Journal of Chromatography, 424 (1988) 111–117
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3957

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CEFOTAXIME, DESACETYLCEFOTAXIME AND CEFTRIAXONE IN RAT PLASMA

L. HAKIM

Department of Pharmacy, University of Queensland, St. Lucia 4067 (Australia)

D.W.A. BOURNE

College of Pharmacy, University of Oklahoma, Oklahoma City, OK 73190 (U.S.A.)

and

E.J. TRIGGS*

Department of Pharmacy, University of Queensland, St. Lucia 4067 (Australia)

(First received July 14th, 1987; revised manuscript received September 10th, 1987)

SUMMARY

A simple and selective high-performance liquid chromatographic method is described for the analysis of the cephalosporins cefotaxime (CXM), desacetylcefotaxime (DACXM) and ceftriaxone (CFX) in rat plasma. Plasma was deproteinized with methanol, and the supernatant was directly injected into the chromatograph and monitored at 254 nm. For determination of the unbound drugs, a centrifugal ultrafiltration method was employed. The calibration curves were linear ($r=0.999$) from 2.5 to 500 μg/ml; the detection limits were 100 ng/ml for DACXM and 250 ng/ml for CXM and CFX. The method was not interfered with by other plasma components, nor by barbital sodium or caffeine, and has been applied to study the pharmacokinetics of the cephalosporins in rats.

INTRODUCTION

Cefotaxime (CXM) and ceftriaxone (CFX) are potent semisynthetic cephalosporin antibiotics that have unusually broad spectra of antibacterial activity and resistance to bacterial β-lactamase [1–3]. A therapeutic combination of the antibiotics may be advantageous since CXM has a short half-life (0.6–2.2 h) with low protein binding (30–50%), and on the other hand CFX has a long half-life (6–8 h) and is highly bound (95%) to plasma protein [4].

In humans, CXM is metabolized to desacetylcefotaxime (DACXM), which to some extent also has antimicrobial action, and several non-active metabolites

0378-4347/88/$03.50

[5]. In rats, although the metabolites occur in urine, only DACXM has been detected in plasma [6]. A metabolite of CFX (microbiologically inactive) has been found in faeces [4].

Microbiological determinations for CXM are not selective since its metabolite DACXM interferes with the assay techniques [7, 8], and thus are not appropriate for pharmacokinetic studies. High-performance liquid chromatographic (HPLC) procedures have recently been performed to overcome this selectivity problem, but it has not been possible to measure simultaneously the parent and metabolite in the course of one analysis [9]. Other disadvantages have occurred when microanalysis and short sample preparation time are required [10, 11]. In our experience with these latter methods, separation of the aqueous layer from the organic is difficult when a small plasma volume (50–100 μl) is employed.

The present report, therefore, offers the advantage of a simultaneous assay of CXM, DACXM and CFX in plasma with reasonable simplicity, sensitivity and selectivity, which subsequently allows pharmacokinetic studies of the drugs to be performed. Precision, accuracy and an application of the method are also presented.

EXPERIMENTAL

Materials and solvents

Cefotaxime sodium, desacetylcefotaxime (Roussel Pharmaceuticals, Castle Hill, Australia) and ceftriaxone sodium (Roche Products, Sydney, Australia) were gifts; cephradine (Squibb and Sons, Melbourne, Australia), barbitone sodium and caffeine (Ajax Chemicals, Sydney, Australia) were used as supplied. The other chemicals (analytical grade), methanol (HPLC grade) and water (distilled in all-glass apparatus) were utilized without further purification. Stock solutions of CXM, DACXM, CFX and cephradrine were prepared weekly and stored frozen ($-20°C$).

Apparatus

The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisted of a pump (Model M-45) connected via a reversed-phase column (RP-8, 10 μm, 250×4.6 mm I.D., Brownlee Labs., Santa Clara, CA, U.S.A.; aged one month) to a UV detector (Model 440) with a fixed wavelength of 254 nm. The column was kept in isopropanol (10% in water) or methanol (100%) when not in use for more than two days. Chromatograms were recorded on an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). An IEC clinical centrifuge (International Equipment, Needham, MA, U.S.A.) was employed to separate protein precipitate from methanolic supernatant.

A spectrophotometer (Varian, DMS 100S, UV–VIS, equipped with a plotter and Epson LX-80 printer) was employed to scan the wavelength maximum of each cephalosporin.

Chromatographic conditions

The mobile phase comprised sodium phosphate buffer (20 m*M*; pH 4.5)–methanol (77:23) and was freshly prepared and degassed by filtration. The

eluent was allowed to flow at 1.0 ml/min at room temperature (25°C), and the chromatogram was monitored at a chart speed of 0.25 cm/min.

Sample preparation

Stock solutions of CXM, CFX (both at 100 mg/ml) and DACXM (50 mg/ml) were prepared in phosphate buffer (20 m*M*; pH 5). A series of blank plasma was spiked with each compound to give final concentrations of 2.5, 10, 50, 200 and 500 μg/ml (for calibration curves) and 10 and 200 μg/ml (for analytical studies), with an internal standard (cephradine, 20–500 μg/ml) added. The overall volume of the drug solutions added to plasma was less than 20 μl.

Rat plasma (100 μl) and methanol (200 μl) were mixed, vortexed for 5 s and centrifuged (2000 *g*; 10 min). The resulting supernatant was then injected (20 μl) into the HPLC system.

Calibration and calculation

Five calibration samples of varying concentration were used to construct a calibration curve by plotting peak-height ratios (drug/internal standard) against the drug concentrations. Methods of precision and accuracy described by Pachla et al. [12] were adopted to validate the assay.

Animal studies

Two male Wistar rats (330 g) were each given an intravenous bolus injection of a mixture of CXM and CFX (each 100 mg/kg) in a sterile, non-pyrogenic saline solution (Travenol Labs., Sydney, Australia). Blood samples (1 ml) were collected by heart puncture (25-gauge needle) at various time intervals of 2, 5, 10, 20, 35, 60, 90, 120, 150, 180, 210 and 240 min after the drug administration.

Plasma was obtained by centrifugation (1500 *g*; 5 min) of blood in tubes containing 65 USP units of lithium heparin (Terumo, Tokyo, Japan) and kept frozen (−20°C; upright position) overnight before analysis of total drug content. A portion of plasma (300 μl) was immediately ultrafiltered (YMT membrane; Amicon, Danvers, MA, U.S.A.) by centrifugation (500 *g*; 15 min) to obtain protein-free plasma water for unbound drug analysis. The filtrate was then stored frozen overnight in an upright position.

RESULTS AND DISCUSSION

Wavelength maxima of the cephalosporins

When CFX (10 μg/ml), CXM (10 μg/ml) and DACXM (20 μg/ml) in the mobile phase were scanned, they gave wavelength maxima (and absorbances) at 240 (0.588), 235 (0.425) and 235 (0.768) nm, respectively. By employing a 254-nm filter, hence, the loss of sensitivity for each compound was less than 10%. With a filter of 280 nm the sensitivities were further reduced, by 14% for CFX and almost 50% for both CXM and DACXM.

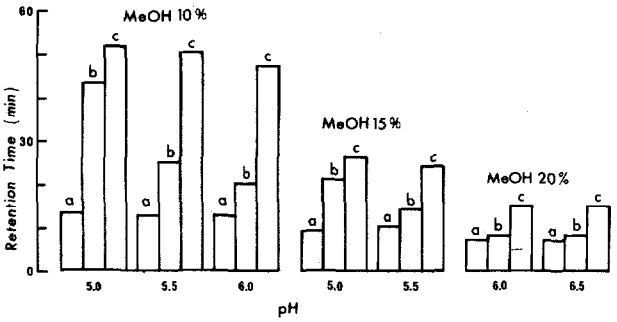

Fig. 1. Effect of pH on the retention time of the cephalosporins. (a) Desacetylcefotaxime; (b) ceftriaxone; (c) cefotaxime.

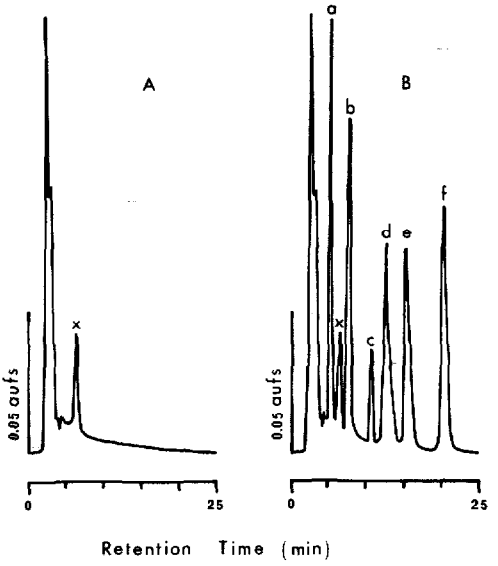

Fig. 2. Chromatographic separation of the cephalosporins. (A) Blank plasma; (B) spiked plasma. Peaks: a=desacetylcefotaxime (5.4 min); b=ceftriaxone (7.6 min); c=internal standard, cephradine (11.6 min); d=cefotaxime (14.6 min); e=sodium barbital (15.4 min); f=caffeine (21.6 min); x=plasma component. The compounds were eluted with sodium phosphate buffer (20 m*M*; pH 4.5)-methanol (77:23) at a flow-rate of 1 ml/min at 25°C, monitored at 254 nm, and recorded at 0.05 a.u.f.s.

Effect of pH and methanol on retention time

At various concentrations of methanol the most affected compound by a change of mobile phase pH is CFX, except at 20% methanol at pH 6.0–6.5 (Fig. 1), whereas CXM and DACXM are not markedly affected. When the pH is raised from 5.0 to 6.0 (10% methanol) there is a two-fold decrease in retention time of CFX, but only a 10% decrease for CXM, and no alteration for DACXM.

These results noted for CFX indicate that at higher pH CFX molecules become more polar and hence less bound to the hydrophobic moieties in the column. CFX

TABLE I

WITHIN-DAY AND DAY-TO-DAY PRECISION OF THE METHOD

Each concentration ($n=10$) was prepared and assayed twice a day.

Compound	Concentration added (μg/ml)	Coefficient of variation (%)	
		Within-day	Day-to-day
Cefotaxime	10	1.12	4.85
	200	0.58	1.80
Desacetylcefotaxime	10	0.90	4.39
	200	0.65	1.55
Ceftriaxone	10	1.42	5.27
	200	0.24	1.58

TABLE II

ACCURACY OF THE METHOD

Compound	Concentration added (μg/ml)	Recovery (mean ± S.D.) (%)	Coefficient of variation (%)
Cefotaxime	10	101.80 ± 4.66	4.58
	200	95.68 ± 0.83	0.87
Desacetylcefotaxime	10	101.62 ± 9.01	8.86
	200	99.61 ± 0.79	0.79
Ceftriaxone	10	95.60 ± 4.97	5.20
	200	98.23 ± 2.39	2.43

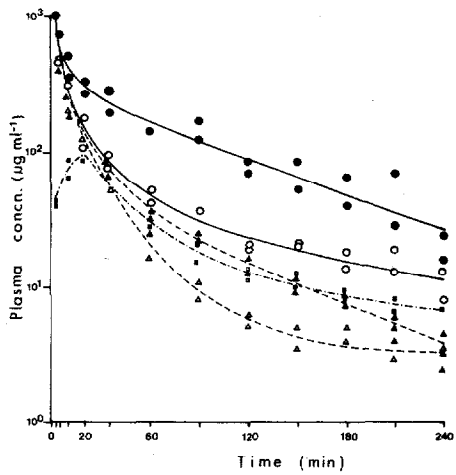

Fig. 3. Semilogarithmic plot of total and free cephalosporins in plasma following a single intravenous injection of CFX plus CXM (each 100 mg/kg) to the rats ($n=2$). (●) Total CFX; (○) free CFX; (▲) total CXM; (△) free CXM; (□) total DACXM.

has been reported to have three pK_a values of about 2.6–3 (COOH), 3.2 (NH_3^+) and 4.1 (enolic OH) [4, 13]. However, with pK_a values of 2.1, 3.4 and 10.9 [14], CXM appears to have the strongest binding to the stationary phase under these conditions as compared with the other cephalosporins.

It was noted that by doubling the concentration of methanol (pH 6.0) CFX and CXM are eluted faster by factors of 2.5- and 3-fold, respectively. DACXM is only moderately decreased in its retention time (by 78%) under this operating condition.

The results indicate that the chromatography of CFX is affected by both pH and methanol concentration, whereas CXM and DACXM are prone to alteration by methanol concentration while remaining relatively unaffected by pH.

Selectivity

The cephalosporins studied are well separated from each other giving retention times of 5.4, 7.6, 11.6 and 14.6 min for DACXM, CFX, cephradine (internal standard) and CXM, respectively. As seen in Fig. 2, no interference is noted from other components of plasma.

Neither barbital (a hypnotic agent) or caffeine (a cardiac stimulant) which may under certain circumstances be present in plasma, interfered with the analysis of the cephalosporins. The retention times for barbital and caffeine were 15.4 and 21.6 min, respectively.

Other metabolites of CXM (DACXM lactone and unknown polar metabolites) which are commonly present in human and dog plasma [6, 15] are absent from rat plasma [6].

Calibration curve

Five calibration curves were performed throughout the studies, and the resulting curves were found to be linear ($r=0.999$) for concentrations ranging from 2.5 to 500 μg/ml for each drug. The coefficients of variation of the slopes were less than 5% (CFX and CXM) or 10.78% (DACXM).

Sensitivity

Under the previously described assay conditions the limits of detection for DACXM, CFX and CXM were 100, 250 and 250 ng/ml of plasma, respectively, at 0.005 a.u.f.s. However, these limits may depend on the condition of the column and detector noise level [10]. In this regard we have found that under normal operating conditions the ratios of signal-to-noise level were 20:1 (DACXM), 5:1 (CFX) and 8:1 (CXM) when a relatively new column (aged one month) was used. In our experience with rats, a sensitivity of 500 ng/ml for each compound is sufficient to measure the cephalosporins in plasma for up to 4 h (more than five plasma half-lives) after a single intravenous injection (100 mg/kg).

Precision and accuracy

The cephalosporins were added to blank plasma as described under sample preparation to give final concentrations of 10 and 200 μg/ml. For within-day vari-

ation studies, ten replicates of each concentration were prepared, and this procedure was repeated three times (day-to-day variation studies).

It can be seen (Table I) that at both concentrations, the difference in the coefficient of variation for each compound which was determined on two occasions on the same day is usually less than 2%. The difference in variation from day to day for each compound is less than 6%. The peak-height variations of the internal standard of 4.6% (at 10 μg/ml) and 6.61% (at 200 μg/ml) might have contributed to these random errors.

The accuracy of the entire determinations is presented in Table II. The amount of each compound recovered from plasma samples is more than 95% of that added. The coefficient of variation was less than 10 and 3% of the added values for the low and high concentrations, respectively. For pharmacokinetic and biopharmaceutical studies, the precision and accuracy obtained by the present method are thus adequate [12].

Animal studies

The results of a preliminary pharmacokinetic study are presented in Fig. 3. Following concomitant administration of CFX and CXM, the plasma concentration–time profile of both drugs and DACXM show a two-compartment decay as judged by non-linear least-squares analysis with the NONLIN program [16] modified to run on a PDP-11 [17].

The total plasma concentrations of CFX, CXM and DACXM were 18.7, 1.28 and 4.77 μg/ml, respectively, 4 h after injection, whereas the respective unbound concentrations were 4.79, 0.66 and 4.65 μg/ml. The results of the pharmacokinetic studies will be reported elsewhere.

REFERENCES

1 M.J. Hall, D. Westmacott and P. Wong-Kai-In, J. Antimicrob. Chemother., 8 (1981) 193.
2 J.D. Coombes, Rev. Infect. Dis., 4 (Suppl.) (1982) S325.
3 J.T. Doluisio, Rev. Infect. Dis., 4 (Suppl.) (1982) S333.
4 L. Balant, P. Dayer and R. Auckenthaler, Clin. Pharmacokin., 10 (1985) 101.
5 H.B. Lassman and J.D. Coombes, Diagn. Microbiol. Infect. Dis., 2 (1984) 3S.
6 J. Chamberlain, J.D. Coombes, D. Dell, J.M. Fromson, R.J. Ings, C.M. Macdonald and J. McEwen, J. Antimicrob. Chemother., 6 (Suppl. A) (1980) 69.
7 T. Bergan and R. Solberg, Chemotherapy, 27 (1981) 155.
8 F. Kees, E. Strehl, K. Seeger, G. Seidel, P. Dominiak and H. Grobecker, Arzneim.-Forsch., 31 (1981) 362.
9 J.B. Lecaillon, M.C. Rouan, C. Souppart, N. Febvre and F. Juge, J. Chromatogr, 228 (1982) 257.
10 D. Dell, J. Chamberlain and F. Coppin, J. Chromatogr., 226 (1981) 431.
11 R.L. Yost and H. Derendorf, J. Chromatogr., 341 (1985) 131.
12 L.A. Pachla, D. Scott Wright and D.L. Reynolds, J. Clin. Pharmacol., 26 (1986) 332.
13 G.G. Raymond and J.L. Born, Drug Intell. Clin. Pharm., 20 (1986) 683.
14 H. Fabre, N.H. Eddine, G. Berge and M.D. Blanchin, J. Pharm. Sci., 74 (1985) 85.
15 D.S. Reeves, L.O.White, H.A. Holt, D. Bahari, M.J. Bywater and R.P. Bax, J. Antimicrob. Chemother, 6 (Suppl. A) (1980) 93.
16 C.M. Metzler, G.L. Elfring and A.J. McEwen, Biometrics, 30 (1974) 562.
17 D.W.A. Bourne and A.W. Wright, Aust. J. Pharm. Sci., 10 (1981) 23.