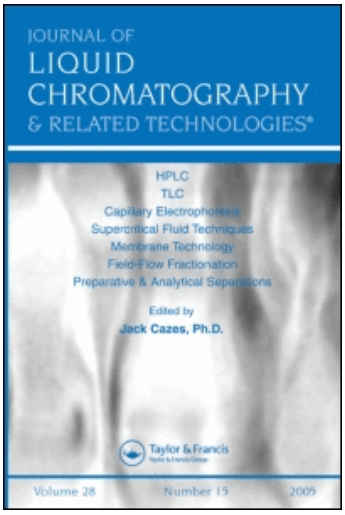

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Extraction and Determination of Cefazolin Sodium and Cefotaxime Sodium in Human Urine with a Weak Ion Exchange Monolithic Column

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Abstract: An online solid phase extraction (SPE) method was developed for the simultaneous screening of cefazolin sodium and cefotaxime sodium in human urine. A short weak anion exchange monolithic column was prepared and used as the selective extraction sorbent. The column exhibited good permeability and biocompatibility. During the online SPE process, most matrices in the urine samples were removed rapidly, while cefazolin sodium and cefotaxime sodium were effectively trapped in this monolithic column. Reversed phase high performance liquid chromatography (HPLC) was performed on a C_{18} column with ultraviolet detection at 254 nm. The mobile phase consisted of methanol-0.005 mol/L phosphate (21/79, *v*/*v*, pH = 5.0) and the flow rate was set to 0.7 mL/min. A good linear relationship was demonstrated at analyte concentrations ranging from 1 to 100 μg/mL. The absolute recovery ranged from 79.4% to 81.5% and the inter- and intra-day relative standard deviations were <5.0%. These results suggest that the online SPE-HPLC method is suitable for the simultaneous monitoring of cefazolin sodium and cefotaxime sodium in human urine samples because it avoids tedious pretreatments and provides a fast, economical, and reproducible method for assaying trace drugs in biological samples.

Keywords: Cefazolin sodium, Cefotaxime sodium, Human urine, Online SPE, Weak ion monolithic column

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INTRODUCTION

Cefazolin sodium and cefotaxime sodium are members of the cephalosporin antibiotic class of drugs with a wide spectrum of activities that are useful as presurgery antibiotics. Their structures are shown in Figure 1. Cefazolin sodium is classified as a first generation cephalosporin with a broad spectrum of activity and exhibits somewhat greater activity against klebsiella pneumonia.[1] The antibiotic is active against many gram-positive aerobic cocci, but has limited activity against gram-negative bacteria. Cefotaxime sodium generally has good coverage against most gram-negative bacteria, with the notable exception being pseudomonas. It is also effective against most gram-positive cocci with the exception being enterococcus. These two antibiotics work by inhibiting bacterial cell wall biosynthesis and are active against a wide range of gram-positive and negative bacteria. They can be used to treat moderately severe bacterial infections involving the lungs, stomach, joints, skin, blood, heart valve, and urinary tract. The side effects include diarrhea, stomach pain, fainting, and vomiting.[2] Their concentration in human urine is a key marker for examining the urinary clearance rate and bioavailability after being used to treat some diseases, and they have some practical significance in their ability to predict the drug metabolism, as well as the type of drug metabolism. For this purpose, a rapid and inexpensive analytical method for their simultaneous determination is needed.

The HPLC detection of drugs in urine samples usually involves sample treatment before being injected into the chromatograph. Typically, this has been achieved by techniques, such as protein precipitation by organic solvents,[3] liquid–liquid extraction[4,5] and off-line SPE.[6,7] These conventional procedures are complicated and environmentally unfriendly, and drugs may be partly lost in the sample preparation steps. On-line SPE has gained popularity as a useful technique for extracting a wide range of analytes from urine, as well as other biological and environmental samples.[8,9] The on-line SPE method is a clean, fast, efficient, and sensitive trace level determination pretreatment

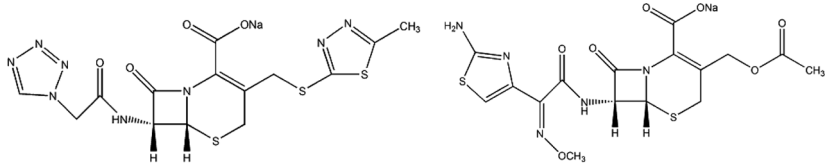

Figure 1. Chemical structures of cefazolin sodium (a) and cefotaxime sodium (b).

procedure,[10] and is particularly attractive because it allows the simultaneous pretreatment of biological samples and an investigation of analytes. A monolithic column has recently become a rapidly interesting field in the preparation of chromatographic stationary phases.[11,12] It exploits the advantage of cheap, easy preparation and excellent properties for the extraction of drugs compared with the conventional C_{18} extraction column.

In this study, a new on-line SPE method with column switching was developed to determine the cefazolin sodium and cefotaxime sodium concentration simultaneously, in human urine solutions was developed using a weak anion monolithic column. The mobile phase was optimized and their effect on the obtained signal was investigated.

EXPERIMENTAL

Reagents and Materials

Cefazolin sodium, cefotaxime sodium, methacrylic acid (MAA), and glycidyl methacrylate (GMA) were bought from Sigma (St Louis, MO, U.S.A.). Ethylene glycol dimethacrylate (EGDMA) was purchased from Fluka (Buchs, Switzerland). Dodecanol was purchased from Acros organics (New Jersey, USA). Cyclohexanol and 2, 2′-azobis (isobutyronitrile) (AIBN) were purchased from Junsei Chemical Co. Ltd. (Japan) and refined before use. Methanol and sodium phosphate was bought from Pure Chemical Co., Ltd (Ansan, Korea). All the other solvents used in the experiment were HPLC or analytical grade.

Apparatus

The chromatography system consisted of Waters 600s Multi solvent Delivery System, Waters 616 liquid chromatography (Waters Associates, Milford, MA, U.S.A.), a Rheodyne injector (20 μL sample loop), and a variable wavelength 2487 UV dual channel detector. Data processing was carried out with a Millenium 3.2 consisted of HP Vectra 500PC. The analysis was performed on an OptimaPak C_{18} column (5 μm, 150×4.6 mm, RS tech Corporation, Daejeon, Korea) with a guard column (10×4.6 mm) packed with C_{18} materials. Deionized water was filtered with a vacuum pump (Division of Millipore, Waters, U.S.A.) and filter (HA-0.45, Division of Millipore, Waters, U.S.A.) before use. All the samples were filtered by using a filter (MFS-25, 0.2 μm TF, WHATMAN, U.S.A.) before injection into the HPLC system.

Preparation of the Weak Ion Monolithic Column

The monolithic column was prepared by an in-situ polymerization. First, a mixture consisting of 0.25 mL MAA, 1.2 mL GMA, 1.5 ml EGDMA, 2.3 mL dodecanol, 2.0 mL cyclohexanol, 2.2 mL methanol, and 0.031 g AIBN were purged with nitrogen for 15 min. Then, the stainless steel column (20×4.6 mm I.D.) sealed at the bottom, was filled with the polymerization mixture and then sealed at the top. After the polymerization was allowed to proceed at 55°C for 24 h, the column was flushed with methanol to remove the porogen and other soluble compounds present in the polymer rod. Then a mixture of ethylenediamine with tetrahydrofuran (1:1, *v*/*v*) solutions and a solution of 15.0% chloroacetic acid (pH 12.0) adjusted by sodium hydroxide were pumped through the column at 0.1 mL/min in steps at 80°C for 24.0 h. Finally, the column was washed with 0.05 mol/L acetate buffer followed by deionized water until the eluent was neutral.

Standard Solutions Used for Method Development

The mixed stock solution of cefazolin sodium and cefotaxime sodium was prepared by dissolving 10 mg of drug in 10 mL of methanol. Blank human urines from healthy volunteers were stored at −18°C and kept at 4°C before use. Any precipitated material was removed by centrifuging the sample at 5000 rpm for 15 min. Different amounts of stock solutions were spiked in an appropriate volume of the urine solution. The stock solutions were diluted in order to obtain three solutions at 80, 30, and 5 μg/mL. These intermediate solutions were used to spike free drug samples at seven concentration levels covering a range of 1∼100 μg/mL for urine samples.

Sample Pretreatment and Chromatographic Separation

The weak ion exchange monolithic column was used as an on-line SPE sorbent, which was placed in the sample loop position of the six-port injector valve and used for sample cleanup. In the "load" position, 0.1 mL urine samples were directly injected into the SPE monolithic column and then deionized water was employed for selected washing at a flow rate of 0.3 mL/min. After 2 min of washing, the six-port valve switched to "inject" position, and the target analytes could be eluted in the back flush mode by the mobile phase at an optimized flow rate of 0.7 mL/min. The chromatogram was monitored at a wavelength of 254 nm, and was obtained on a C_{18} column. Thus, a complete cycle of

the on-line SPE preconcentration and HPLC separation of the analytes was accomplished.

RESULTS AND DISCUSSION

Column Permeability

The mechanical stability of the column material was evaluated by measuring the pressure drop across the column at different flow rates. Figure 2 shows the effect of the low rate on the back pressure when deionized water and methanol were used as the mobile phase. An excellent linear dependence of the column pressure on the flow rate was indicated by $r^2 \geq 0.9995$ for the measured curve.

Influence of Solution pH on Retention Time

Because of dietary variations, the pH of human urine can vary from 4.5 to 9.0, with a mean of approximately 6.0. Mixtures of cefazolin sodium and cefotaxime sodium standard samples at a concentration of 5.0 mg/mL were placed in glass tubes and the pH was adjusted to pH 4.5~9.0 in steps

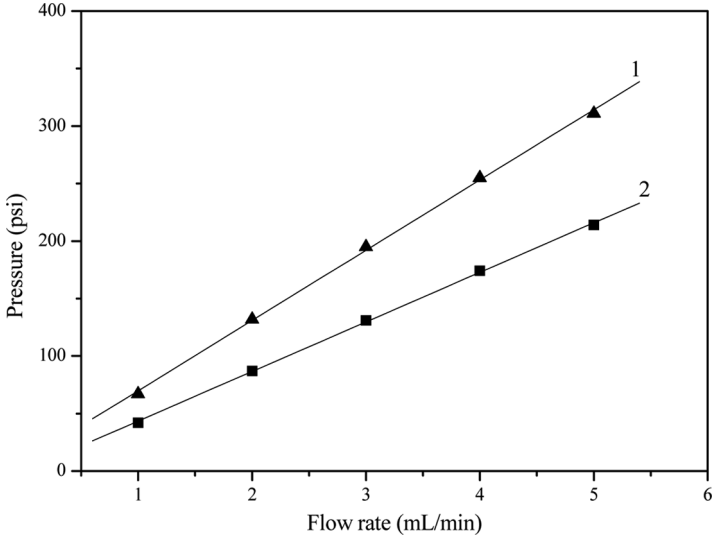

Figure 2. Change of back pressure with different flow rate for monolithic column. Mobile phase: (1) deionized water; (2) methanol.

of 0.5 using the appropriate volumes of 10% HCl or 10% NaOH. Subsequently, 10 μL standard samples of the mixture at different pHs were injected directly into the anion monolithic column connected to the UV detector, monitored at 254 nm with deionized water as the mobile phase. The drug samples with deionized water as the solvent could not be flushed out of the ion exchange monolithic column within 10 min, indicating that the human urine samples did not affect the retention of cefazolin sodium and cefotaxime sodium on the monolithic column when the pH ranged from 4.5~9.0.

Chromatography

The chromatographic conditions were optimized in order to determine the most favorable separation and chromatographic efficiency. The retention times of cefazolin sodium and cefotaxime sodium on the C_{18} column were greatly affected by the content and pH of the phosphate solution in the mobile phase. Chromatograms were obtained after direct injection of a standard solution of cefazolin sodium and cefotaxime sodium at a concentration of 10 μg/mL. The composition of the methanol-phosphate mobile phase was changed from 50/50 (v/v) to 10/90 (v/v) and the pH of the phosphate solution ranged from 3.0 to 9.0. The results showed that methanol-0.005 mol/L phosphate (21/79, v/v, pH = 5.0) was the optimal condition for separating these two drugs on the C_{18} column.

A weak anion exchange monolithic column was used as an SPE column to process the human urine samples in order to completely eliminate the matrix interference and concentrate the analyte. Human urine samples were pretreated by passing through a SPE column with a column switch, with deionized water as the mobile phase. The samples were then analyzed on a C_{18} column with the optimized mobile phase. The retention times for cefotaxime sodium and cefazolin sodium were approximately 18.1 min and 23.2 min, respectively, with an overall run time of 30 min. Blank urine samples were used to determine if there were any interfering peaks around the retention times of cefazolin sodium and cefotaxime sodium. The peaks for cefazolin sodium and cefotaxime sodium showed good separation with no interference, demonstrating the high specificity and sensibility of the above described method. Figure 3 shows the 0.1 mL urine extracts obtained after ion exchange material extraction.

Calibration Curves, LOD and LOQ

The standard calibration curves (concentration as a function of the chromatographic peak area) for cefazolin sodium and cefotaxime sodium

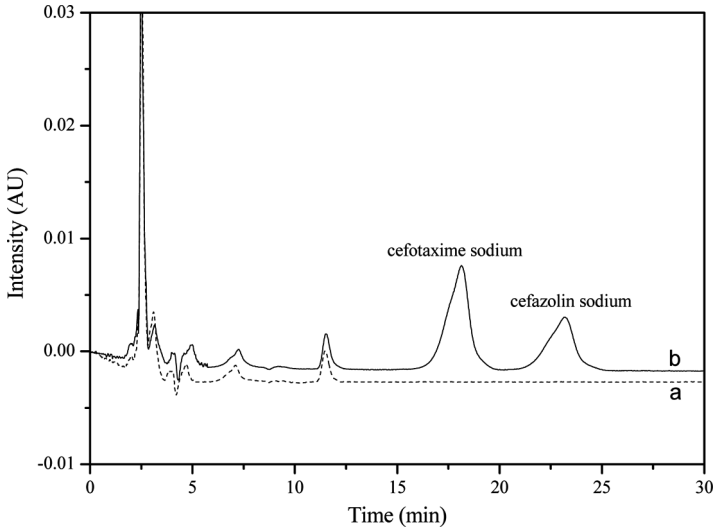

Figure 3. Chromatograms of blank urine sample (a) and a sample spiked with cefazolin sodium and cefotaxime sodium at a concentration of 10 μg/mL (b) (Mobile phase: methanol-0.005 mol/L phosphate (21/79, *v*/*v*, pH = 5.0)).

in the urine sample were linear ($r^2 \geq 0.9997$, $n = 7$) over the concentration range, 1∼100 μg/mL. All analyses were carried out in triplicate. Table 1 shows the calculated results, where a and b are the coefficients of the regression equation $y = ax + b$, x refers to the concentration of the drug (μg/mL), y the peak area, and r^2 the correlation coefficient of the equation. The sensitivity of the method is expressed by the limit of detection (LOD) and the limit of quantification (LOQ). The LOD and LOQ for cefazolin sodium and cefotaxime sodium in the urine sample, which were established as the amounts for which the signal-to-noise ratios were 3:1 and 10:1, respectively, were 0.2 μg/mL and 0.6 μg/mL, respectively.

Table 1. Calibration curve ($n = 7$), LOD and LOQ for the quantifianion of cefazolin sodium and cefotaxime sodium

	Regression equation	r^2	Linear range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Cefazolin sodium	$y = 1.70 \times 10^4 x - 6.07 \times 10^2$	0.9998	1.0∼100.0	0.2	0.6
Cefotaxime sodium	$y = 2.89 \times 10^4 x - 1.07 \times 10^4$	0.9997	1.0∼100.0	0.2	0.6

Table 2. Intra-day and inter-day precisions, accuracies and recoveries of cefazolin sodium and cefotaxime sodium in human urine samples spiked with three different concentrations

		Intra-day			Inter-day			Recovery	
	Concentration (μg/mL)	Measured concentration (μg/mL)	Accuracy (%)	Precision RSD (%)	Measured concentration (μg/mL)	Accuracy (%)	Precision RSD (%)	Absolute recovery (%)	Method recovery (%)
Cefazolin sodium	5.0	5.02	100.4	2.47	5.13	102.6	2.36	80.3	100.4
	30	30.15	100.5	3.26	30.21	100.7	2.28	81.5	100.5
	80	80.52	100.7	2.77	80.86	101.1	1.07	79.4	100.7
Cefotaxime sodium	5.0	4.99	99.8	2.15	4.98	99.6	3.65	80.9	99.8
	30	30.85	102.8	3.51	30.72	102.4	3.74	81.3	102.8
	80	79.75	99.7	3.85	79.92	99.9	3.19	79.8	99.7

Precision and Accuracy

The precision and accuracy of the method were examined by performing replicate analyses of the three levels of QCs against the calibration standards. The precision is presented as the relative standardization deviation (RSD) of the concentrations of the analytes. The accuracy is the quality of being close to the true value. The precision and accuracy of this method are expressed as the intra-day and inter-day variability in the concentration ranges of cefazolin sodium and cefotaxime sodium in the urine samples. All analyses were carried out five times. The precision values of cefazolin sodium and cefotaxime sodium were $<5.0\%$. The intra-day and inter-day accuracy values of these two drugs at three concentrations ranged from 99.7 to 102.8% and from 99.6 to 102.6%, respectively (Table 2). Therefore, the developed analytical method for analyzing biological samples fulfilled the criteria of precision and accuracy.

Recovery

The extraction of drugs in urine samples, which can be expressed as the absolute recovery, is indispensable for successful analysis. The absolute recovery of cefazolin sodium and cefotaxime sodium from the urine samples were examined at low, medium, and high concentrations, respectively, in five replicates. It was determined by comparing the peak area of cefazolin sodium and cefotaxime sodium of the extracted samples with the peak area obtained from direct injections of a standard solution containing the same concentration of the two drugs. The method for recovering cefazolin sodium and cefotaxime sodium at the three different concentrations were determined by comparing the concentrations measured after analyzing the spiked urine samples according to the procedure reported in Sample Pretreatment and Chromatographic Separation section with those of the targeted concentrations. The absolute and method recoveries of these two drugs at the three concentrations ranged from 79.4 to 81.5% and from 99.7 to 102.8%, respectively (Table 2), which confirms the reliability of this method. This suggests that the extraction and determination of these two drugs from human urine were concentration independent over the concentration range evaluated and were acceptable.

CONCLUSIONS

A weak ion exchange monolithic column was used successfully as an on-line SPE material for the simultaneous determination of cefazolin

sodium and cefotaxime sodium in human urine. This developed method showed high sensitivity, as well as the appropriate precision, accuracy, and recovery, which can be used to monitor these drugs in clinical therapy. Furthermore, the cost of analysis can be reduced by reusing the monolithic column up to 100 times without significant changes in the analyte recovery or the column back pressure. The method was suitable for determining the cefazolin sodium and cefotaxime sodium level in human urine samples, which had avoided the tedious pretreatment and provided fast, economical, repeatability, and effective method for assaying trace drugs in biological samples.

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