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ENANTIOSELECTIVE SEPARATION OF OFLOXACIN BY LIGAND EXCHANGE CHROMATOGRAPHY

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Abstract

A ligand exchange HPLC method for enantioseparation of ofloxacin enantiomers was developed by a using C_8 stationary phase. Chromatographic separation was performed on a Zorbax-300SB-C_8 column eluted with the mobile phase consisting of 15 % methanol in water containing 10mM phenylalanin and 5 mM $CuSO_4$ at flow rate of 0.8 mL/min. The method was simple, rapid and suitable for routine analytical studies of ofloxacin enantiomers.

Keywords. Ofloxacin, levofloxacin, ligand exchange HPLC, enantio selective separation.

1. INTRODUCTION

Ofloxacin is one of the most commonly used second-generation fluoroquinolones due to its wide spectrum of antimicrobial activity, low toxicity, long half-life, and high stability. As the compound contains a chiral center at C-3, ofloxacin is a racemic mixture of (*R*)-ofloxacin and (*S*)-ofloxacin (levofloxacin). The *S*-enantiomer shows 8–128 times higher activity than the *R*-form against Gram-positive and Gram-negative bacteria [1, 2]. Therefore, enantiomer selective analysis of ofloxacin is important for the drug quality control, investigation of the pharmacokinetics of the enantiomers in vitro as well as for the design and development of new chiral pharmaceutics. Several analytical methods for determination of ofloxacin enantiomers have been reported including capillary electrophoresis (CE) [3, 4], and high performance liquid chromatography (HPLC) using chiral stationary phases or chiral mobile phase additives [5,6]. HPLC analysis using chiral stationary phase requires a special chiral column, which is expensive and only suitable for several enantiomers in most cases. In contrast, HPLC methods based on chiral mobile phase additives are not only efficient tools for the separation of racemic drugs but also relatively cheap and feasible. Most of reports have shown that the use of RP_{18} columns eluted by ligand exchanged mobile phases consisting of an amino acid such as L-leucine or L-phenylalamine provided the best separation [6-8]. In the present paper, we described the development of a simple and rapid HPLC method for separation of ofloxacin enantiomers by using a reverse phase C8 column and a mobile phase of methanol-water containing 5 mM copper sulfate and 10 mM L-phenylalanine.

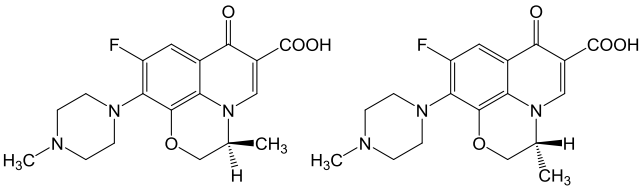

Fig. 1: Structures of levofloxacin and ofloxacin

2. EXPERIMENTAL

2.1. Chemicals and reagents

Racemic ofloxacin và levofloxacin (> 98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol, water, and analytical grade copper sulfate and L-phenylalanine were purchased from Merck (Darmstadt, Germany). Stock standard solutions of ofloxacin và levofloxacin were prepared by dissolving 1 mg powder in 1 mL mixture of methanol-water (1:1 v/v).

2.2. HPLC experiments

HPLC was performed on an Agilent 1200 liquid chromatography system equipped with a diode array detector. Data acquisition, processing and instrument

control were monitored by Microsoft Windows XP based ChemStation software. Chromatographic separation was carried out in a Zorbax-300SB-C_8 column (4.6x250 mm) with a C_8 guard column maintained at 21 °C. The elution was performed with a 30 min. isocratic mobile phase of 15% methanol in water containing 10mM phenylalanin and 5mM $CuSO_4$ at flow rate of 0.8 mL/min. The injection volume was of 10 µL. The DAD acquisition wavelength was set at 290 nm.

Retention factor (k), enantioseparation factor (α), and resolution (R) were calculated using the following equations: $k = (t-t_0)/t_0$, where t and t_0 are the retention times of analyte and unretained solutes, respectively; $\alpha = k_R/k_S$, where k_S and k_R are the retention factors of (*S*)- and (*R*)-enantiomer, respectively; $R = 2(t_R-t_S)/(w_R+w_S)$, where t_S and t_R are retention times of the (*S*)- and (*R*)-enantiomer, respectively, and w_S and w_R are the baseline peak widths of the two enantiomers.

3. RESULTS AND DISCUSSION

3.1. Reverse phase column selection

Previous ligand exchange HPLC methods have used only C_{18} reverse phase columns for enantioseparation of ofloxacin. However, our experiment showed that C18 columns such as Zorbax Eclipse XDB C_{18} column, YMC Pack Pro C_{18} and J'sphere ODS-H80 did not provide separation of ofloxacin enantiomers by using mobile phase of 15 % methanol in water containing 10 mM phenylalanin and 5 mM $CuSO_4$. This could be explained as the amino acid phenylalanin was not sensitive with C_{18} columns [7]. We next tried the Zorbax-300SB-C_8 column with the mobile phase described above. The retention factor (k), enantioseparation factor (α), and resolution (R) obtained from test columns were compared. As showed in table 1, Zorbax-300SB-C_8 column provided highest resolution (R = 1.56), while no separation was found when using C_{18} columns.

3.2. Mobile phase optimization

Among organic solvents such as methanol, acetonitril, ethanol and isopropanol, methanol provided the best separation. The concentration of methanol was also investigated. Increased organic solvent decreased the separation and low methanol concentration caused the extended retention time. Hence, it is necessary to adjust the methanol concentration in the mobile phase in order to obtain good and rapid chromatographic separation of levofloxacin and (R)-enantiomer. Considering the retention time and enantioselectivity, 15% methanol was selected as the organic modifier in this work. The amounts of copper sulfate and L-phenylalanine were set up as 5 and 10 mM, respectively. The pH of the mobile phase is another critical factor for the enantioseparation. In order to investigate the effect of the pH of mobile phase on the enantioselectivity of ofloxacin enantiomers, the pH-dependence of the nantioseparation was investigated in a pH range of 3.0-5.2 using phosphoric acid.

Table 1: Effect of columns on the separation of ofloxacin enantiomers

Columns	k_S	k_R	α	R
Zorbax-300SB-C_8	3.63	4.18	1.15	1.56
Zorbax Eclipse XDB C_{18}	6.92	6.92	1.00	-
YMC Pack Pro C_{18}	7.32	7.41	1.02	<0.1
J'sphere ODS-H80	7.55	7.55	1.00	-

Previous study indicated that the resolution of ofloxacin enantiomers distinctly decreased from 2.35 to 1.10 when the pH of the mobile phase was lowered from 4.0. On the other hand, when the pH of the mobile phase exceeded 5.0, Cu^{2+} would precipitate and block the chromatographic system [7]. Hence, 4.5 were chosen as the optimized pH of the mobile phase. With these conditions, levofloxacin and (*R*)-ofloxacin were well separated at the retention time of 16.6 and 18.7 min, respectively (figure 2).

3.3. Method validation

Calibration curves were constructed using the areas of the chromatographic peaks measured at increasing concentrations in the range of 2–200 µg/mL for ofloxacin enantiomers. The results showed good linearity with the correlation coefficients of 0.9996 and 0.9991 for levofloxacin, and (*R*)-ofloxacin, respectively. The intra- and interday accuracy and precision of the assay assessed as relative standard deviation (RSD) were

determined on the basis of peak-area by assaying the standards at three different concentrations in five replicates in the same day and consecutive days. The results showed that the intraday and interday RSDs of the proposed method were lower than 1.47 % and 2.82 % for levofloxacin, and 1.35 % and 4.01 % for (*R*)-ofloxacin, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were both determined as the concentrations that produced signal-to-noise ratios of 3 and 10, respectively. The value obtained for LOD and LOQ were 1.51 and 5.14 µg/mL, respectively, for two enantiomers.

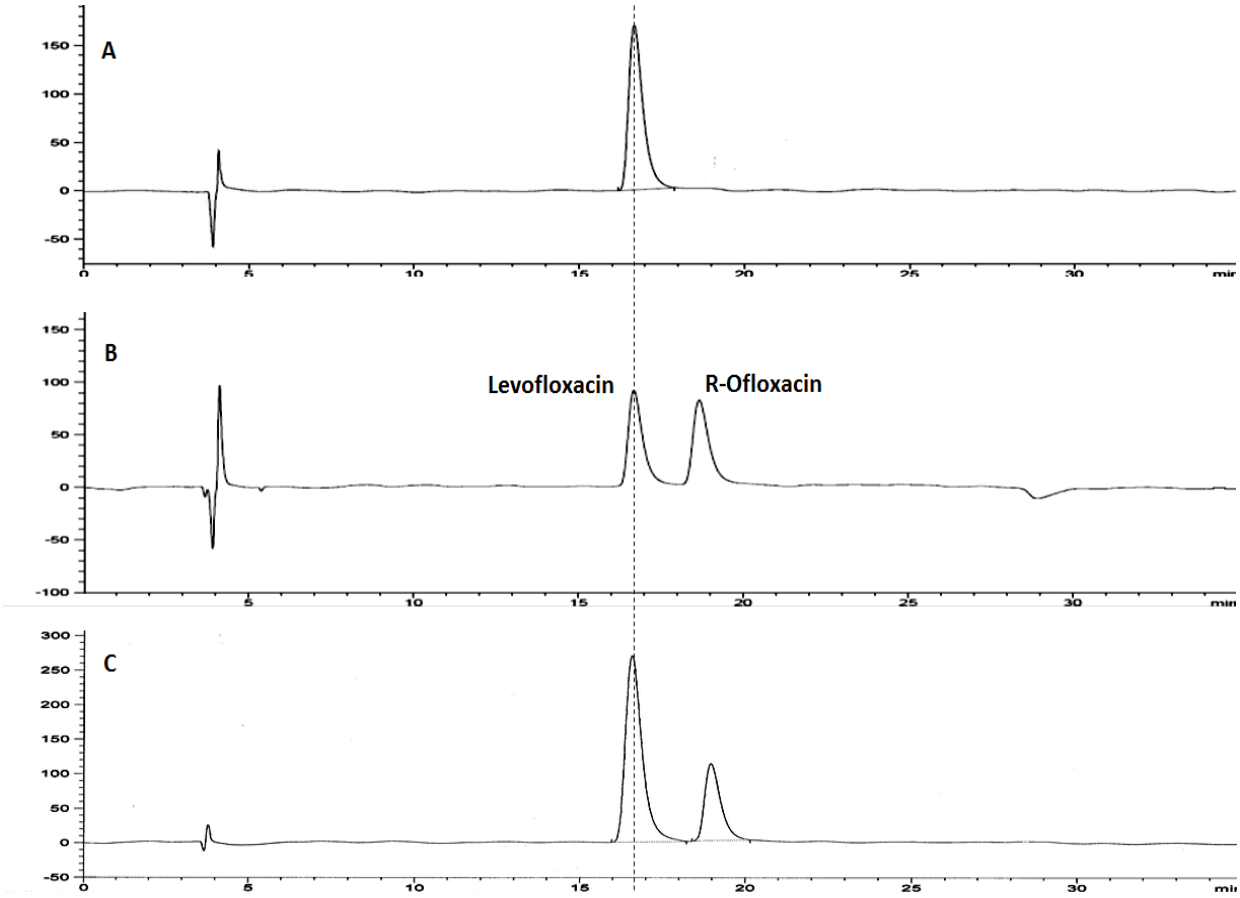

Fig. 2: HPLC chromatograms of levofloxacin (A), ofloxacin racemic (B) and mixture of levofloxacin-ofloxacin racemic (C)

4. CONCLUSION

A sensitive, simple, and accurate method for enantioseparation of ofloxacin enantiomers was developed by chiral ligand exchange RP-HPLC using Zorbax-300SB-C_8 column. The method provided good enantioseparation factor (α = 1.15) and resolution (R = 1.56) with the mobile phase of 15% methanol in water containing 10mM phenylalanin and 5mM $CuSO_4$ at flow rate of 0.8 mL/min. This is the first time that the separation of ofloxacin enantiomers by using a C_8 stationary phase has been reported.

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