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Short Communication

Microanalytical high-performance liquid chromatographic assay for cefpirome in human milk and urine

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ABSTRACT

To permit the characterization of cefpirome disposition in lactating females, a previously published high-performance liquid chromatographic (HPLC) method for determining the drug in serum was adapted for use with milk and urine. This automated, microanalytical technique requires 50 μl of biological matrix, which is subjected to an isopropanol extraction. Chromatography was accomplished using a microbore HPLC system, a reversed-phase C_{18} column and a mobile phase of 0.3% triethylamine in water (pH 5.1). Cefpirome and the internal standard (β-hydroxypropyltheophylline) were monitored using UV detection at 240 nm and had retention times of 2.84 and 5.05 min, respectively. The method was linear up to 500 mg/l for both matrices and had a limit of detection of 0.6 mg/l. The interday variation (relative standard deviation) at concentrations of 5.0, 50.0 and 500.0 mg/l was consistently $< 5\%$ in both urine and breast milk. The method was found to be free from interference by other commonly administered medications and readily adaptable for use in clinical investigations. The ease of sample preparation, small sample volume requirement, short chromatographic time, apparent lack of interferences, analytical sensitivity and high precision and accuracy make this method ideal for use in pharmacokinetic investigations involving the determination of cefpirome in human milk and urine.

INTRODUCTION

Cefpirome (HR 810), 3-[(2,3-cyclopentento-1-pyridinium)methyl]-7-[2-methyoximino-2-(2-aminothiazole-4-yl)acetamido]ceph-3-em-4-carboxylate, is a new aminothiazolyl cephalosporin which has an expanded antimicrobial spectrum as compared with currently marketed third-

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generation cephalosporins [1]. *In vitro* studies have shown that cefpirome is as active as or more active than cefotaxime and ceftazidime against *Haemophilus influenzae, Neisseria* spp. and Enterobacteriacea [1]. While its activity against *Pseudomonas aeruginosa* is reported to be less than that for ceftazidime [1], combined *in vitro* and *in vivo* studies have shown that cefpirome killed methicillin-resistant *Staphylococcus aureus* as rapidly as did vancomycin [2]. Based on its wide antimicrobial spectrum cefpirome would appear to be a potentially valuable antimicrobial agent for the treatment of infections which are common in the postpartum period. Consequently, it will be of clinical importance to characterize the disposition of cefpirome in lactating females and their infants, and to determine the amount of drug excreted into breast milk.

Selective and sensitive high-performance liquid chromatographic (HPLC) methods for the determination of cefpirome in serum [3–6] and urine [3,4] have been developed to permit pharmacokinetic studies in adults. Available methods may have limited utility for performing similar investigations in neonates, infants and children as the serum sample volume requirement is generally $\geq$0.5 ml [3–6]. We previously developed a microanalytical HPLC method for cefpirome which maintained sufficient sensitivity (*i.e.*, 0.6 mg/l) and required only 0.05 ml of serum [7]. We now report the adaptation of this method to urine and human milk.

EXPERIMENTAL

Reagents and standards

Triethylamine buffer (0.3%) was prepared by adding 3.0 ml of triethylamine (Pierce, Rockford, IL, USA) to 1000 ml of analytical-reagent grade water and adjusting the pH to 5.1 with glacial acetic acid (Sigma, St. Louis, MO, USA). The mobile phase was methanol–triethylamine (12:88, v/v) buffer for milk samples and methanol–water (10:90, v/v) for urine. Prior to introduction into the chromatographic system, the triethylamine buffer was filtered through a GA-8 Metricell filter (pore size 0.2 μm; Gelman Sciences, Ann Arbor, MI, USA). Following filtration, the mobile phase was deoxygenated by passing helium through the solution at 100 ml/min for 15 min prior to use and at 5 ml/min during chromatography. All solvents used in sample peparation and/or chromatography were of HPLC grade (American Burdick and Jackson, Muskegon, MI, USA).

The internal standard (I.S.) was prepared by dissolving 10 mg of β-hydroxypropyltheophylline (Sigma) in 10 ml of analytical-reagent grade water. A 2-ml volume of this stock solution was diluted to a total volume of 10 ml with isopropanol to yield a working I.S. concentration of 200 mg/l.

Analytical-reagent grade cefpirome sulfate was donated by the manufacturer (Hoechst-Roussel Pharmaceuticals, Somerville, NJ, USA). A working standard solution was prepared by dissolving 12.3 mg of the drug in 1.0 ml of analytical-reagent grade water to yield a final cefpirome (base) concentration of 10 mg/ml. Cefpirome standards were prepared in drug-free human milk and urine obtained from a single volunteer donor by simple addition and serial dilution. The concentrations of cefpirome in the analytical standards were 500, 200, 100, 50, 25, 10, 5, 2.5, 1.25 and 0.625 mg/l. Triplicate samples at each concentration were used to establish calibration graphs for urine and milk.

Chromatography system

Chromatography was accomplished on a Hewlett-Packard Model 1090L microbe system with a variable-volume injector and a diode-array detector. Separations were performed using an ODS column (10 cm × 2.1 mm I.D.) with a particle size of 5 μm (Hewlett-Packard). The analytical column was maintained at 50°C and the mobile phase flow-rate at 0.5 ml/min. The eluate was monitored at 240 nm and 0.001 a.u.f.s.

Sample preparation

After gentle vortex mixing to homogenize the specimen, milk samples were prepared as described previously for serum [7] by adding 200 μl of the isopropanol–I.S. solution to 50 μl of sample. Samples were vortex mixed for *ca.* 20 s, centrifuged at 2000 g and 4°C for 2 min and the supernatant was removed and dried at 25°C under a gentle stream of nitrogen. Samples were then re-

constituted with 200 μl of the mobile phase, resuspended by vortex mixing and centrifuged as described previously. Following the second centrifugation, 10 μl of the clear supernatant were injected into the chromatograph.

Urine samples were initially centrifuged at 1500 *g* and 4°C for 5 min to sediment any gross particulate matter. A 50-μl volume of this supernatant was added to 200 μl of mobile phase containing 200 μg/ml of the I.S. After vortex mixing for *ca.* 15 s, 10–20 μl of this diluted sample were injected into the chromatograph.

Evaluation of potential interfering substances

Analytical-reagent grade standards obtained from a commercial vendor (Sigma) and, when necessary, from various pharmaceutical manufacturers, were evaluated for possible chromatographic interferences. Solutions of antimicrobial agents which would be expected to be co-administered with cefpirome (*i.e.*, ampicillin, tobramycin, gentamicin, amikacin and ticarcillin) were prepared in the mobile phase at concentrations of 1000 mg/l. Standards of other commonly administered medications (*i.e.*, acetylsalicylic acid, acetaminophen, ibuprofen, theophylline, caffeine, chlorpheniramine, cimetidine, carbamazepine, phenytoin and phenobarbital) were also prepared at a concentration of 200 mg/l in the mobile phase. Each of these standards (25–50 μl) was injected into the HPLC system and monitored for 15 min under the conditions used for the determination of cefpirome in both urine and milk. The retention time of the drug peak was then compared with that for either cefpirome or the I.S. to determine the potential for interference.

RESULTS

Representative chromatograms for high (500 mg/l) and low (0.625 mg/l) concentrations of cefpirome in urine and human milk are shown in Figs. 1 and 2, respectively. There was clear resolution of each compound in both matrices. The retention times for cefpirome and the I.S. in urine were *ca.* 2.8 and 5.0 min, respectively, and in milk *ca.* 2.0 and 4.0 min, respectively. Extraction and chromatographic analysis of triplicate blank samples of both urine and breast milk confirmed the absence of co-eluting peaks with retention times corresponding to those of either cefpirome or the I.S. This is illustrated by the representative chromatograms from blank urine and milk shown in Fig. 3.

Linearity was demonstrated (*i.e.*, *p* value from ANOVA for linearity <0.0001) by analysis of

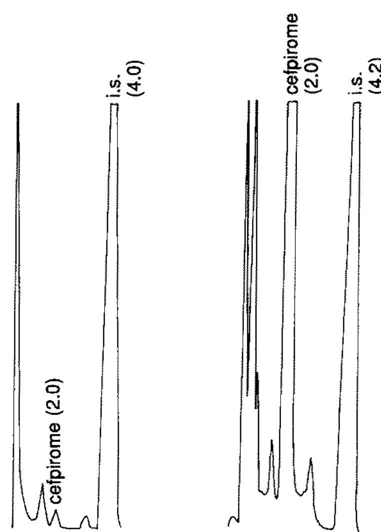

Fig. 1. Chromatograms of breast milk containing (left) 0.625 and (right) 500.0 mg/l cefpirome. Retention times (min) for cefpirome and the internal standard (i.s.) are shown.

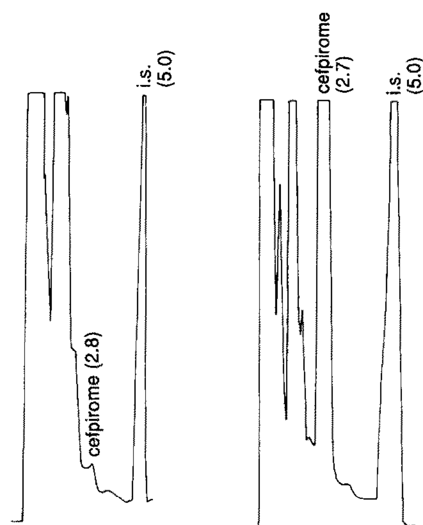

Fig. 2. Chromatograms of urine containing (left) 0.625 and (right) 500.0 mg/l cefpirome. Retention times (min) for cefpirome and the internal standard (i.s.) are shown.

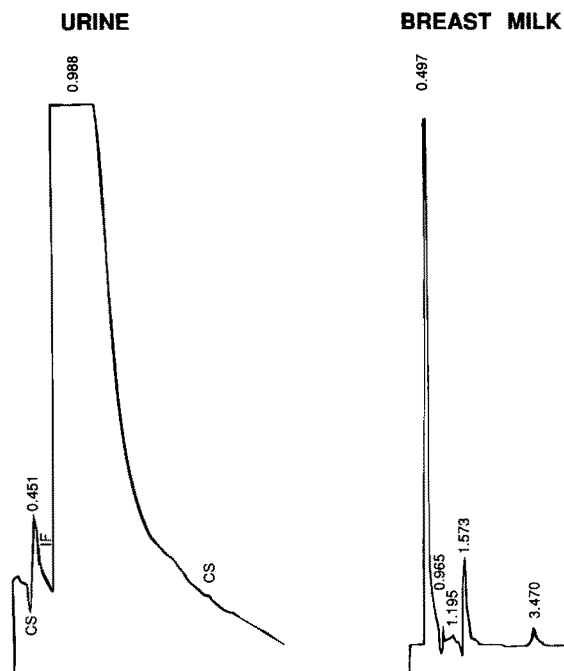

Fig. 3. Chromatograms of drug-free (*i.e.*, blank) urine and breast milk. Retention times (min) for unidentified endogenous substances are shown.

triplicate calibration solutions with cefpirome concentrations ranging from 0.625 to 500 mg/l for both milk and urine. The respective regression parameters from the mean data for each calibration graph were as follows: milk, $y = 0.0065x + 0.00733$, $r^2 = 0.9999$; and urine, $y = 0.006x + 0.0024$, $r^2 = 0.9999$ (y = peak-area ratio; x = cefpirome concentration). For each biologial matrix, the y-intercept of the respective calibration graphs was not significantgly different from zero (*i.e.*, $p > 0.05$ from Student's t-test). The limit of detection was defined *a priori* as the lowest point on the calibration graph that exhibited $<10\%$ bias and $<10\%$ variability, and for this asay was established at 0.625 mg/l. Back-calculation of this point from calibration analyses performed in triplicate revealed $<6\%$ bias and $<4\%$ variability for both biological matrices. These characteristics are in agreement with those found for our initial HPLC assay for cefpirome in serum [7].

The accuracy and precision of the assay were evaluated by preparing replicate samples ($n = 6$ for milk; $n = 8$ for urine) at cefpirome concentrations of 500, 50 and 5 mg/l in both drug-free milk and urine on different days and analyzing them over a 6-week period. The results from this experiment are summarized in Table I. In each instance, the variability was $\leqslant 4.3\%$ with a bias of no more than 4.9%, the latter being associated with the 5.0 mg/l standard. The recovery of cefpirome from milk and urine was assessed by comparing the area of the drug peak from samples ($n = 3$) in each matrix with that obtained from corresponding drug standards prepared in water at concentrations of 500 and 5 mg/l. The analytical recovery was consistently $\geqslant 94\%$ for cefpirome at both concentrations from urine and breast milk.

A number of commonly administered antimicrobial, anticonvulsant, antipyretic, antihistamine and decongestant agents were examined for possible interference with the HPLC method (see Experimental for specific drugs). As reported previously in the evaluation of the method for cefpirome in serum [7], none of the agents examined was found to co-elute with either cefpirome or the internal standard. The storage stability of

TABLE I

ACCURACY AND PRECISION OF THE DETERMINATION OF CEFPIROME IN MILK AND URINE

Data obtained from replicate samples of milk ($n = 6$) and urine ($n = 8$) for each concentration.

Target concentration (mg/l)	Human milk			Urine		
	Mean ± S.D.	R.S.D.[a] (%)	Bias (%)	Mean ± S.D.	R.S.D.[a] (%)	Bias (%)
500	502.4 ± 4.5	0.9	0.9	507.1 ± 6.9	1.4	1.4
50	49.3 ± 0.7	1.3	1.4	48.8 ± 1.0	2.0	2.3
5	5.3 ± 0.2	4.3	4.9	4.9 ± 0.1	2.8	1.2

[a] Relative standard deviation.

the drug in urine and milk was evaluated by preparing replicate samples ($n = 12$) at concentrations of 500, 50 and 5 mg/l, storing them at −10°C and removing duplicate samples for analysis at weekly intervals over a 6-week period. The concentration of cefpirome in the frozen specimens was determined from a calibration graph prepared for each biological matrix on the respective day of analysis. Over this 6-week period, the mean cefpirome concentrations in the frozen breast milk samples were 499.8, 49.3 and 4.9 mg/l and in the urine samples 507.3, 48.3 and 5.1 mg/l. Accordingly, ⩾94% stability of cefpirome in both urine and breast milk stored at −10°C was demonstrated over a 6-week storage period.

DISCUSSION

The modification of our previously reported HPLC method for cefpirome in serum [7] to urine and human milk samples retained both selectivity and sensitivity for this analyte, despite changes in the selection of an ion-pairing agent (triethylamine) and in the composition of the mobile phase. The addition of a second centrifugation step for preparation of the breast milk samples and the deletion of the final filtration step (*i.e.*, passing the supernatant through a 0.2-μm filter in the serum assay) resulted in a satisfactory analytical recovery of cefpirome from both breast milk and urine (*ca.* 94%). The difference in the recovery of cefpirome compared with our previous method for serum [7] should not be of technical and/or clinical importance given the fact that in all instances calibration graphs are prepared with the medium of interest.

Despite the aforementioned modifications made to the assay for milk and urine, commonly used antimicrobial agents, anticonvulsants, antihistamines and decongestants were found not to co-elute with cefpirome or the internal standard. Accordingly, the present method appears to be relatively free from interferences. This assertion must be tempered by the fact that only a limited number of medications were evaluated, and as such, our findings may not be representative of every patient who might receive cefpirome. Additionally, the finding of ⩾94% stability of cefpirome in both biological matrices at −10°C is limited by the 6-week time period over which our investigation was conducted. Further studies of cefpirome stability at −70°C are needed in all biological matrices to be used to characterize its human disposition so that acceptable storage limits for this compound can be determined.

The analytical characteristics of this HPLC method for the determination of cefpirome in urine and human milk appear to render it suitable for use in clinical pharmacokinetic investigations. The small sample volume requirement, ease of sample preparation, short run time, detection limit and specificity of this method make it ideal for application to pharmacokinetic studies in pediatric patients (*i.e.*, where sample volumes must be limited) and in subpopulations of adults when many samples of multiple body fluids may be necessary to characterize completely the disposition characteristics of cefpirome.

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