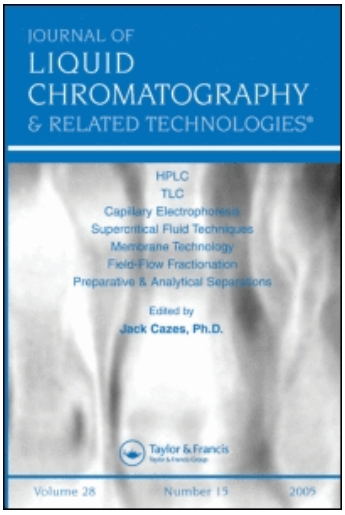

Journal of Liquid Chromatography & Related Technologies

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 Martinez, Luisa Gallo , ns-Falcó, Pilar Campí , Sevillano-Cabeza, Adela and Herráez-Hernández, Rosa(1998) 'Improved Solid Phase Extraction Procedure for Assay of Cephalosporins in Human Urine Samples', Journal of Liquid Chromatography & Related Technologies, 21: 14, 2191 — 2203
To link to this Article: DOI: 10.1080/10826079808006618
URL: http://dx.doi.org/10.1080/10826079808006618

J. LIQ. CHROM. & REL. TECHNOL., 21(14), 2191–2203 (1998)

IMPROVED SOLID PHASE EXTRACTION PROCEDURE FOR ASSAY OF CEPHALOSPORINS IN HUMAN URINE SAMPLES

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ABSTRACT

Solid phase extraction technique has been evaluated for the treatment of urine samples in the analysis of cephalosporins before injection into an HP-Hypersil ODS-C_{18} column. Cephalexin, cefotaxime, cefazolin, cefuroxime, and cefoxitin were tested with seven different reversed-phase extraction column cartridges and the obtained urine extracts were not clean. However, 3M Empore extraction disk cartridges packed with octadecyl (C_{18}) bonded silica provided clean extracts with a single extraction. The recoveries of the five cephalosporins ranged from 56 to 60 % in the 1.25- 500 μg/mL concentration range. The assay was accurate, precise, and adequate for testing the drug content in urine samples. The detection limit found for the five drugs was 0.25 μg/mL after an injection of 10 μL. The reliability of this study was tested by analyzing a urine sample containing cefuroxime after a minimum single dose administration (250 mg).

INTRODUCTION

The application of high performance liquid chromatography (HPLC) to the analysis of antibiotics introduces a powerful tool for therapeutic drug monitoring as well as clinical research. HPLC is to be preferred for the determination of cephalosporins because it offers advantages such as short turnaround time, method reliability, sensitivity, and drug specificity.[1-3]

The analysis of these drugs in biological fluids by HPLC requires sample clean-up procedures before injection into the analytical column. The work-up procedures are usually based on deproteinization or liquid-liquid extraction, which are usually tedious to perform. Moreover, the mixed polarity of cephalosporins makes the usual solvent extraction procedures inefficient[4] and serum protein precipitation is often inadequate due to high protein binding .

Garcia et al.[5] studied the retention mechanism of some cephalosporins as examples of zwitterions solutes in micellar liquid chromatography. This approach could permit direct injection of biological fluids without any previous treatment but this study was only carried out for standards.

Solid phase extraction has also been reported in the literature.[6] This technique is rapid, simple, and generally gives good recoveries of the assayed compunds. However, the degree of polarity of the different cephalosporins varies widely and it is, therefore, difficult to develop a single extraction procedure for all of them.[6] We observed similar behaviour for other drugs such as diuretics and proposed optimized procedures.[7-9]

Lee and Lee[1] developed an automated HPLC method with direct injection of plasma sample, using the column-switching technique, for the simultaneous determination of cefoxitin, cefuroxime, cephalexin, and cephaloridine. The total analysis time for sample was less than 25min. Moore et al.[6] proposed a double solid phase extraction employing C_{18} and NH_2 columns to determine cefazolin and ceftizoxime in human plasma.

The extraction exploits the non-polar and polar properties of these drugs. A single polar extraction is inefficient and a single non polar extraction is not clean. The retention times for the two compounds are 12 and 5 min, respectively.

The present study improves the existing solid phase extraction and HPLC procedures. The cephalosporins with various degrees of polarity included in this study are cefazolin, cephalexin, cefoxitin, cefotaxime, and cefuroxime.

The packing materials tested are used in normal and reversed-phase mode liquid chromatography: C_{18}, C_8, C_2, cyclohexyl (C_H), phenyl (PH), cyano (CN) as column cartridges, and 3M Empore extraction disk cartridges with C_{18} packing. This paper shows that the use of the disk cartridges provides clean extracts with a single extraction.

MATERIALS

Apparatus

The chromatographic system used consisted of a quaternary pump (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA) and an automatic sample injector (Hewlett-Packard, 1050 Series). A diode array (Hewlett-Packard, 1014 Series) detector linked to a data system (Hewlett-Packard HPLC Chem Station, Dos Series) was used for data acquisition and storage. The detector was set to collect a spectrum every 640 ms over the range 220-600 nm, and the chromatographic signal was monitored at 254 nm, 300 nm, and 260 nm. All the assays were carried out at room temperature. The pH was measured with a Crison micropH 2000 pH-meter (Crison Instruments, S.A., Alella, Barcelona, Spain).

Reagents

All the reagents used were of analytical grade. Acetonitrile was HPLC grade (Scharlau, Barcelona, Spain). Water was distilled, deionized, and filtered in nylon membranes 0.45 μm (Teknokroma, Barcelona, Spain). Sodium cefoxitin (Merck Sharpe & Dohme of Spain, Alcala de Henares), sodium cefazolin (Lilly S.A., Alcobendas, Spain), cephalexin hydrate (Sigma, St. Louis, USA), sodium cefuroxime (Glaxo, Aranda de Duero,Spain), and sodium cefotaxime (Hoescht Ibérica S.A., S. Feliu de Llobregat, Spain) were also used.

METHODS

Standard Solutions

Standard solutions of sodium cefoxitin, sodium cefazolin, cephalexin hydrate, and sodium cefuroxime of pharmaceutical grade were prepared by

dissolving 0.0158 g, 0.0158 g, 0.0161 g, 0.0105 g of the respective solid in 10 mL of distilled water. The stock solution of sodium cefotaxime was prepared by dissolving 0.0263 g in 25 mL of distilled water. Working solutions were prepared by dilution as required. The NaH_2PO_4 solution was prepared by dissolving 3.5 g of sodium dihydrogen phosphate (Probus, Barcelona, Spain) in 500 mL of distilled water. The pH was adjusted to 3 by adding a minimum amount of H_3PO_4 50 %.

Urine Standards

Appropriate volumes of stock solutions of sodium cefoxitin, sodium cefazolin, cephalexin hydrate, sodium cefuroxime, and sodium cefotaxime were added to urine to yield concentrations ranging from 1.25 to 500 μg/mL.

Solid Phase Materials

Six different Bond-Elut columns (Scharlau, Barcelona, Spain) 100 mg/mL used to extract cephalosporins from urine samples were evaluated: C_{18}, C_8, C_2, cyclohexyl, phenyl, and cyanopropyl. Another column 3M Empore high performance extraction disk cartridges with octadecyl (C_{18}) bonded silica (10 mm/6 mL cartridges) (Varian , Barcelona, Spain) was also used.

Columns and Mobile Phases

The precolumn (20 mmx2.1 mm I.D.), for the on-line extraction, was dry packed with a Hypersyl ODS C_{18}, 30 μm stationary phase (Hewlett-Packard). A LiChrospher 100PR 18 (125x4 mm I.D.) (Merck, Darmstadt, Germany) column was used as an analytical column. The precolumn was combined with the analytical column by means of a six port valve (Rheodyne model 7000, Cotati, CA, USA).

A gradient of acetonitrile, NaH_2PO_4 $5x10^{-2}$ M (pH = 3), with an acetonitrile content that increased from 10 % at zero time to 20 % at 2 min, 20 % at 6 min and 50 % at 8 min was used.

The solution was prepared daily, filtered through a nylon membrane, 0.45 μm (Teknokroma, Barcelona, Spain) and degassed with helium before use. The flow rate was 0.75 mL/min and 10 μL of each sample were injected.

Extraction Procedure

Solid phase extraction

Columns were conditioned previously by drawing with 1 mL of methanol, followed by 1 mL of buffer (pH = 3). Samples of urine (500 μL) containing H_3PO_4 :H_2O (1:80) (v/v) were transferred to the columns, and washed to eliminate the biological matrix with 1 mL of buffer (pH = 3). Cephalosporins were eluted from the columns with 500 μL of acetonitrile-water mixture 50:50 (v/v).

Recovery studies

The percent recoveries from a particular extraction were calculated comparing the peak areas obtained for cephalosporins in the spiked samples with the respective peak areas obtained by direct injection of aqueous solutions.

Human studies

Urinary excretion studies were performed with a human volunteer after a single dose administration of 250 mg of cefuroxime. Urine samples were collected at appropriate time intervals post-dose, and analyzed as described above. Each sample was assayed in triplicate.

Cephalexin was used as internal standard. The urine samples of cefuroxime were spiked with 0.05 mL of a solution of cephalexin in urine containing 2000 μg/mL.

RESULTS AND DISCUSSION

Initially, we investigated the chromatographic conditions needed to reach a satisfactory resolution of the compounds under study by direct injection of aqueous standard solutions of cephalosporins into the analytical column. A typical chromatogram of the five cephalosporins is shown in Figure 1.

The retention times of the different cephalosporins assayed were: cephalexin 4.7 min, cefotaxime 4.9 min, cefazolin 5.2 min, cefuroxime 5.5 min, and cefoxitin 6.2 min. The detector reponse (peak-area) was linear for all the drugs over the assayed range (1.25-500 μg/mL), and all the graphs had ordinate values statistically consistent with zero.

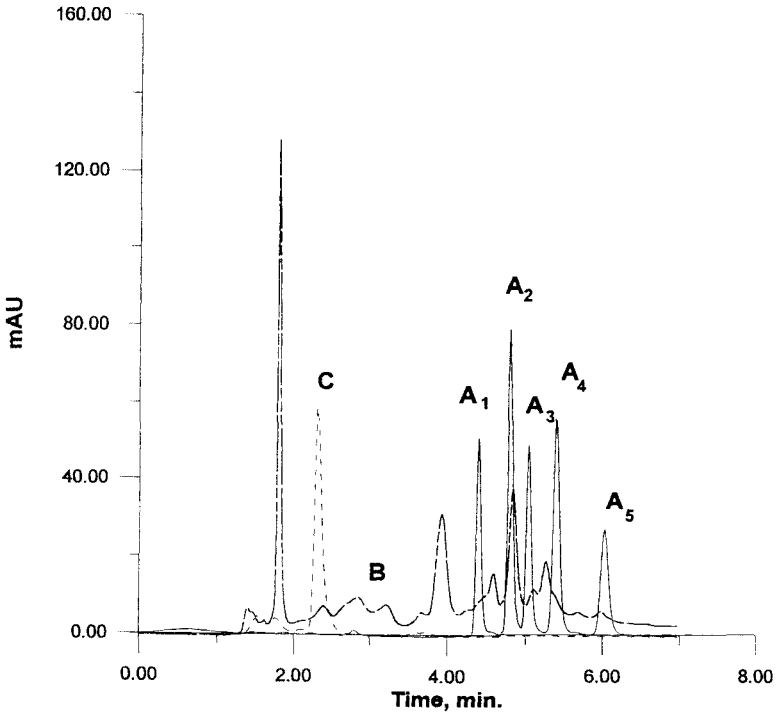

Figure 1. Chromatograms at 254 nm of (A) a mixture of: cephalexin (1), cefotaxime (2), cephazolin (3), cefuroxime (4) and cefoxitin (5) standards; (B) a blank urine sample flushed with a C_{18} column cartridge and (C) a blank urine sample flushed with a C_{18} disk cartridge

Solid Phase Extraction

Due to the widely differing degree of polarity within the cephalosporin group and to the difficulty in obtaining a clean extract using C_{18} columns, we optimized the volume and pH of the flushed samples and the washing step. Table 1 summarizes the results obtained. The best procedure corresponds to the conditions reflected in the last row of Table 1 because for all cephalosporins the recoveries are similar to those obtained without the washing step.

When spiked urine samples were processed the extracts were not clean, as can be seen in Figure 1. The volume of the washing step was increased to 1 mL and the wavelengths were selected in order to improve selectivity. The area values were read at 300 nm for cefotaxime and cefazolin and at 254 nm for

Table 1

Recoveries Obtained for Standards of Cephalosporins in Different Conditions using C_{18} Column Cartridges

Sol'n. Vol. (mL)	Sol'n. Vol. (mL)	Sample Conditions	Washing Solution I*/Taken Vol. (mL)	Cepha-lexin	Cefo-taxime	Cef-azolin	Cefur-oxime	Cef-oxitin
1	1	water	0.05 / 2	30	84	80	53	43
1	1	H_3PO_4:H_2O (1:80)	0.05 / 2	32 ± 4	80 ± 4	77 ± 5	56 ± 10	50 ± 10
1	1	H_3PO_4:H_2O (1:80)	0.1 / 2	31 ± 7	79 ± 8	76 ± 8	62 ± 2	51 ± 1
1	1	H_3PO_4:H_2O (1:80)	0.001 / 2	36 ± 3	80 ± 2	72 ± 6	53 ± 11	46 ± 9
1	1	H_3PO_4:H_2O (1:80)	without	80.2 ± 0.2	90.3 ± 0.3	88.3 ± 0.6	87 ± 2	87 ± 1
0.5	0.5	H_3PO_4:H_2O (1:80)	0.05 / 1	71 ± 3	98 ± 6	96 ± 7	96 ± 6	88 ± 5
0.025	0.025	H_3PO_4:H_2O (1:80)	0.05 / 0.5	82 ± 3	92 ± 3	92 ± 4	94 ± 5	90 ± 4

* I = ionic strength.

cephalexin, cefoxitin, and cefuroxime. The percentage recoveries (n = 5) obtained for cephalexin, cefotaxime, cefazolin, cefuroxime, and cefoxitin in spiked urine samples were: 75±7, 89±5, 90±6, 86±8, and 82±9, respectively. These values are similar to those obtained for standards (see Table 1).

Other different packing materials with different polarities were tested in order to increase selectivity: C_8, C_2, CH, PH, CN. The percentage recoveries obtained for standards of cephalosporins were worse than those reported in Table 1. CN columns are not appropiate for these drugs because they are not retained. Blank urine samples were also analyzed, but the selectivity was not improved.

3M Empore high performance extraction disk cartridges with C_{18} packing were also tested. The disk cartridges provide lower recoveries when working in the selected conditions than do C_{18} columns. The percentage recoveries (n = 3) obtained for standards of cephalexin, cefotaxime, cefazolin, cefuroxime, and cefoxitin were: 37±2 , 61±2 , 63±2 , 62±3, and 64±1. However, the extracts of urine samples were clearer than those obtained with the column cartridges (see Figure 1). It is therefore possible to work at 254 nm for all cephalosporins when the disk cartridges are used and therefore the detection limits of cefotaxime and cefazolin can be improved. For this reason we optimized the most important parameters of the liquid-solid extraction procedure for the disk cartridges. We began by studying the influence of the volume of the washing solution in the possible elution of the retained cephalosporins for standards.

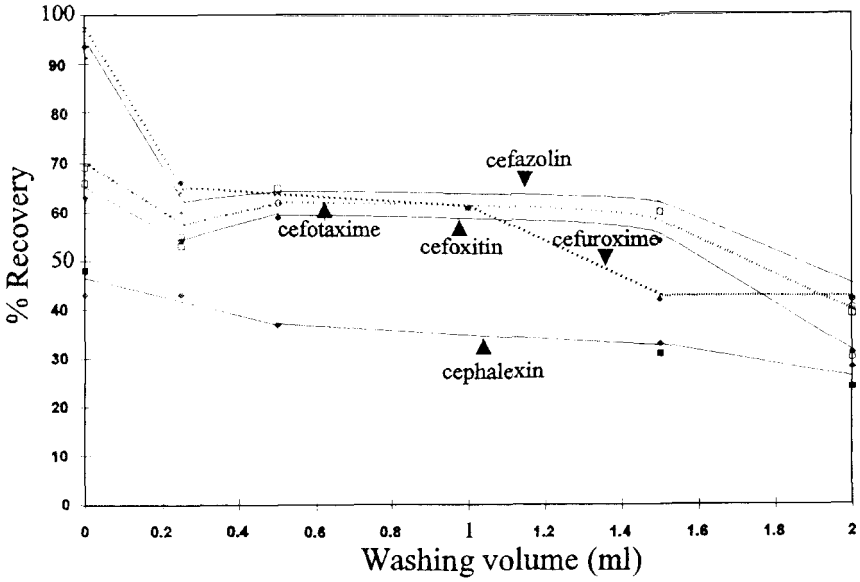

Figure 2. Plotted recoveries vs. washing volume for cefoxitin, cephalexin, cefotaxime, cephazolin and cefuroxime.

Because of their polar character, the recovery decreased rapidly when the washing volume was increased (Figure 2). However, and since the amount of the cephalosporins in urine samples is not a factor limiting the analysis, we optimized the washing volume in order to obtain a proper selectivity. A washing volume of 1 mL was selected as the best option for cleanup giving suitable recoveries.

The influence of the sample volume and ionic strength of the washing solution on the percentage recovery were also studied. When the sample volume was 0.5 mL, cephalexin recovery improved, while the obtained values for the other cephalosporins were similar. The ionic strength of the washing solution did not modify the recoveries observed. We selected a value of 0.05 M.

The percentage recoveries obtained for standards of cephalexin, cefotaxime, cefazolin, cefuroxime, and cefoxitin working in the 1.25-500 μg/mL range of concentrations were : 50±1 (n=18), 59±1 (n = 15), 53±1 (n = 18), 55±1 (n = 18), and 70±1 (n = 15).

We also evaluated the possibility of performing solid phase extraction of the analytes in an on line mode because this procedure has been reported to be succesful in the analysis of some cephalosporins in plasma samples.[1] A precolumn packed with a Hypersil ODS-C_{18}, 30 μm phase was used for retention of the cephalosporins. This precolumn was combined with the analytical column by means of a switching arrangement. Owing to the polarity of the compounds studied, the recoveries were unacceptable when the precolumn was flushed with 0.5-1 mL of the phosphate buffer solution at a flow rate of 0.5 mL/min. Since volumes of washing solvent lower than 1 mL could result in a rapid analytical column lifetime deterioration, extraction into the 3M Empore disks was selected as the best option for the analysis of cephalosporins in urine.

Urine Samples

The disk packing cartridges were employed for the detection and determination of the cephalosporins studied in spiked urine samples. The percentage of extraction recoveries for different concentrations in the 1.25-500 μg/mL range of cephalexin, cefotaxime, cefazolin, cefuroxime, and cefoxitin were: 56±7 (n = 18), 60±8 (n = 15), 56±8 (n = 18), 59±6 (n = 15), and 65±2 (n = 15). The efficiency and precision obtained in the sample cleanup step were sufficient and do not depend on the drug concentration in the interval studied. The percent recovery from a particular extraction was calculated with respect to the direct injection. As can be seen, the values obtained are similar to those reported for cephalosporins standards.

All the calibration graphs of spiked urine were linear in the working range of concentrations (1.25-500 μg/mL). The equations of the calibration graphs at 254 nm (intercept ± s, slope ± s, mean standard deviation of the regression s_{yx}) for cephalexin, cefotaxime, cefazolin, cefuroxime, and cefoxitin were, for three replicates: (29±28, 2.41±0.09, 0.04), (17±20, 4.2±0.2, 0.04), (20±20, 3.4±0.1, 0.02), (-20±20, 3.8±0.1_5, 0.06), and (20±20, 2.7±0.2, 0.03), respectively. The slope values are similar to those obtained with standards. Therefore, a calibration graph with standards can be used to analyze urine samples.

Detection limits were determined as the concentration of compound giving a signal-to-noise ratio greater than 3:1. The limit of detection estimated was 0.25 μg/ mL for the five cephalosporins. The detection limit observed for cefoxitin, cefuroxime, cephalexin in plasma reported by Lee and Lee[1] was 0.5 μg/mL, and the recoveries obtained by their solid phase extraction procedure ranged from 72 to 85 %.

Table 2

Precision And Accuracy Of The Assay For The Five Cephalosporins In Spiked Urine Samples

Compound	C_{added} (μg/mL)	C_{found} (μg/mL) Within Day/ Between Day	% R.S.D. Within Day (n)*	% R.S.D. Between Day (n)	% Relative Error Within Day (n)	% Relative Error Between Day (n)
	25	- /23		10.8 (n=3)		-8.0
Cephalexin	200	220 / 207	8.0 (n=6)	12.0 (n=6)	10.0	3.5
	300	- / 300		8.3 (n=5)		0
	500	- / 500		5.0 (n=9)		0
	25	26 / -	12.0 (n=9)		4.0	
Cefotaxime	200	193 / 180	8.5 (n=6)	8.7 (n=6)	-2.0	-10.0
	300	300 / -	4.2 (n=9)		0	
	25	- / 25		10.0 (n-9)		0
Cefazolin	200	196 / 193	8.4 (n=6)	11.0 (n=6)	-2.0	-3.5
	500	- / 500		2.0 (n=9)		0
	25	- / 26		10.0 (n=9)		4.0
Cefuroxime	200	190 / 198	3.8 (n=6)	6.0 (n=6)	-5.0	-1.0
	300	- / 301		8.3 (n=9)		0.3
	25	- / 25		10.0 (n=9)		0
Cefoxitin	200	204 / 208	7.6 (n=6)	13.0 (n=6)	2.0	4.0
	300	- / 310		9.0 (n=9)		3.3

* n = number of replicates.

The accuracy and precision of the assay were evaluated by analyzing spiked urine samples over the 25-500 μg/ mL concentration range (Table 2). The replicates for each urine sample were assayed on the initial day of preparation and/or were processed on different days. Table 2, also shows intra-day and inter-day precisions. The concentrations found were close to the actual concentrations in all cases tested, as can be seen from the relative errors (%) given in Table 2. From these results it can be established that the accuracy and precision of the method are satisfactory.

Human Studies

The assay described was used to measure the urinary levels of cefuroxime after a single dose of 250 mg to a human volunteer. The urinary excretion-time profile is given in Figure 3. Cephalexin was used as an internal standard

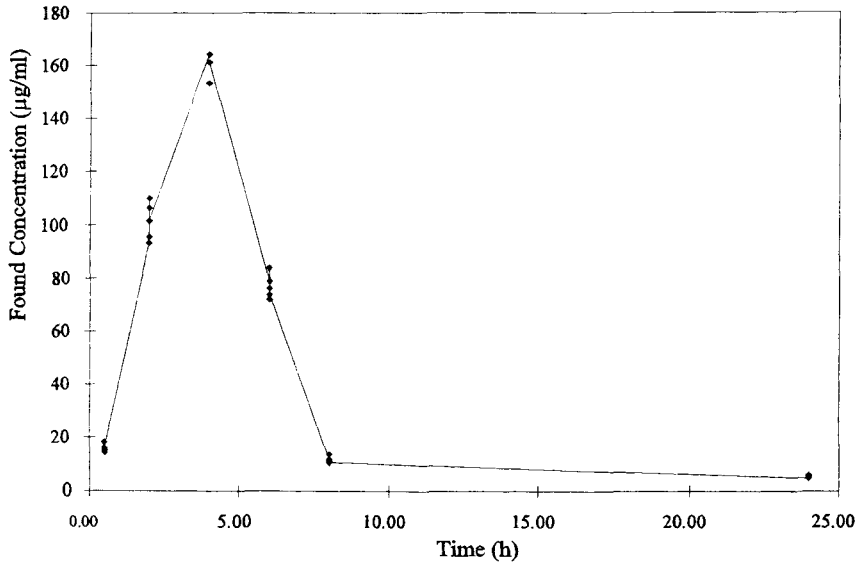

Figure 3. Plotted urine concentration vs.time following dose.

and the processed analytical signal was the ratio between the area values of cefuroxime and cephalexin. We studied the inter and intra-day precision for the real samples. The results obtained are shown in Table 3. The % rsd values are similar to those given in Table 2. The detection limit given above was corroborated.

We have tested the possibility of employing the disk cartridges repeatedly for two concentrations, 25 and 500µg/mL, in urine. The % rsd obtained for ten uses were 5.5 and 7.5, respectively. The values for standards were 4.8 and 4.3. These results indicate that the disk cartridges can be employed repeatedly for at least ten times.

CONCLUSIONS

We have studied several variables affecting the retention/elution of cephalexin, cefotaxime, cefazolin, cefuroxime, and cefoxitin on solid phase extraction cartridges.

Table 3

Inter-Day and Intra-Day Precision for Urine Samples Containing Cefuroxime*

Time Following Dose (Hours)	Within-Day % R.S.D. (n=5)	Between-Day % R.S.D. (n=10)
1/2	5.0	11.1
2	7.0	6.0
4	9.4	9.2
6	6.2	10.1
8	5.7	10.8
24	4.3	13.7

* Internal standard used cephalexin.

We have proven that using disks instead of column cartridges make it possible to obtain clean extracts using C_{18} support. It is therefore not necessary to use two packings, NH_2 and C_{18} columns, as proposed by Moore et al. in [2] for cefazolin.

The injection volume in our procedure is 10 μL. Although volumes of 20 μL[2] or 25 μL[1] have been proposed, the detection limit achieved in this proposed method is better.

The cephalosporins assayed can be determined by our procedure with satisfactory precision, accuracy and sensitivity at therapeutical levels.

ACKNOWLEDGMENT

The authors are grateful to the CICYT for financial support (Project No. SAF95-0586).

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Received June 7, 1997
Accepted November 20, 1997
Manuscript 4518