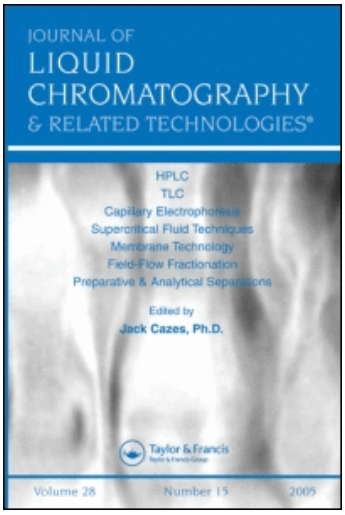

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Steenwyk, R. C. , Brewer, J. E. , Royer, M. E. and Cathcart, K. S.(1991) 'Reversed-Liquid Phase Chromatographic Determination of Cefpodoxime in Human Plasma', Journal of Liquid Chromatography & Related Technologies, 14: 20, 3641 — 3656
To link to this Article: DOI: 10.1080/01483919108049483
URL: http://dx.doi.org/10.1080/01483919108049483

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(20), 3641-3656 (1991)

REVERSED-PHASE LIQUID CHROMATOGRAPHIC DETERMINATION OF CEFPODOXIME IN HUMAN PLASMA

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ABSTRACT

The reversed-phase liquid chromatographic determination of cefpodoxime in human plasma is described. Plasma samples were acidified with 1 mL of phosphoric acid solution containing internal standard, and adsorbed onto a C8 solid phase cassette designed specifically for use with a Varian Advanced Automated Sample Processor. Each cartridge on the cassette was washed with 1 mL of phosphoric acid containing 5% methanol and then was placed into the automated sample processor for direct elution of the retained drugs into the chromatographic system. Chromato-graphy was free from interferences in the analyte and internal standard windows, and yielded excellent linearity, accuracy, and precision in studies performed in this laboratory.

INTRODUCTION

Cepfodoxime proxetil (Doxef) is a broad spectrum, third generation oral cephalosporin antibiotic undergoing Phase II testing. Cefpodoxime proxetil is rapidly deesterified by intestinal wall esterases into its active

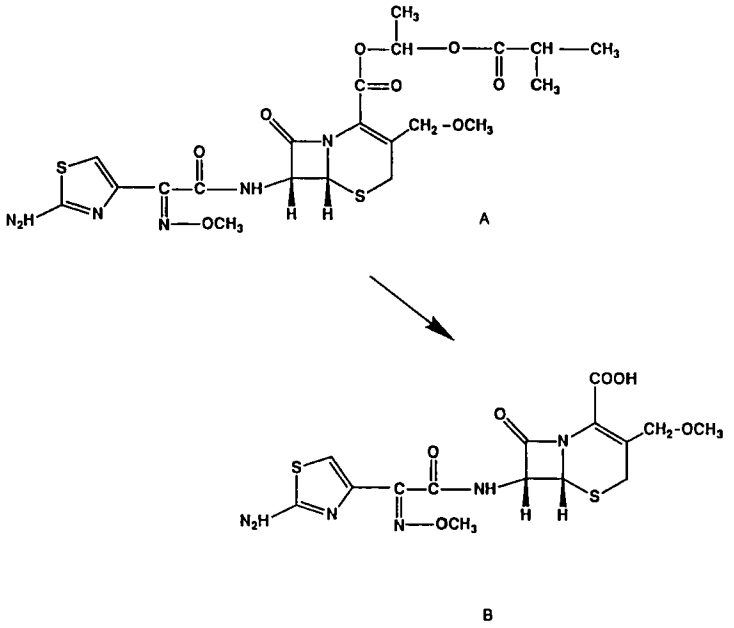

Figure 1. Structure of Cefpodoxime Proxetil (A) and Its Deesterfied Metabolite, Cefpodoxime (B).

metabolite, cefpodoxime (Figure 1) (1), and is well absorbed from the gastrointestinal tract (2). In order to assess the pharmacokinetics of cefpodoxime in humans, a reliable and sensitive method was needed which could be used on large numbers of clinical samples in our laboratory.

Existing microbiological assays used in early Phase II studies for the quantitation of cefpodoxime provided preliminary analytical results until a more specific, HPLC assay could be developed. An unpublished method previously used in our laboratory inadequately resolved peaks in the region of both the analyte and internal standard (β-hydroxyethyltheophylline) in some clinical specimens. The method reported here substantially modifies the mobile phase, the internal standard, and the solid phase extraction

procedure of the initial method such that the resolution of plasma constituent peaks from the cefpodoxime peak and the internal standard peak has been enhanced to permit quantitation down to 10 ng/mL in all plasma specimens tested thus far.

The use of solid phase extraction for the analysis of drugs in plasma is well documented. However, when low drug levels are determined, evaporation and reconstitution of the collected eluent is usually required in order to concentrate the sample prior to injection into the chromatography system such that maximum sensitivity is achieved. Evaporation can be a time consuming step and can occasionally be disadvantageous when dealing with volatile or thermally unstable compounds. The Varian Advanced Automated Sample Processor (AASP) combines semi-automated solid phase extraction with on-line elution of the adsorbed analyte directly into the HPLC column. This on-line elution capability obviates the evaporation step and provides maximal sensitivity by applying the entire sample to the analytical column. Use of the AASP for analysis of pharmaceuticals in clinical samples has been previously documented (3,4), and has provided a simplified technique to prepare and chromatograph large numbers of samples.

We describe here an HPLC technique utilizing a solid phase extraction with an AASP for the analysis of cefpodoxime in human plasma. The primary advantages of the AASP system are the ease of preparing a large number of samples and the ability to introduce all of the retained sample into the chromatography system, allowing quantitation of cefpodoxime as low as 10 ng/mL from 200 µl of plasma.

EXPERIMENTAL

Reagents

All solvents were UV grade and distilled in glass (Burdick and Jackson Laboratories, Muskegon, MI, USA). Sodium hydroxide, glacial

acetic acid, and phosphoric acid (85%) were AR grade (Mallinkrodt, Paris, KY, USA). Water was Milli-Q (Millipore, Milford, MA USA) or equivalent. The AASP cartridge activation solution was methanol:dimethylformamide (90:10) (v/v). The cartridge wash solution was 5% methanol in 1% phosphoric acid (v/v). Calibration standard diluent was 1% phosphoric acid containing 1% acetonitrile (v/v). The AASP purge solvent was 0.1% phosphoric acid. Nafcillin sodium (Bristol-Meyer Co., Evansville IN, USA), Ticarcillin disodium (Beecham Laboratories, Bristol, TN, USA), Ceftriaxone sodium (Roche Laboratories, Nutley, NJ, USA), Cefotaxime sodium (Hoechst-Roussel, Sommerville, NJ, USA), Vancomycin HCL and Gentamicin Sulfate (Elkina-Sinn, Inc, NJ, USA), Tobramycin sulfate and Ceftazidime (Eli Lilly and Company, Indianapolis, IN, USA), Amikacin sulfate (Bristol Laboratories, Evansville, IN, USA), Acetaminophen (McNeilab Inc. Fort Washington, PA, USA), Phenytoin sodium (Park-Davis division of Warner-Lambert, Moris Plains, NJ. USA), Theophylline (Key Pharmaceuticals, Inc., Kenilworth, NJ, USA), and Caffeine (Sigma Chemical Co., St. Louis, MO) were dissolved with Milli-Q water to concentrations of approximately 5000 ng/mL for use in the interference study.

Chromatography System

The chromatographic system consisted of a Spectra-Physics 8800 gradient pump (Spectra-Physics, San Jose, CA, USA), and a Kratos Spectroflow 783 detector (Applied Biosystems, Ramsey, NJ, USA) with a 8 µl cell operated at 254 nm. Sample preparation and on-line elution were accomplished using the Varian Advanced Automated Sample Processor (AASP) (Varian Associates, Inc, Walnut Creek, CA, USA). Solid phase cassettes (C8) designed specifically for the AASP were used for the liquid-solid extraction (Analytichem International, Harbor City, CA, USA). The analytical separation was performed on a Phenomenex IB-SIL C18 column (5 µm; 250 x 4.6 mm) (Phenomenex, Rancho Palos Verdes CA, USA), and the analytical column was protected with a Newguard C8 precolumn (7 µm; 12 x 4.6 mm) (Brownlee Labs., Santa Clara, CA, USA). The mobile phase

was sodium acetate buffer (pH 6.0; 0.05M)/acetonitrile/methanol, 92/4/4, (v/v/v) flowing at 2 mL/min. A 1 mL volume of acetonitrile/water, 90/10 (v/v), was introduced by the gradient pump following elution of the internal standard to facilitate the removal of late eluting peaks from the analytical column. The detector signal was monitored using a Kipp & Zonen BD41 recorder (Kipp & Zonen, Delft, Holland) and peak heights were collected on a Harris 1000 mini computer system (Harris Inc., Ft. Lauderdale, FL, USA). Data were reduced using customized chromatography software (5) residing on the Harris system.

Internal Standard Solution

A working solution containing 1000 ng/mL of Cefaclor (Eli Lilly and Company, Indianapolis, IN, USA) in 1% phosphoric acid (v/v) was used as the internal standard and stored at -20°C in appropriate glass containers in aliquots suitable for each days use. Prior to analysis the frozen aliquot was thawed and mixed well. Thawed internal standard solution remaining after each days sample preparation was discarded.

Calibration standards

Stock standards were prepared by weighing 0.1 and 0.5 mg of cefpodoxime into volumetric glassware and dissolving with 1% phosphoric acid containing 1% acetonitrile (v/v) to achieve solutions containing approximately 1000 and 5000 ng/mL. Calibration curve standards were diluted from the two stock solutions using volumetric glassware to yield working standard concentrations of about 10, 20, 40, 100, 200, 500, 1000, 2500, and 5000 ng/mL. Approximately 0.5 mL of calibration curve standards were aliquoted into 1 dram glass vials (Kimble Div., Owens, Ill, USA), and stored at -20°C until time of analysis. Unused portions of thawed standards were discarded after each days use.

Spiked Plasma Controls

Aliquots of a stock solution containing 100 μg/mL of cefpodoxime (in 1% phosphoric acid containing 1% acetonitrile (v/v)) prepared in volumetric

glassware were diluted 1:10 with blank human plasma to yield a 10,000 ng/mL stock control. Working plasma controls containing approximately 100, 800, and 2000 ng/mL cefpodoxime were prepared in volumetric glassware by making 1:100, 1:12.5, and 1:5 dilutions of the stock control with blank human plasma. Controls were aliquoted into 1 dram glass vials in daily use amounts and stored at -20°C until time of analysis. Unused portions of thawed controls were discarded each day after use.

Sample Preparation

AASP cassettes were pre-conditioned prior to use by passing 1 mL of the cartridge activation solution through each cartridge on the cassette followed by 1 mL of 1% phosphoric acid solution. The solid phase packing bed was not allowed to go dry at any stage prior to addition of the plasma samples. One entire cassette was used to prepare the standards with additional cassettes being used for controls and subject samples. Each cassette containing subject samples also had at least one control present to verify recovery of the analyte from that cassette. Calibration curve sample preparation consisted of adding 1 mL of internal standard, 200 µL of the appropriate calibration standard, and 200 µL of blank human plasma to each cartridge reservoir on the standard containing cassette. Subject sample preparation consisted of adding 1 mL of internal standard, 200 µL of calibration standard diluent, and 200 µL of control or subject plasma. High purity nitrogen was then applied to each cassette (10 psi) forcing the samples to flow completely through the cartridge. The nitrogen flow was stopped, 1 mL of cartridge wash solution was added to each cartridge reservoir, and the nitrogen flow was re-applied to the cassettes (20 psi) forcing the cleanup solvent to pass through the cassettes. Each cartridge was then rinsed with about 0.5 mL of 1% phosphoric acid (v/v), the nitrogen flow was re-applied, and stopped again before the cartridges were allowed to go dry. Cassettes then were placed into the AASP for sample introduction into the chromatographic system. The AASP was programmed for 10 pre-injection and 30 after injection purge strokes (20 µL/stroke) of 0.1%

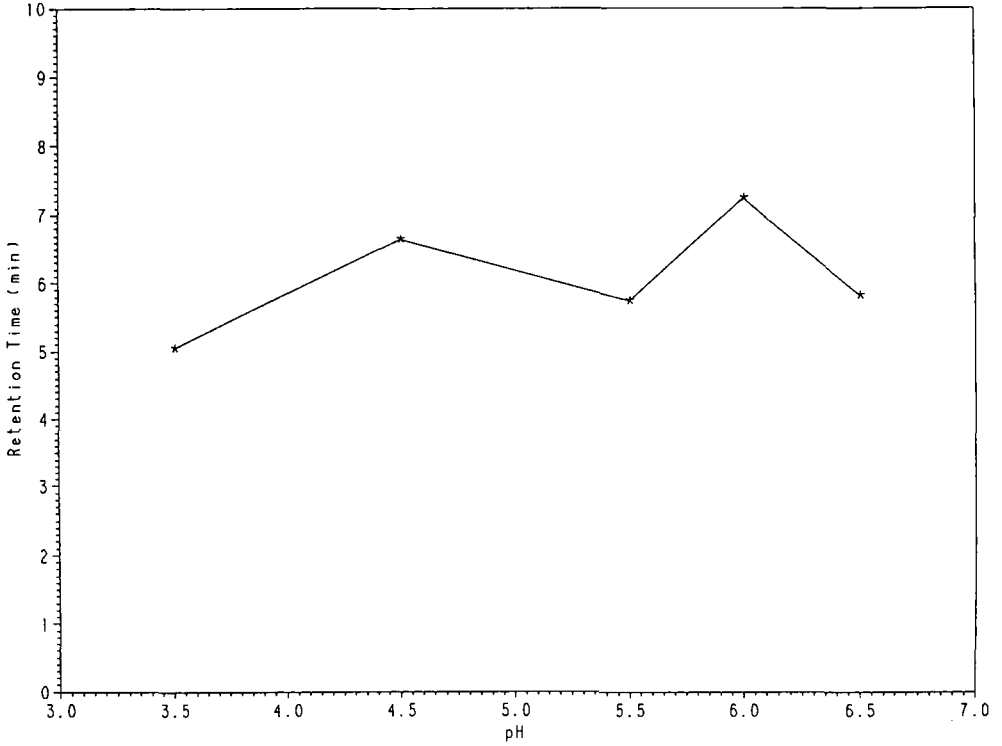

Figure 2. Effect of pH on the Retention Time of Cefpodoxime Mobile Phase: 0.05 M Sodium Acetate:Acetonitrile 92.5:7.5.

phosphoric acid. Other AASP parameters were as follows: run time = 21 minutes, cycle time = 22 minutes, and valve reset time = 1.0 minute.

RESULTS

Method Development

The effect of pH on the retention time of cefpodoxime was examined using a mobile phase composition containing 0.05 M sodium acetate buffer and 7.5% acetonitrile (v/v). The retention time of cefpodoxime was not appreciably altered by pH changes from 3.5 to 6.5 (Figure 2), however the

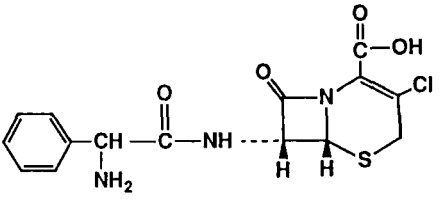

Figure 3. Structure of Cefaclor (internal standard).

plasma constituents which interfered with cefpodoxime were better resolved from the desired analyte peak at pH6 than at lower pH's. Separation of cefpodoxime from all background peaks was further improved by using a ternary mobile phase consisting of 4% methanol and 4% acetonitrile in 0.05 M sodium acetate buffer (pH 6.0). The ternary mobile phase increased the retention time of cefpodoxime to 10.2 minutes, compared to 6.2 minutes using the original binary mobile phase (pH 5.2). The increased retention time resulted in better separation of the analyte from earlier eluting plasma constituents without sacrificing peak shape.

Sample cleanup was studied in order to selectively remove plasma constituents which coeluted with the previously used internal standard (β-hydroxyethyltheophylline). With a sample wash of 1 mL of methanol in 1% phosphoric acid (5/95,v/v) these coeluting constituents were removed, but the recovery of the internal standard was very low. Cefaclor, another semi-synthetic cephalosporin (Figure 3) was substituted as the internal standard, and cartridge cleanup solutions containing 5, 10, 15, and 20% (v/v) methanol were applied during sample preparation (Figure 4). Removal of plasma interferences in the chromatography, and satisfactory recoveries of cefpodoxime and the internal standard (80 and 81 % respectively, relative to a 100% water wash) were obtained using a 5% methanol sample wash. Increasing the sample wash methanol strength above 5% resulted in lower recoveries of both cefpodoxime and the internal standard.

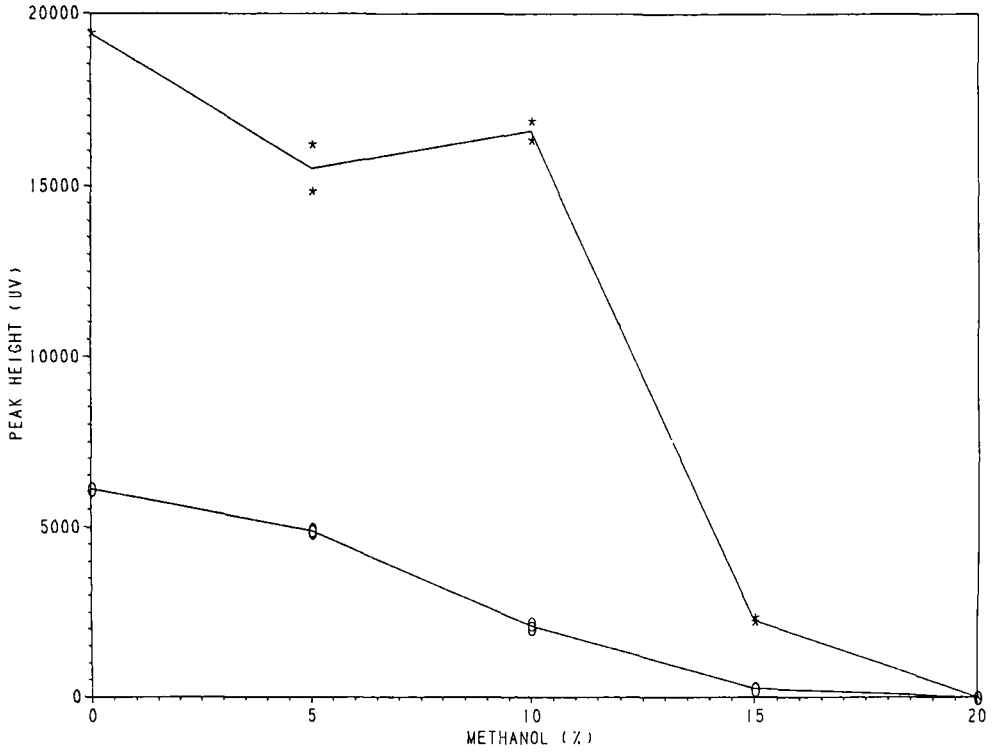

Figure 4. Effect of percent methanol in sample wash solution (0.1% phosphoric acid) on peak height of cefpodoxime (*) and the internal standard (0).

Figure 5 is an overlay chromatogram of a subject predose and post dose sample assayed using the original method, and shows interference peaks present in both the cefpodoxime and internal standard peak windows. In comparison, Figure 6 shows an overlay chromatogram of the same subject predose and postdose plasma samples assayed using the new, improved method. No significant interferences were seen from endogenous components in these chromatograms. In addition, 24 subject predose plasma samples, which had been assayed by the original method and found

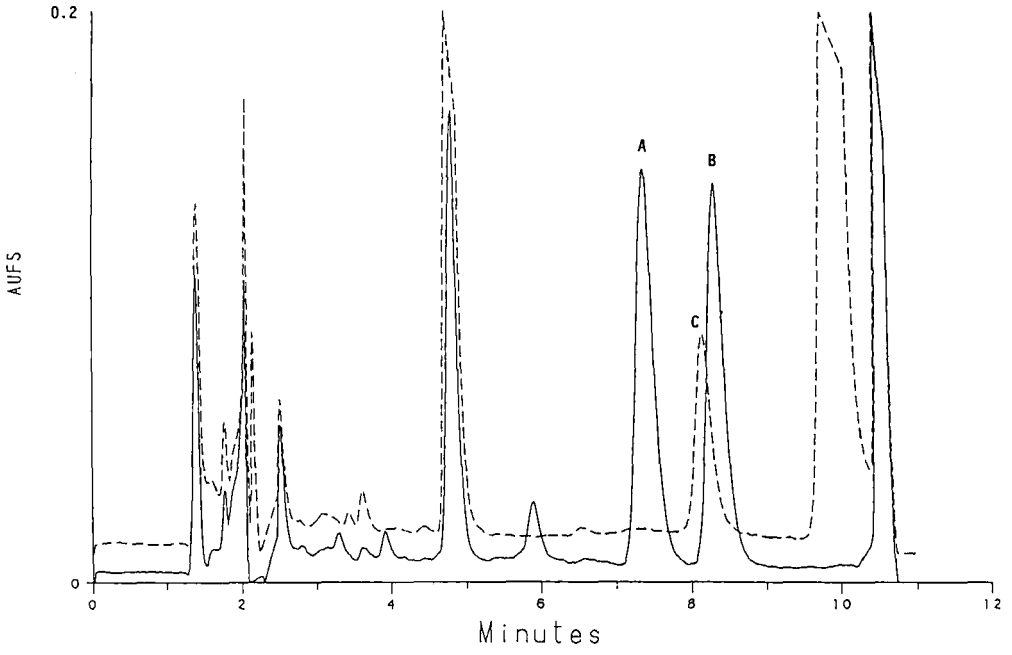

Figure 5. Representative chromatogram from original method showing plasma standard (solid line) containing 2500 ng/mL cefpodoxime (A) and β-hydroxyethyltheophyllic (B); and subject predose sample assayed without internal standard (dashed line), showing interferant (C) in the internal standard peak window.

to have significant interferences, were reassayed by the new, improved procedure. The resulting chromatograms were found to be free of interferences in the retention window for both cefpodoxime and the internal standard.

Linearity and Precision

The linearity of the method was established by unweighted linear regression analysis of 25 calibration curves ranging in concentration from 10 ng/mL to 5054 ng/mL. Intercepts for the standard curves were not

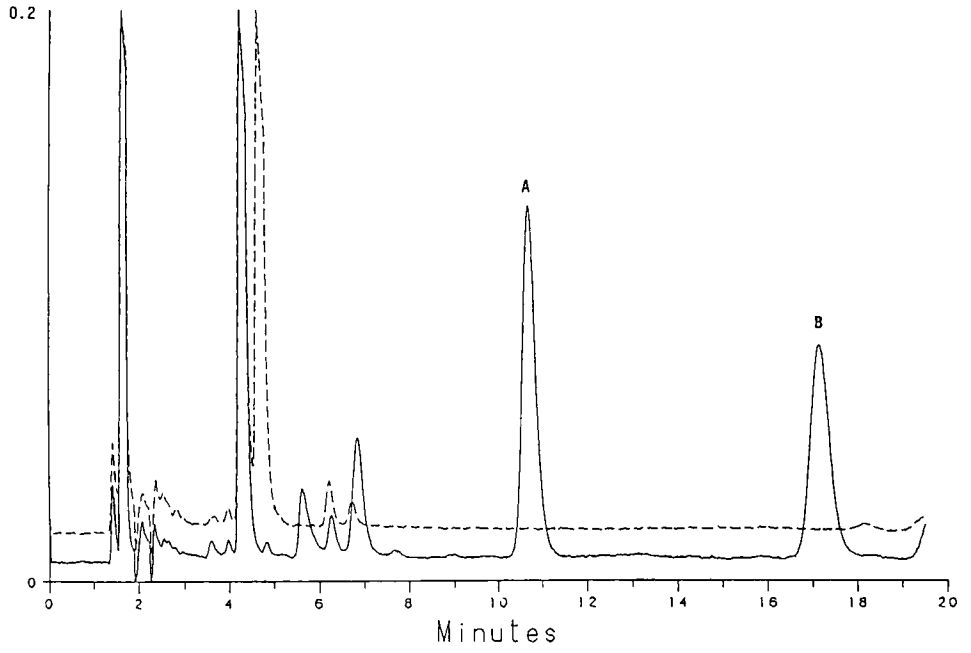

Figure 6. Representative chromatograms from new improved method showing plasma standard (solid line) containing 2500 ng/mL cefpodoxime (A) and cefaclor internal standard (B); and subject predose sample assayed without internal standard (dashed line).

significantly different from zero ($p>0.05$), therefore all calibration curves were analyzed with a forced through-the-origin model. The correlation coefficients for the calibration curves ranged from 0.9995 to 1.0. The within-day precision, expressed as the coefficient of variation (CV) of the slope (forced through the origin) ranged from 0.10 to 0.8%. The between-day combined precision of the analytical method and the chromatographic system expressed as the CV of the mean of the slope of each calibration curve ($n=25$), was 6% (Table 1). The between-day precision of the analytical method was determined by averaging the back-calculated calibration standards and calculating the CV. The precision on these standards averaged 5.5%.

TABLE 1

Plasma Calibration Curve Linearity and Precision (back calculated concentrations).

Label Conc. (ng/mL)	N	Mean Back Calc. Conc.	S.D.	C.V.%
10.01	24	10.9	1.5	14
20.02	25	20.5	2.6	13
40.04	25	39.6	2.3	6
100.1	25	97	4	4
200.2	25	192	7	4
505.4	25	510	15	3
1001	24	990	30	3
2527	25	2540	60	2.2
5054	25	5050	30	0.6
Slope	25	.0054	--	6
Corr. Coef.	25	.99984	--	--

The limit of detection (LOD) and the limit of quantitation (LOQ) were determined by fitting the interday, back-calculated standard deviations of each calibration standard. The Y-intercept was then equal to s_0 (the estimated standard deviation at a concentration of zero). The LOD was defined at $3s_0$ and the LOQ was defined as $10s_0$ (5). The LOD and the LOQ for cefpodoxime were found to be 3 and 11 ng/mL respectively.

Control Precision and Recovery

Three levels of spiked control samples were run in duplicate along with each calibration curve (Table 2). Within-day recovery of duplicated controls at all three levels ranged from 98 to 105% (CV 0.3 to 5.0%). Mean recovery values for all controls at all levels was 101% (4% CV).

Table 2

Spiked Plasma Control Recovery

Label Conc. (ng/mL)	N	Mean (ng/mL)	% Label	CV%
97.53	76	101	104	2.9
780.3	79	782	100	3
1951	79	1940	99	4
Grand Mean	--	--	101	4

TABLE 3

Regression Statistics for Cefpodoxime Stability in Plasma at -20°C.

Concentration (ng/mL)	Slope[a]	Intercept[a]	P
98.3	-.026	99.2	.12
1966.2	-.008	100.8	.65

[a]Based on percent of T_0

Stability

The stability of cefpodoxime was investigated for plasma controls containing 98.3 and 1966.2 ng/mL of cefpodoxime which were stored for a period of 371 days at -20°C. Linear regression of the concentrations normalized to percent of time zero (t_0) versus days on stability yielded slope values not significant from zero ($p>0.05$) for both concentration levels (Table 3). Therefore, cefpodoxime was stable in human plasma for a minimum of 371 days when stored at -20°C.

Table 4

Retention Time of Drugs Tested for Potential Interference[a].

Drug	Retention Time (min)
Cefpodoxime	8.9
Cefaclor	14.1
Nafcillin	ND
Ticarcillin	ND
Ceftriaxone	ND
Cefotaxime	18.2
Ceftazidine	<4
Vancomycin	ND
Amikacin	ND
Gentamicin	ND
Acetaminophen	ND
Tobramycin	ND
Phenytoin	ND
Theophylline	8.1
Caffeine	20.0

[a] Each compound was assayed from plasma fortified to approximately 10 µg/mL.

ND = Not detected.

DISCUSSION

The online injection technique provided by the Varian AASP has allowed for the analysis of up to 50 samples in a 24 hour period in this laboratory. Although the overall run time is relatively long, control samples run after 20 hours on the AASP have shown recovery values similar to controls assayed early in the run. Thus, stability of cefpodoxime in prepared cassettes, at room temperature seems to be acceptable for at least 20 hours.

Ten commonly prescribed antibiotics as well as caffeine, theophylline, acetaminophen, and phenytoin were evaluated as potential interference in this assay. Plasma samples were fortified in a similar fashion to the cefpodoxime standards by addition of 200 μL of aqueous solutions containing 5000 ng/mL of each of the listed compounds (Table 4) to 200 μL of plasma in the AASP prepstation. These plasma samples (equivalent to approximately 10 μg/mL of each compound) were then assayed according to the described method. Only theophylline was found to interfere at the concentration evaluated. Therefore, specimens from subjects known to be treated therapeutically with theophylline cannot be reliably quantitated using this procedure.

The excellent linearity, precision, and sensitivity of this assay, as well as the chromatography free from plasma interferences have allowed this method to be applied to pharmacokinetic studies of cefpodoxime in human subjects.

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Received: April 2, 1991
Accepted: April 15, 1991