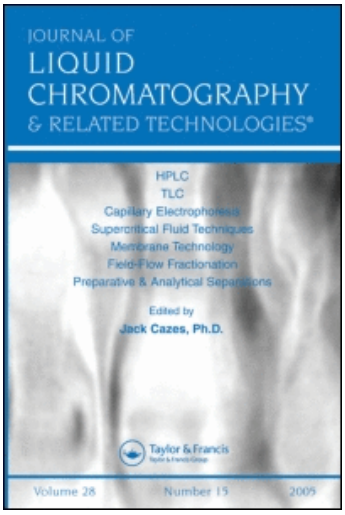

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An Isocratic HPLC Method for the Determination of Cephalosporins in Plasma

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AN ISOCRATIC HPLC METHOD FOR THE DETERMINATION OF CEPHALOSPORINS IN PLASMA

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ABSTRACT

An isocratic reversed-phase liquid chromatographic method for the determination of eight cephalosporins in human plasma using UV detection at 254 nm is described. Plasma proteins were precipitated using acetonitrile prior to injection of a 10 µl aliquot onto an octadecylsilane column. The mobile phases consisted of 6-11% acetonitrile in sodium dihydrogen phosphate (0.01M). The minimum detectable limit for each drug was less than 1 µg/ml of plasma. Possible interference from other drugs which might be administered concurrently is discussed. The reproducibility and precision of the method for cephalosporin assay are shown from the analysis of plasma containing 5-500 µg/ml of plasma. The chromatographic behavior of the eight cephalosporins was examined by varying mobile phase conditions.

INTRODUCTION

Cephalosporins are a family of antibiotics commonly used for the treatment of infections caused by gram-positive cocci and gram-negative bacilli. Toxicities from cephalosporin therapy have been documented in cases of renal impairment (1,2) and during combination therapy with aminoglycosides (3). Monitoring of serum

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concentrations of the cephalosporins ensures adequate drug levels for treatment of infections while avoiding potentially toxic concentrations (4).

Quantitative analyses for cephalosporins in human serum have routinely been done by microbiological assay methods which are subject to interference from other antibiotics commonly used in combination therapy. These assay interferences can be avoided by the use of high performance liquid chromatography (HPLC). A number of recent publications have described HPLC methods for assaying cephalosporins in plasma or serum (5-23). Two review articles discussing the methods of analysis of antibiotics including cephalosporins have recently appeared (24-25). Many of these published methods suffer certain limitations including 1) the use of a lengthy extraction procedure, 2) the lack of an internal standard, 3) the need for ion-pairing reagents, 4) the use of protein precipitation reagents which can cause co-precipitation or degradation of the drugs or are toxic, and 5) the inclusion of limited information on the retention times of other drugs.

The present study was undertaken to meet the need for a single, simple HPLC method to monitor several commonly used cephalosporins without interference from other antibiotics. The drugs studied (cefamandole, cefazolin, cefonicid, cefoperazone, cefotaxime, cefoxitin, cephalothin and cephapirin) represent the three generations of cephalosporins and includes a recently

released product. The method is simple and rapid, requiring only precipitation of proteins with acetonitrile and injection of an aliquot of the supernatant into the chromatograph. The chromatographic behavior of these eight cephalosporins was studied using an octadecylsilane column with combinations of phosphate or acetate buffer and acetonitrile or methanol as mobile phases. The method is applicable to the direct determination of plasma levels in the presence of one or more of several drugs which might be prescribed concurrently. The applicability of the method has been demonstrated by the analysis of serum or plasma from patients receiving cephalosporins.

MATERIALS AND METHODS

Instrumentation

A Hewlett-Packard Model 1084B liquid chromatograph with a variable wavelength UV detector and autoinjector was equipped with an Ultrasphere-ODS (Beckman Instruments) column, 150 mm long and 4.6 mm i.d. The degassed mobile phase was pumped through the column at 2.0 ml/min using isocratic conditions. The column compartment was maintained at 45°C, and the detector was set at a wavelength of 254 nm.

Chemicals and Reagents

Reagent grade sodium dihydrogen phosphate, sodium acetate and acetic acid were used. HPLC grade methanol and acetonitrile were obtained from Fisher Scientific Co. Cefamandole nafate (Mandol^{R}, Eli Lilly and Co.), cefazolin sodium (Ancef^{R}, Smith Kline and

French Labs), cefotaxime sodium (Claforan^{R}, Hoechst-Roussel Pharmaceuticals, Inc.), cefoxitin sodium (Mefoxin^{R}, Merck, Sharp and Dohme), cephalothin sodium (Keflin^{R}, Eli Lilly and Co.) and sodium cephapirin (Bristol Laboratories) were obtained commercially. Cefonicid sodium (Smith Kline and French Labs) and cefoperazone sodium (Pfizer Pharmaceuticals) were obtained courtesy the manufacturers.

Drug Solutions

The powdered drugs were reconstituted to an equivalent of 100 mg/ml water, and aliquots of these solutions were stored frozen for up to one month. Working dilutions of 0.25, 1.0 and 10.0 mg/ml water were prepared daily from the frozen aliquots for each drug.

Internal Standard Solution

A solution of 1.25 mg cephapirin/ml water was prepared and then diluted with acetonitrile to a final concentration of 50 μg/ml.

Mobile Phases

Sodium dihydrogen phosphate, 0.01M, was prepared in deionized distilled water. The pH was not adjusted. Sodium acetate, 0.01M, was prepared in deionized distilled water, and the pH was adjusted to 4.0 with acetic acid. The mobile phases were mixtures of a buffer with methanol or acetonitrile.

Sample Preparation

To 0.5 ml of plasma in a 10x75 mm pyrex tube were added an aliquot (2.5 to 50 μl) of a drug working solution and 1.0 ml of

acetonitrile containing the internal standard. The tubes were vortexed for 10 seconds and centrifuged for 10 minutes at 1500xg. An aliquot of the supernatant was transferred to a polypropylene microvial (P. Weidmann & Co., Romanshorn, Switzerland) before injection of 10 µl into the chromatograph.

Quantitation

A standard curve was constructed for each drug utilizing three replicates simulating concentrations of drugs from 5 to 500 µg/ml of plasma. The mobile phases used were phosphate buffer containing the following percentages of acetonitrile: cefonicid, 6%; cefazolin, cefotaxime and cefoxitin, 7.5%; and cefamandole, cefoperazone and cephalothin, 11%. The chromatograms were recorded at a chart speed of 5 mm/min. The peak heights were measured and the ratios (drug/internal standard) were calculated and plotted versus concentration expressed as micrograms per milliliter of plasma.

Patient Samples

Plasma or serum samples from patients receiving cephalosporin therapy were analyzed in duplicate using the same procedure. The amount of drug in patient samples was calculated by comparison with a standard curve prepared daily.

Interferences

The possible interference of normal plasma constituents was tested by the analysis of blank plasma samples. The interference of other drugs was tested by direct injection of aqueous or methanolic drug solutions.

Recovery

The recovery of each cephalosporin from spiked plasma was compared with that from water.

RESULTS AND DISCUSSION

Deproteinization of plasma samples with acetonitrile containing internal standard is a simple and rapid means of preparing the samples for HPLC. Monitoring the effluent at 254 nm afforded good sensitivity since cephalosporins exhibit appreciable absorbance at this wavelength. Slightly increased sensitivity might be obtained by using the maximum absorbance wavelength (λmax) for each drug (Table 1).

The chromatographic conditions used were chosen after comparison of two buffers and two organic modifiers as the mobile phases on an octadecylsilane reversed-phase column. The buffers tested were sodium acetate (0.01M, pH4) and sodium dihydrogen phosphate (0.01M). The pH of the phosphate solution was not adjusted, but was found to be 4.7. Methanol and acetonitrile were also compared for use in the mobile phase. The chromatographic behavior of the eight cephalosporins was examined and showed that both the magnitude and relative order of the capacity factors of the compounds tested are effected dramatically by the change from acetate to phosphate buffer, by the change from methanol to acetonitrile, and by even a small change in percent of organic modifier (Tables 2a and 2b). The use of phosphate buffer and acetonitrile both contributed to improved resolution of the

TABLE 1. MAXIMUM ABSORBANCE WAVELENGTHS

DRUG	λ_{max}*, nm
Cefamandole	265
Cefazolin	276
Cefonicid	270
Cefoperazone	232
Cefotaxime	238
Cefoxitin	239
Cephalothin	240
Cephapirin	264

* Ultraviolet spectrum of each compound scanned by stop-flow method during chromatography. The mobile phase consisted of 0.01M NaH_2PO_4 and acetonitrile.

cephalosporins, but complete separation of all eight compounds simultaneously was not obtained even with the use of gradient analysis. The cephalosporins would normally not be used concurrently with each other for therapy so patient samples would not be expected to have interference among the cephalosporins. All chromatograms were therefore run isocratically using phosphate buffer and with the percent acetonitrile chosen to provide convenient analysis time for a single drug and internal standard (K between 2 and 10). Elution with only 6% acetonitrile in phosphate buffer was used to resolve cefonicid from the normal plasma constituents. The other cephalosporins were eluted at higher percent acetonitrile to reduce analysis time. Maintaining

TABLE 2a. VARIATION OF CAPACITY FACTOR (K) WITH MOBILE PHASE CHANGE

COMPOUND	NaH_2PO_4, 0.01M					
	+5% CH_3CN	+10% CH_3CN	+15% CH_3CN	+10% CH_3OH	+15% CH_3OH	+20% CH_3OH
Cefonicid	4.08	1.08	0.57	3.32	1.25	0.65
Cefoxitin	12.97	4.23	1.67	11.8	5.32	2.78
Cefotaxime	15.85	3.03	0.98	15.83	5.58	2.40
Cefazolin	16.42	3.17	1.02	18.42	5.78	2.42
Cephapirin	17.63	3.73	1.25	>25	10.75	4.93
Cefamandole	>25	12.42	3.25	>25	20.57	6.15
Cefoperazone	>25	14.42	2.70	>25	22.57	8.02
Cephalothin	>25	18.25	4.85	>25	>25	13.72

TABLE 2b. VARIATION OF CAPACITY FACTOR (K) WITH MOBILE PHASE CHANGE (continued)

COMPOUND	CH_3COONa, 0.01M, pH4					
	+5% CH_3CN	+10% CH_3CN	+15% CH_3CN	+10% CH_3OH	+15% CH_3OH	+20% CH_3OH
Cefonicid	3.63	1.44	1.00	3.45	1.30	0.68
Cefoxitin	10.38	3.74	1.92	11.80	5.55	2.87
Cefotaxime	11.88	2.78	1.41	17.50	6.10	2.57
Cefazolin	11.55	2.89	1.39	16.63	5.82	2.40
Cephapirin	5.96	1.88	1.15	7.43	4.20	2.02
Cefamandole	>25	9.06	3.18	>25	19.37	7.67
Cefoperazone	>25	9.88	2.75	>25	19.90	5.73
Cephalothin	>25	13.07	4.64	>25	>25	13.42

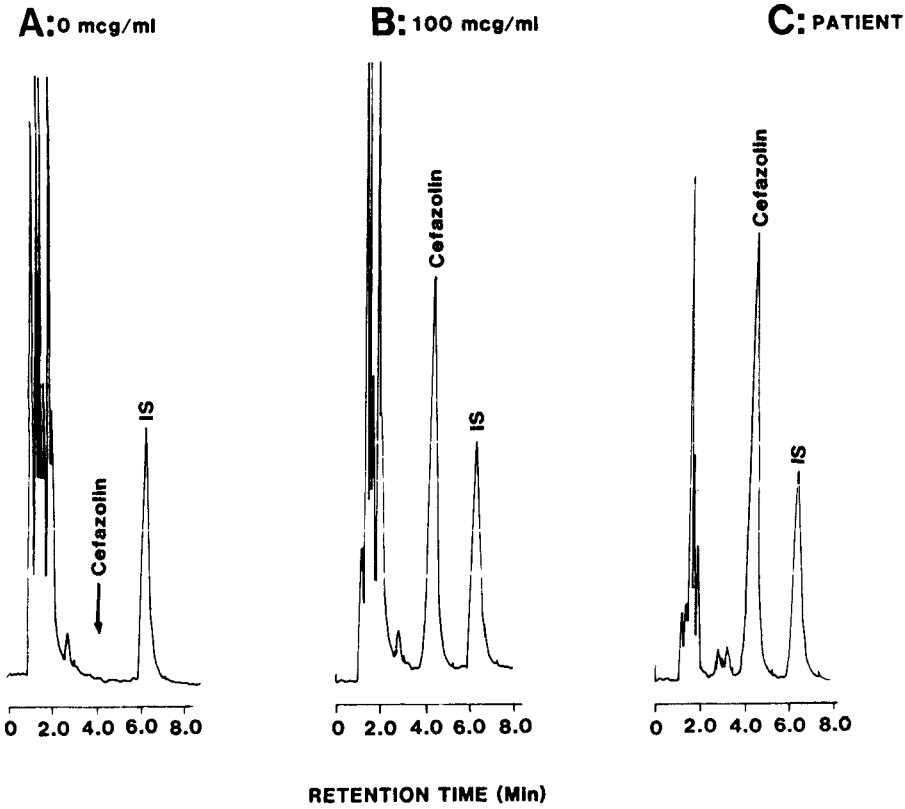

Figure I. Typical Chromatograms of Plasma Extracts

the column at 45°C also contributed to resolution of the compounds. No interference from normal plasma constituents was observed (Figure 1a) and several drugs which might be prescribed concurrently with cephalosporins were chromatographed. Their capacity factors are listed in Table 3.

The use of acetonitrile for precipitation of the serum and plasma proteins has the advantages of having low toxicity, of being the organic modifier in the mobile phase and of resulting in

TABLE 3. CAPACITY FACTORS (K) OF DRUGS WHICH MIGHT BE PRESCRIBED CONCURRENTLY WITH THE CEPHALOSPORINS

COMPOUND	0.01M NaH_2PO_4		
	+5% CH_3CN	+10% CH_3CN	+15% CH_3CN
Moxalactam	0.87 & 1.05 (doublet)	0.35	0.11
Carbenicillin	1.37	0.62	0.18
Ticarcillin	2.92	0.36	0.16
Acetaminophen	4.18	0.77	0.93
Salicylic Acid	4.42	2.37	1.37
Theophylline	6.92	1.15	0.88
Caffeine	9.20	4.15	1.78
Pennicillin G	>25	>25	5.59
Sulfamethoxazole	>25	>25	5.95
Chloramphenicol	>25	>25	11.88
Piperacillin	>25	>25	12.66
Amikacin	>25	>25	>25
Chlortetracycline	>25	>25	>25
Clindamycin	>25	>25	>25
Erythromycin	>25	>25	>25
Gentamicin	>25	>25	>25
Nafcillin	>25	>25	>25
Tetracycline	>25	>25	>25
Tobramycin	>25	>25	>25

TABLE 4. PRECISION OF CEPHALOSPORIN ASSAY

Amount Added ug/ml	Amount Found, ug/ml*			
	Cefamandole	Cefazolin	Cefonicid	Cefoperazone
5	6.6±1.5	5.4± 1.4	6.7±0.3	5.9± 0.7
25	25.5±0.3	24.0± 1.3	27.4±0.8	25.2± 1.0
50	51.7±0.6	51.7± 0.6	52.7±2.2	50.9± 2.1
75	75.0±1.9	74.4± 2.3	75.6±1.2	74.9± 5.1
100	98.9±2.3	101.5± 3.2	97.1±1.7	101.7± 2.2
200	197.5±4.0	206.2± 3.8	207.4±6.3	207.2± 4.6
300	303.6±6.9	293.0± 5.3	302.8±1.7	291.7± 7.8
400	403.8±6.0	397.6±13.1	404.9±3.6	392.0± 4.7
500	495.8±11.5	505.6± 7.4	495.7±8.9	506.3±11.0
Correlation Coefficient	0.9994	0.9987	0.9989	0.9989
Total N	27	27	27	27
R^2	0.9987	0.9974	0.9978	0.9977
y-Intercept	0.0018	0.0208	0.0331	0.0059

* Mean ± Std. dev.

(continued)

high recoveries for most of the cephalosporins. The percentage of recoveries were: cefamandole 96.3%, cefazolin 89.1%, cefonicid 74.7%, cefoperazone 83.3%, cefotaxime 99.1%, cefoxitin 92.4%, and cephalothin 97.3%. The lower recovery of some of the drugs is probably due to co-precipitation of the drugs with protein.

TABLE 4. PRECISION OF CEPHALOSPORIN ASSAY (continued)

Amount Added ug/ml	Amount Found, ug/ml*		
	Cefotaxime	Cefoxitin	Cephalothin
5	4.3± 0.2	5.2± 0.4	5.6± 0.7
25	22.9± 3.1	26.5± 0.2	27.9± 2.0
50	50.7± 1.8	51.7± 0.9	50.4± 3.1
75	77.2± 7.3	74.2± 2.3	76.0± 3.4
100	102.6± 1.2	99.4± 2.3	98.3± 5.7
200	203.8± 4.8	207.3±4.5	210.4± 8.2
300	298.9± 8.2	303.1±6.4	310.6±11.7
400	399.8± 6.5	400.1±3.9	404.9±11.6
500	496.3±10.7	496.2+10.2	491.7± 9.5
Correlation Coefficient	0.9995	0.9990	0.9995
Total N	27	27	27
R^2	0.9990	0.9980	0.9989
y-Intercept	-0.0115	0.0225	0.0144

* Mean ± Std. dev.

The ratios of the peak heights of the drugs to the peak height of the internal standard were calculated. Statistical analysis of the data by linear regression indicated linearity and reproducibility in the range of 5 to 500 μg/ml plasma (Table 4). This range includes the therapeutic range. The minimum detectable limit for the compounds by this method is less than 1 μg/ml plasma

(less than 3 ng/10 µl injection). The injection volume was maintained at 10 µl because larger volumes resulted in peak broadening.

The method has been applied to the analysis of patient samples in our laboratory (Figure 1c). Major advantages of this method for the analysis of cephalosporins in patient samples are its precision, simplicity, sensitivity and rapidity. All the drugs are determined from small volumes (0.5 ml or less) of serum or plasma with minimal sample preparation. The use of an isocratic mobile phase, an internal standard and UV detection at 254 nm contribute to its simplicity. The applicability of the method to cephalosporins from all three generations including a recently released product makes it feasible for routine patient monitoring. In addition, the high sensitivity of quantitation indicates its applicability for pharmacokinetic studies. The method is especially well suited to analysis of samples from patients receiving a combination of antibiotics which would preclude the use of microbiological assay methods.

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