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QUANTITATION OF CEFTETRAME IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for quantifying ceftetrame, the acid metabolite of methylene (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-[(5-methyl-2H-tetrazol-2-yl)-methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate pivalate, an orally active cephalosporin. Sodium benzylpenicillin is added to the plasma as the reference standard. The compounds are extracted from plasma or urine using a Bond Elut phenyl column. An aliquot of the methanol eluate is analyzed by high-performance liquid chromatography using a Waters Nova-Pak phenyl column and a UV detector set to 225 nm. The ratios of the peak heights for ceftetrame and sodium benzylpenicillin are calculated and converted to concentrations of analyte with calibration curves that are generated from the analysis of analyte-free plasma or urine fortified with various amounts of ceftetrame and a fixed amount of sodium benzylpenicillin. For plasma, the limit of quantitation for the assay is 0.48 μg/ml and the inter-assay precision (relative standard deviation) is 9.3%. For urine, the limit of quantitation for the assay is 19.1 μg/ml, and the inter-assay precision is 4.9%.

INTRODUCTION

Ceftetrame, (7*R*,8*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)-acetamido]-3-[(5-methyl-2H-tetrazol-2-yl)methyl]-3-cephem-4-carboxylic acid (compound II, Fig. 1), is the principal metabolite and active form of the oral cephalosporin, methylene (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-[(5-methyl-2H-tetrazol-2-yl)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate pivalate (compound I, Fig. 1). When administered to mice, autoradiographic studies have shown that compound II is well distributed in the body tissues, and is rapidly excreted [1, 2]. In vitro studies have revealed that ceftetrame is highly active against *Enterobacteriaceae, Haemophilus influenzae* [3], *Streptococcus pneumoniae,* and *Nesseria* spp. [4], while inhibiting the chromosomally mediate β-lactamase of *Enterobacter cloacae,* P99 [5].

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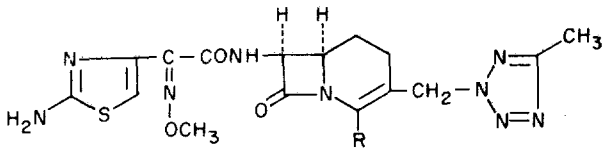
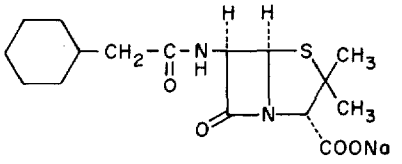


COMPOUND I : R= $COOCH_2OCOC(CH_3)_3$
COMPOUND II : R = COOH



COMPOUND III : Sodium Benzylpenicillin (PEN G)

Fig. 1. Structures of (I) the pivalyl ester of ceftetrame, (II) ceftetrame and (III) penicillin G.

This paper presents a high-performance liquid chromatographic (HPLC) assay for ceftetrame in plasma or urine which utilizes reversed-phase chromatography on a Nova-Pak phenyl column with UV detection at 225 nm. Sample preparation involves the use of solid-phase extraction on a phenyl column with sodium benzylpenicillin (compound III, Fig. 1) added as the reference, i.e., internal standard. The assay was applied to the determination of plasma and urine concentrations of ceftetrame in humans receiving various doses of compound I.

EXPERIMENTAL

Chemicals

Sodium ceftetrame was obtained from the Quality Control Department, Hoffmann-La Roche (Nutley, NJ, U.S.A.). Sodium benzylpenicillin (stock No. PEN-NA) and Trizma base (stock No. T1503) were obtained from Sigma (St.Louis, MO, U.S.A.). Monobasic potassium phosphate and potassium hydroxide were obtained from Baker (Phillipsburg, NJ, U.S.A.), and reagent-grade hydrochloric acid was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Nanograde methanol was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All water was distilled and deionized.

Solutions

Ceftetrame stock solution. Sodium ceftetrame (100 mg) was dissolved in 100 ml water, yielding a solution of 0.954 mg/ml ceftetrame, which was stored at 4°C.

Sodium benzylpenicillin stock solution. Sodium benzylpenicillin (50 mg) was dissolved in 10 ml of methanol (5 mg/ml) and stored at −20°C.

Ceftetrame solutions for the calibration standards. For the plasma assay, individual aliquots of the 0.954 mg/ml stock solution (either 2.0, 1.0, 0.5, 0.2, or 0.1 ml) were added to 10-ml volumetric flasks and brought to volume with water giving solutions with concentrations of 9.54, 4.77, 2.39, 0.95, or 0.48 μg per 50 μl,

respectively. For the urine assay, individual aliquots of the 1 mg/ml stock solution (either 8.0, 4.0, 2.0, 1.0, or 0.4 ml) were added to 10-ml volumetric flasks and brought to volume with water giving solutions with concentrations of 38.16, 19.08, 9.54, 4.77, or 1.91 μg per 50 μl, respectively. All solutions were stored at 4 °C.

Spiking solution for the reference standard. For the plasma assay, 1.0 ml of the 5 mg/ml sodium benzylpenicillin stock solution was added to a 10-ml volumetric flask and brought to volume with methanol to give a solution of 25 μg per 50 μl. For the urine assay, 4 ml of the stock solution were added to a 10-ml volumetric flask and brought to volume with methanol to give a solution of 100 μg per 50 μl. These solutions were stored at −20 °C.

1.0 M Potassium phosphate buffer (pH 5.2). KH_2PO_4 (136 g) was dissolved in 1000 ml water and adjusted to pH 5.2 using 5 *M* potassium hydroxide (28 g in 100 ml water) to give a stock solution which was diluted 1:100 prior to use (0.01 *M*).

1.0 M Tris buffer (pH 7.0). Trizma base (121 g) was dissolved in 1000 ml water and adjusted to pH 7.0 using hydrochloric acid. This solution was diluted 1:100 prior to use (0.01 *M*).

Column

A prepacked 15 cm × 3.9 mm I.D. stainless-steel column containing 4-μm Nova-Pak phenyl packing generating 30 000 plates/m (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.) was used.

Instrumental parameters

The HPLC system consisted of two Model 6000A reciprocating piston pumps (Waters Assoc., Milford, MA, U.S.A.), a Waters Model 710B intelligent sample processor (WISP™), a Waters Model 660 automated gradient controller (Waters Assoc.), and a SpectroMonitor III Model 1204A variable-wavelength detector (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). The UV detector was operated at a wavelength of 225 nm and at a sensitivity of $2 \cdot 10^{-2}$ a.u.f.s. A Hewlett-Packard Model 7132A chart recorder (Hewlett-Packard, San Diego, CA, U.S.A.) was used to record chromatograms and a Waters Model 730 data module was used to record and calculate peak heights of compounds II and III. For the analysis of ceftetrame in plasma, the isocratic mobile phase was 0.01 *M* potassium phosphate buffer (pH 5.2)–methanol (78:22) at a constant flow-rate of 2.0 ml/min for 16 min. Under these conditions, ceftetrame eluted in 5.6 min and sodium benzylpenicillin in 12.8 min. In the analysis of ceftetrame from urine, the ratio of buffer to methanol was changed to 81:19 in order to separate ceftetrame from endogenous chemicals. Following each run the column was flushed with 100% methanol for 4 min, in order to wash the column of late-eluting peaks which would otherwise build up and have a detrimental effect on the chromatography. The column was allowed to equilibrate at the original conditions for 6 min before the next injection.

Solid-phase extractions

Solid-phase extractions were performed using Bond Elut phenyl columns (500 mg per 2.8 ml size) on a Vac Elut vacuum manifold (Analytichem International, Harbor City, CA, U.S.A.) at a pressure of approximately 100 Torr.

Procedure

Calibration curve samples (prepared in duplicate). For the plasma assay, 1 ml of analyte-free control plasma was transferred to each of ten 100×13 mm glass test tubes containing either 0.48, 0.95, 2.39, 4.77 or 9.54 μg of ceftetrame together with 25 μg sodium benzylpenicillin. Analyte-free plasma samples were used as plasma controls and for preparation of the external standards. For the urine assay, 0.1 ml of analyte-free urine was transferred to each of ten 100×113 mm glass test tubes containing either 1.91, 4.77, 9.54, 19.08, or 38.16 μg of ceftetrame together with 100 μg sodium benzylpenicillin. Analyte-free urine samples were used as urine controls and for preparation of the external standards.

Experimental samples. Blood samples were collected into vacutainers (B-D Cat. No. 6470) containing oxalate as the anticoagulant and sodium fluoride as a preservative, centrifuged, and the plasma was placed in scintillation vials, while urine samples were collected and placed in polypropylene tubes. All samples were stored at −70°C until assayed. A quality assurance sample was prepared by combining aliquots from experimental samples. Enough plasma and urine was combined so that duplicate aliquots could be analysed with each set of samples over the course of the analyses. The quality assurance sample was also used to determine long-term stability of the compound in plasma and urine and to check the reproducibility of the assay.

Blood collection device. The blood collection device experiments were done as follows. A volume of approximately 60 ml of blood was collected into a heparinized vacutainer (B-D Cat. No. 6527, 10 ml capacity). The blood was pooled and spiked with ceftetrame to give a concentration of approximately 2.5 μg/ml. Aliquots (5 ml) of the spiked blood were transferred to three vacutainers (B-D Cat. No. 6470) and three siliconized glass tubes. All the tubes were shaken for 30 min, and 1-ml aliquots of sample from each tube were analyzed for ceftetrame by comparison against a calibration curve for the analyte in blood. The remaining fortified blood was centrifuged to separate the plasma. Plasma (1 ml) from each tube and vacutainer was analyzed using a calibration curve for ceftetrame in plasma.

Extraction. Plasma samples were thawed, and 2 ml of 0.01 *M* Tris buffer (pH 7.0) were added to each tube, which was vortexed. A Bond Elut column was placed in the Vac Elut manifold and was washed with 3 ml methanol followed by 3 ml of 0.01 *M* Tris buffer. The column was drained between washings. The sample was loaded onto the column and drawn through by applying vacuum. The column was washed with 3 ml of 0.01 *M* Tris buffer and allowed to drain well. The compound of interest was eluted with 0.5 ml methanol and collected in a small tube. The eluate was transferred to a WISP vial for injection. An injection volume of 75 μl was sufficient to produce adequate peak heights for analysis of the chromatograms.

Calculations. The peak heights in the HPLC profiles were measured, and the

ratio of the peak height for ceftetrame to the peak height for sodium benzylpenicillin was calculated. A calibration curve was established comparing the peak-height ratio against the concentration of ceftetrame in the standards. The slope and intercept of the calibration curve were calculated using weighted ($1/y$) linear regression. Concentrations of ceftetrame in the experimental samples were calculated using the equation $x\ (\mu g) = (R-b)/m$, where R is the ratio of the ceftetrame to sodium benzylpenicillin in an experimental sample and b (intercept) and m (slope) were constants generated by the linear regression analysis of the calibration curve data.

RESULTS AND DISCUSSION

Fig. 2. shows chromatograms at a wavelength of 225 nm from the analysis of an experimental plasma from a subject prior to dosing and 0.5 h after receiving a

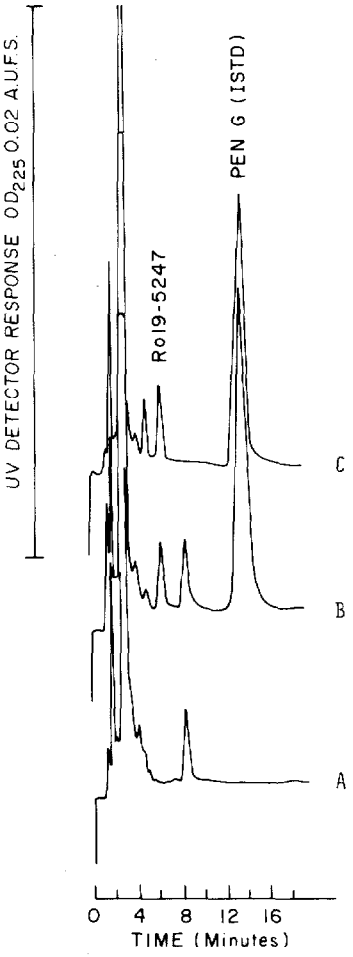


Fig. 2. Typical chromatograms from (A) control human plasma, (B) human plasma spiked with ceftetrame and the reference standard (concentration of ceftetrame is 0.95 μg/ml) and (C) plasma from a subject 0.5 h after administration of 400 mg of compound I (concentration of ceftetrame is 1.3 μg/ml).

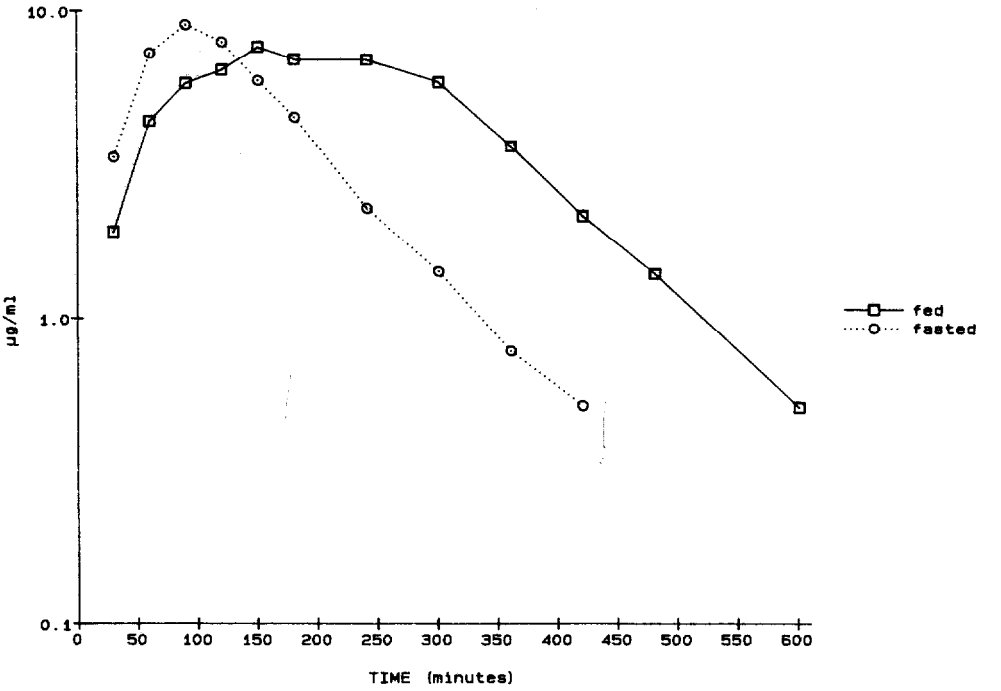


Fig. 3. Plasma level versus time curve for a subject after a 400-mg dose of compound I.

TABLE I

INTER-ASSAY PRECISION FROM A CONSIDERATION OF THE CALIBRATION DATA

Fit of back-calculated concentrations to calibration line.

Ceftetrame added (µg/ml)	Concentration found (mean ± S.D.) (µg/ml)	Relative standard deviation (R.S.D.) (%)
0.48	0.48 ± 0.03	5.2
0.95	0.94 ± 0.03	3.1
2.39	2.39 ± 0.07	3.1
4.77	4.89 ± 0.19	3.8
9.54	9.54 ± 0.24	2.5
Overall R.S.D.		3.5

200-mg oral dose of compound I in tablet form. Typically, no interfering peaks were observed in chromatograms from analyte-free plasma at the retention time of authentic ceftetrame. However, in certain plasma samples a small endogenous peak was observed, and it is the occasional appearance of this peak which limits the sensitivity of the assay to 0.48 µg/ml.

The plasma concentration versus time curve for ceftetrame in a male volunteer given 400 mg of compound I is shown in Fig. 3.

The inter-assay precision of the method in plasma was evaluated over a con-

TABLE II

INTRA-ASSAY PRECISION FROM A CONSIDERATION OF THE CALIBRATION DATA

Ratio of duplicate analyses.

Ceftetrame added (μg/ml)	Ratio of determinations (mean ± S.D.)	Relative standard deviation (R.S.D.) (%)
0.48	0.984 ± 0.069	7.0
0.95	0.979 ± 0.041	4.2
2.39	1.002 ± 0.047	6.2
4.77	0.994 ± 0.047	4.7
9.54	1.001 ± 0.062	6.2
Quality assurance sample	1.005 ± 0.082	8.2
Overall R.S.D.		6.1

TABLE III

EFFECT OF VACUTAINER ON THE CONCENTRATION OF CEFTETRAME

Values in parentheses are relative standard deviations.

Sample	Ceftetrame concentration (mean ± S.D.) (μg/ml)	
	Vacutainer	Glass tube
Human blood	2.07 ± 0.20 (9.5%)	2.01 ± 0.02 (1.2%)
Human plasma	3.30 ± 0.02 (0.8%)	3.88 ± 0.05 (1.4%)

centration range of 0.48–9.54 μg/ml (Table I). Duplicate samples at each concentration in this range were taken through the analytical procedure. The method demonstrated excellent precision over the concentration range with a mean relative standard deviation of 3.5% for the inter-assay precision. The mean correlation coefficient for the fit to the linear equation was 0.9987, indicating a good fit of the data to the model. The recovery of ceftetrame from plasma (determined by adding 2.39 μg of analyte to the processed external standards) was 88%, and the limit of quantitation was 0.48 μg/ml. Intra-assay precision for the method (determined by dividing one member of the set of duplicates by the other) for the calibration curve and the quality assurance sample was 6.1% (Table II).

The inter-assay precision of the method in urine was evaluated over the concentration range 19.1–382.4 μg/ml ceftetrame in urine. The mean overall relative standard deviation of the inter-assay precision was 4.9% and the mean correlation coefficient for the fit of the data to the linear equation was 0.9934. Intra-assay precision for the method was 10.5%. The recovery of ceftetrame from urine (determined by adding 19.12–382.4 μg of analyte to the processed external standard) was 76.4%.

Table III shows the results of the experiment to ascertain if the vacutainer was having any effect on the concentration of ceftetrame in the plasma or the red blood cell to plasma partitioning. The results indicate that there was no signifi-

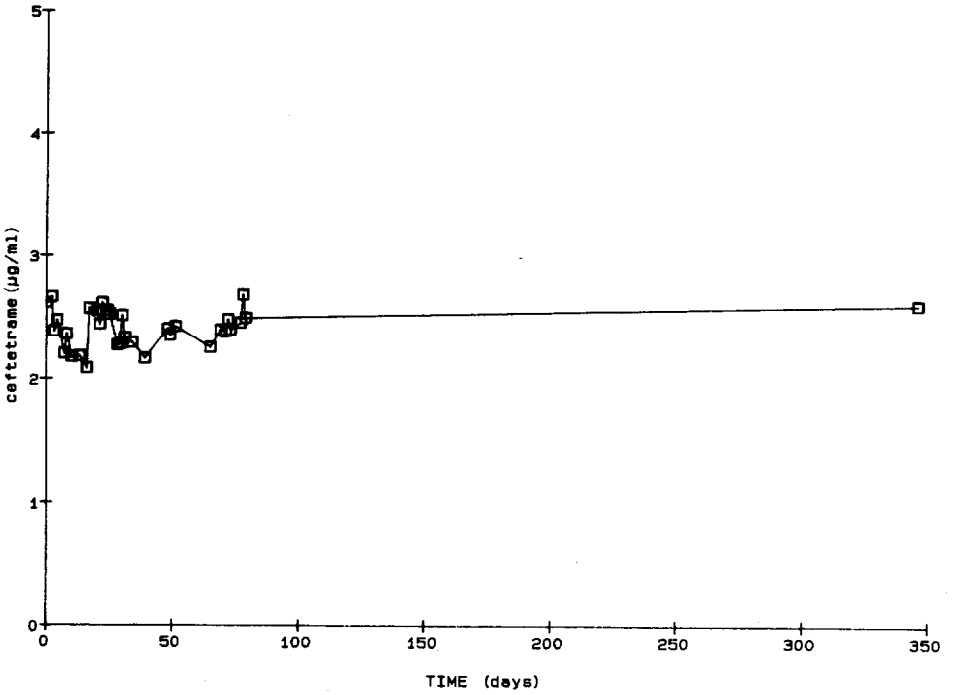


Fig. 4. Stability of ceftetrame in the human plasma quality assurance sample.

TABLE IV

BENCHTOP STABILITY OF CEFTETRAME ($n=3$)

Time (h)	Concentration of ceftetrame (mean ± S.D.) (µg/ml)	Relative standard deviation (%)
0	3.08 ± 0.06	1.9
3	3.06 ± 0.07	2.6
6	3.22 ± 0.11	3.4

cant effect of the vacutainer when compared to a glass tube and that over 85% of the drug is in the plasma fraction of the blood.

Fig. 4 contains the results of the assay of the quality assurance sample of ceftetrame in human plasma. Aliquots of a pooled experimental sample were analyzed after being frozen at −70°C for up to eleven months. These data indicate that when stored at −70°C, ceftetrame shows no degradation for up to eleven months. The variability shown is within 10% of the mean value and is thus considered to be due to experimental error. Table IV contains data showing the concentration of analyte in the same sample left on the bench top at room temperature (24°C) for up to 6 h. Samples were analyzed and compared to a 0-h sample. The data indicate that the compound is stable in human plasma for 6 h at room temperature.

CONCLUSIONS

A sensitive and specific HPLC assay has been developed for the analysis of ceftetrame in plasma and urine. The method may also be applied to samples from other species besides man. The assay is simple and rapid, and requires a minimum of organic solvents. The assay was validated over the range of 0.48–9.54 μg/ml in plasma and 19.1–381.6 μg/ml in urine.

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