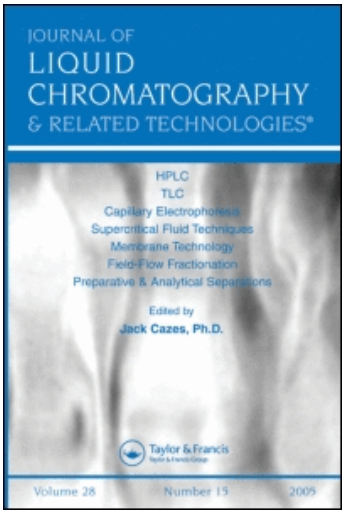

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DETERMINATION OF CEFPODOXIME LEVELS AND CEFPODOXIME STABILITY IN HUMAN URINE BY DIRECT INJECTION HPLC WITH COLUMN-SWITCHING

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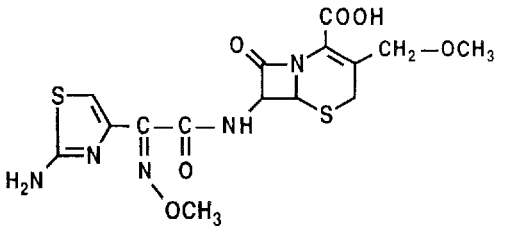
ABSTRACT

A semi-automated method providing on-line sample extraction and quantitative analysis for cefpodoxime in human urine, injected directly into the HPLC, is reported.

Samples were filtered by the analyst, injected into the HPLC system with an autosampler and loaded onto a 3 cm RP-18 precolumn with a mobile phase consisting of 10% methanol in 0.2% phosphoric acid and then automatically eluted onto a RP-18 analytical column using a mobile phase containing 7% acetonitrile in pH 5.2 sodium acetate buffer. The mean between-day precision of the standards was ± 4.2%. Spiked urine control recovery averaged 96 ± 6% for controls ranging from 1.0 to 20.0 μg/mL. The limit of quantitation for the method was 0.11 μg/mL.



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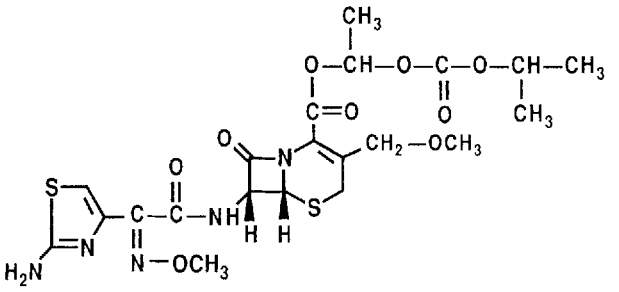

II

FIGURE 1: Chemical Structure of Cefpodoxime (I) and Cefpodoxime Proxetil (II).

INTRODUCTION

Cefpodoxime (I, Figure 1) is the deesterified, active moiety of cefpodoxime proxetil (II, Figure 1), a new, third generation cephalosporin oral antibiotic undergoing development and Phase I and II testing by The Upjohn Company. The drug is active against a wide range of both gram-negative and gram-positive organisms, primarily through a mechanism of interfering with cell wall

synthesis. The drug is resistant to penicillinases and cephalosporinases and does not induce β-lactamases.

Phase I and II studies investigating the pharmacokinetics of cefpodoxime required methodology to quantitate cefpodoxime in both urine and plasma. As was typical of these types of studies, large numbers of samples were anticipated (5000-8000), as well as a need for highly sensitive and precise methodology. Several recent publications describe the extraction and analysis of second and third generation cephalosporins in plasma and urine (1, 2), however, these are generally for compounds administered intravenously at doses exceeding 2 g, where circulating blood levels are in the high μg/mL range with subsequent urine concentrations in the mg/mL range. Cefpodoxime proxetil, given by the oral route, is an extremely potent antibiotic, and has been investigated at single doses as low as 100 mg and up to thrice daily doses of 400 mg resulting in total daily doses of between 100 to 1200 mg. Consequently, these low doses, given orally, required methodology accurate at much lower concentrations than methods previously described for cephalosporin antibiotics.

Methods involving liquid-liquid extraction have generally not been favored for cephalosporins due to their polar nature, while methods employing protein precipitation did not afford the sensitivity or selectivity required for this drug. Traditional liquid-solid extraction (LSE) methods can provide selectivity and the required sensitivity, but the speed of sample preparation, and the degree of "art" involved in the procedure make it a less appropriate technique in a high volume laboratory. Our approach of using direct injection of the sample in matrix, coupled with an on-line, column-switching extraction reduced the need for operator knowledge of LSE "art" and

maximized sample through-put, while simultaneously providing sufficient sensitivity for pharmacokinetic studies.

This paper describes the procedure used to quantitate cefpodoxime levels in human urine and using this procedure, describes the stability of cefpodoxime in human urine.

MATERIALS

All water was reagent grade Milli-Q (Millipore Corp., Bedford, MA, USA) freshly prepared each day. The sodium acetate buffer was prepared by making a 0.05 M solution of sodium hydroxide (Mallinkrodt Inc., Paris, KY, USA) and adjusting the pH to 5.2 with glacial acetic acid (99.7% purity, Aldrich Chemical Company Inc., Milwaukee, WI, USA). Acetonitrile and methanol were UV Grade (Burdick and Jackson, Muskegon, MI, USA).

The analytical mobile phase consisted of 7% acetonitrile in 0.05 M sodium acetate buffer which was filtered (0.4 μm, Nuclepore Corp., Pleasanton, CA, USA) and then degassed (by helium sparging for 10 minutes) prior to use. The sample loading mobile phase consisted of 10% methanol in 0.2% phosphoric acid (pH 2.0), and the column wash mobile phase consisted of 50% methanol in water. Both of these mobile phases were filtered and degassed identically to the analytical mobile phase.

The chromatographic system was comprised of a Waters (Waters Chromatography Division, Millipore Corp., Milford, MA, USA) 590 Programmable pump (Pump 1), a Waters Model 484 variable wavelength UV detector operated at 254 nm and a Waters

Automated Valve Station (WAVS). A second pump (Pump 2) used for the loading mobile phase and the wash phase was an Anspec Model SM909 (Anspec Company, Inc., Ann Arbor, MI, USA). Samples were introduced into the chromatograph with a Perkin Elmer ISS-100 autosampler (Perkin-Elmer Corp., Norwalk, CT, USA) equipped with a 50 µL loop and a refrigerated sample tray. The sample tray was cooled to 4°C with a Neslab constant temperature circulating bath (Neslab Instruments, Portsmouth, NH, USA). The sample loading column was a Brownlee C18 (Brownlee Labs, Santa Clara, CA, USA), 30 x 4.6 mm, 5 µm column, and the analytical column was a Phenomenex IB-SIL C18 (Phenomenex, Rancho Palos Verdes, CA, USA), 250 x 4.6 mm, 5 µm column.

Cefpodoxime calibration standard stock solutions were prepared in fresh, chromatographically screened human urine. Approximately 10 mg of reference standard purity cefpodoxime free acid (Sankyo Company Limited, Tokyo, Japan) were accurately weighed into a 100 mL volumetric flask. One milliliter of acetonitrile, 200 µL of 85% phosphoric acid, and 1 mL of blank human urine were added to the flask which was gently swirled to ensure solubility of the drug. Once the drug had dissolved, the flask was brought to volume with blank human urine and mixed by inverting several times. A second standard stock solution was prepared by weighing 0.5 mg of reference standard purity cefpodoxime (free acid) into a 100 mL volumetric flask and dissolving and diluting as before. Using volumetric pipets and flasks, the following standards were prepared from the 100 µg/mL stock: 50, 40, 20, and 10 µg/mL. Using the 5 µg/mL stock the following standards were prepared in a similar fashion: 5, 1, 0.5, 0.2, and 0.1 µg/mL. All standards were diluted with fresh, blank human urine and mixed well by inverting the

flasks. Calibration standards were frozen in 2 mL aliquots at -20°C until the time of analysis (but no longer than 60 days) or used fresh.

Spiked controls were prepared by accurately weighing 0.1 mg, 1.0 mg and 2.0 mg of cefpodoxime (free acid) into separate 100 mL volumetric flasks, and dissolving and diluting by the addition of 1 mL of acetonitrile, 200 μL of 85% phosphoric acid, and 1 mL of blank human urine. Following dissolution of the drug, the flasks were brought to volume with blank human urine, mixed well, and frozen at -20°C in 2 mL aliquots until the time of analysis (but no longer than 60 days).

Prior to chromatography, standards, controls and unknowns were thawed, gently mixed by swirling, and filtered using 0.45 μm Acro LC13 disposable filters (Gelman Sciences, Ann Arbor, MI, USA). Unused portions of thawed standards and controls were not refrozen and were discarded.

A sample loading column test solution was also prepared, similar to the calibration standards, by weighing approximately 5.0 mg of cefpodoxime (free acid) into a 100 mL flask but diluted with Milli-Q water instead of urine to yield a final concentration of 50 μg/mL. Two mL aliquots were frozen at -20°C until the time of analysis.

Controls for the stability study were prepared in the same fashion as those described above, except one set was stored at 4°C while another set was stored at -20°C until analysis. On each stability study day, fresh standards were prepared and assayed along with the controls.

METHODS

Sample Loading Column Evaluation

Sample loading column retention was evaluated each time a new column was installed, since the retention of the drug was critical to valve switching-times on the WAVS. A new sample loading column was first wetted with 30 mL of methanol followed by about 30 mL of 50% methanol in water. The sample loading column was connected directly to the detector and the sample loading mobile phase was switched into the column. It was allowed to equilibrate at 2.0 mL/min for 20 minutes. Once the sample loading column was equilibrated, the retention of cefpodoxime was measured by injecting 50 µL of the test solution. The sample loading column was rejected if cefpodoxime was not completely retained for at least 12.0 minutes. Once cefpodoxime had eluted from the column, another test injection was made, and, exactly at 12.0 minutes, event #1 on the WAVS was switched to the "off" position, placing the sample loading column in the analytical side with the analytical mobile phase. Within 3.0 minutes of switching into the analytical side, cefpodoxime should have been completely eluted from the sample loading column. If the elution time differed from 3.0 minutes, then the WAVS event times were modified to allow complete elution of cefpodoxime.

Column-Switching Program

On-line sample extraction via column-switching was defined as five separate operations.

LOAD - Pump 2 delivered the injected sample onto the sample loading column using the sample loading mobile phase at 2.0 mL/min.

ELUTE - The WAVS switched the sample loading column in line with the analytical mobile phase and analytical column at 10.0 minutes. The analytical mobile phase, pumped by Pump 1 at 2.0 mL/min, eluted the sample from the sample loading column in 5.0 minutes and onto the analytical column.

ANALYZE - While the separation proceeded on the analytical column, the WAVS switched the sample loading column back to the load position at an elapsed time of 15.0 minutes.

WASH - At the beginning of the sample elution step (at 10 min elapsed time), the WAVS switched the solvent for Pump 2 from the sample loading mobile phase to the wash phase, 50% methanol in water. This event allowed Pump 2 and its associated tubing to become fully primed with the wash phase, so that after 5 minutes (elapsed time 15 minutes) the sample loading column was washed for 2.0 minutes with 50% methanol in water.

RE-EQUILIBRATION - At an elapsed time of 17.0 minutes, the sample loading mobile phase was switched in to the sample loading column and

reequilibrated this column for 6.0 minutes before the next sample was injected.

The total run time was 23 minutes per injection.

Specimen Analysis

Specimen analysis consisted of simply thawing subject specimens, calibration standards and controls, gently mixing each vial and filtering prior to placing on the autosampler.

RESULTS

Sample Loading Column Retention

The critical parameter of the column-switching extraction was the retention of cefpodoxime on the 30 mm C18 column. The retention of cefpodoxime was evaluated versus pH by measuring k′ of the analyte using mobile phases containing 10% methanol and buffers of 1) 0.2% phosphoric acid (pH 1.63), 2) 0.1 M acetic acid (pH 2.65), 3) 0.05 M sodium acetate to pH 4.0 with acetic acid, 4) 0.05 M sodium acetate to pH 5.0 with acetic acid, 5) 0.05 M monobasic sodium phosphate to pH 6.0 with phosphoric acid. Figure 2 illustrates the relation of k′ versus pH. The retention of cefpodoxime is at a minimum around pH 4.0 and becomes very large as the pH approaches 1.5. Consequently, by loading the sample at a pH of less than 2.0 for about 10 minutes, a large amount of endogenous, potentially interfering material was shunted to waste.

Figure 3 illustrates the need for sample clean-up. A 50 μL sample of fresh, filtered human urine was injected directly onto the

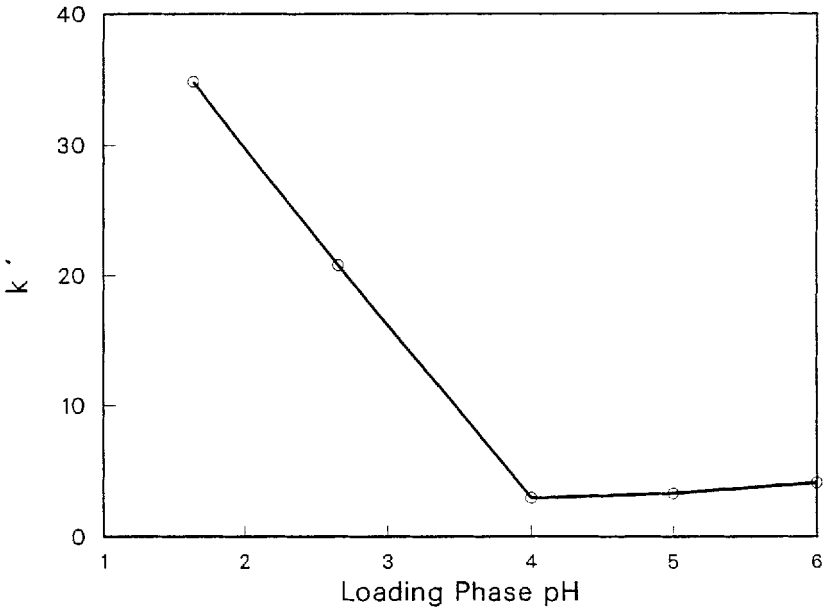

FIGURE 2: Relationship of the pH of the Mobile Phase to k′ of Cefpodoxime.

analytical column (solid line). The dashed line is the same matrix spiked with 0.2 μg/mL of cefpodoxime injected directly onto the analytical column. As the figure illustrates, there was an enormous amount of endogenous material present in urine under these chromatographic conditions that would prohibit quantitation of the cefpodoxime peak.

Figure 4 demonstrates the power of the column-switching clean-up. The solid line is the chromatogram of a subject predose urine, and the dashed line is the chromatogram of the same subject's urine from the 0-2 hours post-dose collection interval.

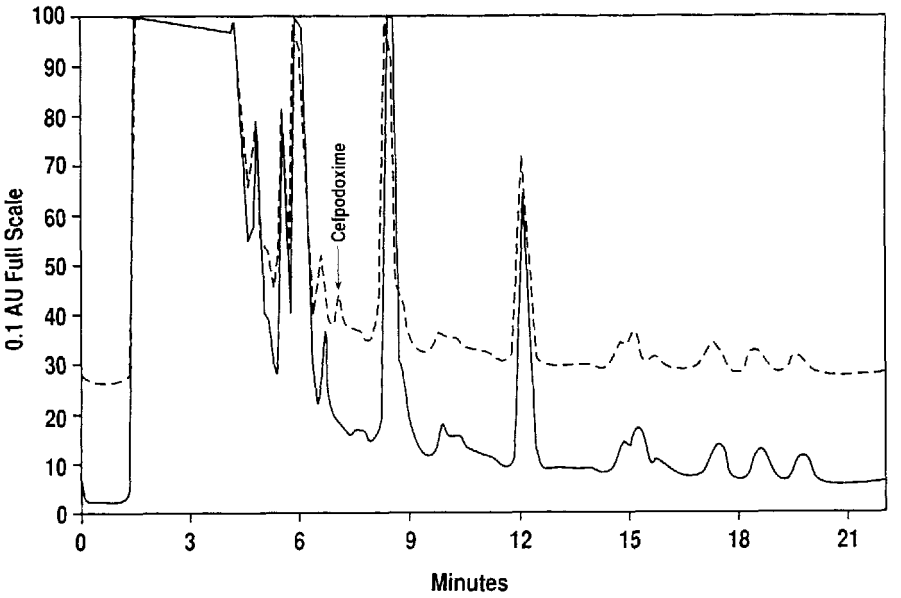

FIGURE 3: Chromatogram of Human Urine Without Column-Switching

The solid line chromatogram is 50 µl of blank human urine and the dashed line chromatogram is 50 µL of the same urine spiked with 0.2 µg/mL cefpodoxime.

Absolute Recovery

The recovery of cefpodoxime with and without using the column-switching procedure, and in urine versus water was determined. A reference curve was prepared as outlined in the Materials section above, except that instead of using fresh human urine as the diluent, Milli-Q water was used. These standards were then chromatographed directly on the analytical column, without using the precolumn or the column-switching clean-up. The reference standards were then chromatographed a second time using the

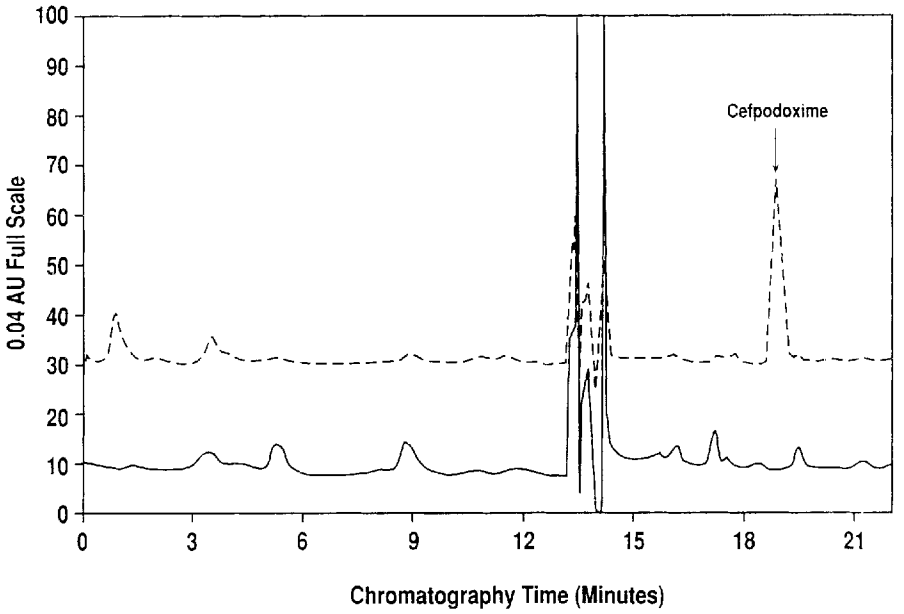

FIGURE 4: Chromatogram of Human Urine with Column-Switching

The solid line chromatogram is of a subject predose urine and the dashed line chromatogram is of the same subject's 0-2 hour post-dose collection interval.

complete column-switching routine. A second standard curve, prepared with human urine, was also chromatographed using the complete, column-switching routine. In order to normalize for differing retention times (with versus without column-switching) and slightly different stock weighings, the curves were calculated using peak area, and the average relative weight response (RWR) was determined. The RWR was calculated for each standard (except the blank) by dividing the peak area by the theoretical concentration. The RWR for all standards were then averaged and a mean RWR obtained.

Table 1
Calibration Curve Summary

Theor. Conc. (μg/mL)	N	Mean (μg/mL)	SD (μg/mL)	CV (%)
0.0992	6	0.089	0.016	18
0.1984	6	0.197	0.020	10
0.4960	6	0.492	0.014	2.8
0.992	6	1.008	0.018	1.8
4.960	6	5.04	0.06	1.3
10.01	6	10.21	0.09	0.9
20.02	6	20.44	0.23	1.1
40.04	6	40.1	0.4	1.0
50.05	6	49.8	0.4	0.8

Compared to the reference curve analyzed without column-switching (mean RWR = 8797 ± 203), the reference curve analyzed with column-switching had a mean RWR of 8755 ± 483, or 99.5 ± 5.5% that of the reference curve analyzed without column-switching. Thus, for cefpodoxime in water, recovery was essentially 100% with the column-switching extraction.

When the standards were prepared in human urine and chromatographed with the column-switching clean-up, the mean RWR was 8777 ± 248, or 99.8 ± 2.8% that of the reference curve directly injected. Again, recovery was essentially 100% for cefpodoxime in urine.

Accuracy and Precision of Calibration Standards

The precision of the method was determined by calculating the mean and standard deviation of back-calculated calibration curve

Table 2
Spiked Control Recovery

Label (μg/mL)	Found (μg/mL)	SD (Mean)	Recovery (%)	CV (%)
1.00	0.925	0.058	92.5	6.3
9.78	9.66	0.465	98.8	4.8
19.8	19.2	0.696	96.8	3.6

concentrations over six analytical runs on separate days. Tabulated data (Table 1) from these assays show an expected decrease in the CV from 18% to 0.8% with increasing concentrations from 0.1 to 50 μg/mL. The mean CV (averaged over all concentrations) was 4.2%. The mean of the through-the-origin slopes was 25800 ± 600. The precision of the analytical method and the system was represented by the CV of the between-day slope, an excellent 2.4%.

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined by plotting the between run standard deviations of the back-calculated concentrations of standards and performing a linear regression analysis (4). The LOD was defined as three times the intercept ($3s_o$), or 0.034 μg/mL, and the LOQ was defined as $10s_o$, or 0.11 μg/mL. On-column amounts were then 1.7 ng and 5.8 ng for the LOD and LOQ, respectively.

Recovery and Precision of Controls

Recovery of cefpodoxime from the urine was determined by quantitating recoveries from spiked controls analyzed during each chromatographic run. Table 2 shows the excellent recovery obtained over six analytical runs for all three concentrations of controls.

Table 3
Stability of Cefpodoxime in Urine at 4°C

Days on Stability	Recovery (% of Time Zero)	
	0.97 μg/mL	25.1 μg/mL
0	100.0 ± 0.8	100.0 ± 0.8
9	102.5 ± 0.5	94.7 ± 0.4
16	93.9 ± 0.7	89.1 ± 0.6
23	88.0 ± 0.1	84.7 ± 0.3
30	82.6 ± 0.7	80.6 ± 0.4

Grand recovery, over all three concentrations, was 96 ± 6%. Within-day precision of duplicate controls (at each concentration level) ranged from ± 0.3% to ± 10%.

Stability of Cefpodoxime in Urine

At 4°C, the stability of cefpodoxime in urine was examined out to 30 days. In that interval, a significant change in concentration occurred (as measured by the significance of the slope). Table 3 shows the time-dependent decline in cefpodoxime concentration for controls of 0.97 μg/mL and 25.1 μg/mL. The low and the high controls declined at approximately the same rate (slopes = -0.65 and -0.66 for low and high controls, respectively) with both controls having slopes significantly different from zero ($p < 0.024$ for the low control and $p < 0.00005$ for the high control). The time for each control to reach 95% (t_{95}) of its time zero value (at 4°C) was estimated to be 13.2 days for the low control and 7.7 days for the high control.

Table 4
Stability of Cefpodoxime in Urine at -20°C

Days on Stability	Recovery (% of Time Zero)	
	0.97 μg/mL	25.1 μg/mL
0	100.0 ± 0.8	100.0 ± 0.8
9	112.2 ± 2.3	102.3 ± 1.7
16	106.0 ± 0.3	100.2 ± 1.4
30	102.2 ± 1.4	100.5 ± 0.5
77	106.0 ± 0.3	94.6 ± 7.3
97	106.2 ± 0.4	100.6 ± 0.4
176	103.1 ± 0.3	100.2 ± 0.2
393	93.5 ± 0.8	102.3 ± 2.8

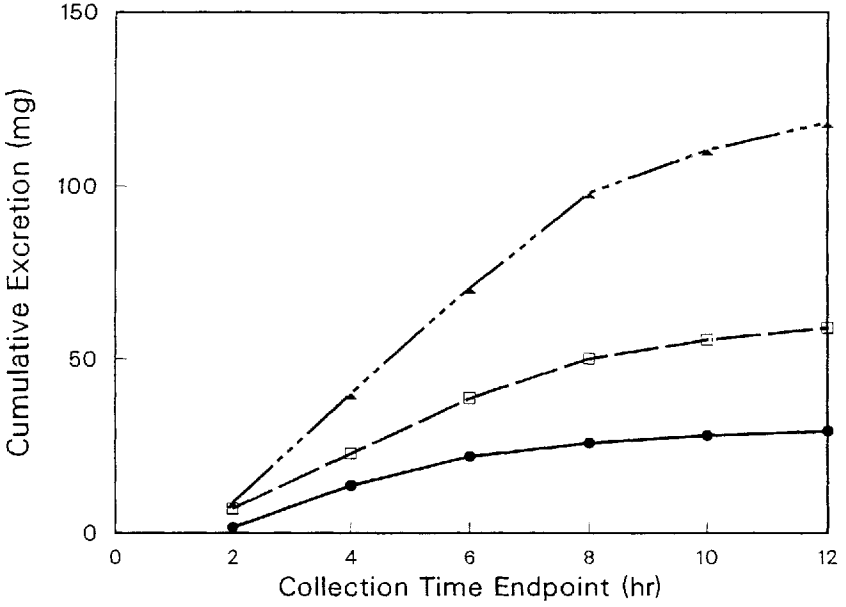

FIGURE 5: Plot of Urinary Excretion

The line of solid circles represents mean cumulative excretion following a dose of 100 mg t.i.d.; open squares, 200 mg t.i.d.; solid triangles, 400 mg t.i.d.

As expected, at -20°C, neither control showed a significant decrease in concentration to the end of the study. Table 4 shows the tabulated data from this study. While the slope was not statistically significant from zero ($p > 0.49$ and $p > 0.96$ for the low and high controls, respectively), a plot of the data for the 0.97 μg/mL control suggests a decrease in recovery between 176 and 393 days. These low results for the 393 day sample may indicate a stability problem or it may indicate an analytical problem in that sample. Unfortunately, insufficient sample remained to verify the recovery. Therefore, while the statistics indicated that the drug was stable in urine for up to 393 days when stored at -20°C, caution should be exercised for specimens containing low levels of cefpodoxime when storage for more than 176 days is anticipated.

Utility of the Method

The utility of the procedure was demonstrated by assaying specimens from a multiple dose tolerance and pharmacokinetic study with cefpodoxime. Normal human volunteers were dosed thrice daily with either 100, 200 or 400 mg of cefpodoxime proxetil. Mean cumulative excretion (Figure 5) at 12 hours was 29.5 mg, 58.6 mg, and 112.4 mg for doses of 100, 200 and 400 mg of cefpodoxime. These data demonstrate that this procedure obtains sufficient sensitivity and precision for the support of pharmacokinetic calculations in clinical and pre-clinical studies.

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