Research Article

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Investigation of ofloxacin enantioseparation by ligand exchange chromatography

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Abstract

BACKGROUND: There is much interest in the recognition and determination the two ofloxacin enantiomers, not only from the point of view of investigating the pharmacokinetics of the enantiomers *in vitro* but also in the design and development of new chiral pharmaceutics.

RESULTS: Chiral separation was performed on a C_{18} column, in which the mobile phase consisted of a methanol–water solution (containing different concentrations of L-phenylalanine and copper sulfate) and its flow rate was set at 0.7 mL min^{-1}. The effect of different kinds and concentration of ligands, bivalent copper ion, organic modifier, ionic liquid modifier, pH of mobile phase, and temperature on enantioseparation were evaluated and the results show that the enantioselectivity was strongly affected by the pH and ligand concentration of the mobile phase. Under optimal conditions, baseline separation of the two enantiomers was obtained with a resolution of 4.69 in less than 40 min.

CONCLUSION: The mechanism of chiral discrimination is based on the stabilities of the copper(II) binary complexes and their ternary diastereomeric complexes with amino acids formed in solution and stationary phase. The proposed method could be used for the quality evaluation of ofloxacin enantiomers.

Keywords: enantioseparation; ofloxacin enantiomers; chiral recognition; ligand exchange chromatography

INTRODUCTION

In recent years, reports on the vast differences in pharmacological effects and pharmacokinetics between the two enantomeric forms of many drugs have highlighted the need for enantioselective separation and determination of chiral medicaments. For many racemic drugs, the desired pharmacologic effect is due largely to one enantiomer, while its antipode may be responsible for significant undesirable side effects. Enantioseparation of chiral drugs has been extensively studied by gas chromatography, capillary electrophoresis, and high-performance liquid chromatography (HPLC) using chiral stationary phases or chiral mobile phase additives.[1–6] Among the chromatographic methods developed thus far, HPLC methods using chiral stationary phase require a special chiral column, which is expensive and can only separate a couple of enantiomers in most cases.[7] In contrast, HPLC methods based on chiral mobile phase additives (such as β-cyclodextrin or chiral ligand salts) are not only efficient tools for the separation of racemic drugs but are also relatively cheap and feasible.[8] Enantioselectivity is based on the formation of a kind of ternary complex which conjoins with the achiral stationary phase to generate a secondary chemical equilibrium.[9–11] Any difference in the stability or energy of these diasteromeric complexes will affect the chemical equilibrium, resulting in different chromatographic behaviors; as a result, the enantiomers can be separated on a conventional HPLC column. Retention of a given species is directly related to the stability of the mixed ligand complex and it forms with the metal ion complex immobilized on a chromatographic support.[12,13] The use of an optically active counter-ion often results in the formation of diastereomeric ion pairs which can be easily separated on conventional reversed-phase columns.[14]

Enantioselective ligand exchange chromatography was the liquid chromatography technique that provided a complete and reliable separation of stereoisomers of the most important classes of natural and synthetic compounds, such as α-amino acids, hydroxy acids, amino alcohols and some others. Until now, many chiral ligand agents including different amino acids had been investigated and L-leucine and L-phenylalanine were the most widely applied. Different ligand ions such as Mg(II), Cd(II) Zn(II), Co(II), and Cu(II) ions had been attempted and Zn(II) ions performed well with hydroxyl-containing amino acids; however, no advantage over Cu(II) ions and Cu(II) was most universally used due to the rapid formation and excellent stability of their diastereomeric complexes.

Ofloxacin, (±)-9-fluoro-2,3,-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H- pyrido-[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid, is one of the most commonly used second-generation fluoroquinolone broad-spectrum antimicrobials, with enhanced antimicrobial activities due to its high potency, low minimal inhibitory concentration, low toxicity, long half-life, and high stability.[15–17] (*S*)-Ofloxacin, the bacteriologically active (*S*) isomer

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of racemic ofloxacin, shows 8- to 128-fold higher activity than the (*R*) isomer, with different *in vitro* bacterial strains.[18,19] Therefore, there is much interest in the recognition and determination of 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. However, there are only a few reports on the chrial separation of ofloxacin enantiomers.[20–22]

The aim of this study was to develop a simple and rapid HPLC assay for measuring ofloxacin enantiomers and optimizing its enantioseparation selectivity by using a low concentration of chiral mobile-phase additives on a conventional C_{18} column. The effects of different kinds and concentration of ligands, bivalent copper ion, organic modifier, ionic liquid modifier, pH of mobile phase, and chiral recognition mechanisms were also investigated.

EXPERIMENTAL

Materials

L-Tyrosine, L-phenylalanine, L-proline, L-leucine, L-aspartic acid, L-arginine, L-isoleucine, (*S*)-ofloxacin, and (*R*)-ofloxacin were obtained from Sigma (St Louis, MO, USA). The structures of these molecules are shown in Fig. 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. Four ionic liquids, [BMIm][BF_4], [OMIm][BF_4], [EMIm][MS] and [EMIm][BF_4], were from C-TRI (Suwon, Korea). Double-deionized water was filtered through a 0.45 μm filter membrane before use.

Chiral ligand exchange 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, and a Rheodyne injection valve (IDEX Health & Science, Oak Harbor, WA, USA) (20 μL sample loop). Autochro-2000 software (Young Lin Co. Ltd, Anyang, Korea) was used as data acquisition system. The analytical column (250 mm × 4.6 mm i.d.) was packed with C_{18} stationary phase (particle size 5 μm, RStech, Daejeon, Korea). The solution of chiral mobile phase additive (CMPA) consisted of 0.6 mmol L^{-1} L-phenylalanine mixed with 0.4 mmol L^{-1} cupric sulfate in water. The mobile phase consisted of CMPA solution–methanol (80 : 20 v/v). The flow rate of the mobile phase was set at 0.7 mL min^{-1}. The chromatographic assay was carried out at ambient temperature and UV wavelength was set at 293 nm. The concentration of the mixture of the ofloxacin enantiomers was 0.5 mg mL^{-1}.

The retention factor was calculated from the equation $k = (t - t_0)/t_0$, where t and t_0 (min) are the retention times of analyte and unretained solutes, respectively. The enantioseparation factor (α) was calculated from the equation $\alpha = k_R/k_S$, where k_S and k_R are the retention factors of (*S*)-ofloxacin and (*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 (min) are the baseline peak widths of the two enantiomers. The number of theoretical plates (N) was calculated from the equation $N = 16(t/w)^2$.

Experimental procedure

1.0 mmol L^{-1} of L-tyrosine, L-phenylalanine, L-proline, L-leucine, L-aspartic acid, L-arginine, and L-isoleucine were used as ligand agents to investigate the effects of different ligands on enantioseparation. At the optimum ligand concentration, different Cu^{2+} concentrations in the range of 0.0–6.0 mmol L^{-1} were investigated for their effects on enantioseparation. Different concentrations of acetonitrile, methanol, tetrahydrofuran and four different ionic liquids were then used as the mobile phase modifier to investigate the effects on enantioseparation. Additionally, the effect of pH of mobile phase and quantitative evaluation of ofloxacin enantiomers were also investigated.

RESULTS AND DISCUSSION

Enantioseparation mechanism

Ofloxacin possesses two relevant ionizable functional groups: a basic piperazinyl group and a carboxylic acid group. The carboxylic and 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

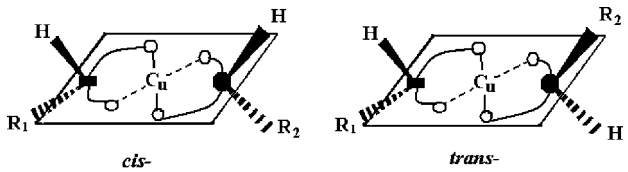

Figure 2. Schematic structure of ligand complex of ofloxacin, L-phenylalanine and Cu^{2+}. ■, chiral center of L-phenylalanine; ●, chiral center of ofloxacin; ○, functional groups on the chiral centers.

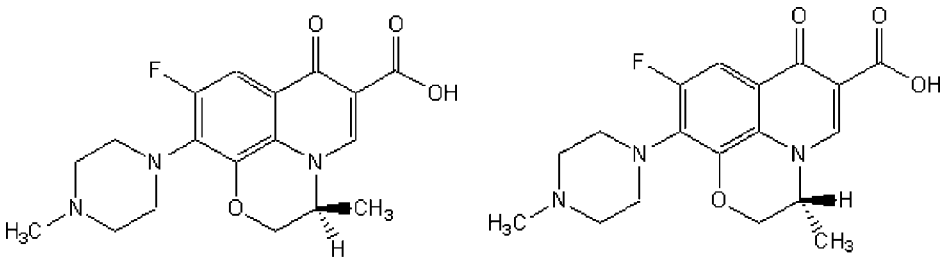

Figure 1. Molecular structures of (*S*)-ofloxacin and (*R*)-ofloxacin.

complexes with different configurations. The enantioselectivity depends on the differences in the relative stabilities, energy, and affinity to the stationary phase of the two complexes. These kinds of complexes formed in mobile phase and conjoined with an achiral stationary phase to generate equilibrium. Any difference in the stability or energy of these diasteromeric 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 Fig. 2.

The effects of different kinds and concentration of ligands

As shown in Table 1, L-phenylalanine ($R = 4.69$) showed better resolution than L-leucine ($R = 3.04$) and L-valine ($R = 2.23$); L-tyrosine and L-proline hardly showed any enantioselectivity. This indicated that the chiral ligand for enantioseparation should possess a larger group to produce the space exclude function and also should possess certain lipophilia to be retained by the reverse stationary phase.

Most previous reports concerning enantioseparation by ligand exchange chromatography commonly use 6.0–10.0 mmol L^{-1} ligand reagent and 3.0–5.0 mmol L^{-1} metal ions in the mobile phase. In order to decrease the use of expensive chiral reagent and improve enantioselectivity, relatively low concentrations of L-phenylalanine in the mobile phase were investigated, in the range of 0.0–3.0 mmol L^{-1}, with the concentration of Cu^{2+} kept at 1.0 mmol L^{-1}. Figure 3 indicates that the retention time of the two enantiomers decreased and enantioseparation factor increased with increasing L-phenylalanine concentration in the mobile phase. However, the enantioseparation factor decreased when the concentration of L-phenylalanine was higher than 0.6 mmol L^{-1}. Considering the retention and selectivity, 0.6 mmol L^{-1} L-phenylalanine was used in further investigations.

The effects of Cu^{2+} concentration

The effects of Cu^{2+} concentration on enantioseparation are shown in Fig. 4. With increasing Cu^{2+} concentration in the mobile phase, both retention and resolution of the two enantiomers decreased.

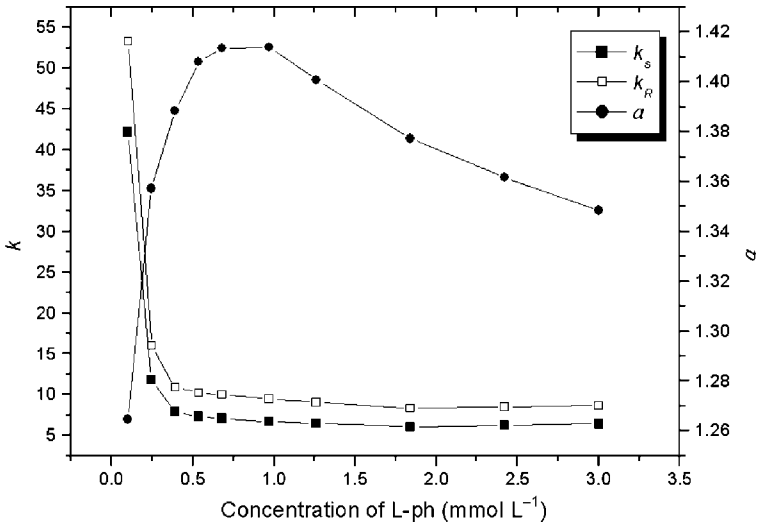

Figure 3. Effect of retention factors (k) and enantioseparation factor (α) of two enantiomers, using different concentrations of L-phenylalanine in the mobile phase, on enantioseparation.

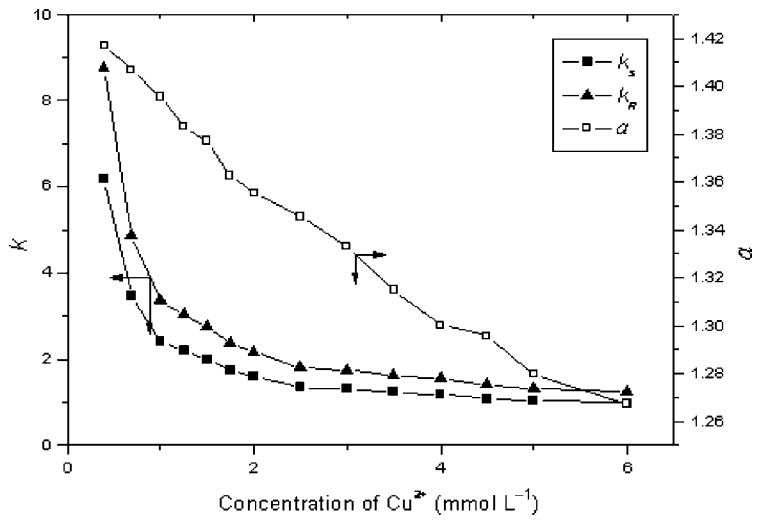

Figure 4. Effect of retention factors (k) and enantioseparation factor (α) of two enantiomers, using different concentrations of Cu^{2+} in the mobile phase, on enantioseparation.

Table 1. Effect of retention factor, selectivity and resolution of the two enantiomers by using different ligands on enantioseparation

Different ligands	Retention factor k_S	k_R	Selectivity (α)	Resolution (R)
L-Tyrosine	2.95	3.49	1.18	0.72
L-Phenylalanine	7.04	10.77	1.53	4.69
L-Proline	3.59	4.29	1.20	1.16
L-Leucine	5.96	8.43	1.41	3.03
L-Aspartic acid	5.81	5.81	1.00	0.00
L-Arginine	2.16	2.16	1.00	0.00
L-Isoleucine	4.78	7.13	1.49	2.56

However, when the concentration of Cu^{2+} was near zero, ofloxacin enantiomers could not be washed out within 60 min. Considering the retention time and selectivity, 0.4 mmol L^{-1} was used as optimum Cu^{2+} concentration (t = 21.96 and 28.87 (min), and R = 1.74). Under these conditions, with only a small increase in methanol in the mobile phase, the enantioseparation of ofloxacin enantiomers could result in similar enantioselectivity compared with the previous results using high concentrations of chiral ligand and copper ion (Fig. 5).

The effects of the organic modifier

The results of experiments using different organic modifiers showed that acetonitrile and tetrahydrofuran utilized as the organic modifier showed little enantioselectivity, while methanol show better resolution and retention ability. The effect of methanol concentration on enantioseparation is shown in Fig. 6. Both the retention and resolution 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 enantioselectivity increases with a decreased amount of organic modifier, the retention time became longer with increasing concentration of methanol. Hence, in order to obtain good and rapid chromatographic separation of (*S*)-ofloxacin and its (*R*) isomer, 20% methanol was selected as the organic modifier.

Effects of ionic liquid modifiers

In this work, four different ionic liquids ranging from 0.0 to 5.0 mmol L^{-1} were investigated as mobile phase modifier for enantioseparation. With increasing ionic liquid concentration, the retention and resolution of ofloxacin enantiomers all decreased (e.g., k_S changed from 5.93 to 1.91, and α from 1.21 to 1.07) when [OMIm][BF_4] was used as modifier. For [BMIm][BF_4], [EMIm][MS], and [EMIm][BF_4], the retention factor of the two enantiomers increased (from 4.10 to 5.75 for k_S; from 3.22 to 4.87 for k_D) with increasing concentration of ionic liquid, but enantioselectivity decreased (α from 1.35 to 1.09). These results indicated that different kinds of ionic liquid have different interactions with analytes and that their metabolism needs to be further investigated.

Effects of the pH of the mobile phase

The pH dependence of enantioseparation was investigated in a pH range of 3.0–5.4 using phosphoric acid, acetic acid, and trifluoroacetic acid as pH adjuster, respectively. In contrast to the previous study, which reported that changing the pH of the mobile phase slightly influenced the resolution, our results showed that the resolution of the two enantiomers distinctly decreased from 4.69 to 1.23 when the pH of the mobile phase was reduced to 3.5. Moreover, no enantioselectivity was observed when the pH of the mobile phase was lower than 3.0. 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 a value of 4.9 was chosen as the optimized pH of the mobile phase.

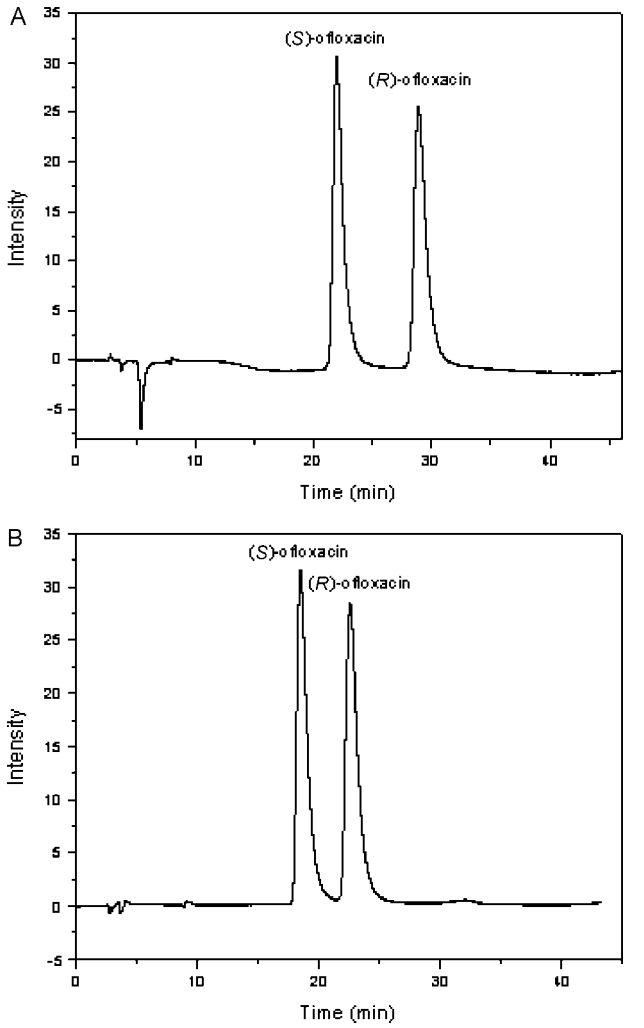

Figure 5. Chromatogram of ofloxacin enantiomers under different conditions. (A) Mobile phase: methanol–water (80 : 20 v/v, containing 0.6 mmol L^{-1} L-phenylalanine and 0.4 mmol L^{-1} copper sulfate. (B) Mobile phase: methanol–water (88 : 12 v/v, containing 8.0 mmol L^{-1} L-phenylalanine and 4.0 mmol L^{-1} copper sulfate).

Validation of the method

Calibration curves were constructed using the areas of the chromatographic peaks measured at eight increasing concentrations, in the range from 0.8 to 400 μg 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 $Y = 173.4X + 537.3$ ($r^2 = 0.999$) for (*S*)-ofloxacin and $Y = 168.5X + 644.6$ ($r^2 = 0.999$) for the

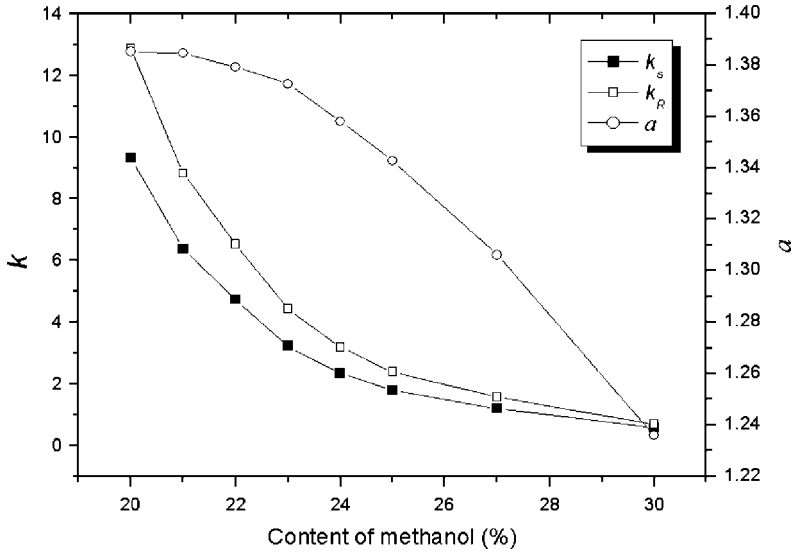

Figure 6. Effect of retention factors (k) and enantioseparation factor (α) of two enantiomers, using different concentrations of organic modifier in the mobile phase, on enantioseparation.

(*R*) enantiomer, respectively. The intra- and inter-assay accuracy and precision of the assay assessed as relative standard deviation (RSD) were determined by assaying the ofloxacin samples at three different concentrations in five replicates on the same day and on consecutive days. The results showed that the intra-assay and inter-assay RSDs of the proposed method were lower than 2.9% and 3.4% for (*S*)-ofloxacin, and 2.8% and 3.3% for (*R*)-ofloxacin, respectively.

CONCLUSIONS

A sensitive, simple, and accurate method for determination of ofloxacin enantiomers was developed by chiral ligand-exchange reversed-phase HPLC. Stereospecificity was achieved in the ligand exchange mode by incorporating chiral reagents directly into the HPLC mobile phase. The effects of different concentrations of ligand and pH of the mobile phase were investigated; 0.6 mmol L^{-1} L-phenylalanine and 0.4 mmol L^{-1} Cu^{2+} under pH 4.9 of mobile phase was established as the optimum separation conditions. The proposed method could be applied to investigate the stereoselectivity and pharmacokinetics of chiral drugs in ligand-exchange chromatography.

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REFERENCES

1 Bhushan R and Kumar V, Indirect enantioseparation of α-amino acids by reversed-phase liquid chromatography using new chiral derivatizing reagents synthesized from s-triazine chloride. *J Chromatogr A* **1201**:35–42 (2008).
2 Berkecz R, Ilisz I, Fülöp F, Pataj Z, Hyun MH and Péter A, High-performance liquid chromatographic enantioseparation of β-3-homo-amino acid stereoisomers on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase. *J Chromatogr A* **1189**:285–291 (2008).
3 Chu BL, Guo B, Zuo H, Wang Z and Lin JM, Simultaneous enantioseparation of antiparkinsonian medication Rotigotine and related chiral impurities by capillary zone electrophoresis using dual cyclodextrin system. *J Pharm Biomed Anal* **46**:854–859 (2008).
4 Lee KA, Yeo S, Kim KH, Lee W and Kang JS, Enantioseparation of *N*-fluorenylmethoxycarbonyl α-amino acids on polysaccharide-derived chiral stationary phases by reverse mode liquid chromatography. *J Pharm Biomed Anal* **46**:914–919 (2008).
5 Wang Z, Ouyang J and Baeyens WRG, Recent developments of enantioseparation techniques for adrenergic drugs using liquid chromatography and capillary electrophoresis: a review. *J Chromatogr B* **862**:1–14 (2008).
6 Kwon C, Park H and Jung S, Enantioseparation of some chiral flavanones using microbial cyclic β-(1 $\rightarrow$ 3),(1 $\rightarrow$ 6)-glucans as novel chiral additives in capillary electrophoresis. *Carbohydr Res* **342**:762–766 (2007).
7 Péter A, Török G, Armstrong DW, Toth G and Tourwe D, High-performance liquid chromatographic separation of enantiomers of synthetic amino acids on a ristocetin A chiral stationary phase. *J Chromatogr A* **904**:1–15 (2000).
8 Taylor DR and Maher K, Chiral separations by high-performance liquid chromatography. *J Chromatogr Sci* **30**:67–85 (1992).
9 Hyun MH, Han SC, Lee CW and Lee YK, Preparation and application of a new ligand exchange chiral stationary phase for the liquid chromatographic resolution of α-amino acid enantiomers. *J Chromatogr A* **950**:55–63 (2002).
10 Davankov VA, Enantioselective ligand exchange in modern separation techniques. *J Chromatogr A* **1000**:891–915 (2003).
11 Schmid MG, Schreiner K, Reisinger D and Gübitz G, Fast chiral separation by ligand-exchange HPLC using a dynamically coated monolithic column. *J Sep Sci* **29**:1470–1475 (2006).
12 Zheng ZX, Lin JM and Qu F, Chiral separation of underivatized and dansyl amino acids by ligand-exchange micellar electrokinetic capillary chromatography using a copper(II)-L-valine complex as selector. *J Chromatogr A* **1007**:189–196 (2003).
13 Chen Z, Lin J, Uchiyama K and Hobo T, Reversal behaviors of the enantiomer migration order and the stereo-selectivity of Cu(II) complex with amino acid enantiomers in ligand exchange-micellar electrokinetic chromatography. *Anal Sci* **16**:131–137 (2000).
14 Stefansson M and Westerlund D, Ligand-exchange chromatography of carbohydrates and glycoconjugates. *J Chromatogr A* **720**:127–136 (1996).
15 Andriole CL and Andriole VT, Are all quinolones created equal? *Medicguide Infect Dis* **21**:1–5 (2002).
16 Wang H, Liao ZX, Chen M and Hu XL, Effects of hepatic fibrosis on ofloxacin pharmacokinetics in rats. *Pharmacol Res* **53**:28–34 (2006).
17 Andersson MI and MacGowan AP, Development of the quinolones. *J Antimicrob Chemother* **51**:1–11 (2003).

18 Hayakawa I, Atarashi S, Yokohama S, Imamura M, Sakano K and Furukawa M, Synthesis and antibacterial activities of optically active ofloxacin. *Antimicrob Agents Chemother* **29**:163–164 (1986).
19 Drew RH and Gallis HA, Ofloxacin: its pharmacology, pharmacokinetics, and potential for clinical application. *Pharmacotherapy* **8**:35–46 (1988).
20 Awadallah B, Schmidt PC and Wahl MA, Quantitation of the enantiomers of ofloxacin by capillary electrophoresis in the parts per billion concentration range for *in vitro* drug absorption studies. *J Chromatogr A* **988**:135–143 (2003).
21 Zeng S, Zhong L, Pan L and Li Y, HPLC separation and quantitation of ofloxacin enantiomers in rat microsomes. *J Chromatogr B* **728**:151–155 (1999).
22 Gong QJ, Quiao JL, Du LM and Dong C, Recognition and simultaneous determination of ofloxacin enantiomers by synchronization–1st derivative fluorescence spectroscopy. *Talanta* **53**:359–365 (2000).

