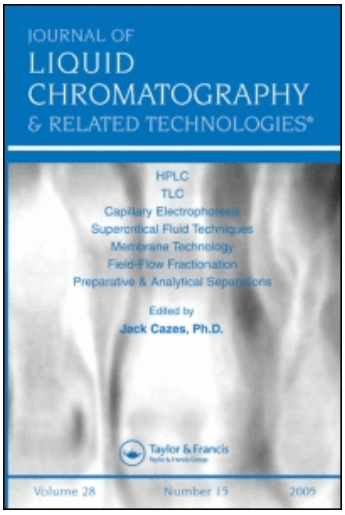

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND DETERMINATION OF CIPROFLOXACIN AND ITS METABOLITES IN URINE

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ABSTRACT

An improved HPLC procedure for separating ciprofloxacin and its four metabolites, M1, M2, M3, M4 in urine, was developed. The procedure used is reversed phase chromatography, the stationary phase being the C_{18}-column, followed by quantification using a UV detector. The mobile phase is a mixture of iso-propanol, acetonitrile, tetrabutylammonium bromide (TBABr), heptanesulfonic acid (HSA), and 0.05% triethylamine, (adjusted to pH=3.0 with phosphoric acid.) In the process of determining the optimum separation condition, the effect of each ion-pairing reagent on the retention of all analytes was investigated and explained as well. This method involves an off-line solid phase extraction, using C_{18} Sep-Pak cartridges for ciprofloxacin and its metabolites in urine. The desorption condition is investigated by using different kinds of organic solvents, or a mixture of organic solvents and phosphate buffer, adjusted to different values of pH. At the optimum desorption condition, all analytes show a recovery rate over 90%. Based on this optimum condition, this method was applied to pharmacokinetic study of solutes in urine sample.

INTRODUCTION

Ciprofloxacin is a kind of quinolone carboxylic acid derivative from Gram-negative and Gram-positive bacteria that are resistant to penicillins, cephalosphorins, aminoglycosides, β-lactams and tetracyclins.[1-4] Recently, ciprofloxacin has been widely used in the treatment of urinary and respiratory tract infections, and also in gastrointestinal diseases. It is known to react with DNA gyrase inhibition, similar to nalidxic acid and norfloxacin.[5] The structures of ciprofloxacin and its metabolites are depicted in Figure 1.

Much research has been conducted about the separation and detection methods of ciprofloxacin and its metabolites in biological fluids by the use of HPLC, however, previous methods, in most cases, had problems related to resolution, sensitivity, and the efficiency of sample preparation. Krol et al. has developed the isocratic separation method of ciprofloxacin and its metabolites in saliva, urine, and serum by using polystyrene-divinylbenzene (PSDVB) and the C_{18} column as stationary phases.[6] The PRP-1 cartridge was especially used for eliminating the sample matrix in the analysis of serum. Myers and Blumer have used the isocratic separation method for ciprofloxacin and the gradient method for its metabolites in saliva, urine, and serum by using fluorescence and a UV detector.[7] So, the developers could not separate the four kinds of analytes simultaneously in one condition. Mack et al. has also developed the gradient separation method of ciprofloxacin and its metabolites in saliva, urine, serum, using the C_{18} stationary phase, and the UV detector.[8] But, because of baseline drift, they had quantitative limits. Both Myer's and Mack's technique required a liquid-liquid extraction as a sample preparation. Sholl et al. has described the separation and detection method of ciprofloxacin and its metabolites in saliva, urine, serum, bile, using the C_{18} column, and fluorescence detector.[9] But the researchers went through a complicated procedure, including thermolysis, photolysis and post-column derivatization to improve the detection limits of M2, M3, and M4.

This study describes a simple separation condition of five solutes in urine and an off-line solid phase extraction procedure for the sample preparation work. Ciprofloxacin and its four metabolites in urine were isocratically separated in 16 minutes by using ion-pairing reagents, the C_{18} column stationary phase, and UV detector. In the process of determining the optimum separation condition, the effect of each ion-pairing reagent on the retention of analytes was investigated and explained as well. The sample preparation work involved a C_{18} Sep-Pak pre-treatment. The efficiency of the desorption step was investigated by varying the organic solvent or the pH of the mixture of organic solvent and phosphate buffer. The optimum desorption condition, showing over a 90% recovery of all analytes was found. This optimum C_{18} Sep-Pak pre-treatment procedure to a pharmacokinetic study of ciprofloxacin and its metabolites in urine after an oral administration of ciprofloxacin was applied.

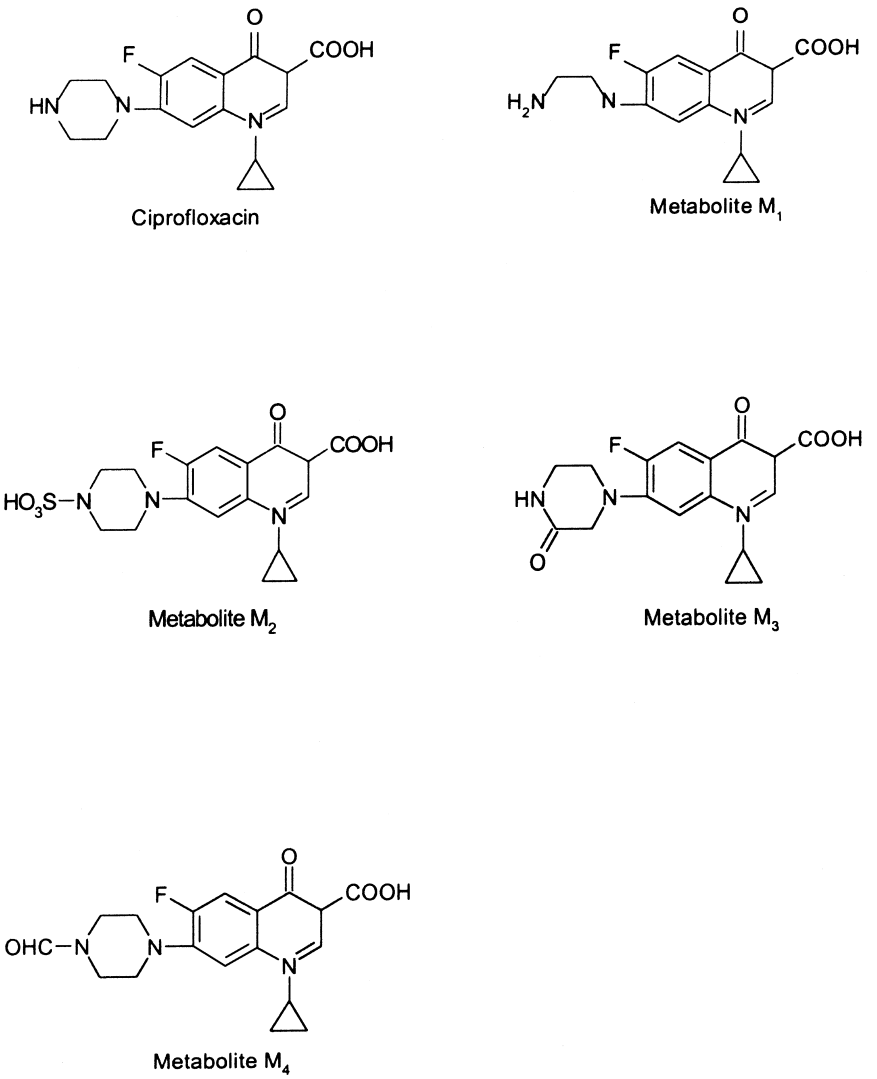

Figure 1. The structures of ciprofloxacin and its metabolites.

EXPERIMENTAL

Chemicals

Ciprofloxacin was purchased from Sigma, and its four kinds of metabolites (M1, M2, M3, M4) were obtained from Bayer AG (Wuppertal, Germany). The solvents used in this experiment were all HPLC grades. The heptanesulfonic acid (HSA), tetraethylammonium bromide (TEABr), tetrabutylammonium bromide (TBABr), and tetrapentylammonium bromide (TPABr) were purchased from Sigma, and tetraethylammonium chloride(TEACl), tetrabutylammonium chloride(TBACl), and tetrapentylammonium chloride (TPACl) were from Tokyo Chemical Industry (Tokyo, Japan). Sodium dihydrogen phosphate and sodium hydrogen phosphate were obtained from Merck.

Instrumentation

The chromatographic equipment used in this study was a Samsung electronic device system, which is composed of an intelligent pump, Rheodyne injector (20 μL loop), wavelength variable UV detector, column oven, and degasser. The data was collected by using Chromate (Windows version) program from Interface Engineering Company. Separation was carried out on a 25cm x 4.6mm packed supheriorex ODS column. The sorbent used for the sample pre-treatment was C_{18} Sep-Pak from Waters.

Sample Preparation

The stock solutions were prepared to 100 ppm by dissolving 1mg of each sample in 2.0 mL of acetonitrile, diluting with 0.5 mL of 99.9% phosphoric acid and afterwards with water to a final volume of 10 mL. After that, the stock solution was diluted one after another with 0.1N phosphoric acid to 50, 20, 10, 5, 2, 1, 0.5, 0.1 ppm. After the urine sample was diluted to 1:100 with water, 1 mL of diluted urine was diluted again with 4 mL of 0.01M sodium hydrogen phosphate buffer solution, and then spiked with 0.1 mL of 10 ppm standard solution, followed by Sep-Pak pre-treatment.

The 20 μL of the total elution volume desorbed from the C_{18} Sep-Pak cartridge was injected into the HPLC. The recovery of each analyte was investigated by varying the kinds of organic solvents themselves or the mixture of organic solvent and phosphate buffer, adjusted to different values of pH with phosphoric acid: 2.5, 4.1, 5.7, 7.4. Sep-Pak pre-treatment procedures involved four steps: including pre-condition, sample loading, washing, and desorption First, the cartridge was pre-conditioned with 1mL of methanol, followed by 1 mL of 0.01M sodium hydrogen phosphate (pH=7.0). Second, sample loading on the cartridge was followed by washing matrix components with 3 mL of

0.01M sodium hydrogen phosphate (pH=7.0). Finally, the retained analytes on the C_{18} Sep-Pak cartridge were eluted by different kinds of desorption solvents mentioned above.

Chromatography

The optimum separation condition, volume to volume ratios of isopropanol to acetonitrile to 0.007M HSA to 0.005M TBABr, was 11:5:42:42 plus 0.05% triethylamine with a pH of 3.0 adjusted with 99.9% phosphoric acid. The mobile phase flow rate was 1.4 mL/min, the column temperature was 40°C, and the wavelength of UV detection was 276 nm.

Quantitation

Peak area ratios were used to quantify ciprofloxacin and its metabolites. Quantification of ciprofloxacin and its metabolites was based on calibration curves obtained with external standard samples containing different concentrations of each sample. The concentrations of each sample ranged from 0.1 ppm to 50.0 ppm.

RESULTS AND DISCUSSION

The Effects of Cationic or Anionic Ion-Pairing Reagent on Retention

Ciprofloxacin and its metabolites have ionizable functional groups, such as carboxylic groups at C3 position, amino group at C7 position in common, so that adding an appropriate kind of ion pairing reagent is expected to control the retention of each analyte. Table 1 shows the retention of each analyte, when using the various concentrations of cationic ion-pairing reagents, tetraalkylammonium bromide (TEABr, TBABr, TPABr), tetraalkylammonium chloride (TEACl, TBACl, TPACl), and 1-hepatanesulfonic acid (HSA). The variation of the concentration of TEABr (or TEACl) gave little retention selectivity among analytes. As alternatives to TEABr (or TEACl), more hydrophobic cationic ion-pairing reagents, TBABr (or TBACl), and TPABr (or TPACl), were used to investigate the effect of retention selectivity and resolution. Compared with other analytes, the retention increase of M2 was pronounced as the concentration of TBABr (or TBACl) or TPABr (or TPACl) increased in the range of investigations. This phenomenon could be explained by hydrophobic ion-pair formation between the M2 sulfonyl group and quaternary ammonium cation of TBABr (or TBACl).

Throughout the experimental tetraalkylammonium salts, the resolution of M1 and CFX was poor. The similar retention trend of M1 and CFX is thought

Table 1

Dependence of the Capacity Factor of Ciprofloxacin and Its Metabolites on the Concentration of Tetraalkylammonium Bromide, Tetraalkylammonium Chloride and 1-Heptanesulfonic Acid

	k'			k'			k'			k'		
	Conc.			Conc.			Conc.			Conc.	HSA	
Cmpd.	(mM)	TEABr	TEACl[a]	(mM)	TBABr[a]	TBACl[a]	(mM)	TPABr[a]	TPACl[a]	(mM)	i-PrOH[b]	MeOH[c]
CFX	0	1.672	0.140	0	1.672	1.140	0	0.140	0.140	2.0	5.210	2.109
	5.0	1.031	0.078	5.0	0.210	0.287	0.2	0.184	0190	7.0	7.951	3.673
	10.0	0.939	0.116	10.0	0.179	0.377	0.5	0.169	0.062	10.0	9.902	3.961
	20.0	0.840	0.220	20.0	0.210	0.451	1.0	0.159	0.152			
M1	0	1.340	0.047	0	1.340	0.047	0	0.047	0.047	2.0	4.262	1.872
	5.0	0.802	0.023	5.0	0.137	0.195	0.2	0.115	0.400	7.0	6.740	3.308
	10.0	0.729	0.049	10.0	0.122	0.257	0.5	0.121	0.013	10.0	8.385	3.523
	20.0	0.634	0.122	20.0	0.122	0.313	1.0	0.119	0.103			
M2	0	2.447	5.617	0	2.447	5.617	0	5.617	5.617	2.0	0.504	0.620
	5.0	2.630	8.403	5.0	6.996	3.601	0.2	13.92	14.59	7.0	0.310	0.392
	10.0	2.340	10.354	10.0	8.618	3.496	0.5	23.30	21.39	10.0	3.325	0.349
	20.0	2.389	12.398	20.0	11.095	3.060	1.0	37.08	36.10			
M3	0	3.107	3.051	0	3.107	3.051	0	3.051	3.051	2.0	3.401	2.530
	5.0	3.176	3.078	5.0	3.172	2.889	0.2	3.908	4.030	7.0	3.098	2.665
	10.0	3.0574	3.277	10.0	3.191	3.177	0.5	4.251	3.800	10.0	3.282	2.473
	20.0	3.134	3.506	20.0	3.435	3.111	1.0	4.706	4.583			

Table 1 (Continued)

		k'			k'			k'			k'	
Cmpd.	Conc. (mM)	TEABr	TEACl[a]	Conc. (mM)	TBABr[a]	TBACl[a]	Conc. (mM)	TPABr[a]	TPACl[a]	Conc. (mM)	HSA i-PrOH[b]	MeO
M4	0	4.958	4.924	0	4.958	4.924	0	4.924	4.924	2.0	5.413	4.109
	5.0	5.073	4.950	5.0	5.034	4.685	0.2	6.143	6.342	7.0	4.869	4.304
	10.0	4.885	5.257	10.0	5.061	5.158	0.5	6.643	5.982	10.0	5.137	3.961
	20.0	5.015	5.608	20.0	5.447	5.073	1.0	7.299	7.098			

[a] Mobile phase; i-PrOH: TEABr(Cl), TBABr(CL), TPABr(Cl) = 15:85 with 0.05% triethylamine (pH = 3.0), Temp.: 40°C; Flow rate: 1.0 mL/min. [b] Mobile phase; i-PrOH:HAS = 15:85 with 0.05% triethylamine (pH = 3.0), Temp.: 40°C; Flow rate: 1.0 mL/min. [c] Mobile phase; MeOH:HAS = 45:55 with 0.05% triethylamine (pH = 3.0), Temp.: 40°C; Flow rate: 1.0 mL/min.

to be due to the similar polarity on piperazinyl or ethylenediamine moiety. The counter ion effect was shown to make little difference in the retention of each analyte. As a result, varying the type and concentration of cationic ion-pairing reagent made a slight improvement on selectivity, but the condition for baseline separation of all analytes was not discovered. In order to improve the separation of M1 and CFX, a kind of anionic ion-pairing reagents, HSA, was selected and added into the mobile phase in a range of 2-10 mM. The retention increase of M1 and CFX can be interpreted as hydrophobic ion-pair formation between the protonated form of M1 on ethylenediamine or that of CFX on the piperazinyl group and the sulfonyl group on HSA.

Using isopropanol as an organic modifier, all analytes were completely resolved as the concentration of HSA increased from 2 mM to 10 mM. Even though complete separation was possible, it was thought that the early eluting M2 would be affected by interfering matrix components, if applied to biological fluids such as urine. So the important factor is to consider the retention time of matrix components, possible decomposition of analytes, and total analysis time. It was decided to set the total analysis time gap between 5 and 15 minutes, completely separating five kinds of analytes.

The Effect of Adding Both Cationic and Anionic Ion-Pairing Reagents into the Mobile Phase on Retention

As mentioned above, adding a single ion-pairing reagent to the mobile phase has limitations for completely separating five kinds of analytes in a range from 5 to 15 minutes. However, the effect of the cationic or anionic ion-pairing reagent on retention is somewhat different in that it could be expected that co-addition of cationic and anionic ion-pairing reagent to the mobile phase would achieve a desirable degree of separation between all analytes in a range from 5 to 15 minutes. It was found that the retention selectivity of M2 could be controlled by changing the concentration of TBABr (or TBACl) or TPABr (or TPACl). In the case of TPABr (or TPACl), the retention of M2 was so sensitive to slight changes of TPABr (or TPACl) concentration that reproducible data could not be obtained. Therefore, TBABr was selected as the cationic ion-pairing reagent, because the counter ion effect was negligible.

As explained in the effect of HSA on retention, it was found that the addition of 7.0 mM HSA gave a complete separation, eluting M2 in matrix interfering time. The addition of 5.0mM TBABr into the mobile phase, containing 7.0mM HSA, made the retention of M2 increase enough to avoid the interference, and the resolution of M1 and CFX improved. But M3 was partially eluted together with M1. The variation of isopropanol concentration gave no other selectivity. In order to give polar selectivity, another modifier, acetonitrile, was added into the mobile phase from 5% to 10%. Upon 7 or 10% addition of ace-

tonitrile, M2 was partially overlapped with M3, while improving the resolution of M1 and CFX. But, 5% addition of acetonitrile gave the chromatogram of baseline separation condition, eluting M1, CFX between M2 and M3. At this condition, all analytes were eluted between 12 and 30 minutes. As a result, it was found that the resolution among analytes improved as the acetonitrile concentration decreased from 10% to 5%. Considering the analysis time and the avoidance of matrix interferences, the flow rate was controlled from 1.0 mL/min to 1.4mL/min. The final optimum mobile phase condition of isopropanol to acetonitrile to 0.007M HSA to 0.005M TBABr had the volume to volume ratio of 11:5:42:42 plus 0.05% triethylamine, with a pH of 3.0, adjusted with 99.9% phosphoric acid.

The Result of Sep-Pak Pre-Treatment

The urine samples were pre-treated with the C_{18} Sep-Pak cartridge. The C_{18} Sep-Pak cartridge was desorbed by different kinds of organic solvent or the mixture of organic solvents and phosphate buffer, adjusted to different values of pH, with phosphoric acid: 2.5, 4.1, 5.7, 7.4. The recovery of each analyte was investigated in the above conditions, and Table 2 shows the recovery of ciprofloxacin and its metabolites when using various kinds of eluting solvent for desorption from C_{18} Sep-Pak cartridge.

When methanol was used as a desorption solvent, in the average recovery of the five kinds of analytes, it was the highest, followed by isopropanol, and acetonitrile, being the last. In the end, however, methanol had an average recovery, which was less than 80%. When these organic solvents were mixed with 0.01M sodium dihydrogen (hydrogen) phosphate buffer, adjusted to a more acidic condition, the average recovery improved more than organic solvents themselves or the mixture solvents in more basic conditions. It is thought that the protonated species might be easily desorbed from the C_{18} Sep-Pak cartridge at the acidic pH condition. But when the desorption is performed in a neutral pH condition, the analytes have relatively higher portions of neutral character, which are more difficult to be desorbed, as well as a zwitterionic character.

In the case of isopropanol, the peak tailing was so serious that the increase of peak area seemed to make recovery higher. Therefore, it was decided to use as the optimum desorption solvent, a mixture of methanol and sodium dihydrogen phosphate buffer, adjusted to the pH value of 2.5. Based on this optimum desorption condition, the urine spiked sample was also introduced to the C_{18} Sep-Pak pre-treatment. Figure 2 shows the chromatogram of both the blank urine and the urine-spiked sample by Sep-Pak pre-treatment. It showed no special kind of matrix peak interference with the analyte peaks.

Table 2

The Recovery of Ciprofloxacin and its Metabolites when Using Each Kind of Eluting Solvent for Desorption from C_{18} Sep-Pak Cartridge

Compounds	Recovery (%) (n = 3) Methanol	Isopropanol	Acetonitrile
Ciprofloxacin	72 ± 2	34 ± 4	12 ± 2
M1	74 ± 2	46 ± 5	33 ± 8
M2	68 ± 1	105 ± 18	97 ± 1
M3	93 ± 6	46 ± 10	45 ± 5
M4	70 ± 3	66 ± 6	66 ± 6
Average	75	59	45

Compounds	Recovery (%) (n = 3) Methanol: 0.01M NaH_2PO_4 = 80:20 pH 2.5	pH 4.1	pH.5.7	pH 7.4
Ciprofloxacin	95 ± 3	86 ± 4	82 ± 3	77 ± 11
M1	97 ± 2	88 ± 1	86 ± 3	79 ± 4
M2	92 ± 2	118 ± 7	108 ± 1	96 ± 17
M3	93 ± 0	68 ± 4	62 ± 15	46 ± 2
M4	100 ± 2	101 ± 7	73 ± 10	86 ± 3
Average	95	92	82	77

	Recovery (%) (n = 3) Acetonitrile: 0.01M NaH_2PO_4 = 70:30			
Ciprofloxacin	90 ± 0	85 ± 1	83 ± 4	47 ± 9
M1	101 ± 1	88 ± 2	88 ± 3	74 ± 7
M2	108 ± 3	104 ± 3	87 ± 7	96 ± 1
M3	105 ± 11	47 ± 11	32 ± 15	39 ± 6
M4	100 ± 2	41 ± 6	37 ± 6	30 ± 6
Average	95	73	65	57

	Recovery (%) (n = 3) Isopropanol: 0.01M NaH_2PO_4 = 70:30			
Ciprofloxacin	98 ± 3	94 ± 2	97 ± 1	97 ± 1
M1	96 ± 3	96 ± 1	95 ± 4	97 ± 1
M2	107 ± 4	97 ± 0	99 ± 6	95 ± 1
M3	110 ± 8	100 ± 2	64 ± 4	67 ± 10
M4	95 ± 3	77 ± 0	67 ± 4	50 ± 13
Average	101	93	84	81

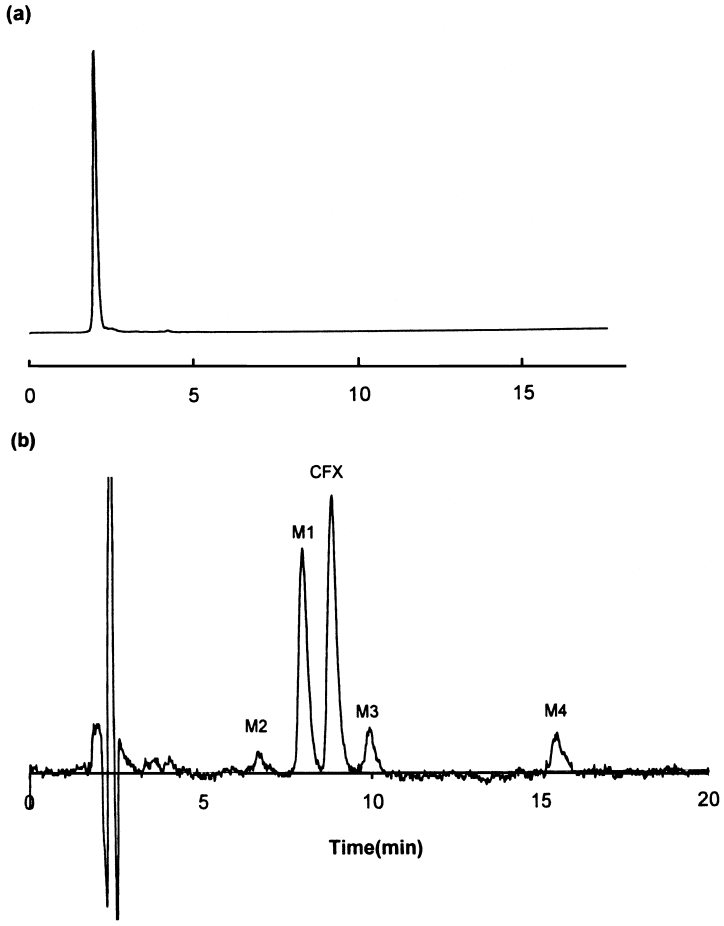

Figure 2. Representative chromatograms of (a) blank diluted urine after C_{18} Sep-Pak pretreatment, (b) urine standard, spiked to 1μg /mL after C_{18} Sep-Pak pretreatment.

Pharmacokinetic Study

In this study, a volunteer (age 26) was administered a 250 mg dose of ciprofloxacin. Urine was collected periodically and diluted to 1:100 with filtered water, followed by C_{18} Sep-Pak pre-treatment, and 20 μL of total elution volume was injected into the HPLC. The concentration of each analyte as a function of time was calculated by using the pre-made calibration curves. Good

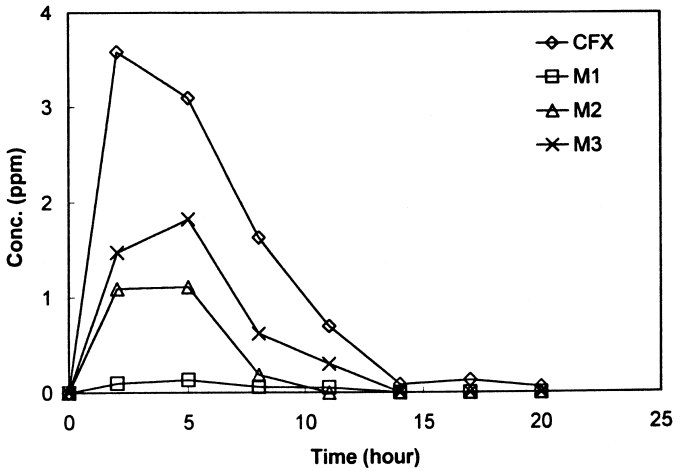

Figure 3. Plot of time as a function of concentration of ciprofloxacin and its metabolites in urine matrix after a single oral dose (250mg) of ciprofloxacin.

linearity was observed for all analytes in the range from 0.1 ppm to 50.0 ppm ($r^2 > 0.99$). Figure 3 shows the plotting of the pharmacokinetic study as a function of time. After ciprofloxacin was administered, within 2 hours it was found that the parent compound, M1, M2, and M3 existed at 3.6, 0.1, 1.1, 1.5 ppm, respectively. This information suggests that the metabolism within the body occurs in 2 hours. The concentration of ciprofloxacin increased rapidly between 0 and 2 hours, and decreased after 2 hours. The concentration of M2 and M3 increased between 2 and 5 hours, and decreased after 5 hours. But the maximum concentration of M2, and M3 was lower than that of CFX. M1 was eliminated from the body at a constant rate, showing little change of concentration. After almost 20 hours, all kinds of analytes were shown to be eliminated from the body.

CONCLUSION

Here, the simple separation condition for ciprofloxacin and its metabolites by using both TBABr, cationic ion-pairing reagent and HSA, anionic ion-pairing reagent simultaneously has been created. In addition, the efficient procedure of C_{18} Sep-Pak pre-treatment was used for recovering the five kinds of analytes, showing over 90% recovery. The pharmacokinetic study was applied to quantify the five kinds of analytes in urine.

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