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Note

Micromethod for the determination of cefotaxime and desacetylcefotaxime in plasma and urine by high-performance liquid chromatography

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Cefotaxime (CTX), a third generation cephalosporin, has a broad antibacterial spectrum and resistance to β-lactamases. Its main metabolite, desacetylcefotaxime (dCTX), is a microbiologically active product and its pharmacokinetics are different from CTX [1]. Therefore, it is of particular interest to determine CTX and dCTX concentrations in biological fluids. High-performance liquid chromatography (HPLC) presents an advantage in comparison with microbiological assays, which have poor specificity when CTX is determined in the presence of dCTX or other antibiotics.

Several HPLC methods have been described [2—8]. Among these techniques, only two require a small volume of biological sample (100—300 μl) [6, 7]. Kees et al. [6] determined the concentrations of CTX and dCTX using an external standard and no results on accuracy and precision were reported. In the technique described by Lecaillon et al. [7], CTX and its metabolite were not analysed under the same conditions.

We report a micromethod allowing the simultaneous determination of CTX and dCTX in plasma and urine in the presence of an internal standard, cephaloridine. Since the aqueous solubility of cephalosporins is too high to allow their extraction by organic solvents, we have chosen a sample pretreatment which obviates the need for an extraction stage. This two-step method comprises first protein precipitation by propanol-2, then removal of remaining endogenous compounds by an organic solvent (chloroform), leaving the drugs in an aqueous phase of suitable pH to prevent degradation of cephalosporins. This sample pretreatment is short (6 min for each sample). Separation is carried out by ion-pair chromatography on a Radial-Pak C_{18} column within 7 min.

The precision and accuracy of this method were studied, and the coefficients

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of variation were lower than 7% over all concentrations investigated. This technique has been used to analyse samples obtained following pharmacokinetic studies on patients with renal failure.

MATERIAL AND METHODS

Reagents

Cefotaxime and desacetylcefotaxime were obtained from Roussel Uclaf Labs. (France); cephaloridine was from Eli Lilly (France). Reagent-grade propanol-2, chloroform, isoamyl alcohol, ammonium acetate, acetic acid, acetonitrile (E. Merck, Darmstadt, F.R.G.) were used without further purification. Pic-A (tetrabutylammonium hydroxide in phosphate buffer) was obtained from Waters Assoc. (Milford, MA, U.S.A.). Water was glass-distilled deionized.

Apparatus and chromatographic conditions

A Waters Assoc. liquid chromatograph was used. The instrument was equipped with a Model M 45 pump, a Lambda-Max Model 480 ultraviolet detector at 270 nm, a Wisp Model 710 B automatic injector, and a radial compression Z module. The column was a Radial-Pak C_{18} (Waters Assoc.) (100 mm × 8 mm I.D.; 10 μm particle size). Detector output was recorded on a Model 730 data module. The mobile phase was prepared by dissolving one vial of Pic-A in 1 l of acetonitrile—water (17:83, v/v). The working pressure of the pump was 35 bars at a flow-rate of 4 ml/min.

Standard solutions

Stock solutions of CTX, dCTX and cephaloridine were prepared in bidistilled water and stored at −20° C.

Plasma standards. Blank human plasma was spiked daily with CTX and dCTX stock solutions to yield concentrations ranging from 2 to 50 μg/ml. An appropriate dilution of cephaloridine stock solution was prepared in 0.1 *M* acetate buffer (pH 5) to provide a concentration of 50 μg/ml.

Urine standards. From stock solutions of CTX and dCTX, appropriate dilutions were prepared in blank urine to yield concentrations ranging from 5 to 100 μg/ml. The internal standard was at a concentration of 100 μg/ml.

Calibration curves. The calibration curves were constructed by plotting peak height ratios of CTX or dCTX to cephaloridine against concentration of CTX and dCTX in plasma or urine. Standard solutions were prepared daily.

Sample preparation

Plasma and urine samples are stored at −20° C until analysis.

In a 1.5-ml conical centrifuge tube, 100 μl of plasma or urine and 50 μl of 0.1 *M* ammonium acetate buffer (pH 5) containing cephaloridine were mixed with 500 μl of propanol-2; the mixture was vortexed for 30 sec and centrifuged for 2 min at 8700 *g* (Beckman Microfuge). The clear supernatant was transferred into another conical tube (1.5 ml) and 500 μl of chloroform—isoamyl alcohol (100:4, v/v) were added. The tube was vortexed for 30 sec and centrifuged for 2 min at 8700 *g*. A 5—20-μl portion of the upper aqueous phase was injected into the chromatograph.

RESULTS

Under the described chromatographic conditions, CTX, dCTX and cephaloridine were well resolved. Endogenous plasma or urine components did not give any interfering peaks (Fig. 1). Potential interference of some other drugs was investigated (Table I). None of these substances interfered with the quantitation of CTX and dCTX.

The reproducibility (intra-day assay, $n = 8$) and the repeatability (inter-day assay, $n = 8$) of the HPLC procedure were tested with plasma and urine of subjects who intravenously received 1 g of CTX. For reproducibility, the coefficients of variation (C.V., %) were: in plasma, 2.2% for CTX and 3.0% for dCTX; in urine, 2.4% for CTX and 4.0% for dCTX. The study of repeatability has led to the following results (C.V., %): in plasma, 2.4% for CTX and 3.7% for dCTX; in urine, 2.4% for CTX and 5.6% for dCTX.

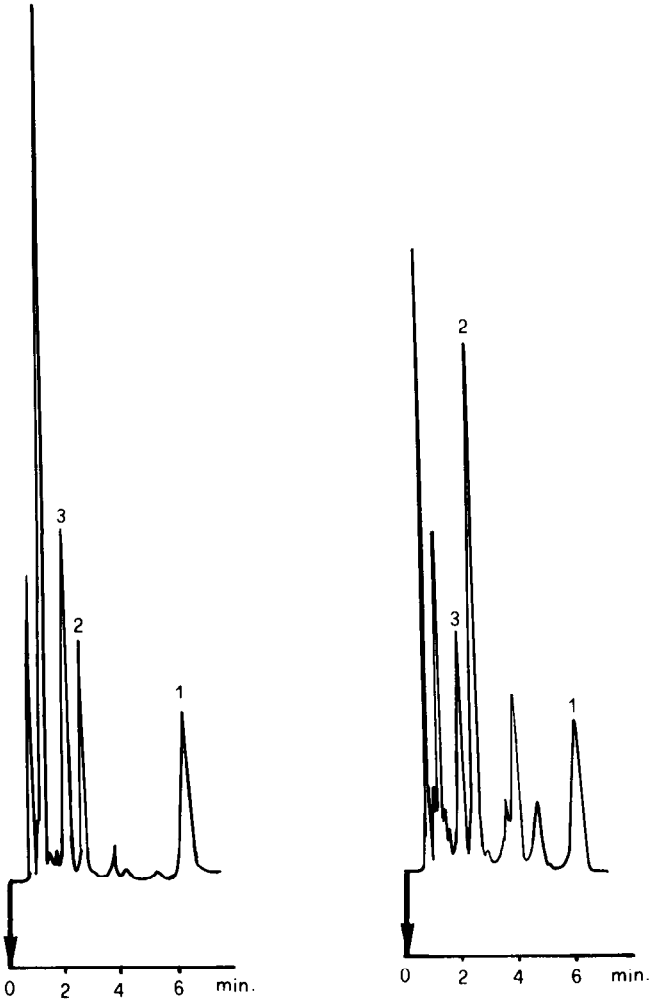

Fig. 1. Chromatograms of a plasma sample (left) and of a urine sample (right) from a patient after a single intravenous dose of CTX. Peaks: 1 = cefotaxime; 2 = desacetylcefotaxime; 3 = internal standard, cephaloridine.

TABLE I

RETENTION TIMES OF SOME DRUGS (1 μg INJECTED)

Drug	Retention time (min)
Cefotaxime	6.03
Desacetylcefotaxime	2.50
Desacetylcefotaxime lactone	2.61
Cephaloridine	1.95
Cephalexin	6.73
Cefazolin	7.24
Cephalothin	26.92
Moxalactam	Two peaks: 10.12 and 10.85
Theophylline	1.45
Caffeine	1.64
Acetaminophen	1.50
Salicylic acid	14.0
Phenobarbital	8.21
Phenacetin	8.04
Amobarbital	No response
Indomethacin	No response
Flufenamic acid	No response
Mefenamic acid	No response
Niflumic acid	No response
Ketoprofen	No response

The accuracy and precision were evaluated by adding known amounts of CTX and dCTX to blank serum and urine. For plasma, the precision of the method was determined by eight replicate assays at concentrations of 7.5 μg/ml and 25 μg/ml for CTX and dCTX, respectively. The coefficients of variation were, respectively: for CTX, 1.9% and 1.0% (intra-day assay) and 2.6% and 1.5% (inter-day assay); for dCTX, 2.3% and 2.2% (intra-day assay) and 2.9% and 6.8% (inter-day assay). In urine, a similar study was performed from two spiked concentrations: 12.5 μg/ml and 25 μg/ml, respectively. The coefficients of variation were, respectively: for CTX, 1.9% and 2.2% (intra-day assay, $n = 8$) and 2.6% and 3.0% (inter-day assay, $n = 8$); for dCTX, 4.5% and 3.1% (intra-day assay, $n = 8$) and 4.6% and 5.8% (inter-day assay, $n = 8$).

In plasma the calibrations curves were linear within the range of 2—50 μg/ml [$Y = 0.034X + 0.013$ ($r = 0.995$) for CTX ($n = 8$); $Y = 0.069X + 0.006$ ($r = 0.998$) for dCTX ($n = 8$)]. In urine, the relationship between concentration and peak height ratio was linear in the calibration range 5—100 μg/ml [$Y = 0.017X + 0.011$ ($r = 0.998$) for CTX, ($n = 8$); $Y = 0.039X - 0.023$ ($r = 0.995$) for dCTX ($n = 8$)].

Under these conditions the detection limit was 0.5 μg/ml for CTX and 0.25 μg/ml for dCTX (signal-to-noise ratio $\geqslant 2$).

DISCUSSION

In this study, potential degradation of cephalosporins during sample conservation, extraction procedure or automatic injection has been carefully taken into consideration, leading us to find analysis conditions to avoid this degradation.

After centrifugation, plasma and urine were stored at −20°C over a short period not exceeding ten days, and thawed just prior to analysis. A plasma sample was kept during 21 days at −20°C: no degradation of CTX and dCTX was noted (Table II). For the storage of samples over several months, a temperature of −70°C was necessary [6]. Blood samples must not be haemolysed. Indeed, blood esterases rapidly hydrolyse CTX and dCTX and this may explain the unexpectedly high levels of metabolite [9].

For sample preparation, a single protein precipitation by methanol or acetonitrile was not satisfactory: endogenous compounds prevented the determination of CTX and dCTX. However, a double purification of sample first by protein precipitation with propanol-2 then by extraction, which removes the

TABLE II

STABILITY OF CTX AND dCTX IN PLASMA FROZEN AT −20°C

Day	Concentration of CTX (μg/ml)	Concentration of dCTX (μg/ml)
0	7.5	7.5
1	7.5	7
2	7.0	7.5
3	7.0	7.5
8	7.5	7.0
21	7.5	7.5

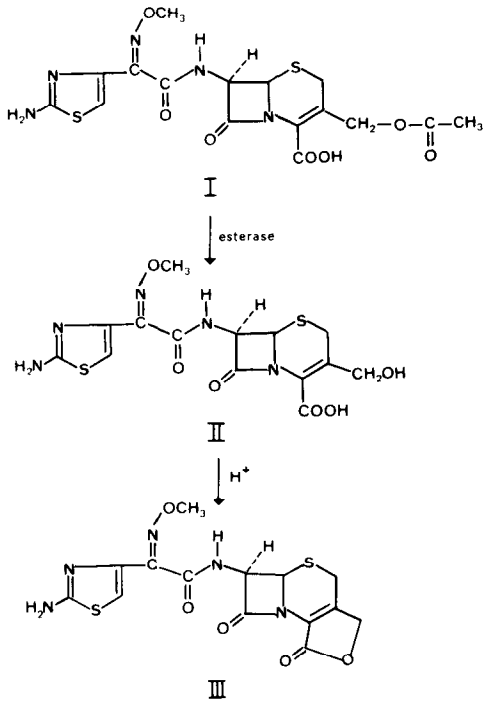

Fig. 2. Transformation of cefotaxime (I) into desacetylcefotaxime (II) and into desacetylcefotaxime lactone (III).

remaining endogenous compounds and the initial solvent, provided a good sample for HPLC. Using the indicated proportions of propanol-2—chloroform (1:1, v/v), the upper aqueous phase containing the drugs was clear.

The recovery of drugs in the upper aqueous phase was assessed by comparing the peak heights of CTX and dCTX obtained after processing plasma samples containing known concentrations of drugs (1, 5, 10, 25, 50 μg/ml for CTX and dCTX). The results obtained were: 92.0 ± 5.2% for CTX and 100.5 ± 4.7% for dCTX.

Under the described conditions, automatic injection of 50 samples required only 7 h. We have studied a possible degradation of CTX into dCTX then into its lactone (Fig. 2) in the final aqueous layer. No degradation was observed (Fig. 3). Several authors have studied the stability of 3-acetoxymethylcephalosporins in aqueous solution [10—12]. They have shown that at 25°C, in the pH range 4.5—6.5, the stability of CTX was maximal and desacetylcefotaxime lactone only appeared below pH 4.

This method allowed us to determine serum concentrations of cefotaxime

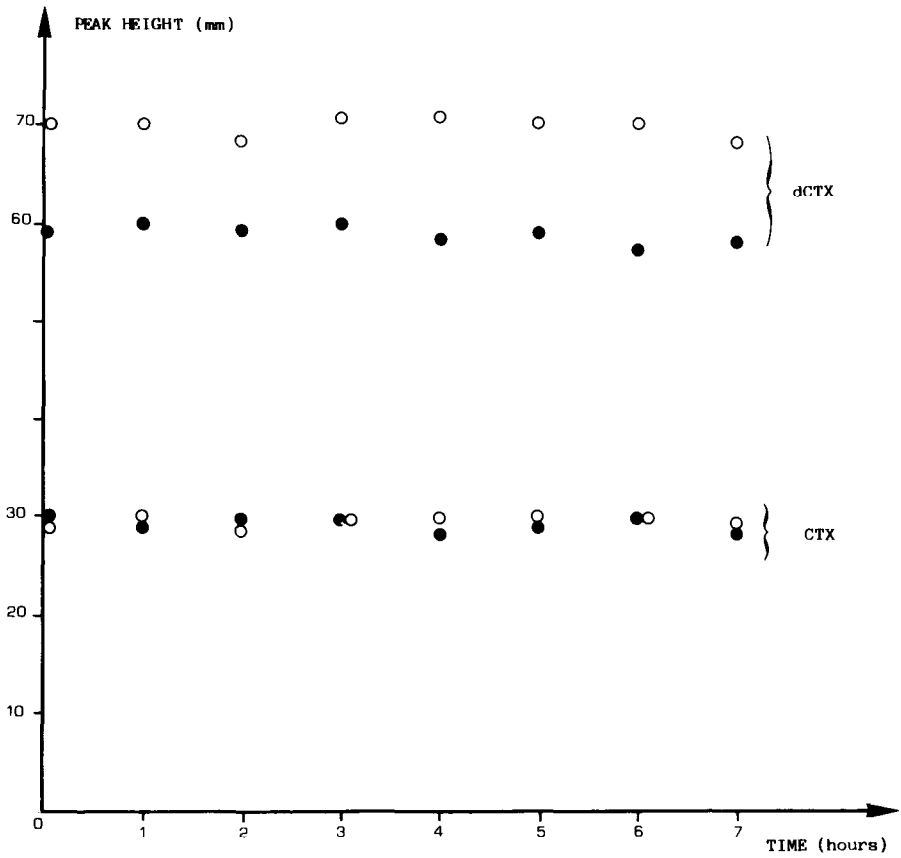

Fig. 3. Stability of CTX and dCTX during 7 h at 25°C in the final aqueous phase. (•), Blank plasma spiked with 12.5 μg/ml each of CTX and dCTX; (○), blank urine spiked with 75 μg/ml each of CTX and dCTX.

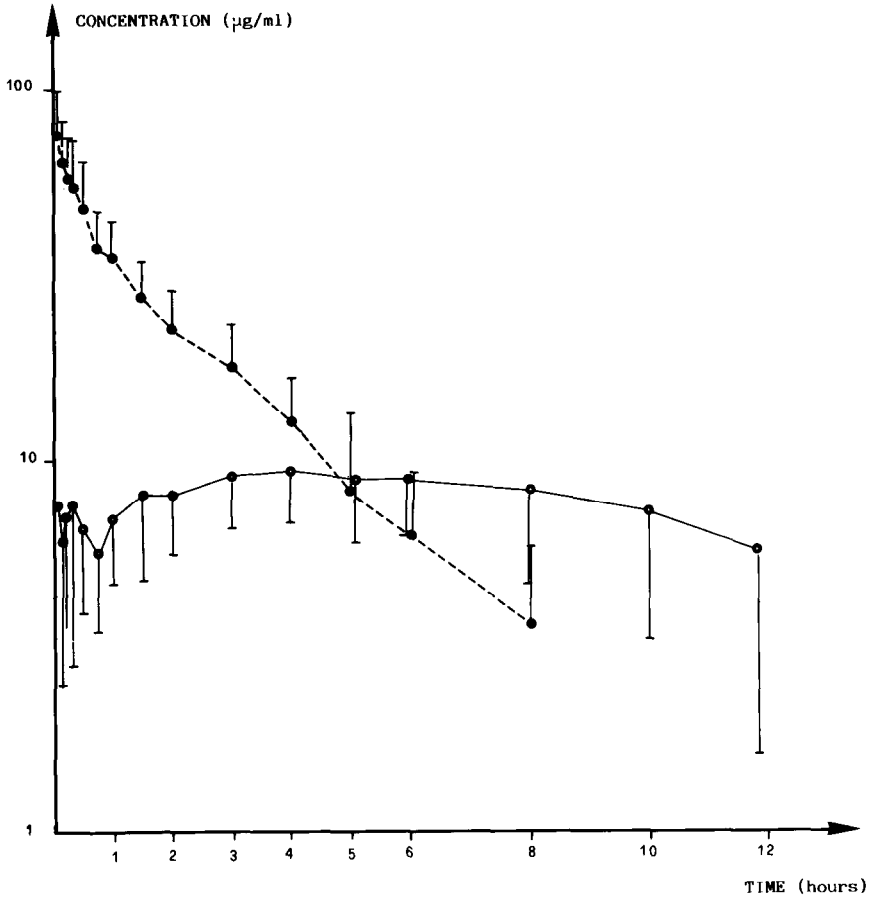

Fig. 4. Mean plasma concentrations of cefotaxime (•- - -•) and desacetylcefotaxime (○—○) in eight patients following administration of 1 g of cefotaxime intravenously.

and desacetylcefotaxime in eight patients undergoing peritoneal dialysis with creatinine clearance below 5 ml/min. These subjects received a slow intravenous injection (3 min) of 1 g of cefotaxime (Fig. 4).

CONCLUSIONS

The present paper describes a rapid and sensitive quantitative micromethod for CTX and dCTX determination in plasma and urine. The presence of an internal standard is not absolutely necessary since there is no extraction of drugs from biological fluids. However, as the chromatographic or detection conditions may undergo fluctuations, the use of an internal standard seems important to enhance the assay precision and reproducibility.

This micromethod is particularly useful in paediatrics and in patients with renal failure where sample size is of major concern.

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