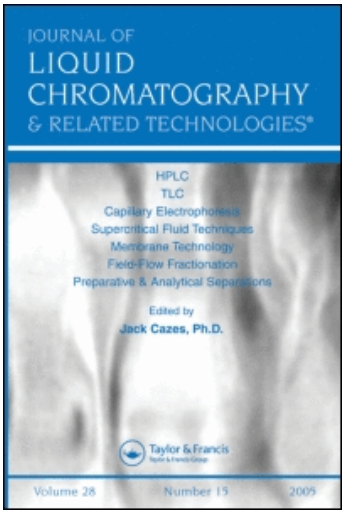

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Direct Determination of Ofloxacin Enantiomers in Human Urine by Ligand Exchange Chromatography

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Abstract: A sensitive and simple method for the determination of ofloxacin enantiomers in human urine was developed by ligand exchange high performance liquid chromatography. Chiral separation was performed on a C_{18} column, where the mobile phase consisted of a methanol-water solution (containing1.2 mmol L^{-1} L-phenylalanine and 1.0 mmol L^{-1} copper sulphate) (15:85, v/v) and its flow rate was set at 1.0 mL min^{-1}. After centrifugation, the human urine was injected into a C_{18} column directly. Baseline separation of ofloxacin enantiomers in human urine were obtained with a resolution of 3.24 in less than 25 min, and no interference by the protein or endogenous compounds were observed. The effects of different separation conditions were investigated and the concentration of ligand and pH of the mobile phase play a critical role in the enantioseparation. The standard curves showed excellent linearity over the concentration range from 0.8 to 400 μg mL^{-1} for ofloxacin enantiomers. The linear correlation equations are: $Y = 171.11X + 504.13$ ($r = 0.9995$) and $Y = 169.01X + 631.59$ ($r = 0.9994$) for (S)-ofloxacin and (R)-ofloxacin, respectively. The average recovery of ofloxacin enantiomers from human urine samples was more than 96%. The procedure developed was successfully applied to investigate the stereoselectivity and pharmacokinetics of ofloxacin enantiomers in the human body.

Keywords: Enantioseparation, Ofloxacin enantiomers, Human urine, Ligand exchange chromatography

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INTRODUCTION

In recent years, reports on the vast differences in pharmacological effects and pharmacokinetics between the two enantiomeric forms of many drugs have highlighted the need for enantioselective separation and determination of chiral drugs. For many chiral drugs, the desired pharmacologic effect is due largely to one enantiomer, while its antipode may be responsible for significant undesirable side effects. Ofloxacin, (±)-9-fluoro-2,3,-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)- 7-oxo-7H-pyrido [1,2,3 –de]–[1,4]–benzox-azine-6-carboxylic acid, is one of the most commonly used second generation fluoroquinolone broad spectrum antimicrobial with enhanced antimicrobial activities due to its high potency, low minimal inhibitory concentration, low toxicity, long half life, and high stability.[1,2] The mechanism of the bactericidal effect of ofloxacin is based on the inhibition of the bacterial enzymes, DNA gyrase and DNA topoisomerase IV, to exhibit broad activity against Gram-negative bacteria and marginal activity against Gram-positive bacteria.[3] It is often used in the treatment of a range of illnesses, including respiratory tract, urinary tract, and tissue based infections. Following oral administration to man, approximately 75–80% of ofloxacin in oral doses is excreted in the urine unchanged. (S)-ofloxacin, the bacteriologically active (S)-isomer of the racemic ofloxacin, shows 8- to 128-fold higher activity than its (R)-isomer with different in vitro bacterial strains.[4,5] Therefore, it will be of great interest to recognize and determine the two ofloxacin enantiomers, not only in the investigation of pharmacokinetics of the enantiomers in vitro, but also in design and development of new chiral pharmaceutics.

Several techniques have been proposed for the determination of ofloxacin in biological fluids, including chemiluminescence,[6] solid phase spectrofluorimetry,[7] pulse polarography,[8] spectrophotometry,[9] capillary electrophoresis,[10,11] and high performance liquid chromatography.[12–15] However, there have been few reports on the chiral separation of ofloxacin enantiomers.[16–18] Assays pertaining to ofloxacin are generally complex and lengthy, mostly because of the sample preparation, like solid phase extraction, liquid–liquid extraction, protein precipitation combined with time consuming centrifugation steps, or expensive automated sample analysis requiring column switching.

The aim of this study was to develop and validate a simple and rapid HPLC assay for measuring ofloxacin enantiomers in human urine by using a low concentration of chiral mobile phase additives on a conventional C_{18} column. Stereospecificity was achieved in the ligand exchange mode by incorporating chiral reagents directly into the mobile phase. A relatively lower concentration of L-phenylalanine was used as a ligand agent and Cu^{2+} as a ligand ion. The effects of different separation conditions, such as kinds and concentration of ligands, organic modifier, and the pH of the mobile phase on enantioseparation, were investigated. The proposed method was successfully applied to investigate the stereoselectivity and pharmacokinetics of ofloxacin enantiomers in the human body.

EXPERIMENTAL

Materials

L-Phenylalanine was purchased from Sigma (Louis, MO, USA). (S)-ofloxacin and (R)-ofloxacin were kindly donated by Hebei University (Baoding, China). The structures of these molecules are shown in Figure 1. Anhydrous cupric sulfate (Extra Pure grade) was purchased from Tedia Company, Inc. (Fairfield, OH, USA). Acetonitrile, tetrahydrofuran, and methanol were all of HPLC grade and were obtained from Duksan Pure Chemical Co., LTD (Ansan, Korea). All the other reagents used in the experiment were of the highest grade commercially available. Double deionized water was filtered with a 0.45 μm filter membrane before use.

Chiral Ligand Exchange RP-HPLC System

HPLC analysis was performed using a liquid chromatography system containing a Waters 600s Multisolvent Delivery System and a Waters 616 pump (Waters, Milford, MA, USA), a Waters 486 Tunable Absorbance UV detector (Waters, Milford, MA, USA), and a Rheodyne injection valve (20 μL sample loop). Autochro-2000 software (Younglin Co. Ltd., Korea) was used as the data acquisition system. The analytical column (150 mm × 4.6 mm I.D.) was packed with C_{18} stationary phase (OptimaPak, particle size 5 μm, RStech, Daejeon, Korea).

The solution of chiral mobile phase additive (CMPA) was comprised of 1.2 mmol L^{-1} L-phenylalanine mixed with 1.0 mmol L^{-1} cupric sulfate in water. The mobile phase consisted of CMPA solution–methanol (85:15, v/v). The flow rate of the mobile phase was set at 1.0 mL min^{-1}. The chromatographic assay was carried out at ambient temperature. UV wavelength was set at 293 nm.

The retention factor was calculated from the equation $k = (t - t_0)/t_0$, where t and t_0 are the retention times of analyte and unretained solutes, respectively. The enantioseparation factor was calculated from the equation $\alpha = k_R/k_S$, where k_S and k_R are the retention factors of (S)-ofloxacin and

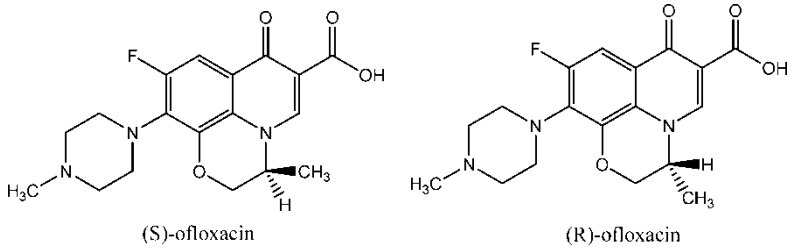

Figure 1. The molecular structures of (S)-ofloxacin and D-enantiomer.

(R)-ofloxacin, respectively. Resolution was calculated from the equation $R = 2(t_R - t_S)/(w_R + w_S)$, where t_S and t_R are the retention times of (S)-ofloxacin and (R)-ofloxacin, respectively, and w_S and w_R are the baseline peak widths of the two enantiomers. The number of theoretical plates (N) was calculated by the equation $N = 16(t/w)^2$.

Sample Treatment

Urine samples were obtained from fasting healthy volunteers. The samples were centrifuged for 15 min at 10000 rpm and then filtered through a cellulose acetate filter (0.25 μm pore size, Advantec MFS Inc. CA, USA). The filtrate were collected in glass containers that had been carefully cleaned with hydrochloric acid and washed with deionised water and stored at -20°C until analysis was performed, with the minimum possible delay.

Stock standard solutions of ofloxacin enantiomers were prepared in the mixture of methanol:water (15:85, v/v). Further dilution steps were made by human urine. Working standard solutions were prepared by adding appropriate volumes of ofloxacin solution and the volume added was always less than 2% of the final urine volume to preserve the integrity of the samples. After aliquoting, the urine samples were stored at −20°C until analysis.

RESULTS AND DISCUSSIONS

Enantioseparation Mechanism

Ligand exchange chromatography exploits the rapid and reversible formation of metal ion complexes to separate compounds which can donate electrons and coordinate to the immobilized metal ions. Solvent components occupying coordination sites on the metal centers are displaced by ligands from the sample. Retention of a given species is directly related to the stability of the mixed ligand complex it forms with the metal ion complex immobilized on a chromatographic support. Ligand exchange with soluble metal complexes that partition between the mobile and solid phases also affects retention. The use of an optically active counterion often results in the formation of diastereomeric ion pairs, which can be easily separated on conventional reversed phase columns.

Ofloxacin possesses two relevant ionizable functional groups: a basic piperazinyl group and a carboxylic acid group. The carboxylic and the carbonyl groups are required for antimicrobial activity and it is in these groups that the chelation interaction with various cations takes place. Ofloxacin enantiomers, bivalent copper cation, and L-phenylalanine could form two kinds of ternary complexes with different configurations. The enantioselectivity depends on the differences in the relative stabilities, the energy, and the affinity to the stationary phase of the two complexes. These kinds of

complexes formed in mobile phase and conjunct with an achiral stationary phase to generate an equilibrium. Any difference in the stability or energy of these diastereomeric complexes will affect the chemical equilibrium, thereby resulting in different chromatographic behavior. As a result, the enantiomers can be separated on a C_{18} column. The possible structure of the ternary complex is shown in Figure 2.

The Effects of the Kinds and Concentration of Ligand

In order to investigate the effects of different ligands on the chiral separation, L-leucine, L-phenylalanine, L-serine, and L-histidine were used as ligand agents, respectively. As shown in Table 1, L-phenylalanine (R = 3.24) showed better resolution than L-leucine (R = 2.49), and L-histidine and L-serine hardly showed any enantioselectivity. This indicated that the chiral ligand for enantioseparation should possess a larger group to produce space exclude function and also should possess certain lipophilia to be retained by the reverse stationary phase.

Most previous reports[18,19] about enantioseparation by ligand exchange chromatography often use 6-10 mmol L^{-1} chiral ligand reagent and 3–5 mol L^{-1} metal ions in the mobile phase. In order to decrease the use of expensive

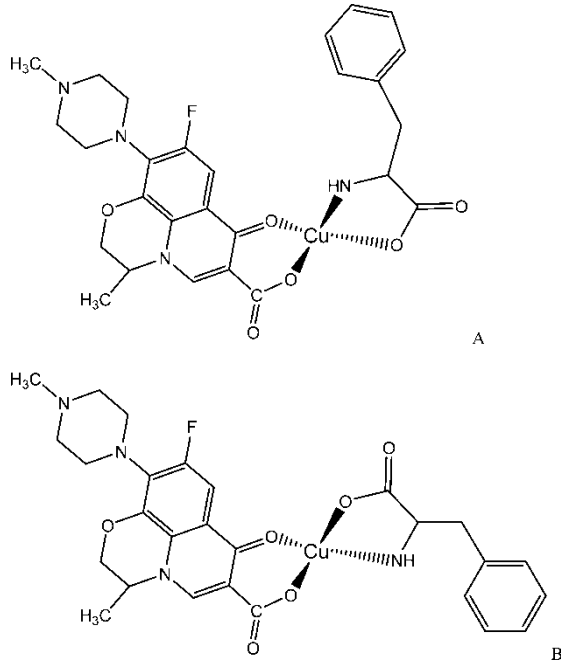

Figure 2. Proposed structure of ligand complex of ofloxacin enantiomers, L-phenylalanine and Cu^{2+}.

Table 1. Effect of different ligands on the enantioseparation

Different ligands	Retention factor		Selectivity (α)	Resolution (R)	Efficiency	
	k_S	k_R			N_S	N_R
L-leucine	8.67	10.71	1.23	2.49	2983	2516
L-phenylalanine	9.36	13.03	1.39	3.24	3261	3178
L-serine	9.24	9.24	1.00	0.00	1459	1459
L-histidine	7.12	7.12	1.00	0.00	1532	1532

chiral reagents and improve the enantioselectivity, relatively low concentrations of L-phenylalanine in the mobile phases were investigated in a range of 0 to 4 mmol L^{-1} when the concentration of Cu^{2+} was kept at 4.0 mmol L^{-1}. Figure 3 indicates that the retention time of the two enantiomers increased, but the separation factor decreased with the increasing of L-phenylalanine concentration in the mobile phase. Considering the retention and selectivity, 1.2 mmol L^{-1} L-phenylalanine was using in further investigation.

The Effects of the Concentration of Bivalent Copper Ion

In order to investigate the effects of Cu^{2+} concentration on the enantioseparation, different Cu^{2+} concentrations were investigated in the range from 0 to 5 mmol L^{-1} and the results were shown in Figure 4. With the decreasing of Cu^{2+} concentration in the mobile phase, both retention and separation of

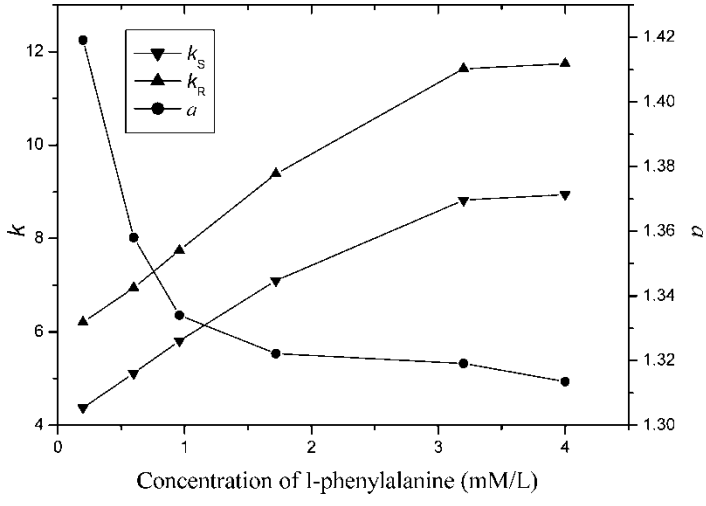

Figure 3. Effect of L-phenylalanine in the mobile phase on enantioseparation.

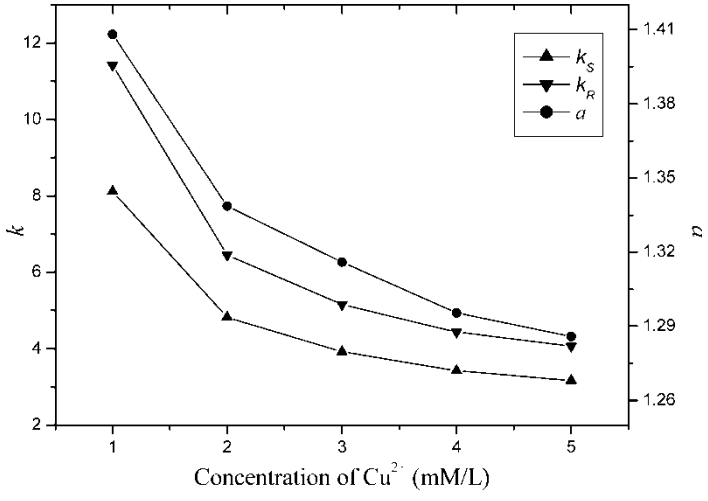

Figure 4. Effect of Cu^{2+} concentration in the mobile phase on enantioseparation.

the two enantiomers increased. The resolution of the two enanitomers increased from 2.32 to 4.34 when Cu^{2+} concentration was changed from 5 to 0.8 mmol L^{-1}. However, when the concentration of Cu^{2+} was near zero, ofloxacin enantiomers can't be washed out within 80 min. Considering the retention time and selectivity, 1.0 mmol L^{-1} was used as optimum Cu^{2+} concentration. Under this condition, only a small increase in the content of methanol in the mobile phase, the enantioseparation of ofloxacin enanitomers could obtain similar enantioselectivity compared with using a high concentration of chiral ligand and copper ion (10 mmol L^{-1} L-phenylalanine + 5 mmol L^{-1} Cu^{2+}) (Figure 5).

The Effects of the Organic Modifier

Different concentrations of methanol, tetrahydrofuran, and acetonitrile were used as the organic modifier in the mobile phase to investigate the effects of organic modifier on enantioseparation. The results showed that acetonitrile and tetrahydrofuran utilized as the organic modifier, only showed a little enantioselectivity, while methanol can give better resolution and retention ability. The effect of methanol concentration on enantioseparation is shown in Figure 6. Both the retention and separation of the two enantiomers decreased when the concentration of methanol in the mobile phase increased. This is attributed to the high concentration of organic solution, which results in precipitation of the electrolyte in the mobile phase. Although the enantioselectivity increases with a decreased amount of organic modifier, the retention time became longer with the increase of the methanol concentration. Hence, in

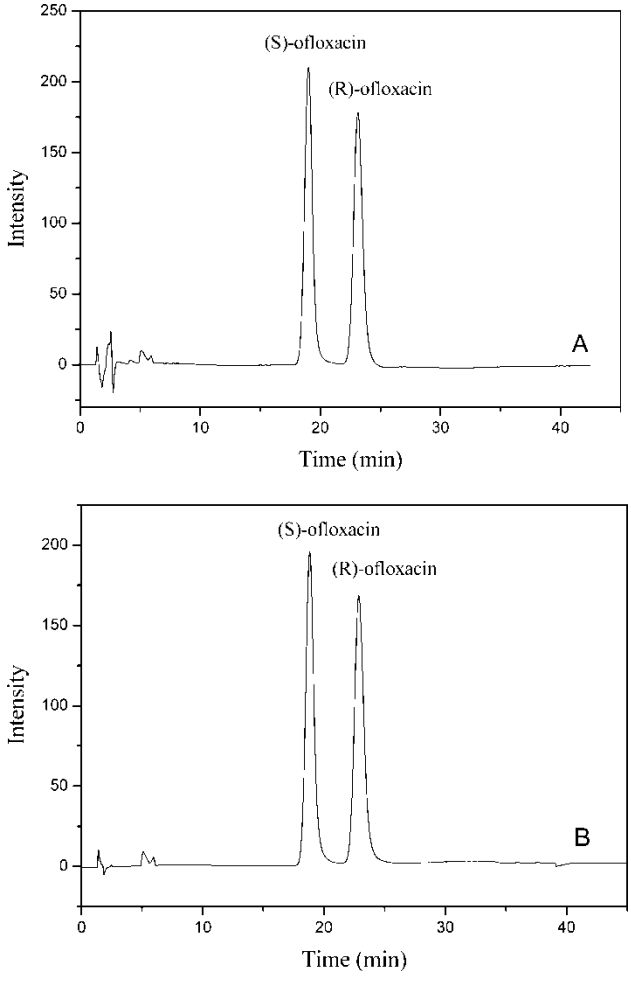

Figure 5. Chromatogram of ofloxacin enantiomers under different ligand concentration. A: Mobile phase: water-methanol (85:15, v/v) (containing 1.2 mmol L^{-1} L-phenylalanine and 1.0 mmol L^{-1} copper sulphate); B: Mobile phase: water-methanol (86:14, v/v) (containing 10 mmol L^{-1} L-phenylalanine and 5 mmol L-1 copper sulphate).

order to obtain good and rapid chromatographic separation of (S)-ofloxacin and its (R)-isomer, 15% methanol was selected as the organic modifier.

Effects of the pH of the Mobile Phase

In order to investigate the effect of the pH of mobile phase on the enantioselectivity, the pH dependence of the enantioseparation was investigated in a pH

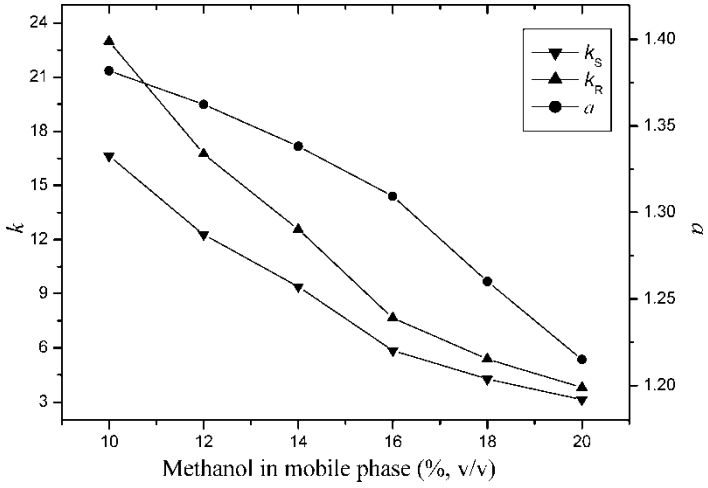

Figure 6. Effect of organic modifier in the mobile phase on enantioseparation.

range of 3.0 to 5.5 using phosphoric acid, hydrochloric acid, and trifluoroacetic acid as pH adjuster, respectively. In contrast with the previous study that reported changing the pH of the mobile phase slightly influenced the resolution, our results showed that the resolution of ofloxacin enantiomers distinctly decreased from 3.24 to 1.06 when the pH of the mobile phase was lowered from 4.9 to 3.8. No enantioselectivity was observed when the pH of the mobile phase was adjusted to be lower than 3.8. On the other hand, when the pH of the mobile phase exceeded 5.0, Cu^{2+} was easily precipitated; this would block the chromatographic system. Hence, 4.9 was chosen as the optimized pH of the mobile phase.

Pretreatment of Human Urine Sample

Because biofluid samples contain large amounts of proteins, it can generate interfering peaks and lead to disadvantageous matrix effects. Therefore, the sample preparing process of biofluid samples was considered very complicating and time consuming. We investigated several previously published methods to pretreat the urine sample, such as protein precipitation with trichloroactic acid, methanol, and acetonitrile. Due to ofloxacin having low solubility and not easily dissolved in both organic and water, the yield was not sufficient. Therefore, we investigated the feasibility of a urine sample to be pretreated only by centrifuging 15 min at 10000 rpm, and then injected directly into the HPLC.

Five randomly selected control drug free human urine samples were processed directly into the chromatographic system and analyzed to

determine the extent to which endogenous components may contribute to interfere with the retention time of the drug. No interference for endogenous compounds was found in the physiological matrices.

Linearity of Calibration

Calibration curves were constructed using the areas of the chromatographic peaks measured at eight increasing concentrations, in the range from 0.8 to 400 $\mu g\ mL^{-1}$ for (S)-ofloxacin and (R)-ofloxacin. To construct the calibration curves the average peak area for each sample was plotted against the concentration of each compound in the solution. The results showed good linearity throughout the concentration for both enantiomers. The linear correlation equations were: $Y = 171.11X + 504.13$ ($r = 0.9995$) for (S)-ofloxacin and $Y = 169.01X + 631.59$ ($r = 0.9994$) for (R)-enantiomer, respectively. (X: The concentration of ofloxacin enantiomer; Y: the peak area of the enantiomers). The standard curves were then used to calculate concentrations of the analytes in urine sample from the measured peak area.

Intra- and Inter-assay Accuracy and Precision

The accuracy and precision of the analyses were assessed by performing replicate analyses of spiked samples against calibration curves. The intra- and inter-assay accuracy and precision of the assay were determined by assaying the spiked urine samples at three different concentrations of ofloxacin in five replicates in the same day and consecutive days. The precision and accuracy of the method were calculated as the relative standard deviation (RSD). The results showed that the intra-assay relative standard deviations and inter-assay relative standard deviations of the proposed method were lower than 2.9% and 3.2% for (S)-ofloxacin and 3.1% and 3.4% for (R)-ofloxacin, respectively.

Determination of Ofloxacin Enantiomers in Human Urine

For the determination of ofloxacin enantiomers in human urine, samples were collected from healthy volunteers before (blank) administration of ofloxacin and during the 25 hours following oral administration of a pharmaceutical compound containing 300 mg of ofloxacin enantiomers. The urine samples were centrifuged for 15 min at 10000 rpm and injected directly into the chromatograph without any other treatment than filtration. The urine samples were analyzed at the optimized separation conditions and the concentration of each sample was calculated from the calibration curve. No interference from endogenous compounds was observed (Figure 7). The recovery was

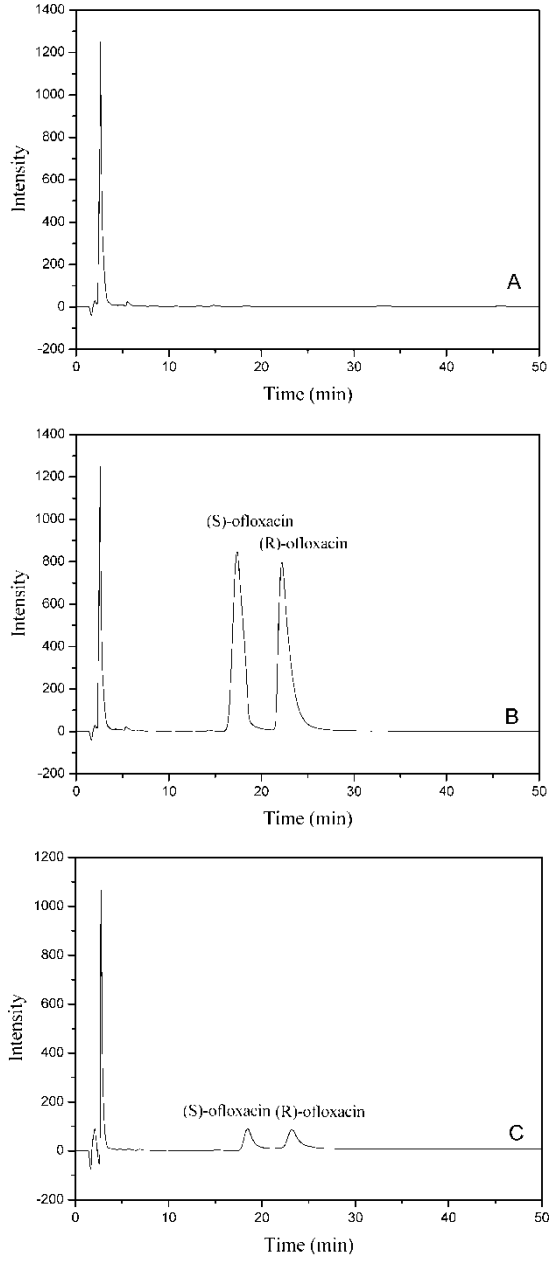

Figure 7. Chromatogram of ofloxacin enantiomers in human urine. (Mobile phase: water-methanol (85:15, v/v) (containing 1.2 mmol L^{-1} L-phenylalanine and 1.0 mmol L^{-1} copper sulphate); flow rate: 1.0 mL min^{-1}; A: blank urine; B: blank urine spike with ofloxacin enantiomers; C: urine sample after oral administration ofloxacin 10 hours.)

Table 2. Recovery of ofloxacin enantiomers in human urine samples

	Content ($\mu g\ mL^{-1}$)		Added ($\mu g\ mL^{-1}$)		Measured ($\mu g\ mL^{-1}$)		Recovery (%)		RSD (%, n = 3)	
No.	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)
1	43.90	45.75	40.00	40.00	82.93	84.75	97.6	97.5	2.9	2.7
2	22.06	17.52	20.00	20.00	41.77	37.26	98.6	98.7	2.1	2.2
3	8.12	2.55	8.00	8.00	15.81	10.25	96.1	96.3	2.9	3.1

Note: (S): (S)-ofloxacin; (R): (R)-ofloxacin.

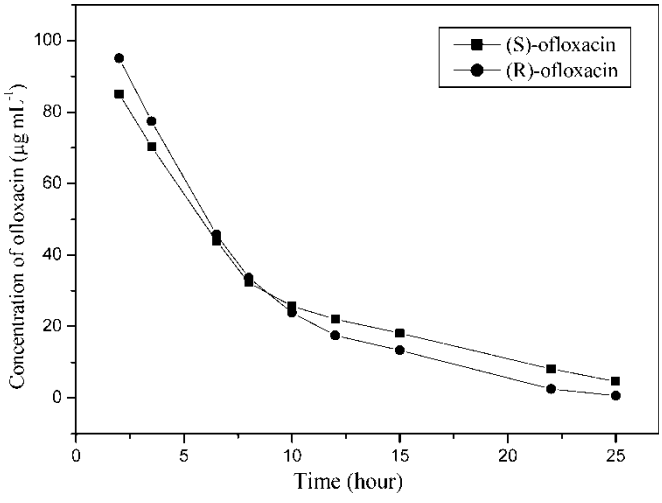

Figure 8. Concentration-time curve of ofloxacin enantiomers in human urine.

determined by calculating the ratio of the amount of ofloxacin enanitomers from urine spiked with known amounts of ofloxacin. The recovery data of ofloxacin enanitomers in human urine are listed in Table 2. Figure 8 showed the concentration of (S)-ofloxacin in human urine was lower than (R)-ofloxacin during the first nine hours after oral administration. Moreover, the ratio of (S)-ofloxacin increased with the time increase, and after nine hours the concentration of (S)-ofloxacin in urine sample was higher than that of (R)-ofloxacin. It indicated the metabolism of ofloxacin enantiomers in the human body has some kind of stereoselectivity. Further investigation about the stereoselectivity and metabolism of ofloxacin enantiomers in the human body will be done in the future.

CONCLUSIONS

A sensitive, simple, and accurate method for determination of ofloxacin enantiomers in human urine was developed by chiral ligand-exchange RP-HPLC. Stereospecificity was achieved in the ligand exchange mode by incorporating a relatively low concentration of chiral reagents directly into the HPLC mobile phase. Baseline separations of ofloxacin enantiomers on a C_{18} column were obtained, and no interference by the protein or endogenous compounds was observed. The effects of different separation conditions were investigated and concentration of ligand and pH of the mobile phase play an important role in the enantioseparation. The proposed method was successfully applied to investigate the stereoselectivity and pharmacokinetics of ofloxacin enantiomers in the human body. This expedient greatly simplified

the overall procedure, resulting in a rapid and efficient sample analysis while maintaining precision and accuracy.

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