Journal of Chromatography, 228 (1982) 257—267
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1160

DETERMINATION OF CEFSULODIN, CEFOTIAM, CEPHALEXIN, CEFOTAXIME, DESACETYL-CEFOTAXIME, CEFUROXIME AND CEFROXADIN IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J.B. LECAILLON*, M.C. ROUAN, C. SOUPPART, N. FEBVRE and F. JUGE

Ciba-Geigy, Centre de Recherche Biopharmaceutique, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

(Received September 2nd, 1981)

SUMMARY

Closely related methods for the determination of several cephalosporins in plasma and urine are described. Deproteinized plasma or diluted urine is directly injected on a RP-8 or RP-18 bonded-material column. Chromatography is performed either in the reversed-phase or the ion-pair mode. The limits of sensitivity range from 0.4 to 2 μmol of cephalosporins per liter of plasma, and from 20 to 100 μmol per liter of urine. The sensitivity may be improved two to five times by using precolumn loading, direct sample clean-up and automatic injection. The stability of the cephalosporins in plasma, urine and water and the reproducibility and accuracy of the methods are reported.

INTRODUCTION

Pharmacokinetic studies on cephalosporins have mostly been carried out up to now by microbiological determination of the concentrations in plasma and urine. This technique appears convenient when drug concentrations are higher than 2 μg/ml, but it becomes less accurate at lower concentrations. In addition, its lack of specificity often does not permit the determination of the unchanged drug in the presence of its metabolites, or when several antibiotics are administered concomitantly. High-performance liquid chromatography (HPLC) can solve these problems, and several HPLC methods for determining cephalosporin concentrations in biological fluids have been already reported [1—6]. This paper describes a set of methods for the determination of cephalosporins either simultaneously or individually in plasma and urine. The sample pretreatment is short and the injection into the column automatically controlled.

0378-4347/82/0000—0000/$02.75

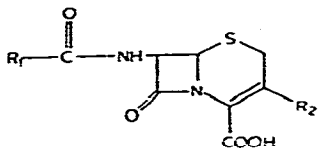
EXPERIMENTAL

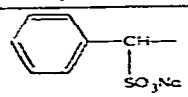
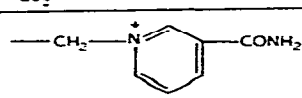
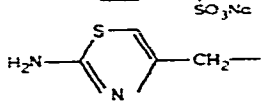
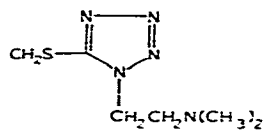
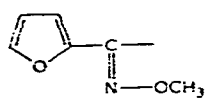
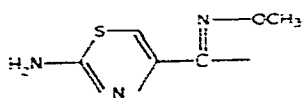
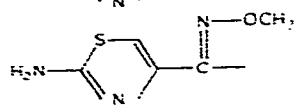
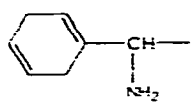
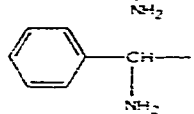
Chemicals and reagents

Cefsulodin, cefotiam, cephalexin, cefotaxime and its desacetyl metabolite, cefuroxime and cefroxadin were used as supplied (Table I). Tetrabutylammonium hydrogen sulphate (THBS) was obtained from Sigma (St. Louis, MO, U.S.A.) and sodium dodecyl sulphate (SDS) from Fluka (Bucks, Switzerland). All other chemicals were of analytical grade.

TABLE I

CHEMICAL STRUCTURE OF THE STUDIED COMPOUNDS



Cephalosporins	R_1	R_2	Supplier
Cefsulodin			Ciba-Geigy, Basle, Switzerland
Cefotiam			Ciba-Geigy, Basle, Switzerland
Cefuroxime		$-CH_2-O-\overset{O}{\overset{\|}{C}}-NH_2$	Glaxo, Paris, France
Cefotaxime		$-CH_2-O-\overset{O}{\overset{\|}{C}}-CH_3$	Glaxo, Hoechst, Frankfurt, G.F.R.
Desacetyl-cefotaxime		$-CH_2-OH$	Roussel Uclaf, Romainville, France
Cefroxadin		$-O-CH_3$	Ciba-Geigy, Basle, Switzerland
Cephalexin		$-CH_3$	Glaxo, Paris, France

Sample treatment

Two aliquots of the plasma or urine sample are introduced into two 10-ml conical glass centrifuge tubes. Plasma proteins are precipitated and urine diluted with convenient solutions in the ratios reported in Table II. In methods

TABLE II

PLASMA DEPROTEINIZATION AND URINE DILUTION

Method*	Volumes of reagents added to:						
	200 µl of plasma				10 µl of urine		
	H_3PO_4 0.45 *N* (µl)	Methanol (µl)	TCA** 10% (w/v) (µl)	Internal standard solution (µl)	H_2O (µl)	TCA** 10% (w/v) (µl)	Internal standard solution (µl)
1	20	100	—	20	1000		20
2	20	100	—	20	500		20
3	20	100	—	20	500		20
4	20	100	—	—	1000		—
5	—	—	70	—	100	10	—
6—7	—	—	70	20	100		20

*See Table III.
**Trichloracetic acid aqueous solution.

1, 2, 3, 6 and 7 (Table III), cephalexin (270 µmol/l) is added as internal standard. No internal standard is used for methods 4 and 5.

After 15 sec on a Vortex mixer and 3 min centrifugation for plasma, 100 µl of the clear solution are transferred to a small conical injection vial; 20 µl of this solution are automatically injected on the column.

Chromatography

Chromatography is performed on a Hewlett-Packard high-pressure liquid chromatograph (Model 1081B) equipped with a fixed-wavelength (254 nm) UV detector, connected to a Spectra-Physics computing integrator (Model 4100).

A precolumn (stainless-steel tube, 10 cm × 4.7 mm I.D., tap-filled with Co:Pell ODS; Whatman, Ferrières, France) is used to protect the analytical column.

The analytical column is filled with LiChrosorb RP-18 (or RP-8 for cefroxadin and cefsulodin) 5 µm particle size (Merck, Paris, France). The slurry is made by suspending 17% (w/v) of the reversed-phase material in the solvent methanol—sodium acetate (10^{-2} mol/l aqueous solution) (80:20, v/v). The filling is performed with a pneumatic Haskel pump using methanol under 300 bars as pressurizing solvent. Before use, the precolumn and the column, connected to the chromatograph, are rinsed with water (except for cefotiam and desacetyl-cefotaxime).

Column and mobile-phase characteristics are given in Table III. The salt aqueous solution is filtered through a Millipore filter (0.65 µm pore size, type DAWP) before being mixed with methanol. The mobile phase is warmed and degassed under vacuum before use. Chromatography is performed at room temperature except for cefotiam (method 5, Table III) for which the column is maintained at 40°C. The flow-rate of the mobile phase is 2 ml/min for cefroxadin and 1 ml/min for all other compounds.

The retention times of the cephalosporins recorded in the various methods

TABLE III

CHROMATOGRAPHIC CONDITIONS

Method	Compounds	Column characteristics			Mobile phase	
		Length (cm)	Diameter (mm)	Stationary phase	Methanol—salt aqueous solution (v/v)	Salt aqueous solution (mol/l)
1	Cefotiam Cefsulodin Cephalexin*	15	4.7	RP-8	15:85	THBS 10^{-1} K_3PO_4 $9 \cdot 10^{-2}$
2	Cefotiam Cefuroxime Cephalexin*	15	4.7	RP-18	23:77	THBS $2 \cdot 10^{-2}$ K_3PO_4 $2.4 \cdot 10^{-2}$ KH_2PO_4 $1.6 \cdot 10^{-2}$
3	Cefotiam Cefotaxime Cephalexin*	12	4.7	RP-18	20:80	THBS 10^{-1} K_3PO_4 $2 \cdot 10^{-2}$ KH_2PO_4 $2 \cdot 10^{-2}$
4	Desacetyl-cefotaxime	15	4.7	RP-18	15:85	THBS 10^{-2} K_3PO_4 $9 \cdot 10^{-3}$
5	Cefotiam	20	4.7	RP-18	24:76	NaH_2PO_4 $1.4 \cdot 10^{-3}$ Na_2HPO_4 $2.5 \cdot 10^{-2}$
6	Cefroxadin Cephalexin* in plasma	10	7.5	RP-8	28:72	H_3PO_4 $2 \cdot 10^{-3}$
7	Cefroxadin Cephalexin* in urine	10	7.5	RP-8	30:70	H_3PO_4 10^{-3}

*Internal standard.

TABLE IV

RETENTION TIMES

Method*	Retention time (min)						
	Cefotiam	Cefuroxime	Cefotaxime	Desacetyl-cefotaxime	Cephalexin	Cefsulodin	Cefroxadin
1	26				16	8	14
2	23	12			19		
3	23		10	4	14		
4			40	12			
5	12						
6					18		12
7					18		14

*See Table III.

are reported in Table IV. Typical chromatograms are shown in Figs. 1—6.

Calibrations and calculations

Plasma and urine are spiked with convenient cephalosporin solutions to produce concentrations in the range of 1—400 μmol/l for plasma and 20—8000 μmol/l for urine. Calibration samples are then worked up as described for the clinical samples (Table II). Four to five calibration samples are handled to construct the calibration curve which is obtained by plotting the peak-area ratios of cephalosporin (or the peak areas of the cephalosporin for methods 4 and 5) against the concentrations on a log—log graph. The regression line obtained in most cases had a correlation coefficient better than 0.9990.

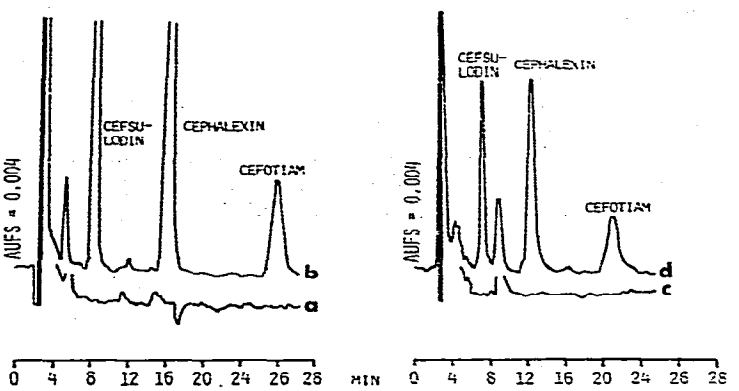


Fig. 1. Cefsulodin/cefotiam, method 1. Column: RP-8, 5 μm, 15 cm × 4.7 mm I.D. Mobile phase: methanol 15% in salt aqueous solution (THBS 10^{-1} mol/l, K_3PO_4 $9 \cdot 10^{-2}$ mol/l). Temperature 25°C. (a) Plasma blank; (b) plasma spiked with cefsulodin (100 μmol/l), cephalexin (270 μmol/l) and cefotiam (40 μmol/l); (c) urine blank; (d) urine spiked with cefsulodin (900 μmol/l), cephalexin (2700 μmol/l) and cefotiam (650 μmol/l).

RESULTS

Specificity

The cephalosporins studied were well separated from plasma or urine components (Figs. 1—6). In most cases, they did not interfere with one another,

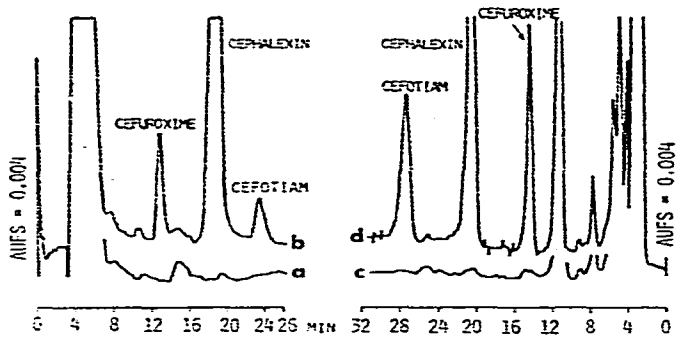


Fig. 2. Cefotiam/cefuroxime, method 2. Column RP-18, 5 μm, 15 cm × 4.7 mm I.D. Mobile phase: methanol 23% in salt aqueous solution (THBS 2.10^{-2} mol/l, K_3PO_4 $2.4\cdot10^{-2}$ mol/l, KH_2PO_4 $1.6\cdot10^{-2}$ mol/l). Temperature 25°C. (a) Plasma blank; (b) plasma spiked with cefuroxime (6 μmol/l), cephalexin (270 μmol/l) and cefotiam (3.25 μmol/l); (c) urine blank; (d) urine spiked with cefuroxime (600 μmol/l), cephalexin (2700 μmol/l) and cefotiam (650 μmol/l).

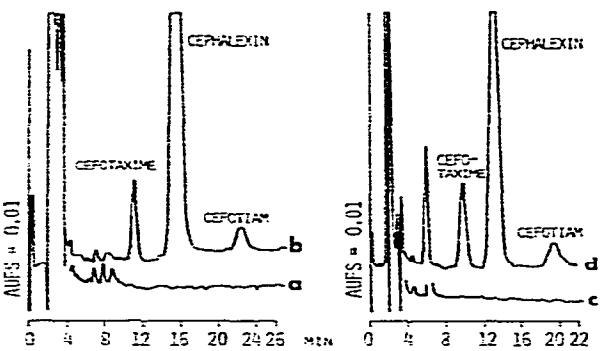


Fig. 3. Cefotiam/cefotaxime, method 3. Column: RP-18, 5 μm, 12 cm × 4.7 mm I.D. Mobile phase: methanol 20% in salt aqueous solution (THBS 10^{-2} mol/l, K_3PO_4 $2\cdot10^{-2}$ mol/l, KH_2PO_4 $2\cdot10^{-2}$ mol/l). Temperature 25°C. (a) Plasma blank; (b) plasma spiked with cefotaxime (5.5 μmol/l), cephalexin (270 μmol/l) and cefotiam (3.25 μmol/l); (c) urine blank; (d) urine spiked with cefotaxime (220 μmol/l), cephalexin (2700 μmol/l) and cefotiam (130 μmol/l).

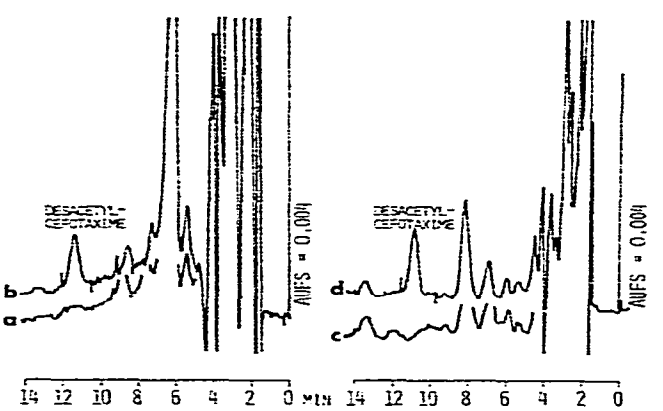


Fig. 4. Desacetyl-cefotaxime, method 4. Column RP-18, 5 μm, 15 cm × 4.7 mm I.D. Mobile phase: methanol 15% in salt aqueous solution (THBS 10^{-2} mol/l, K_3PO_4 $9\cdot10^{-3}$ mol/l). Temperature 25°C. (a) Plasma blank; (b) plasma spiked with desacetyl-cefotaxime (1.2 μmol/l); (c) urine blank; (d) urine spiked with desacetyl-cefotaxime (120 μmol/l).

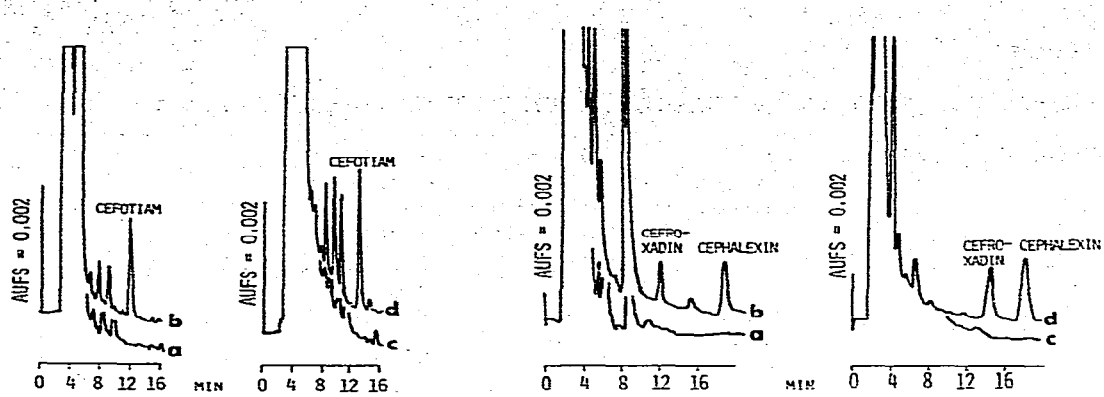


Fig. 5. Cefotiam, method 5. Column RP-18, 5 μm, 20 cm × 4.7 mm I.D. Mobile phase: methanol 24% in salt aqueous solution (NaH_2PO_4 $1.4 \cdot 10^{-3}$ mol/l, Na_2HPO_4 $2.5 \cdot 10^{-2}$ mol/l). Temperature 40°C. (a) Plasma blank; (b) plasma spiked with cefotiam (2 μmol/l); (c) urine blank; (d) urine spiked with cefotiam (20 μmol/l).

Fig. 6. Cefroxadin, methods 6 and 7. Column: RP-8, 5 μm, 10 cm × 7.5 mm I.D. Mobile phase: methanol 28% for plasma and 30% for urine in phosphoric acid solution ($2 \cdot 10^{-3}$ mol/l for plasma and 10^{-3} mol/l for urine). (a) Plasma blank; (b) plasma spiked with cefroxadin (1.2 μmol/l) and cephalexin (1.4 μmol/l); (c) urine blank; (d) urine spiked with cefroxadin (50 μmol/l) and cephalexin (58 μmol/l).

and could be determined simultaneously by several methods. A small number of patients, mainly suffering from renal insufficiency, presented chromatographic peaks in plasma or urine that interfered with those of the cephalosporins to be determined. In these cases, the chromatographic method was changed: for example, cefroxadin can be determined by methods 6 and 1, cefotiam by methods 5 and 2, etc. Most interference problems have been solved in this way.

Reproducibility, accuracy and sensitivity

Several series of six assays of different plasma and urine samples spiked with the same concentrations were determined. Table V shows the reproducibility of the methods. As the concentration decreased, individual determinations became more scattered. The sensitivity limit was taken as the lowest concentration that gave a coefficient of variation inferior or equal to 10% for six assays. The limits were 1 μmol per liter of plasma and around 50 μmol per liter of urine.

Comparison with the microbiological method

Series of samples derived from clinical experiments with cefsulodin, cefotiam, cefroxadin and cephalexin were assayed by both the HPLC and the microbiological methods.

Correlation coefficients given by linear regression analysis of the results obtained with the two methods ranged from 0.983 to 0.996, and the slopes of the regression lines from 0.95 to 1.07. Concentrations below 3 μmol per liter of plasma were below the limit of sensitivity of the microbiological method and were not included in this comparison.

TABLE V

REPRODUCIBILITY AND ACCURACY OF DETERMINATIONS IN PLASMA

Compound	Method	Added concentration (μmol/l)	Mean % of actual concentration	Coefficient of variation (%) (n = 6)
Cefsulodin	1	36	96	1.9
		1.8	100	6.1
		0.9	98	11.5
Cefuroxime	2	329	99	1.4
		11.2	106	2.9
		2.3	105	9.3
Cefotaxime	3	421	100	0.6
		2.3	106	3.4
		1.2	103	7.2
Desacetyl-cefotaxime	4	121	102	2.8
		2.4	98	8.6
		1.2	102	9.1
Cefotiam	5	38	99	2.0
		1.0	102	6.8
		0.4	100	10
Cefroxadin	6	31	103	4.0
		6.2	101	1.8
		1.6	96	2.5
Cephalexin*	6	23	101	2.9
		2.9	99	5.1
		1.4	100	9.7

*Without internal standard, but if needed cefroxadin can be used as internal standard.

TABLE VI

MAXIMUM STORAGE TIME OF CEPHALOSPORIN SOLUTIONS WHICH PRODUCED LESS THAN 5% DEGRADATION

Compound	Calibration solutions at 5°C (days)	Treated sample on the autosampler		Frozen sample at −20°C	
		Plasma (hours)	Urine (hours)	Plasma (months)	Urine (months)
Cefsulodin	7	4	10	1.5	>3
Cefotiam	14	5	5	>9	>9
Cefuroxime	14	>5	>5	>3	>2
Cefotaxime	14	>5	>5	>3	>3
Desacetyl-cefotaxime	14	>5	>5	—	—
Cefroxadin	21	>6	12	9	3
Cephalexin	21	>6	>6	9	3

Storage stability

The stability of cephalosporin solutions depends on several factors: solvent temperature, pH of the solution and addition of sodium dodecyl sulphate [7, 8]. The stability was studied in three instances: in the calibration solutions, in the worked-up clinical samples stored on the automatic sampler, and in frozen plasma and urine samples. The storage times that produced a degradation of around 5% are reported in Table VI. It appears that cefsulodin plasma samples are stable for only about 1.5 months at −20°C, but it is possible to increase the storage stability by pH adjustment: addition of a pH 5 buffer to cefsulodin plasma samples before freezing keeps the samples stable for three months.

DISCUSSION

The chromatographic behaviour of cephalosporins on reversed-phase material as a function of the composition of the mobile phase has been reported previously [9—11]. The chromatographic conditions mentioned above were the best compromise found between specificity and duration of analysis. The addition of an ion-pair reagent in methods 1—4 permitted the separation of the most polar compounds (cefsulodin and desacetyl-cefotaxime) from plasma components. It also made it possible to determine simultaneously two cephalosporins that are difficult to separate in the reversed-phase mode. As there was no extraction of the drug from the biological fluid, and as the injected volume was found to be reproducible from one injection to another, an internal standard is not absolutely necessary. Nevertheless, as the chromatographic or detection conditions might undergo fluctuations not discernible without reference to an internal standard, in methods 4 and 5 without an internal standard, a calibration curve was constructed each day, and validation samples prepared from solutions different from those used for calibration were assayed at the start and the end of each series of determinations. With an internal standard, calibration was only performed every two or three weeks, and the method validated only at the start of the series of determinations.

The sensitivities reported for the proposed procedures were largely related to the volume of sample injected on the column. This volume was chosen to permit a column life of about three weeks (about 400 injections). It is possible to inject a larger volume with column protection as shown in Fig. 7. In this way, the sensitivity can be increased by a factor of 2—5. A small precolumn (10 mm × 7.5 mm I.D.) was introduced in place of the loop of a Valco valve. Then 50—100 µl of plasma with internal standard added were injected with a Technicon autosampler, loaded on the precolumn head, and rinsed with water at a flow-rate of 100—300 µl/min with a Technicon peristaltic pump. Automatization of the whole system and the start of integration were actuated by a Valco digital valve-sequence programmer. The compounds were then back-eluted with the mobile phase by means of the valve pneumatically actuated by the programmer.

The precolumn dry-packed with LiChroprep RP-8, 25—40 µm, was changed every day (20—30 injections). In the case of method 1, a negative peak due to the water rinse interfered with the internal standard; this method was thus used without internal standard.

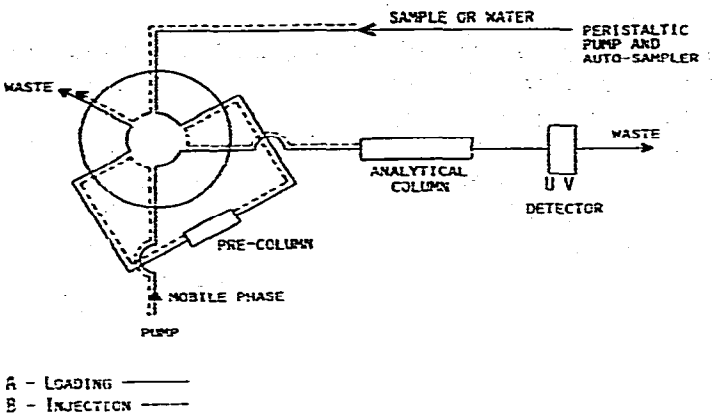


Fig. 7. Precolumn loading: (A) 100-µl sample loaded on the precolumn (RP-8, 25—40 µm, 1 cm × 7.5 mm I.D.) and rinsed with water; (B) sample back-eluted in the inject position and injected on the analytical column.

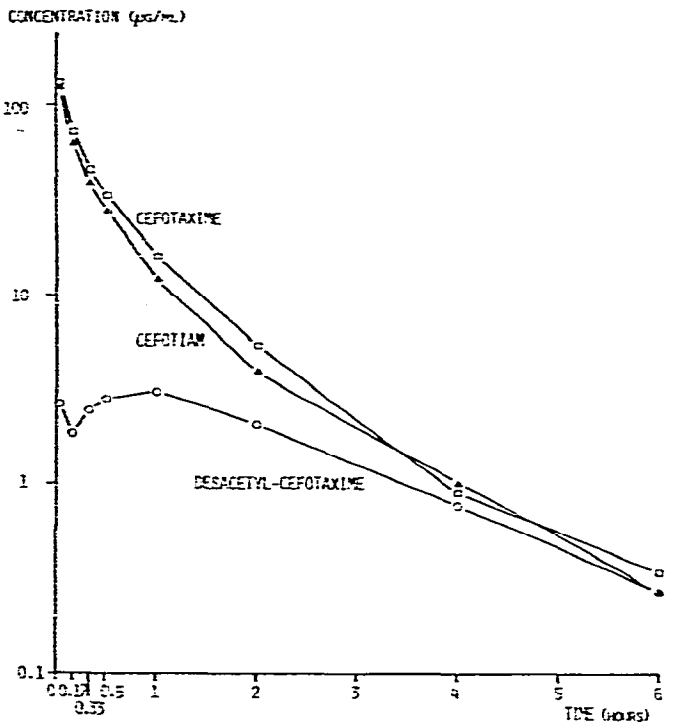


Fig. 8. Semi-logarithmic plots of concentrations in plasma after concomitant intravenous administration of 1 g of cefotiam and 1 g of cefotaxime to one healthy volunteer.

CONCLUSION

The sensitivity and specificity of the described methods make them advantageous when precise analyses have to be made of the pharmacokinetics of antibiotics administered singly or concomitantly [12, 13]. An example

is given in Fig. 8, which shows the plasma concentration—time curves obtained for cefotiam, cefotaxime and its desacetylated metabolite, when the two cephalosporins were administered simultaneously to a volunteer.

REFERENCES

1 L.O. White, H.A. Holt, D.S. Reeves, M.J. Bywater and R.P. Bax, in J.D. Nelson and C. Grassi (Editors), Proceedings of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, The American Society for Microbiology, Washington, DC, 1980, p. 153.
2 N. Wright and R. Wise, in J.D. Nelson and C. Grassi (Editors), Proceedings of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, The American Society for Microbiology, Washington, DC, 1980, p. 133.
3 I. Nilsson-Ehle and P. Nilsson-Ehle, Clin. Chem., 24 (1978) 365.
4 T. Nakagawa, J. Haginaka, K. Yamaoka and T. Uno, J. Antibiot., 31 (1978) 769.
5 M.A. Carroll, E.R. White, Z. Jancsik and J.E. Zarembo, J. Antibiot., 30 (1977) 397.
6 D. Dell and R.M.J. Ings, Arzneim.-Forsch., 28 (1978) 940.
7 T. Yamana and A. Tsuji, J. Pharm. Sci., 65 (1976) 1563.
8 J.M. Broughall, M.J. Bywater, H.A. Holt and D.S. Reeves, J. Antimicrob. Chemother., 5 (1979) 471.
9 M.C. Rouan, F. Abadie and F. Jugé, Vth International Symposium on Column Liquid Chromatography, Avignon, 1981.
10 F. Salto, J.G. Prieto and M.T. Alemany, J. Pharm. Sci., 69 (1980) 501.
11 E.R. White, M.A. Carroll and J.E. Zarembo, J. Antibiot., 30 (1977) 811.
12 J.L. Hirtz, J.B. Lecaillon, A. Gerardin, J.P. Schoeller, G. Humbert and J. Guibert, in J.D. Nelson and C. Grassi (Editors), Proceedings of the 11th International Congress of Chemotherapy and the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, The American Society for Microbiology, Washington, DC, 1980, p. 211.
13 J.B. Lecaillon, J.L. Hirtz, J.P. Schoeller, G. Humbert and W. Vischer, Antimicrob. Ag. Chemother., 18 (1980) 656.