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DETERMINATION OF CEFOTAXIME AND DESACETYLCEFOTAXIME IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the analysis of the anti-bacterial agent cefotaxime and desacetylcefotaxime in physiological fluids. Plasma or serum samples were mixed with chloroform—acetone to remove proteins and most lipid material. The aqueous phase was then freeze-dried, reconstituted in mobile phase and chromatographed on a reversed-phase column using UV detection at 262 nm. Urine was analysed directly after centrifugation to remove particulate matter. The detection limit was 0.5—1.0 μg/ml for serum and 5 μg/ml for urine. The method has been applied to the analyses of cefotaxime and desacetylcefotaxime in plasma, serum, urine, cerebrospinal fluid, saliva, and pus from infected wound secretions. Two additional metabolites, which are lactones in which the β-lactam ring has been opened, could be separated by this method.

INTRODUCTION

Cefotaxime (sodium 7-[{[2(2-amino-4-thiazolyl)-2-methoxyimino]acetyl}-amino]-3-acetoxymethyl-8-oxo-5-thia-1-azatricyclo-[*4,2,0*]-octene 2-carboxylate, developed by Hoechst, Frankfurt, G.F.R., and Roussel-Uclaf, Paris, France) is a semi-synthetic cephalosporin, active against a variety of gram-positive and gram-negative bacteria, including some strains resistant to other antibiotics such as ampicillin, tetracycline and chloramphenicol [1]. Microbiological methods are widely used for the analysis of penicillins and cephalosporins. For cefotaxime, the organism *Escherichia coli* V6311 provides a sensitive (0.1 μg/ml) assay method [2] but it is not completely specific since the organism is sensitive to desacetylcefotaxime. *Proteus morganii* NCTC 11354 is metabolite resistant and will measure cefotaxime even in the presence of high metabolite concentrations [3].

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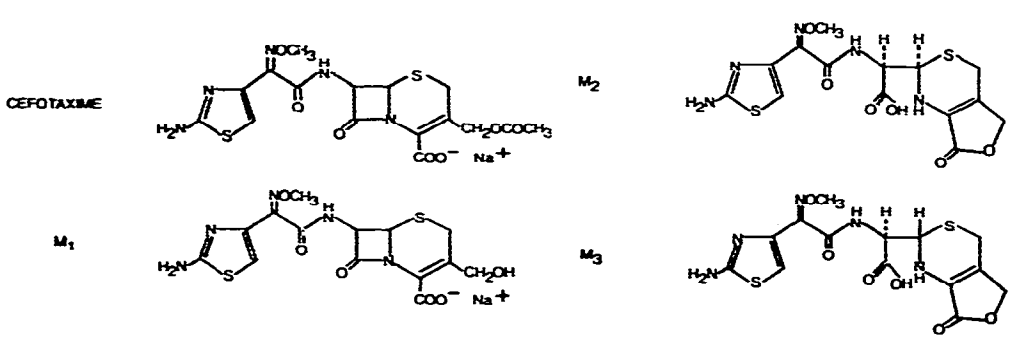
An alternative method of assay which would allow the determination of metabolites in the presence of cefotaxime was required for biological fluids. A reversed-phase high-performance liquid chromatographic (HPLC) method was developed. HPLC has been used for the determination in biological fluids of most of the commonly prescribed cephalosporins including cephaloridine [4], cephalothin [5], cefazolin [5], cefuroxime [6, 7], cefoxitin [8], cefatrizine [9], cephaloglycin [10], and cephalexin [11, 12]. Although the aqueous solubility of cephalosporins is generally too high to allow their extraction by organic solvents from biological fluids, an ion-pair method [13] in which cephalothin and desacetylcephalothin are extracted from biological specimens with tetraheptylammonium chloride has been used to determine these compounds in serum. In our laboratories a reversed-phase method was developed for the analysis of a semi-synthetic cephalosporin, HR 580 (Hoechst), in serum and in urine [14]. Preliminary details of methods for the analysis of cefotaxime and metabolites have been reported [3, 15—17].

In the methods described by Reeves et al. [15], White et al. [3] and Borner and Lode [16], accuracy, precision and recovery data are not reported. Bergan and Solberg [17] report accuracy data for cefotaxime at 10 μg/ml. The present paper reports accuracy and precision for both cefotaxime and desacetylcefotaxime in plasma and urine over a wide concentration range. Plasma sample preparation procedures are discussed in relation to the suitability of the various methods for use with autoinjectors. The reversed-phase method described allows separation of cefotaxime from the metabolites M_2 and M_3 as well as from desacetylcefotaxime (M_1). Evidence for the structure of the epimeric lactones M_2 and M_3 has been presented in a separate publication [18].

The method reported below has been applied to the analysis of cefotaxime, M_1, M_2, and M_3 in plasma, serum, urine, cerebrospinal fluid, saliva and pus from infected wound secretions, from patients receiving intramuscular and intravenous doses of cefotaxime.



EXPERIMENTAL

Apparatus and materials

The chromatographic system consisted of a reciprocating pump, a variable-wavelength detector (262 nm), an automatic injector (WISP, Waters Assoc., Northwich, Great Britain), and a computing integrator (SP 4100, Spectra

Physics, St. Albans, Great Britain). The separation column was a 100 mm × 3 mm I.D. stainless-steel tube packed with ODS Spherisorb (5 μm diameter, Phase Separations, Queensferry, Great Britain). The mobile phase consisted of a mixture of methanol—water—glacial acetic acid (12:87:1, v/v). All chemicals and solvents were of analytical reagent grade (Fison's Scientific Apparatus, Loughborough, Great Britain) and were used without further treatment. Water was distilled in all glass apparatus. Stock aqueous solutions of cefotaxime and desacetylcefotaxime (M_1) were prepared weekly and stored at 0—4°C.

Preparation of columns

Packing material (1.1 g) was added to isopropanol (10 ml) and the mixture was poured into a Micromeritics column packing chamber (Micromeritics, Norcross, GA, U.S.A.). The column was packed by forcing the slurry into the column tube by means of a constant-pressure pump (MCP 71, Olin Energy, Sunderland, Great Britain) set at 20,700 kPa.

Analysis of plasma samples

Plasma or serum (1 ml) was mixed with chloroform—acetone (1:3, v/v) (8 ml) on a vortex mixer for 30 sec. After centrifuging (2000 *g*, 5 min) a measured volume from the upper aqueous layer was freeze-dried. The dry residue was reconstituted in mobile phase (100 μl) and, after centrifuging (2000 *g*, 5 min), 20 μl of the supernatant were injected. To obtain the concentrations of cefotaxime, M_1, M_2, and M_3, the external standard, the non-linear calibration programme of the SP 4100 was utilized. Standard samples for use with this programme were prepared by adding cefotaxime and M_1 to control human plasma to give six samples within the concentration range 0—200 μg/ml; these samples were processed and chromatographed as described above. The SP 4100 computed equations relating peak area to concentration which best fitted the data and applied these to calculate the concentrations of cefotaxime and M_1 in test samples. Pure samples of M_2 and M_3 were not available, so the concentrations of these metabolites were computed from the cefotaxime standard curve. A typical chromatogram is illustrated in Fig. 1.

Analysis of urine samples

Urine samples were centrifuged (2000 *g*, 5 min) and a portion (20 μl) was analysed as described. Calibration samples were prepared by adding known amounts of cefotaxime and M_1 to control urine. A typical chromatogram is illustrated in Fig. 2. All biological samples were stored at —20°C until required for analysis.

RESULTS AND DISCUSSION

Chromatography

Typically, for plasma samples using the conditions described, the retention times for cefotaxime, M_1, M_2, and M_3 were 13.5, 3.9, 6.2 and 6.9 min, respectively, at a flow-rate of 1.1 ml/min (Fig. 1). For urine samples, in order

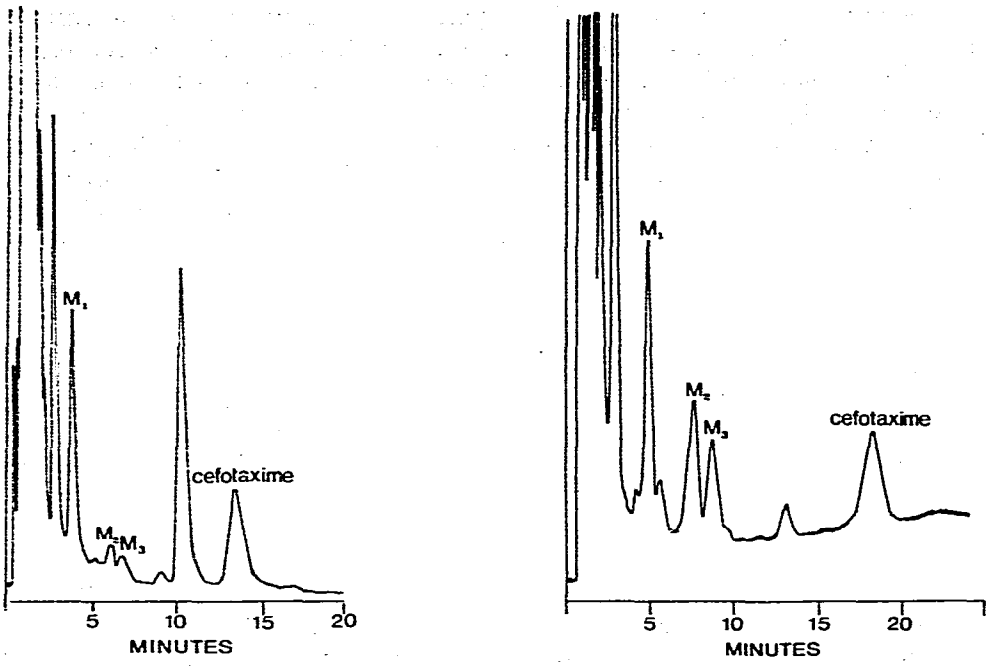


Fig. 1. Chromatogram of a plasma sample from a uraemic patient 1 h after receiving a 1-g intravenous dose of cefotaxime. 12% methanol in mobile phase. Concentrations (μg/ml): cefotaxime, 29; M_1, 70; M_2, 2; M_3, 1.

Fig. 2. Chromatogram of a urine sample from a uraemic patient, 8—12 h after receiving a 1-g intramuscular dose of cefotaxime. 10% methanol in mobile phase. Concentrations (μg/ml): cefotaxime, 127; M_1, 86; M_2, 72; M_3, 50.

to separate M_1 from early eluting endogenous components, it was necessary to lower the methanol content to 10% (Fig. 2). The concomitant increase in overall retention time, however, resulted in a mobile phase composition which was a compromise between adequate separation and a rapid analysis time. It was necessary from time to time to make small changes in the proportions of the mobile phase components in order to accommodate variations in the endogenous background in the large number of biological samples analysed.

Nucleosil ODS (Macherey-Nagel, Camlab, Cambridge, Great Britain) was a suitable alternative to Spherisorb ODS, although the retention times were longer using the former material. On Hypersil ODS (Shandon Southern, Runcorn, Great Britain) M_2 could not be separated from M_3; this material, however, gave the most efficient columns and would be the column of choice in circumstances where separation of M_2 and M_3 was not required. These selectivity differences are illustrated in Fig. 3. Heptanesulphonic acid (0.005 *M*) is a suitable alternative to acetic acid in the mobile phase and adequate separations have been obtained using sulphonic acids with columns of either ODS Spherisorb, ODS Nucleosil or μBondapak C_{18} (Waters Assoc.). In addition, acetonitrile has been used instead of methanol, the advantage of the former solvent being the lower back pressures that result from its use com-

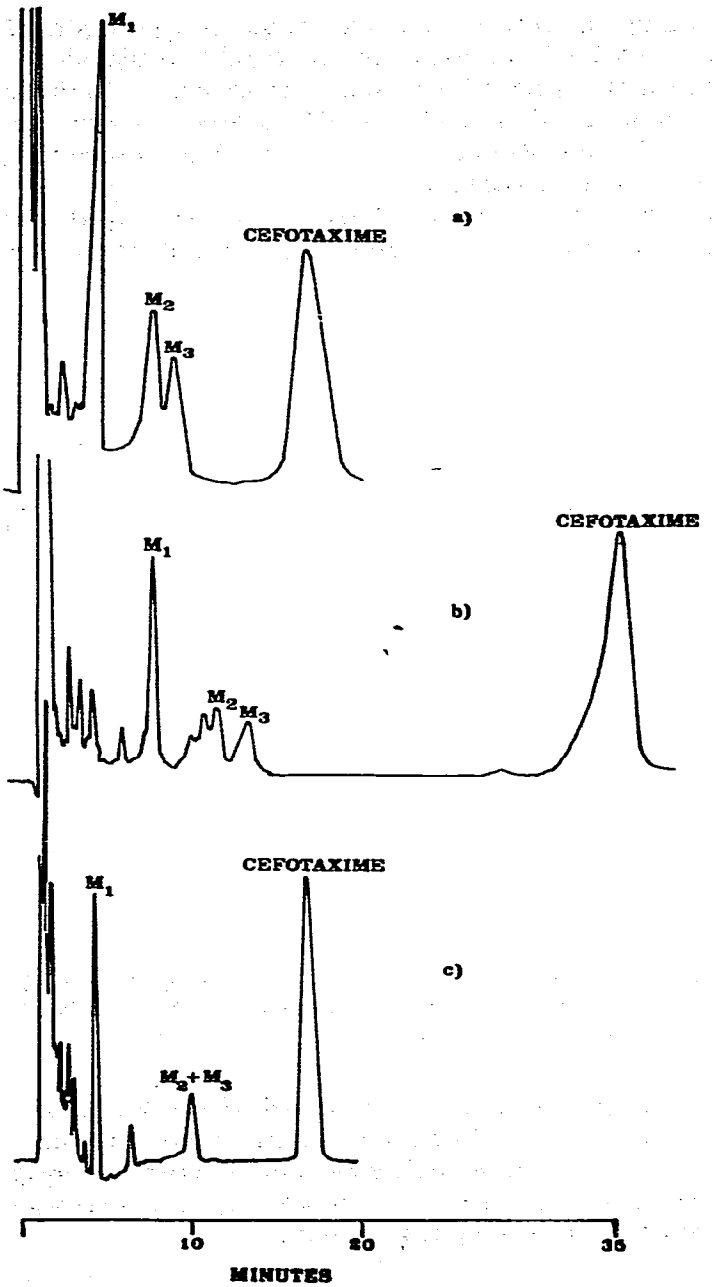


Fig. 3. Chromatograms of a urine sample from a human subject receiving cefotaxime, demonstrating selectivity differences between various reversed-phase columns. (a) Spherisorb ODS, 10 μm, 10 cm × 3 mm. Mobile phase: methanol—water—acetic acid (12:87:1). (b) Nucleosil ODS, 10 μm, 10 cm × 3 mm. Mobile phase: acetonitrile—water—acetic acid (6:93:1). (c) Hypersil ODS, 5 μm, 20 cm × 4 mm. Mobile phase: acetonitrile—water—acetic acid (10:89:1).

pared with methanol. It is likely, therefore, that an adequate separation of all four components can be obtained on most commercially available C_{18} columns, using a mobile phase consisting of a mixture of water, acetonitrile or methanol and a suitable acidic modifier giving a pH of 3—4. In any new system, the retentions of M_1, M_2 and M_3 must be determined to ensure that these metabolites do not co-elute with cefotaxime.

A strong anion-exchange column (Nucleosil 5SB, Macherey-Nagel) has also been used for plasma analysis (Fig. 4). The mobile phase was acetonitrile—

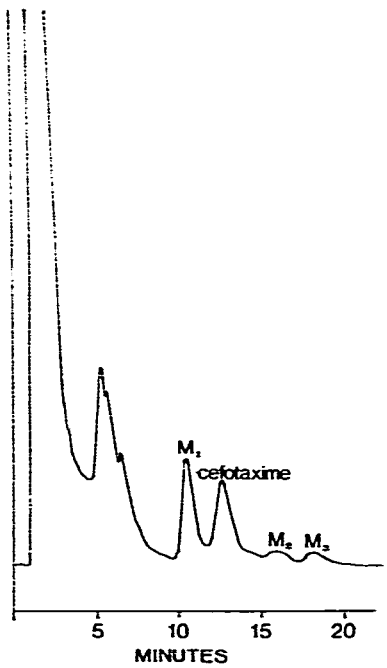


Fig. 4. Chromatogram of a plasma sample from a dog 4 h after receiving a 1500 mg/kg intravenous dose of cefotaxime. Concentrations (μg/ml): cefotaxime, 74; M_1, 84; M_2, 5; M_3, 10. Chromatography on an anion-exchange column (see text).

0.4% ammonium dihydrogen phosphate (1:4, v/v) adjusted to pH 4.7. Under these conditions the elution order is M_1, cefotaxime, M_2, M_3. Since the selectivity is very different from reversed-phase columns, anion exchange may provide a useful alternative when separation of the analytes from endogenous components cannot be achieved on a reversed-phase column.

Sample preparation

An important advantage of HPLC over gas—liquid chromatography is that aqueous biological samples can be injected directly on to a column, obviating the need for an extraction stage. In order to realise this advantage, it is necessary to remove proteins and desirable to remove lipids from plasma samples prior to analysis on a reversed-phase column. The removal of both proteins and neutral lipids was achieved by extraction of the plasma samples with a chloroform—acetone mixture. Using the proportions indicated,

the plasma, free of protein and lipid, separated as a clear upper aqueous layer after mixing and centrifuging to remove the precipitated protein. The highly water-soluble cephalosporin remained in the aqueous phase. In order to achieve acceptable precision below 5 μg/ml it was necessary to concentrate the sample by freeze-drying. A fivefold increase in final concentration was obtained by freeze-drying 0.5 ml of the aqueous phase and re-constituting in 0.1 ml.

The recovery of cefotaxime in the aqueous layer following this procedure was assessed by comparing the peak areas of cefotaxime obtained after processing plasma samples containing known concentrations of the compound with peak areas following analysis of standard aqueous solutions of cefotaxime. The recovery was quantitative (Table I).

TABLE I

THE RECOVERY OF CEFOTAXIME FOLLOWING THE PLASMA SAMPLE PREPARATION PROCEDURE

Cefotaxime added (μg/ml)	Cefotaxime found (μg/ml)	Mean recovery (%)
100.0	100.7, 96.9	99
19.9	20.4, 20.2	103
9.9	10.9, 11.8	115
5.0	4.8, 4.1	94
3.0	2.4, 2.8	87
2.0	1.6, 1.7	83
1.0	0.9, 1.0	95
Overall mean recovery		96 ± 11%

If automatic injection systems are to be used, consideration must be given to the fact that a sample may be standing in solution at room temperature for up to 16 h before analysis, in the case of an overnight run. Cefotaxime was found to be stable under these conditions, following the preparation procedure described in this report and the acetonitrile procedure. The use of acidic protein-precipitation reagents, however, causes significant degradation of cefotaxime. When 0.1 ml of 70% (w/v) trichloroacetic acid was added to 1 ml of plasma containing cefotaxime, the half-life of the drug was 7 h at room temperature.

Detection limit

In the mobile phase used, λ_{max} was 262 nm (ϵ 1.7 × 10^4). At any time, the sensitivity of the method was dependent upon the condition of the column and detector noise level. Over a period of three years, during which time several thousand assays have been carried out, plasma samples containing 0.5 μg/ml cefotaxime gave an average signal-to-noise ratio of 4:1 at 0.01 absorption units full scale deflection. The precision at this concentration was 10—15%.

Specificity

The sample preparation procedure for plasma imparted specificity as far as interference from most lipophilic compounds was concerned, since these would have partitioned into the chloroform—acetone phase. Separation of cefotaxime from the metabolites and endogenous components was achieved on reversed-phase columns as well as on a strong anion-exchange column. When lack of specificity was encountered as a result of interference from endogenous components, it was usually possible to select a different reversed-phase or anion-exchange column on which separation could be achieved.

Precision and accuracy

Cefotaxime and M_1 were added to control plasma to give six samples in the concentration range 0.5—120 μg/ml. Sufficient plasma was prepared to allow six replicate samples to be analysed at each concentration. The procedure was repeated for urine over the concentration range 5—500 μg/ml. The results are presented in Tables II and III. Accuracy, here, is defined as (amount found/amount added) × 100 (%).

TABLE II

PRECISION AND ACCURACY FOR CEFOTAXIME

Cefotaxime added (μg/ml)	Cefotaxime found (μg/ml)	Mean	S.D.	C.V. (%)	$\frac{\text{Found}}{\text{Added}} \times 100$
Plasma					
0.50	0.60, 0.54, 0.63, 0.69, 0.53, 0.54	0.59	0.06	10.8	118
1.0	1.1, 1.3, 1.2, 1.1, 1.0, 1.2	1.2	0.10	9.1	120
4.6	4.9, 4.8, 5.1, 4.8, 4.9, 4.8	4.7	0.14	3.0	107
11.8	12.1, 13.1, 15.1, 12.6, 12.5, 12.3	13.0	1.1	8.5	108
52.2	58.3, 55.9, 59.8 58.6, 57.1, 56.8	57.8	1.4	2.4	98
118.4	113.8, 125.1, 125.3, 120.6, 126.6, 116.8	121.4	5.2	4.3	103
Urine					
580	516, 538, 523 539, 531, 534	530	9.0	1.7	91
230	227, 235, 247, 241, 213, 213	229	14.3	6.2	100
115	100, 102, 116, 114, 116, 114	110	7.3	6.6	96
58	64, 58, 58, 55, 66, 64	60.8	4.4	7.2	105
8.3	7.5, 7.2, 8.0, 6.9, 7.2, 8.3	7.5	0.5	7.1	90
4.6	3.9, 4.7, 3.6, 2.5, 4.2, 3.0	3.7	0.8	22.0	80

TABLE III

PRECISION AND ACCURACY FOR DESACETYLCEFOTAXIME

Desacetylcefotaxime added (μg/ml)	Desacetylcefotaxime found (μg/ml)	Mean	S.D.	C.V. (%)	$\frac{\text{Found}}{\text{Added}} \times 100$
Plasma					
0.49	0.47, 0.50, 0.51, 0.44, 0.49, 0.49	0.48	0.025	5.2	98
0.96	1.0, 0.8, 1.1, 1.2, 1.1, 1.1	1.05	0.14	13.1	110
5.4	4.6, 4.9, 5.2, 5.4, 5.5, 4.5	5.0	0.4	8.0	93
10.8	9.1, 9.4, 9.3, 7.9, 9.4, 9.8	9.2	0.7	7.6	85
16.1	16.5, 15.6, 16.9, 16.3, 16.6	16.4	0.5	3.0	102
21.5	20.7, 23.0, 23.7, 23.1, 23.2, 22.2	22.7	1.1	4.8	105
Urine					
519	503, 502, 501, 513, 513, 511	507	5.7	1.1	98
206	200, 210, 222, 221, 197, 195	208	12.0	5.8	101
103	92, 93, 104, 105, 107, 105	101	6.7	6.6	98
51	58, 52, 50, 47, 59, 58	54	5.0	9.3	106
8.2	9.3, 8.9, 10.1, 8.9, 9.5, 10.1	9.5	0.5	5.7	116
4.5	4.1, 4.9, 3.4, 2.7, 4.6, 3.0	3.8	0.9	23.5	84

Application

The method has been applied to the analysis of cefotaxime and the three metabolites in various physiological fluids including plasma, serum, urine, cerebrospinal fluid, saliva, and pus from infected wound secretions. A typical concentration—time course curve for the elimination of cefotaxime and its metabolites from the plasma of a patient receiving cefotaxime is shown in Fig. 5.

The chloroform—acetone treatment for plasma improved column longevity, compared with our previous plasma preparation procedure, which involved freeze drying and re-constitution in methanol [13]. Using the procedure described in this paper, deterioration in column performance, as judged by peak broadening accompanied by loss of sensitivity, occurred after approximately five hundred injections of biological fluid. A column could normally be regenerated by replacing the top 3 mm of packing material.

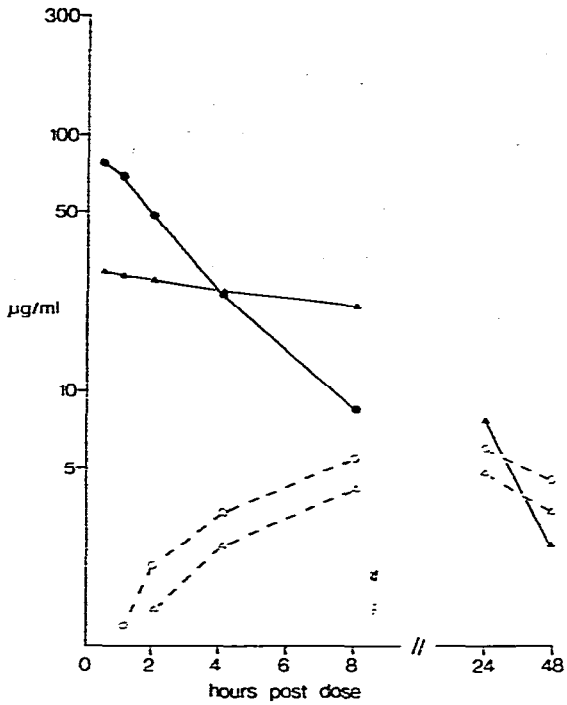


Fig. 5. The concentration—time course of cefotaxime and metabolites in the plasma of a uraemic patient after receiving a 2-g intravenous dose of cefotaxime.

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