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Note

Rapid analysis of ceftetrame in human plasma using sorbent extraction and high-performance liquid chromatography

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Ceftetrame is the acid metabolite of the orally active cephalosporin pivaloyloxymethyl(6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-[(5-methyl-2H-tetrazol-2-yl)methyl]-3-cephem-4-carboxylate. The in vitro activity of ceftetrame against several organisms has previously been demonstrated [1–3]. Ceftetrame has been analyzed in plasma or urine using a high-performance liquid chromatographic (HPLC) method which utilized reversed-phase chromatography with UV detection at 225 nm [4]. The sensitivity of the method in plasma was limited to 0.48 μg/ml, however, and a lengthy rinse cycle was necessary after each injection in order to prevent detrimental effects on chromatography, resulting in long run times.

In this paper we describe modifications which resulted in an increase in sensitivity to a limit of quantitation of 59.8 ng/ml and a shortening of analysis time from approximately 26 min to 14 min per sample. The results obtained for extraction from human plasma are described, but the method has also been successfully applied to human urine, dog plasma and rat plasma.

EXPERIMENTAL

Chemicals and reagents

Sodium ceftetrame was obtained from the Quality Control Department, Hoffmann-La Roche (Nutley, NJ, U.S.A.). Sodium benzylpenicillin (penicil-

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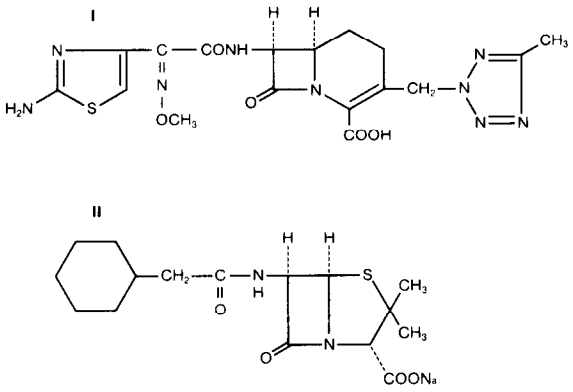

Fig. 1. Structures of sodium ceftetrame (I) and sodium benzylpenicillin (penicillin G) (II).

lin G) (Fig. 1) and Trizma base [tris(hydroxymethyl)aminomethane] were obtained from Sigma (St. Louis, MO, U.S.A.). Monobasic potassium phosphate was obtained from Mallinckrodt (Paris, KY, U.S.A.). Potassium hydroxide was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Hydrochloric acid (reagent ACS grade) and methanol (HPLC grade) were obtained from Fisher Scientific (Springfield, NJ, U.S.A.). All water was distilled and deionized.

Monobasic potassium phosphate (136 g) was dissolved in 1000 ml distilled water and the pH was adjusted to 5.2 using potassium hydroxide (28 g in 100 ml water). This stock solution was diluted 1:100 to obtain a solution of 0.01 *M* potassium phosphate.

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

Standard solutions

Ceftetrame stock solution. Sodium ceftetrame (100 mg) was dissolved in 100 ml of water to obtain a solution of 0.956 mg/ml ceftetrame (salt corrected), which was stored at 4°C.

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

Ceftetrame spiking solutions. Aliquots of 1000, 500, 200, 100, 50 and 25 µl of the 0.956 mg/ml ceftetrame stock solution were placed in 10-ml volumetric flasks and brought to volume with distilled water to give spiking solutions of 95.6, 47.8, 19.1, 9.56, 4.78 and 2.39 µg/ml, respectively. A seventh solution of 1.20 µg/ml was prepared by taking an aliquot of 125 µl of the 95.6 µg/ml spiking

solution and diluting to 10 ml with distilled water. All solutions were stored at 4°C.

Sodium benzylpenicillin spiking solution. A 1-ml volume 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 500 μg/ml, which was stored at −20°C.

Chromatographic conditions

The HPLC system consisted of a Kratos Spectroflow 400 pump (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.) with a Perkin-Elmer ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.). The separation was performed on a prepacked 15 cm × 3.9 mm I.D. stainless-steel column containing 4-μm Nova-Pak phenyl packing (Waters Assoc., Milford, MA, U.S.A.). The UV detector was a Kratos Spectroflow 783 absorbance detector (ABI Analytical), and was operated at a wavelength of 225 nm and a sensitivity of $1 \cdot 10^{-1}$ a.u.f.s. A Spectra-Physics SP4290 integrator was used to record chromatograms and calculate peak heights of ceftetrame and sodium benzylpenicillin. The isocratic mobile phase was 0.01 *M* potassium phosphate buffer (pH 5.2)–methanol (80:20, v/v) at a constant flow-rate of 0.9 ml/min for 14 min. The column temperature was kept constant at 50°C using an SSI Model CH 20-C column heater (Scientific Systems, State College, PA, U.S.A.) resulting in a column pressure of 83 bar. Under these conditions, ceftetrame eluted in 4.6 min and sodium benzylpenicillin in 12 min.

Procedure

Calibration curve samples (prepared in duplicate). To 1 ml of drug-free control human plasma were added 50-μl aliquots of the appropriate ceftetrame and internal standard spiking solutions in 100 mm × 13 mm disposable culture tubes. The resulting ceftetrame concentrations were 4.78, 2.39, 0.956, 0.478, 0.239, 0.120 or 0.0598 μg/ml and 25 μg/ml sodium benzylpenicillin.

Quality assurance (QA) samples. Samples of known concentrations were prepared at two levels, low and high, in order to check the accuracy of the method. The QA low samples were prepared by taking a 30-μl aliquot of the 95.6 μg/ml ceftetrame spiking solution and diluting it to 10 ml with drug-free control human plasma, resulting in a concentration of 0.2868 μg/ml ceftetrame. The QA high samples were prepared by taking a 30-μl aliquot of the 0.956 mg/ml ceftetrame stock solution and diluting it to 10 ml with drug-free control human plasma, resulting in a concentration of 2.868 μg/ml. Sufficient QA samples were prepared to allow for duplicate aliquots to be analyzed with each set of samples throughout the analyses. Aliquots (1 ml) of these samples were extracted along with each standard curve and the concentrations were calculated based on the linear regression analysis of the standard curve. Cal-

culated concentrations were then compared to the theoretical concentrations to check the method.

Extraction

Samples were thawed, 2 ml of 0.01 *M* Tris buffer (pH 7.0) were added to each tube along with 25 μg of sodium benzylpenicillin, and the contents were vortexed. Bond Elut phenyl columns (Analytichem International, Harbor City, CA, U.S.A.) were placed in a Vac Elut manifold (Analytichem International) and washed with 3 ml of methanol followed by 3 ml of 0.01 *M* Tris buffer. The samples were then loaded onto the columns and drawn through by applying 254 mmHg vacuum. The flow-rate was kept constant by controlling the vacuum. The columns were again washed with 0.01 *M* Tris buffer and allowed to drain completely. Ceftetrame and sodium benzylpenicillin were eluted into collection tubes using 0.5 ml of 90% methanol in 0.01 *M* monobasic potassium phosphate (pH 5.2). The eluate was transferred to autosampler vials for injection. An injection volume of 25 μl was used the analyze the samples.

RESULTS AND DISCUSSION

Typical chromatograms from (A) control human plasma and (B) human plasma spiked with ceftetrame (2.39 μg/ml) and sodium benzylpenicillin (25 μg/ml) as internal standard are shown in Fig. 2. There were no interfering peaks in any control plasma samples at either the retention time of ceftetrame or internal standard.

The inter-assay precision of the method in plasma was evaluated over a concentration range of 0.0598–4.78 μg/ml (Table I).

The mean, standard deviation (S.D.) and relative standard deviation (R.S.D.) were calculated based on three separate standard curves with two samples at each concentration, giving a total of six values at each concentration. The overall R.S.D. was found to be 4.5%, indicating good precision over this concentration range. The correlation coefficient for each of the standard curves analyzed was found to be >0.9995 showing excellent linearity of the method. The limit of quantitation, defined as the lowest concentration which can be accurately and reliably quantitated (R.S.D. $\leq 15\%$), was 59.8 ng/ml.

The intra-assay precision was also determined for the three standard curves analyzed, by dividing one member of each set of duplicates by the other. The overall intra-assay precision was found to be 4.0%.

The average measured concentrations of the QA low and QA high samples were determined from the analyses of six replicate samples. The QA low was 0.279 μg/ml (actual concentration 0.287 μg/ml) with an average relative error of 6.3%. The QA high was 2.77 μg/ml (actual concentration 2.87 μg/ml) with an average relative error of 5.1%. Both of these errors are within typical experimental error and show good accuracy of the method.

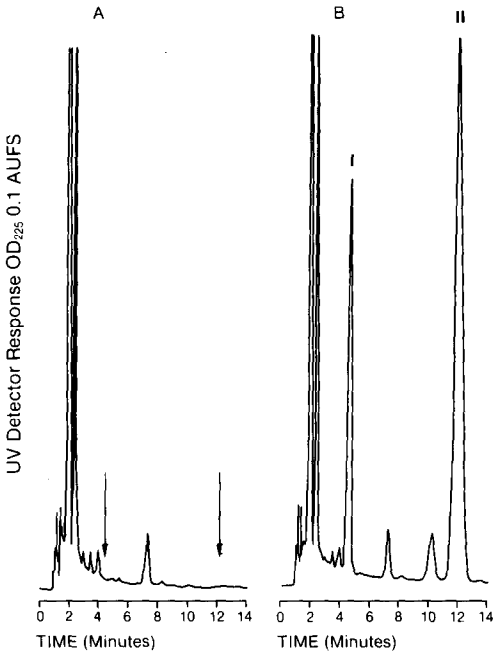

Fig. 2. Chromatograms of ceftetrame and sodium benzylpenicillin. (A) Control human plasma; arrows indicate retention time of ceftetrame and sodium benzylpenicillin. (B) Control human plasma spiked with 2.39 μg/ml ceftetrame (I) and 25 μg/ml sodium benzylpenicillin (II).

TABLE I

INTER-ASSAY PRECISION FROM CONSIDERATION OF THE CALIBRATION DATA

Fit of back-calculated concentrations to calibration line.

Ceftetrame added (μg/ml)	Concentration found (mean ± S.D.) (μg/ml)	R.S.D. (%)
0.0598	0.0587 ± 0.0068	11.5
0.120	0.119 ± 0.005	4.5
0.239	0.243 ± 0.014	5.8
0.478	0.475 ± 0.019	3.9
0.956	0.967 ± 0.021	2.2
2.39	2.38 ± 0.06	2.3
4.78	4.79 ± 0.08	1.6
Overall R.S.D.		4.5

The previously described HPLC method for ceftetrame analysis described the use of 100% methanol to elute the compounds of interest off of a Bond Elut column [4]. This resulted in interfering peaks which limited the sensitivity of the assay, as well as significant late eluting peaks which interfered with subsequent analyses. To correct this, a 4–6 min column wash using 100% methanol was required followed by an additional 6 min at original column conditions to re-equilibrate the column. The result was a total run time of at least 26 min for each specimen. The modification of the elution solvent to 90% methanol in 0.01 *M* monobasic potassium phosphate (pH 5.2) eliminated all interfering peaks as well as late eluting peaks. This had the dual advantage of reducing the run time approximately 50% (14 min) for each specimen by eliminating the need for a column wash and re-equilibration step, as well as eliminating the need for an HPLC system with gradient capability.

The flow-rate was reduced from 2 to 0.9 ml/min and the column temperature was raised from ambient to 50°C, thus improving peak shape and resolution while reducing column pressure to a more desirable 83 bar. This significantly aided daily operations, since the previously reported operating pressure of > 207 bar [4] resulted in frequent system 'shut downs' due to excessive pressure.

As a result of the modifications described above, the sensitivity of the assay was greatly increased from a limit of quantitation of 480 to 59.8 ng/ml. This will prove advantageous in future pharmacokinetic studies enabling drug detection at later time periods following dosing.

CONCLUSIONS

We have described modifications to a previously reported HPLC method for determining ceftetrame in human plasma which have shortened the run time significantly, improved the system stability and lowered the limit of quantitation by a factor of 10. Although only described for human plasma, this modified method has been successfully applied to human urine, as well as dog and rat plasma with only minimal changes to the composition of the organic modifier in the mobile phase. The increased sensitivity will prove valuable in pharmacokinetic studies.

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