urine. The coefficients of variation (C.V.) ranged from 1.1 to 7.5%, and the

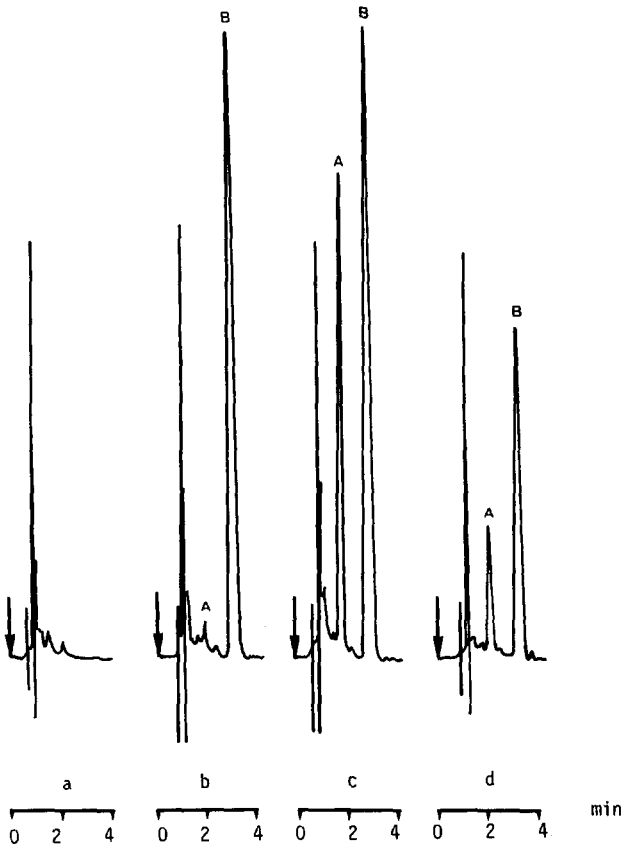

Fig. 4. Chromatograms of human drug-free urine (a); human urine spiked with 1 μg/ml (b) and 20 μg/ml (c) cefpiramide; and urine sample from a subject who received 1 g by intravenous route (10 μg/ml cefpiramide) (d). Peaks: A = cefpiramide, B = cefoperazone.

accuracy, defined as (amount found/amount added) × 100, was ca. 100% for all concentrations investigated (Table II).

Stability

The stability of cefpiramide and cefoperazone during automatic injection was studied on spiked plasma and urine samples (50 and 20 μg/ml, respectively). These samples, treated as described above [diluted with 0.1 *M* acetate buffer (pH 5) containing internal standard at 10 μg/ml] were kept at 25°C for 6 h. Four times an hour, an aliquot was injected and the peak heights of cefpiramide and cefoperazone were measured. Coefficients of variation ($n=28$) were 3.6% (plasma, 50 μg/ml) and 3.3% (urine, 20 μg/ml) for cefpiramide and 3.6% for cefoperazone in each case. These results allowed us to use the automatic system for several hours.

By analogy with previous studies, we have chosen to dilute samples in pH 5 0.1 *M* acetate buffer to prevent the degradation of β-lactam ring [8,9].

TABLE I

RETENTION TIMES OF SOME CEPHALOSPORINS

Amount injected, 2 μg; retention times relative to cefpiramide (2.33 min).

Compound	Relative retention time
Ceftazidime	0.50
Desacetylcefotaxime	0.53
Ceftriaxone	0.54
Cefotaxime	0.67
Cephaloridine	0.85
Ceforanide	0.85
Moxalactam	0.85
Cefazolin	0.96
Cefonicid	0.96
Cefoperazone	1.57
Cephalothin	3.28
Cefotiam	No response
Cefadroxil	No response

Comparison of the automatic HPLC technique with a method including sample pretreatment

For determination of cephalosporins in biological fluids we commonly used a method previously described, requiring a sample pretreatment [6]. Fifteen plasma samples obtained from a clinical study were analysed by both methods. The correlation coefficient, given by linear regression analysis of results obtained from the two assays, was 0.998; the slope of the regression line was 0.93 ± 0.05 and the intercept 2.18 ± 7.87.

Clinical study

This fully automated HPLC method was used for analysis of plasma and urine samples from an healthy subject who received 1 g of cefpiramide by intravenous

TABLE II

ACCURACY AND PRECISION RESULTS FOR PLASMA AND URINE SAMPLES SPIKED WITH CEFPIRAMIDE

Spiked concentration (μg/ml)	Within-day determinations ($n=10$)			Between-day determinations ($n=10$)		
	Mean found (μg/ml)	C.V. (%)	Accuracy (%)	Mean found (μg/ml)	C.V. (%)	Accuracy (%)
Plasma						
0.5	0.52	1.7	105.2	0.48	7.5	96.4
35	33.5	1.3	95.9	34.4	5.5	98.5
360	353.1	1.5	98.1	355.0	2.2	98.6
Urine						
1	0.99	4.0	99.0	1.03	6.8	103.0
70	69.1	1.1	98.7	67.9	5.8	97.0

route. The plasma concentration was 272 μg/ml 5 min after injection, and then decreased to 15 μg/ml after 12 h and to 0.7 μg/ml at 36 h; 23.7% of the dose was excreted as unchanged drug in the 0–36 h urine.

Our results agree with the previous pharmacokinetic studies. In twelve healthy volunteers, peak concentrations after 1 g bolus ranged from 187 to 254 μg/ml, and 25% of the dose was excreted unchanged in urine [4]. In 21 volunteers, the average cefpiramide concentration in plasma 5 min after bolus intravenous injection of 1 g was 272 μg/ml, and concentrations slowly declined to 25 μg/ml at 12 h; urinary excretion of unchanged cefpiramide over a 24-h period was 22.5% [3].

DISCUSSION

In the past few years, several automated methods have been described for quantitation of drugs in biological fluids, allowing many applications in the fields of drug monitoring and pharmacokinetics [7,10–19]. The main advantage of these HPLC systems is that they do not need any sample treatment prior to injection. We have chosen the system described by Roth et al. [7,16], with two six-way valves and two precolumns mounted in parallel. This alternating precolumn enrichment technique reduces the time lost in analysis due to the sample washing step, it protects the analytical column with a precolumn, and it provides an equilibration step before each injection. This system is particularly useful in pharmacokinetic studies where rapidity (100 analyses performed per day), sensitivity and selectivity are required.

The presence of an internal standard is of great importance in HPLC determinations with classical sample pretreatment. But in the technique described here, it use seems important to give information about the stability of the whole automated system and to enhance the linearity and accuracy of the method.

Under the chromatographic conditions described, precolumns have a lifetime of 300 injections. They can be easily packed in the laboratory with C_{18} Corasil material. In this paper, we have shown that cefpiramide can be efficiently analysed by an automatic HPLC technique which may be applicable to other cephalosporins.

ACKNOWLEDGEMENT

The authors thank Mrs. E. Deridet for her technical assistance during the development of the method.

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