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ORIGINAL PAPER

Chiral separation of ofloxacin enantiomers by ligand exchange chromatography

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Abstract The enantioseparation conditions of ligand exchange chromatography were examined using ofloxacin enantiomers. A C_{18} column was used with the mobile phase consisting of a methanol–water solution (containing different concentrations of L-isoleucine and copper sulfate) at flow rate of 0.5 cm^3 min^{-1}. The effect of different kinds and concentrations of ligands, bivalent ligand ions, and organic modifier, and temperature on enantioseparation were evaluated; the results showed that enantioselectivity was strongly affected by the ligand concentration of the mobile phase. Under the optimum conditions (methanol/water 20:80 v/v, containing 2.5 mmol dm^{-3} L-isoleucine and 0.6 mmol dm^{-3} Cu^{2+}, room temperature), baseline separation of the two enantiomers was obtained with resolution of 1.32 in less than 30 min. The separation method was used to analyze the ofloxacin enantiomers in different commercial medicines.

Keywords Enantioseparation · Ofloxacin enantiomers · Bivalent ion · Ligand exchange chromatography

Introduction

In recent years, reports on vast differences in the pharmacological effects and pharmacokinetics between the two enantomeric forms of many drugs have highlighted the need for enantioselective separation and enantiomer quantification of chiral medications. For many racemic drugs, the desired pharmacologic effect is largely due to one enantiomer, but its antipode may be responsible for significant undesirable side-effects. Enantioseparation of chiral drugs has been extensively studied by gas chromatography (GC), capillary electrophoresis (CE), 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 separations using a chiral stationary phase require special chiral columns, such as Chirobiotic R SCP (bonded with ristocetin A [7]) and β-cyclodextrin chiral stationary phase [8]. These are expensive, and a high proportion of methanol in the mobile phase was used. In contrast, HPLC methods based on cheap and feasible chiral mobile phase additives (such as β-cyclodextrin or chiral ligand salts) are efficient tools for separating racemic drugs [9]. The enantioselectivity is based on the formation of a type of ternary complex composed of chiral ligand, central metal ion, and the analyte [10, 11]. Any difference in the stability or energy of these diastereomeric complexes will affect the chemical equilibrium, resulting in different chromatographic behavior. As a result, the enantiomers can be separated on a conventional HPLC column. The retentions of the enantiomers are related to the stabilities of metal ion complexes and their ternary diastereomeric complexes [12, 13].

Enantioselective ligand exchange chromatography is a liquid chromatography technique that has provided complete and reliable separation of stereoisomers of the most important classes of natural and synthetic compounds, such as amino acids, hydroxy acids, amino alcohols, and some others [10]. To date, many chiral ligand agents including

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different amino acids have been investigated and different ligand ions have been attempted [14].

Ofloxacin, (±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid (Fig. 1), is one of the most commonly used second-generation fluoroquinolones due to its high potency, low minimal inhibitory concentration, low toxicity, long half-life, and high stability [15]. Ofloxacin is finally eliminated mainly through renal clearance [16]. Because of its reduced side-effects and wide spectrum of antimicrobial activity, ofloxacin has been widely used instead of drugs with hepatotoxicity [17]. (*S*)-Ofloxacin shows 8–128 times higher activity than (*R*)-ofloxacin against Gram-positive and Gram-negative bacteria [18]. Therefore, there is considerable interest in recognizing and determining the two ofloxacin enantiomers not only for investigation of the pharmacokinetics of the enantiomers in vitro but also for the design and development of new chiral pharmaceutics. However, there are only a few reports of chiral separation of ofloxacin enantiomers. Very low concentration of ofloxacin was detected by using the CE method [19]; also a ligand exchange reversed-phase (RP)-HPLC method was used to detect the ofloxacin in rat plasma with high concentration of amino acid and $CuSO_4$ in the mobile phase [20].

The aims of this study were to develop a simple and rapid HPLC assay for separating and detecting the ofloxacin enantiomers by using low concentrations of chiral mobile phase additives in a conventional C_{18} column. The result was obtained by using relatively low concentrations of L-isoleucine and Cu^{2+} as ligand agent and ligand ion, respectively. The proposed method was used to analyze the ofloxacin enantiomers in different commercial medicines which can cure inflammation of the eyes and trachea.

Results and discussion

Ligand exchange mechanism

Ligand exchange chromatography exploits the rapid formation of metal ion complexes in order to separate drugs that can donate electrons and coordinate to the immobilized metal ions. Ofloxacin has two relevant ionizable functional groups: a basic piperazinyl group and a carboxylic acid group. The chelation interaction with various cations takes place on the carboxylic and the carbonyl groups. Ofloxacin enantiomers, bivalent copper cation, and ligand can form two types of ternary complexes with different configurations. The enantioselectivity depends on the different interactions between the stationary phase and the complex. Any difference in the stability constant of the interaction will result in different retention factors. As a result, the enantiomers can be separated on a C_{18} column.

Effects of different kinds and concentrations of ligands

Figure 2 shows the schematic structure of ligand exchange [21]. L-Isoleucine, L-lysine, L-methionine, L-threonine, L-valine, L-glutamine, and L-arginine were used as ligand agents to determine the effects of the different ligands on separation. In Table 1, L-isoleucine ($R = 1.02$) showed the best resolution and the selectivity was much better than for L-valine. Other ligand agents hardly showed any selectivity of the enantiomers ($R < 0.1$, $\alpha = 1.0$). Therefore L-isoleucine was selected for further experiments.

Most previous reports on enantioseparation by ligand exchange chromatography used 6.0–10.0 mmol dm^{-3} ligand reagent and 3.0–5.0 mmol dm^{-3} metal ions in the mobile phase [22]. In order to decrease the use of expensive chiral reagents and improve the enantioselectivity, relatively low concentrations of L-isoleucine in the mobile phase were examined over the range 0.0–6.0 mmol dm^{-3} with the Cu^{2+} concentration fixed at 1.0 mmol dm^{-3}. Figure 3 shows that the retention factors of the two enantiomers decreased and the enantioseparation factor increased with increasing L-isoleucine concentration in the mobile phase. However, the enantioseparation factor decreased when the L-isoleucine concentration was higher than 2.5 mmol dm^{-3}. Considering the retention and selectivity, 2.5 mmol dm^{-3} L-isoleucine was using for further investigations.

Effects of the different kinds and concentrations of divalent ions

In the periodic table, Fe, Cu, and Zn are in the same period, which means that their chemical characteristics are similar.

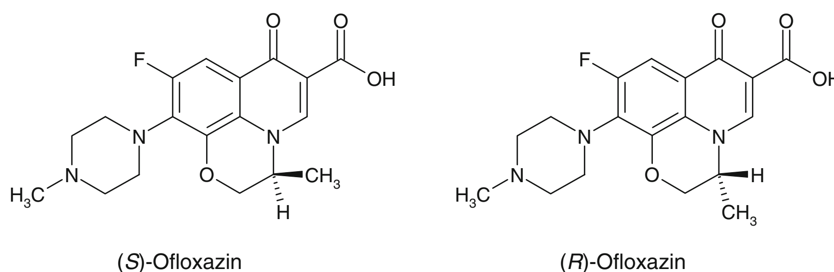


Fig. 1 The molecular structures of (*S*)-ofloxacin and (*R*)-ofloxacin

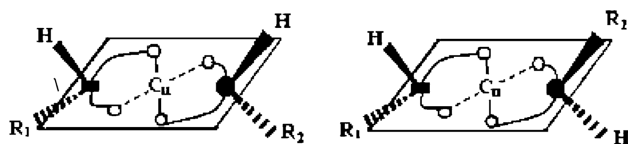


Fig. 2 Schematic structures of ligand complex of ofloxacin, amino acid, and metal ion

Table 1 Effect of different ligands on the separation

Ligand	Retention factor		Resolution (R)	Selectivity (α)
	k_S	k_R		
L-Isoleucine	10.95	12.68	1.02	1.16
L-Lysine	12.26	12.31	<0.1	1.00
L-Threonine	11.25	11.25	<0.1	1.00
L-Methionine	10.53	10.68	<0.1	1.02
L-Glutamine	8.86	8.86	<0.1	1.00
L-Valine	6.97	7.25	0.21	1.04
L-Arginine	8.51	8.51	<0.1	1.00

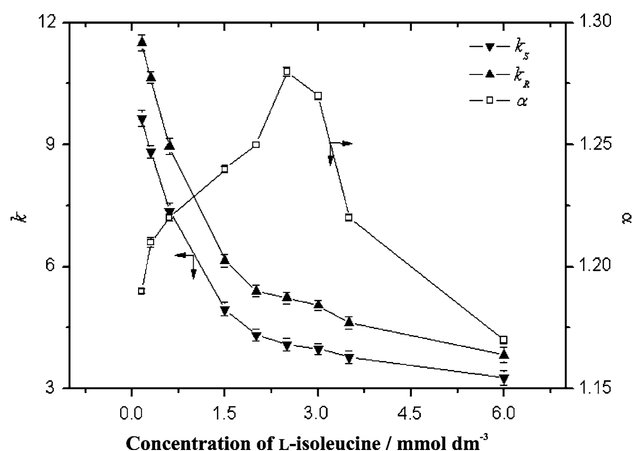


Fig. 3 Effect of retention factors (k) and enantioseparation factor (α) of two enantiomers, using different concentrations of L-isoleucine in the mobile phase

Also, all of them have divalent ions and can be easily obtained. Therefore, different concentrations of Fe^{2+}, Cu^{2+}, and Zn^{2+} were individually added to the mobile phase with 2.5 mmol dm^{-3} L-isoleucine.

According to the mechanism of ligand exchange, only ternary complexes containing ofloxacin enantiomers, a bivalent copper cation, and ligand with the optimum concentration ratio can be separated. When the concentration of divalent ions increased, the excess ions took the place of the carboxylic acid groups of the amino acid. In this case the following equilibria in the mobile phase [23] occurred, the ternary complexes were destroyed, and the retention factor and resolution decreased.

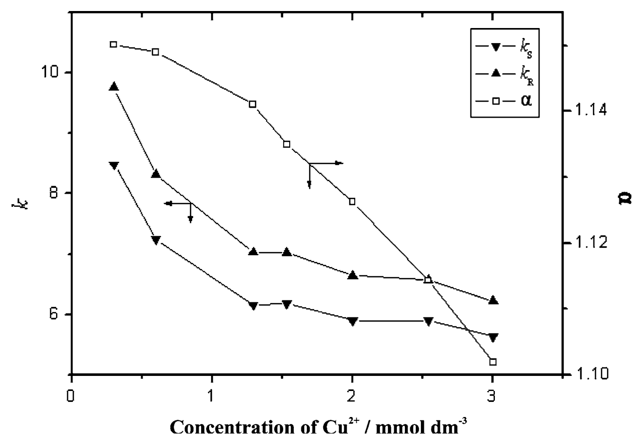
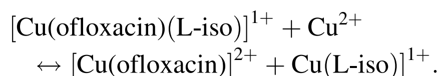


Fig. 4 Effect of Cu^{2+} concentration in the mobile phase on enantioseparation



$$[\mathrm{Cu(ofloxacin)(L\text{-}iso)}]^{1+} + \mathrm{Cu}^{2+} \leftrightarrow [\mathrm{Cu(ofloxacin)}]^{2+} + \mathrm{Cu(L\text{-}iso)}]^{1+}.$$

However, for Fe^{2+} the two enantiomers could not be separated over the range 0.3–6.0 mmol dm^{-3}. When the Zn^{2+} concentration was lower than 1.0 mmol dm^{-3}, the two enantiomers could be baseline separated. However, the retention times of the two enantiomers were still more than 30 min, even if the concentration was 6.0 mmol dm^{-3}. These data indicated that Fe^{2+} and Zn^{2+} were not suitable for enantioseparation under these concentrations.

The effects of Cu^{2+} concentration were examined using different concentrations ranging from 0.0 to 6.0 mmol dm^{-3}; the results are shown in Fig. 4. However, when the concentration of Cu^{2+} was 0.3 mmol dm^{-3}, the ofloxacin enantiomers could not be eluted within 30 min. Considering retention factors and selectivity, 0.6 mmol dm^{-3} was used as optimum Cu^{2+} concentration.

Effects of the organic modifier

Different concentrations of methanol, ethanol, and 1-propanol were used as the organic modifier in the mobile phase to investigate the effects of organic modifier on enantioseparation.

When 1-propanol was used, the two enantiomers could not be separated, even if the proportion was lower than 5 vol%. Moreover, a mobile phase which contains less than 5 vol% organic modifier is harmful for the C_{18} stationary phase. So 1-propanol was not suitable for use as the modifier.

With the proportion of ethanol in the mobile phase decreasing from 30 to 5 vol%, the resolution and selectivity increased from 0.0 to 0.76 and from 1.0 to 1.18, respectively. The results showed that ethanol utilized as the organic modifier showed little enantioselectivity. Furthermore, the

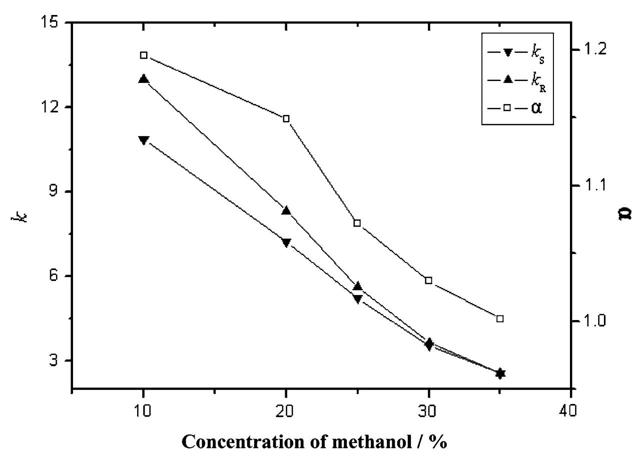


Fig. 5 Effect of methanol concentration in the mobile phase on enantioseparation

experimental data indicated that, with decreasing polarity of the organic modifier, the enantioseparation decreased.

Figure 5 shows the effect of the proportion of methanol on enantioseparation. Although the enantioselectivity increases with decreasing amount of organic modifier, both the retentions and resolution of the two enantiomers decreased with increasing proportion of methanol in the mobile phase. Therefore, 20% methanol was selected as the organic modifier to obtain a combination of high selectivity and fast analysis time.

Effects of temperature

The temperature of the column was increased gradually from 15 °C to 45 °C. With increasing temperature, the selectivities remained almost the same but the resolution of the two enantiomers decreased from 1.12 to 0.91. The results show that, with increasing temperature, the two enantiomers could not be baseline separated. So, a low temperature of 15 °C was selected as the optimized condition for further research.

Validation of the method

Calibration curves were constructed using the areas of the chromatographic peaks measured at increasing concentrations, ranging from 0.0008 to 0.5 mg cm^{-3}. The least-squares regression equation and the coefficient of determination were calculated, resulting in: $y = 19{,}975x + 175.75$ ($r^2 = 0.999$) for (*S*)-ofloxacin and $y = 19{,}909x + 167.46$ ($r^2 = 0.999$) for the (*R*)-enantiomer, respectively. The intra- and interday 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 in the same day and consecutive days. The results showed that the intraday relative standard deviations and interday relative standard deviations of the proposed method were lower than 4.25% and 5.14% for (*S*)-ofloxacin, and 4.36% and 5.21% for (*R*)-ofloxacin, respectively.

Analysis of ofloxacin in different medicines

Under the optimized enantioseparation conditions, three kinds of commercial ofloxacin medicines were dissolved in mobile phase and detected after injection into the HPLC. Fig. 6 shows that the ofloxacins can be detected successfully and the amounts of ofloxacins were 0.39 mg cm^{-3} for (*S*)-ofloxacin and (*R*)-ofloxacin in ofloxacin tablets, 0.90 mg cm^{-3} for (*S*)-ofloxacin and 0.016 mg cm^{-3} for (*R*)-ofloxacin in levofloxacin hydrochloride tablets, and 0.44 mg cm^{-3} for (*S*)-ofloxacin and 0.43 mg cm^{-3} for (*R*)-ofloxacin in ofloxacin eye drops.

Conclusions

A simple method with optimum conditions for determination of ofloxacin enantiomers was developed by chiral ligand exchange RP-HPLC. The effects of different separation conditions were examined, and the concentrations of the ligand of the mobile phase were found to play an important role in enantioseparation. Cu^{2+} and L-isoleucine with low concentrations (0.6 and 2.5 mmol dm^{-3}, respectively) were added to methanol/water (20:80 v/v) as the mobile phase, which was successfully used to analyze the ofloxacin enantiomers in different anti-inflammation medicines. Further investigation into the analysis of ofloxacin enantiomers in human serum albumin and urine samples is currently underway.

Experimental

Materials

L-Isoleucine, L-lysine, L-methionine, L-threonine, L-valine, L-glutamine, and L-arginine (all of them 98%), (*S*)-ofloxacin, and (*R*)-ofloxacin (≥98%) were obtained from Sigma (St. Louis, MO, USA). Copper sulfate pentahydrate, zinc sulfate heptahydrate, iron sulfate heptahydrate (Extra Pure grade), methanol, ethanol, and 1-propanol (HPLC grade) were obtained from Duksan Pure Chemical Co., Ltd. (Ansan, Korea). Double deionized water (Woongjin Chemical Co., Ltd., Seoul, Korea) was filtered with a 0.45-µm filter membrane before use.

Ofloxacin tablets (Gailuoxian®) and levofloxacin hydrochloride tablets (Weilisu®) were purchased from Baiyunshan

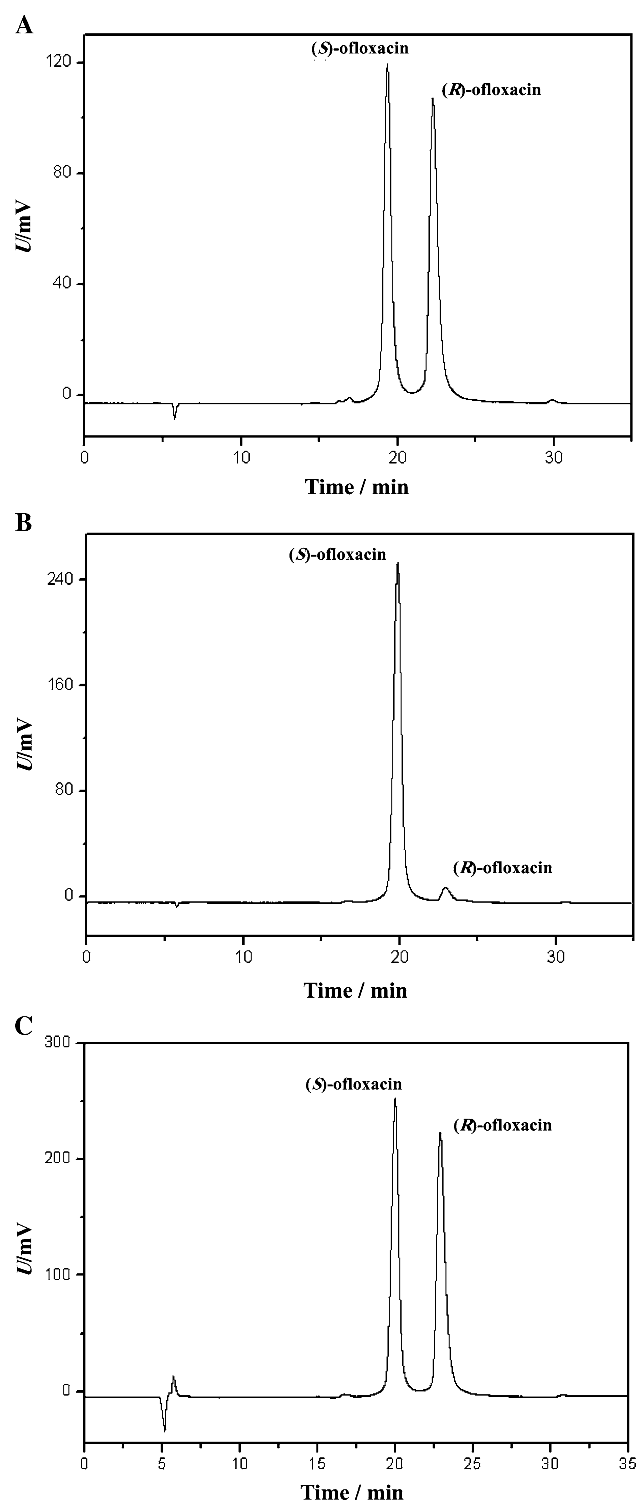


Fig. 6 Chromatogram of three ofloxacin medicines on enantioseparation: **a** ofloxacin tablets, **b** levofloxacin hydrochloride tablets, and **c** ofloxacin eye drops

pharmaceutical factory (Guangzhou, China) and ground to powder. Ofloxacin eye drops (Sanke®) was from KWIN Pharma Co., Ltd. (Jiangsu, China). Medicine (2.0 mg) was mixed with 5.0 cm^3 methanol for 4 h. Then the methanol solutions were filtered (0.45 μm) and subjected to HPLC analysis.

Chiral ligand exchange system

The HPLC system comprised a M930 solvent delivery pump (Young Lin Co. Korea), an ultraviolet (UV) detector (M 720 absorbance detector, Young-In Scientific Co., Korea), an integrated data system (Autochrowin Ver. 1.42, Young Lin Co., Korea), and a Rheodyne injection valve (20 mm^3 sample loop). Autochro-2000 software (Younglin Co. Ltd., Korea) was used as data acquisition system. The analytical column (250 mm × 4.6 mm) was packed with C_{18} stationary phase (5 μm, RStech, Daejeon, Korea). The mobile phase comprised methanol/water (20:80 v/v) containing 2.5 mmol dm^{-3} L-isoleucine and 0.6 mmol dm^{-3} cupric sulfate. The flow rate of the mobile phase was set at 0.5 cm^3 min^{-1}. The chromatographic assay was carried out at ambient temperature, and UV wavelength was set at 293 nm. The concentration of the ofloxacin enantiomer was 0.5 mg cm^{-3}, and the injection volume was 2 mm^3.

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 (*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.

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