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Article

Semi-Preparative Enantiomeric Separation of Ofloxacin by HPLC

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A direct semi-preparative high performance liquid chromatography (HPLC) enantioseparation of ofloxacin was performed on chemically immobilized cyclodextrin derivative–mono (6A-azido-6A-deoxy)-per(p-chlorophenyl carbamoylated) β-CD chiral stationary phase. Conditions for semi-preparative separations were established using a 250 × 4.6 mm i.d. column and subsequently extended to a 250 × 10.0 mm i.d. column that enabled separations on a milligram scale. Optimization of the chromatographic conditions (mobile phase and column load) with respect to better efficiency, resolution and peak retention resulted in a system capable of separating up to 304 mg of (−)-(*S*)-ofloxacin and 56 mg of (+)-(*R*)-ofloxacin of the racemate over 6 h. The purities of the separated enantiomers were determined by HPLC. Moreover, both separated enantiomers were characterized by mass spectrometry; then, the absolute configuration of the products was clearly confirmed by polarimetry.

Introduction

Because of the pharmacodynamic, pharmacokinetic and toxicological differences between enantiomers, stereochemistry has become a hot topic issue in drug research and development (1). In recent years, the production of enantiopure pharmaceuticals has rapidly increased and a significant number of established drugs marketed as racemates have been authorized from regulatory agencies as single enantiomers (2). 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 a kind of fluorinated quinolone antibacterial that targets inhibition of the bacterial enzymes DNA gyrase and DNA topoisomerase IV (3), and is a chiral compound due to the methyl group at the C-3 position of the oxazine ring. The anti-bacterial activity of the (*S*)-isomer (levofloxacin) is 8–128 times higher than that of the (*R*)-isomer, which is harmful to liver and kidney functions. Additionally, they show obvious differences in *in vitro* bacterial strains (4–5). However, it is inevitable that both products may contain levofloxacin synthesis. Hence, it is necessary to establish a powerful method to obtain an entiopure product for the two enantiomers.

Some techniques have been proposed for the determination of ofloxacin, including voltammetry (6), nuclear magnetic resonance spectroscopy (7), spectrophotometry (8), potentiometry (9), capillary electrophoresis (10) and high-performance liquid chromatography (HPLC) using a low concentration of chiral compound as a chiral mobile phase (11–14). In addition, it is difficult to extend the application in the semipreparative separation of ofloxacin for HPLC with chiral mobile phase additives (such as cyclodextrin or chiral ligand salts) and counter-current extraction with protein (15) because they include complicated after-treatment procedures with low yield. Direct HPLC using an immobilized cyclodextrin (CD)-based chiral stationary phases (CSPs) has shown many advantages in enantioseparation of chiral compounds, such as flexibility, broad selectivity, efficiency and relatively easy to operate characteristics, and has been widely applied in enantiomeric separations and analyses. Most importantly, the chiral HPLC technique as a preparative tool also exhibits good industrial prospects. However, to best of our knowledge, the enantioseparation and semi-preparative separation techniques of ofloxacin using a chiral HPLC technique using chiral CSPs (16) have rarely been investigated in recent years.

In our previous work (17), we reported a novel and simple synthesis of a series of structurally well-defined CD-based CSPs by immobilization of monoazido-perfunctionalized β-CD on aminized silica gel (5 μm, 10 nm) via a single stable urea linkage using the Staudinger reaction. Herein, we focused our efforts on developing a high-enantioselective semi-preparative HPLC system based on CSPs for achieving industrial separation of lefloxacin. The results indicated that enantioseparation of levofloxacin and its (+)-(*R*)-isomer have been successful achieved using CSPs under similar reverse-phase conditions. With maximum loading, (−)-(*S*)-ofloxacin (304 mg) and (+)-(*R*)-ofloxacin (56 mg) were obtained in 6 h. The chiral purities of both products were determined up to 98%. Moreover, absolute configuration of the obtained enantiomers was confirmed by polarimetry.

Materials and Methods

Mono (6A-azido-6A-deoxy)-per(p-chloro phenylcarbamoylated) β-CD (Cl-Ph-β-CD) immobilized CSPs were prepared according to the reported literature method. Ofloxacin obtained from South China Agricultural University was dissolved in acetonitrile. After filtering through a 0.45-μm microporous membrane, ofloxacin in acetonitrile solution (1.52 mg/mL) was stored in a refrigerator (approximately 4°C). Acetonitrile (ACN), hexane, 2-propanol (IPA) and methanol (MeOH) of HPLC grade were used throughout the study. All other chemicals were of analytical grade and were dried and purified before used.

Cl-Ph-β-CD immobilized CSPs were slurried in acetone and packed into standard stainless steel columns (analytical column: 250 × 4.6 mm i.d.; preparative column: 250 × 10.0 mm i.d.) on an Alltech HPLC packer (Alltech Associates, Bannockburn, IL) at a pressure of 8,000 psi. All HPLC enantioseparations were

performed using two stainless steel columns. With biphenyl as a test probe under normal phase (hexane and IPA in a 90:10 v/v ratio), two columns gave an efficiency of 40,000 plates/m (analytical column) and 1,290 plates/m (semi-preparative column), respectively (18).

Evaluation of the column was performed using an HPLC system composed of a Shimadzu SPD-10AV HPLC system, a Shimadzu ultraviolet-visible (UV–vis) detector and a 7725i injector equipped with a 20-μL sample loop. The UV absorbance detection was performed at 293 nm. In analytical enantioseparations, a 20-μL sample solution was injected. All chromatographic experiments were carried out at room temperature.

The retention factor (k), separation factor (α) and resolution factor (R_s) were calculated according to the IUPAC *Nomenclature for Chromatography* (20). The dead time, t_0, for the analytical column was determined with 1,3,5-tri-tert-butylbenzene in the normal-phase mode and with sodium nitrate in the reversed-phase mode. The dead volume of the column was 1.8 mL. The elution time is the average of triplicate determinations.

Mass spectrometry (MS) spectra of the compounds were determined using a Thermo LCQ DECA XP MAX LC–MS spectrometer with electrospray ionization (ESI) source at room temperature.

The optical rotation of enantiomers was determined using an P850 Automatic polarimeter (Hanon Instruments, China) with $CHCl_3$ as solvent.

Results and Discussion

Analytical HPLC

First, separations of ofloxacin were performed on the analytical column (250 × 4.6 mm i.d.). In addition, the analytical column was employed to determine the enantiomeric purities of the fractions during the semi-preparative separations. As shown in the Table I, the chromatographic analysis results indicated that the Cl-Ph-β-CD-immobilized CSPs exhibited an excellent enantioseparation ability for ofloxacin with R_s up to 6.574.

Table I
Effect of Different Mobile Phases on Enantioseparation

Condition	Mobile phase (v/v)	k_1	α	R_s
1	ACN–TEAA (95:5)	—	—	0.000
2	ACN–TEAA (90:10)	0.19	1.21	0.874
3	ACN–TEAA (85:15)	0.23	2.75	6.574
4	ACN–TEAA =(80:20)	0.25	1.89	1.466
5	ACN–TEAA (75:25)	0.14	1.47	0.573

In addition, the results indicated that the progressive addition of 1% aqueous triethylammonium acetate (TEA) into the ACN solution led to a remarkable increase in retention, enantioselectivity and resolution. However, with a further increase of TEAA concentration in ACN (from 20 to 25% in Table I), the enantioselectivity and resolution of the chiral column showed a downward trend instead of increasing. Meanwhile, the symmetry of chromatographic peaks grew worse with the increase of the TEAA ratio in ACN solution.

Elution of the two enantiomers was observed within 3.0 min [the first effluent fraction, (+)-(*R*)-isomer] and 4.8 min (the second effluent fraction, levofloxacin), respectively, using ACN–TEAA 85:15 (v/v) as eluent at a flow rate of 1.0 mL/min (Figure 1).

Thus, the ACN–TEAA (85:15, v/v) mixtures were identified as the most suitable eluents for the enantioseparation of ofloxacin enantiomers on the semi-preparative chiral column (250 × 10.0 mm. i.d.), based on the aforementioned results.

Semi-preparative HPLC

The column loading is a critical factor in preparative separation. In this work, the column loading studies were performed at a

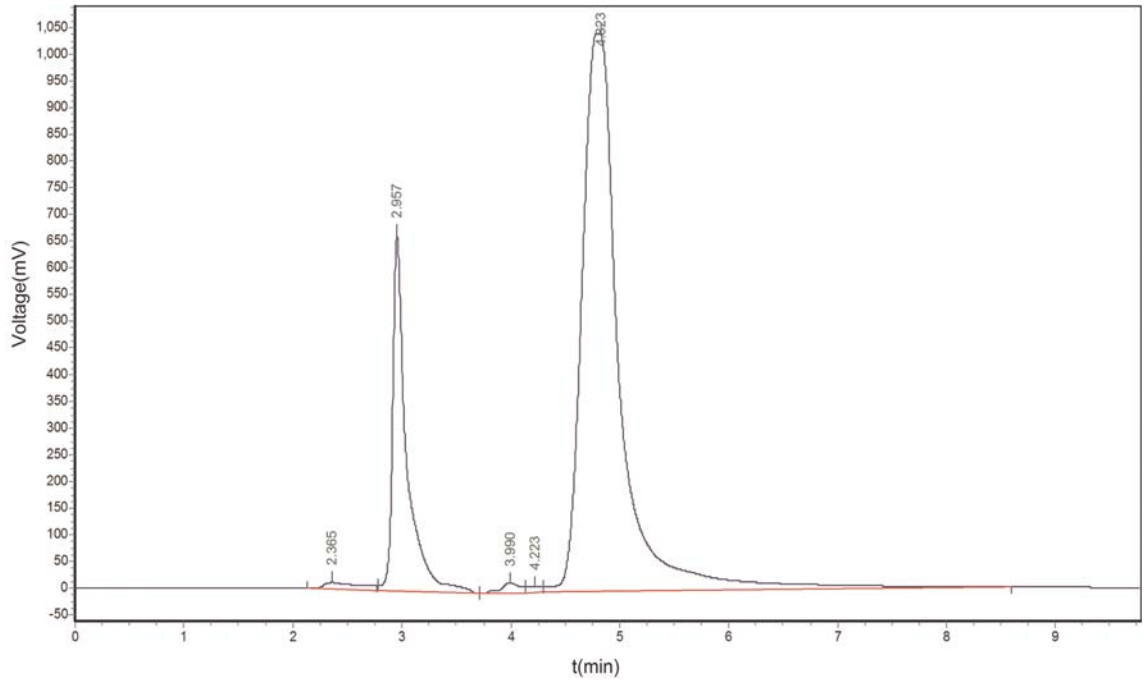
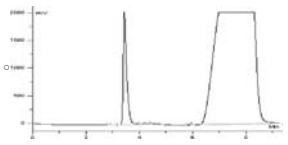
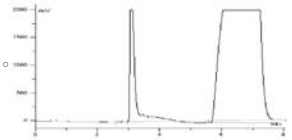
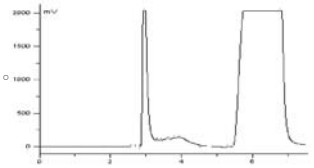
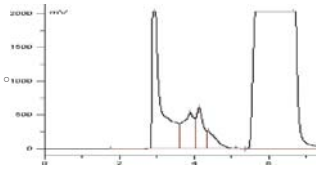
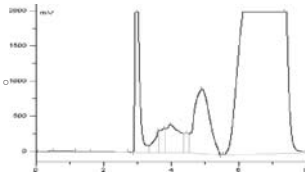


Figure 1. Chromatogram of ofloxacin crude product on analytical column.

3 mL/min flow rate with injections of 3, 4, 5, 6 and 7 mL of ofloxacin in ACN solution, respectively. The results obtained from different working conditions are summarized in Table II and Figure 2.

Table II
Chromatograms of Ofloxacin Enantiomers using the Different Injection Amounts on a Semi-Preparative Column with Injection Concentration of 1.52 mg/mL

Number	Injection	Chromatograms
A	3 mL	
B	4 mL	
C	5 mL	
D	6 mL	
E	7 mL	

As illustrated in Figure 2, the resolution decreased rapidly along with the increase of column loads. At the same time, the worse chromatographic peak shapes and lower purity of ofloxacin enantiomers were observed.

Based on these results, resolution of ofloxacin enantiomers on a semi-preparative column was high enough to obtain a batch of fractions containing practically pure single enantiomers. After comprehensively considering various factors such as retention, resolution, chromatographic peak shape, production rate and the purity of enantiomers, a feasible solution was chosen to be applied in the semi-preparative enantioseparation of ofloxacin.

Further separations were performed with injections of ofloxacin enantiomers (5 mL, 7.6 mg), ACN–TEAA (85:15, v/v) as the eluting mobile phase and appropriate flow rate (3 mL/min) at room temperature. Repetitive injections of ofloxacin solution (5 mL each time) were performed to the same chiral column every 7 min. Because the collection time is closely related to the purity of the product in the semi-preparative process, (+)-(*R*)-ofloxacin and levofloxacin was collected at 2.6 ~ 4.3 min (for the first fraction) and 5.1 ~ 6.9 min (for the second

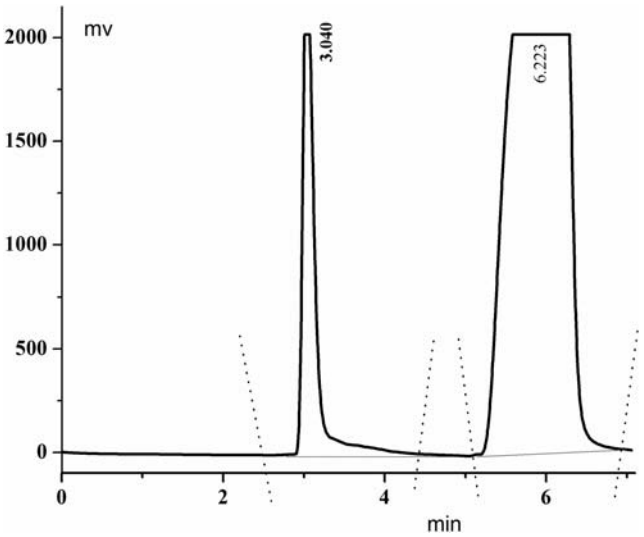


Figure 3. Chromatogram of ofloxacin on semi-preparative column.

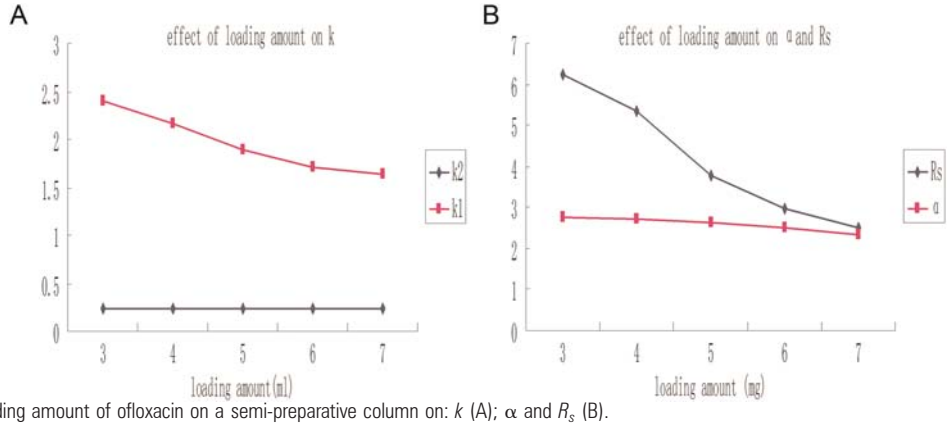


Figure 2. Effects of the loading amount of ofloxacin on a semi-preparative column on: *k* (A); α and R_s (B).

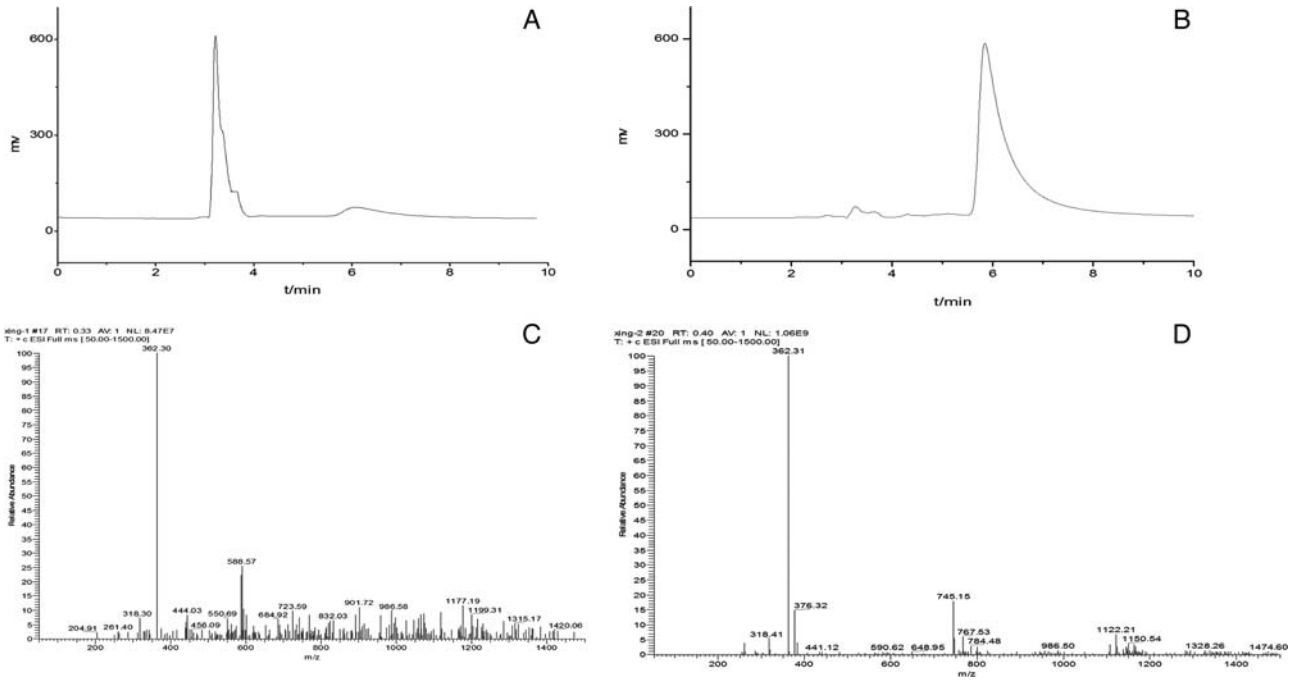


Figure 4. Chromatograms and EI-MS spectra: chromatogram of the first effluent fraction [(+)-(*R*)-ofloxacin] on analytical column (A); chromatogram of the second effluent fraction [(−)-(*S*)-ofloxacin] on analytical column (B); EI-MS spectrum of the first effluent fraction [(+)-(*R*)-ofloxacin] (C); EI-MS spectrum of the second effluent fraction [(−)-(*S*)-ofloxacin] (D).

fraction), respectively (Figure 3). The rest if the effluent contained fewer enantiomers and was not further purified through additional recycling steps.

Through extracting with CH_2Cl_2, distilling and drying *in vacuo*, light yellow solid powders, namely (−)-(*S*)-ofloxacin (304 mg) and (+)-(*R*)-ofloxacin (56 mg), were obtained from the collected elution in 6 h.

For the products [(+)-(*R*)- and (−)-(*S*)-ofloxacin] obtained from semi-preparative chromatography, the enantiomeric purity was investigated using the chiral HPLC technique, as shown in Figure 4. The results indicated that the chromatographic peaks for the stereoisomers were observed with the similar retention times and their enantiomeric purities reached or exceeded 98%.

Determination of the absolute configuration of the products

ESI-MS spectra of two enantiomers are shown in Figure 4. The ESI-MS spectra show the following: MS 361.30 [the first effluent fraction, (+)-(*R*)-ofloxacin]; MS 361.31 [the second effluent fraction, (−)-(*S*)-ofloxacin].

As listed in Table III, off-line polarimetric analysis results indicated that the first eluting enantiomer from ofloxacin showed a dextral structure under the experimental conditions. The optical rotations of measured enantiomers were accordance with those measured by Mitscher *et al.* (19).

Table III
Chromatographic and Polarimetric Analysis of the Pooled Fractions Containing the first (F1) and Second (F2) Eluted Enantiomers of Ofloxacin*

			F1†	F2†
Compound	Mobile phase	Amount‡	Eluted enantiomer (%) $[\alpha]_D^{25}$	Eluted enantiomer (%) $[\alpha]_D^{25}$
Ofloxacin	ACN–TEAA 85:15 (v/v)	7.6	>98 +68°	>98 -43°

*Note: Column, 250 × 10 mm i.d.; flow rate, 3.0 mL/min; detector, UV at 293 nm; temperature, 25°C. †Enantiomeric purity and polarimetric data for the pooled fractions containing the F1 and F2 eluted enantiomers.
‡Amount of sample (in mg) resolved in a single semipreparative run.

Conclusion

In summary, the enantioselective HPLC technique based on chemically immobilized β-cyclodextrin derivative CSPs provided a fast and efficient approach for obtaining optically pure enantiomers from ofloxacin with an ACN–TEAA mixture as the eluent. With maximum loading, (−)-(*S*)-ofloxacin (304 mg) and (+)-(*R*)-ofloxacin (56 mg) were obtained in 6 h. The optimized CSP/eluent/column load system has promising applications in the separation of ofloxacin and evaluation of levofloxacin. Other characterizations such as MS and optical rotation measurements further confirmed that (+)-(*R*)-ofloxacin and (−)-(*S*)-ofloxacin were successfully separated by the optimized HPLC system. Separation, semi-preparation, purification and characterization techniques for optically pure enantiomers of ofloxacin might play a key role in further *in vivo* studies on enantioselectivity of metabolism.

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