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DETERMINATION OF CEFIXIME IN BIOLOGICAL SAMPLES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, accurate and precise isocratic reversed-phase high-performance liquid chromatographic method has been developed and validated for the determination of a new cephalosporin in human serum and urine. Human serum samples, calibration standards and quality control samples (250 μl) were combined with an equal volume of 6% trichloroacetic acid (TCA). Human urine (0.1 ml) was combined with 6% TCA solution containing the internal standard. The compounds were detected by ultraviolet absorbance set to 280 nm for the serum assay and 313 nm for the urine assay. The method for the determination of cefixime in serum was linear from 100 ng/ml to 30.0 μg/ml ($r=0.999$), and for the urine assay from 5 μg/ml to 100 μg/ml ($r=0.999$). The minimum reportable quantity for the serum assay was 0.05 μg/ml. The within- and between-day assay variation for both assays were found to be less than 10% in an extensive assay validation scheme. Results of a storage stability study indicated that human serum and urine samples could be safely stored for up to six months at $-18°C$ and three months at $-10°C$, respectively.

INTRODUCTION

Cefixime, [6*R*-[6α,7β(*z*)]]-7-[[(2-amino-4-thiazolyl)[(carboxymethoxy)-imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-

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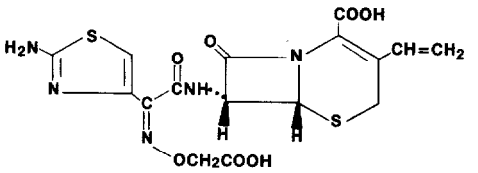

Fig. 1. Chemical structure of cefixime.

carboxylic acid (Fig. 1), is a new orally effective semi-synthetic cephalosporin, which provides antibacterial activity against the most commonly isolated gram-negative bacteria [1]. This compound has been shown to be more potent than other orally active β-lactam antibiotics including cephalexin, cefaclor and amoxicillin [2].

A number of recent publications have described high-performance liquid chromatographic (HPLC) methods for assaying cephalosporins in plasma or serum [3–6]. These methods commonly employ solvent extraction or protein precipitation with acetonitrile prior to reversed phase LC analysis with ultraviolet detection [3,4]. However, these methods are not generally applicable to the determination of the highly polar cefixime due to: (1) lack of a suitable extraction procedure, such as conventional two phase liquid–liquid extraction techniques or ion-pair extraction with quaternary ammonium salts; (2) need for submicrogram sensitivity; and (3) lack of a suitable internal standard which is resistant to decomposition under the conditions of sample preparation. An automated column-switching HPLC procedure has also been described for the determination of cefixime in human serum and urine [7].

The present study was undertaken to develop a rapid, sensitive and precise isocratic reversed-phase HPLC assay for the determination of cefixime in serum and urine. The sample preparation procedure is simple and rapid, requiring only precipitation of proteins with a 6% solution of trichloroacetic acid (TCA). A 75-μl aliquot of the supernatant is used for each HPLC determination. The internal standard used in the assay is 7-hydroxycoumarin. The assay has been used to ascertain the stability of cefixime in human serum and urine stored at -18 and $-10°C$, respectively. The method has been shown to be applicable to the determination of cefixime in serum and urine, and is free of interferences from many drugs which might be prescribed concomitantly.

EXPERIMENTAL

Reagents and materials

The powder reference standard of cefixime was prepared by Fujisawa Pharmaceutical (Osaka, Japan), and was determined to contain 88.1% cefixime by weight and 11.9% water of hydration. Reagent-grade monobasic sodium phosphate, 85% phosphoric acid and TCA, and HPLC-grade acetonitrile and methanol were obtained from Baker (Phillipsburg, NJ, U.S.A.). The water used in the preparation of stock solutions and mobile phases was purified with a Milli-Q

water purification system (Millipore, Bedford, MA, U.S.A.). The internal standard, 7-hydroxycoumarin, was purchased from Aldrich (Milwaukee, WI, U.S.A.).

Apparatus

The liquid chromatograph was assembled from components primarily from Waters Assoc. (Milford, MA, U.S.A.) which included a WISP Model 710B sample processor, a Model 590 high-pressure pump, a Model 440 ultraviolet absorbance detector fitted with a 280-nm or a 313-nm bandpass interference filter for the serum or urine assay procedures, respectively. The detector was operated at a sensitivity range of 0.05 a.u.f.s. An RCM-100 Nova-Pak C_{18} (10 cm × 8 mm I.D., 5 μm particle size) analytical column was used to perform the chromatographic separation at ambient temperature. The isocratic mobile phase was pumped at 2.0 ml/min and was filtered and degassed prior to use on each day of analysis. Data acquisition and peak quantitation were carried out by a Hewlett-Packard 3357 laboratory automation system.

Cefixime standard solutions

Stock solutions of cefixime are prepared by dissolving 11.4 mg of the compound (equivalent to 10.0 mg of cefixime corrected for purity) in 1.0 ml of HPLC-grade methanol and diluting the mixture to 10.0 ml with distilled water in volumetric glassware. This stock solution, equivalent to 1.0 mg/ml, was stored at 3 °C for up to one week. The 1.0 mg/ml stock was further diluted 1:10 with water to prepare an additional standard that was 100 μg/ml.

Serum and urine calibration standards

Calibration standards for the serum and urine assay were prepared by adding microliter amounts of the 100 μg/ml or 1.0 mg/ml stock cefixime solutions to the appropriate volume of drug-free biological fluids. Serum cefixime calibration standards were prepared at concentrations of 0.05, 0.5, 1.0, 2.0, 10.0, 15.0, 20.0 and 30.0 μg/ml. The urine cefixime calibration standards were prepared at concentrations of 5.0, 10.0, 20.0, 40.0, 60.0, 90.0 and 100 μg/ml.

Internal standard solution

A stock solution of the internal standard, 7-hydroxycoumarin, was prepared by dissolving 10.0 mg of the compound in 100 ml of methanol. The stock solution was stored at 3 °C and was stable for up to one month. A working solution for the serum assay was prepared fresh on each day of analysis by dissolving 0.6 g of TCA in 10.0 ml of distilled water containing 400 μl of the stock internal standard solution. A similar reagent for the urine assay was prepared by adding 0.25 ml of the stock internal standard solution to 100 ml of 6% TCA in water resulting in a final internal standard concentration of 0.25 μg/ml.

Mobile phases

Mobile phase was prepared fresh on the day of analysis and was filtered and degassed by vacuum. Mobile phase A (used for the serum assay) was prepared by combining 170 ml of acetonitrile, 1.36 g of monobasic sodium phosphate, 2 ml

of 85% phosphoric acid and 828 ml of distilled water. Mobile phase B (used for the urine assay) was prepared by combining 200 ml of acetonitrile, 1.36 g of monobasic sodium phosphate, 2 ml of 85% phosphoric acid and 798 ml of distilled water. The resulting pH of both mobile phase solutions was approximately 2.7.

Serum and urine sample preparation

A 250-μl aliquot of the serum calibration standard or unknown sample was transferred to a polypropylene 1.5-ml snap-cap conical-bottom centrifuge tube (Eppendorf, Hamburg, F.R.G.) to which were added 250 μl of the working 6% TCA reagent containing 1.0 μg of the internal standard. The tubes were vortexed at high speed for 15 s and centrifuged at 30 000 *g* for 2 min. The urine sample preparation procedure was similar except that 0.1 ml of sample was diluted with 2.0 ml of the 6% TCA solution containing 0.25 μg/ml internal standard. A 200-μl portion of the clear supernatant was transferred to an autosampler microvial, 75 μl of which were injected for each analysis. These samples were found to be stable for at least 48 h at room temperature.

Quantitation

Drug concentrations in the unknown serum and urine samples were calculated by interpolation from the linear least-squares regression line of the multi-level standard curve plot of peak-height ratio (height drug/height internal standard) versus cefixime concentration in the calibration standards. Data acquisition and reduction were processed by an HP-3357 laboratory automation system.

RESULTS

Separation

Chromatograms from the isocratic HPLC analysis of human serum and urine samples are shown in Figs. 2 and 3, respectively. Fig. 2D shows the chromatogram from the analysis of blank human serum, Fig. 2A and B show the resulting chromatograms from the analysis of serum samples supplemented with cefixime to yield concentrations of 5.0 and 0.1 μg/ml, respectively, and Fig. 2C shows a chromatogram obtained from a subject receiving a 400-mg oral dose of the drug. Fig. 3D shows the chromatogram from the analysis of blank human urine, Fig. 3A and B show the resulting chromatograms from the analysis of urine samples supplemented with cefixime to yield concentrations of 40.0 and 4.0 μg/ml, respectively, and Fig. 3C shows a chromatogram obtained from a subject receiving a 400-mg oral dose of the drug. The detection wavelength and sensitivity were individually adjusted for these sample types and are indicated in the figures. The chromatographic peaks have baseline resolution and are also well resolved from other peaks in the chromatogram. No significant peaks were observed after the retention time of the internal standard and the baselines were not adversely affected by small changes in the composition of the mobile phase. However, maintaining the mobile phase pH at 2.7 was found to be an important consideration for achieving acceptable cefixime chromatographic resolution.

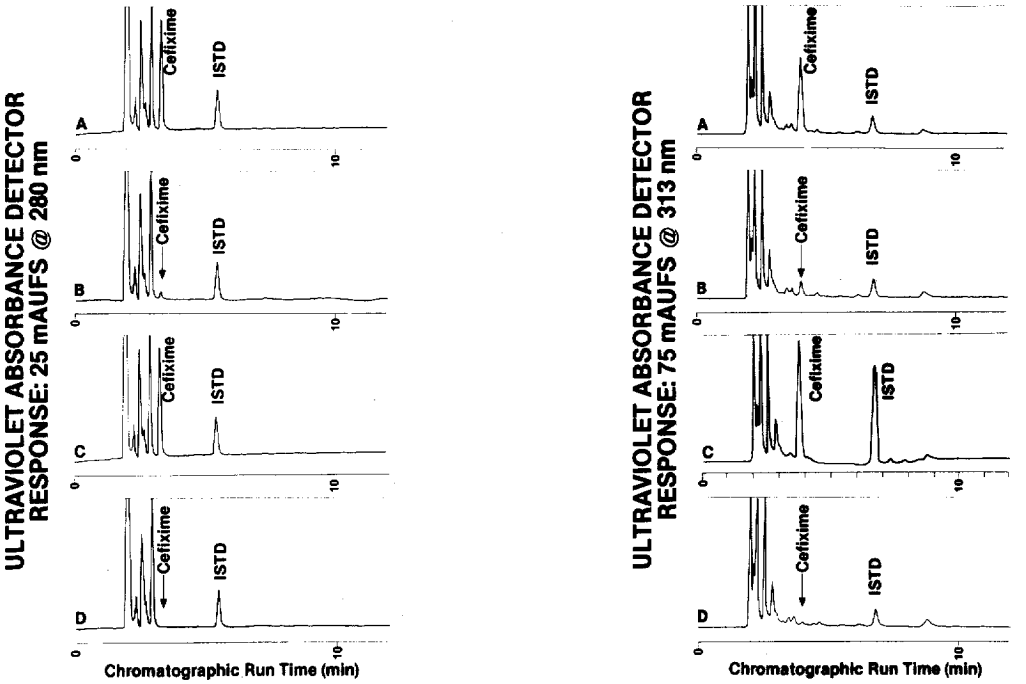

Fig. 2. Typical chromatograms of (A, B) human serum samples supplemented with cefixime to yield concentrations of 5.0 μg/ml (A) and 0.1 μg/ml (B), (C) from a patient receiving a 400-mg oral dose and (D) blank human serum. ISTD = internal standard.

Fig. 3. Typical chromatograms of (A, B) human urine samples supplemented with cefixime to yield concentrations of 40 μg/ml (A) and 4.0 μg/ml (B), (C) from a patient receiving a 400-mg oral dose and (D) blank human urine. ISTD = internal standard.

Recovery

The intra- and inter-subject variability in the recovery of cefixime from human serum samples was investigated. Cefixime was added to serum obtained from twelve human subjects to yield samples that contained 1.0 μg/ml. Samples were assayed, and recovery was calculated with respect to an aqueous sample prepared in the same way. Results of the recovery analysis indicate a mean inter-subject ($n = 12$) recovery of cefixime from human serum of 60.2% using the TCA sample preparation procedure. The intra-subject mean recovery ($n = 6$) was found to be 65.6% with a coefficient of variation (C.V.) of 1.6%.

Assay performance

The performance characteristics for the determination of cefixime in human serum and urine using the prescribed assay procedure are presented in Table I.

Assay validation results

The design of the validation study was selected to evaluate the major contributory factors to overall assay variability, including analysts, time and instruments. For the validation, two analysts, using two HPLC systems, assayed two

TABLE I

TYPICAL ASSAY PERFORMANCE DATA FROM CALIBRATION CURVES FOR THE DETERMINATION OF CEFIXIME IN HUMAN SERUM AND URINE

Sample	Linear concentration range (μg/ml)	UV (nm)	Slope	Intercept	Correlation coefficient	MRQ* (μg/ml)
Serum	0.1- 30	280	0.266	0.0036	0.9999	0.05
Urine	5.0-100	313	0.011	0.0002	0.9999	1.0

*MRQ = Minimum reportable quantity.

TABLE II

SUMMARY OF VALIDATION DATA FOR THE DETERMINATION OF CEFIXIME IN SERUM AND URINE

Concentration added (μg/ml)	Concentration found (mean ± S.D., $n=24$) (μg/ml)	Coefficient of variation (%)
Serum		
0.1	0.102 ± 0.007	6.8
0.2	0.200 ± 0.008	3.8
0.4	0.402 ± 0.020	5.0
1.0	1.02 ± 0.04	4.1
5.0	5.12 ± 0.21	4.2
15.0	14.9 ± 0.55	3.7
25.0	28.8 ± 1.24	4.8
Urine		
10.0	9.38 ± 0.82	8.7
30.0	27.8 ± 0.83	3.0
50.0	51.4 ± 1.33	2.6
90.0	88.6 ± 3.19	3.6

standard curves, one prior to and one following each day's run, and seven unknown concentrations of the validation samples in duplicate, randomized over a three-day period. Results of the validation study for serum and urine are shown in Table II.

The serum assay demonstrated linearity between 0.1 and 30 μg/ml ($r > 0.999$, $p < 0.001$) for cefixime. The minimum reportable concentration was 0.05 μg/ml. A series of serum samples with known amounts of cefixime added (validation samples) were found to have an overall C.V. of 4.6% and the difference between the observed and targeted concentrations was less than 3.2%.

The urine assay was observed to be linear ($r > 0.99$, $p < 0.01$) from 5 to 100 μg/ml cefixime with less than 9% C.V. for concentrations between 10 and 90 μg/ml.

Storage stability

The in-vitro storage stability of cefixime was evaluated at −18°C in serum and −10°C in urine. Results of the stability data are presented in Table III.

TABLE III

IN VITRO STORAGE STABILITY DATA FOR CEFIXIME IN HUMAN SERUM AND URINE

Human serum storage stability at −18°C; target serum cefixime concentration 0.50 μg/ml. Human urine storage stability at −10°C; target urine cefixime concentration 5.0 μg/ml.

Storage time (days)	Found concentration (μg/ml)	C.V. ($n=3$) (%)	Change* (%)
Serum			
Initial	0.50	5.5	–
30	0.48	1.7	−4.0
60	0.49	4.2	−2.0
90	0.40	3.1	−20.0
180	0.47	4.1	−12.0
Urine			
Initial	4.62	5.1	+2.0
14	5.23	11.9	+13.2
35	4.29	0.6	−7.1
90	5.66	10.0	+22.5

*Reported as percent of initial concentration.

The serum and urine stability samples were assayed initially and stored at −18 and −10°C, respectively, in the dark. Periodically, samples were removed from the freezer, equilibrated to room temperature and assayed in triplicate using freshly prepared serum and urine calibration standards.

Results of the storage stability investigation indicate that serum samples may be safely stored for up to six months at −18°C and urine samples may be stored for up to three months at −10°C.

CONCLUSION

An accurate and precise isocratic HPLC procedure has been developed for the determination of cefixime in human serum and urine. The minimum reportable concentration is 0.05 and 1 μg/ml in serum and urine, respectively. This procedure involves the direct injection of the supernatant resulting from deproteinizing biological samples using 6% TCA onto an isocratic reversed-phase HPLC system with ultraviolet detection. The recovery of cefixime from serum was found to be 65% and was consistent (C.V.=4.5%) in an intra-subject ($n=12$) evaluation.

The reproducibility in the determination of cefixime was found to be improved by peak-height ratio calibration with the use of the internal standard, 7-hydroxycoumarin. The detection wavelength for the determination of cefixime in serum (280 nm) was selected for maximum sensitivity. No significant interfering peaks were observed in the elution area of cefixime. Selectivity for the determination of cefixime in human urine samples was greatly enhanced by increasing the detection wavelength to 313 nm, although sensitivity was reduced by this change. However, since urine cefixime concentrations are generally much greater than

serum concentrations, the compromised sensitivity in urine samples is not important.

Endogenous substances normally present in serum and several commonly administered drugs, including acetominophen [retention time (t_R) = 0.99 min], salicylate (t_R = 2.83 min), theophylline (t_R = 0.80 min) and caffeine (t_R = 1.07 min) did not interfere with the assays. This procedure has been used for the determination of cefixime in biological samples from studies in humans, as well as in the dog, rat and monkey.

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