## Letter to the Editor

# Preparative Resolution of Gatifloxacin Enantiomers with Pre-Column Esterification Strategy and Comparing Their Enantioselectivity to Bacteria and Antibody*

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**Gatifloxacin (GFX) is a kind of chiral fluoroquinolones compound due to the methyl group at the C-3 position of the piperazine ring[1]. Although the enantiomers of GFX show similar levels of antimicrobial activity and pharmacokinetics[2], the other biological activities (i.e., toxicity or enantioselective recognition to various receptors *in vivo*) of GFX enantiomers have not yet been studied. With this in mind, we developed a rapid and cost-effective high performance liquid chromatographic (HPLC) separation procedure for GFX enantiomers with a pre-column esterification strategy. With significant enhancement of drug solubility and optimization for chromatographic conditions, the proposed method was scaled up to preparative HPLC to obtain optical active *S*-(−)- and *R*-(+)-GFX. The antibacterial activities of GFX enantiomers after preparative separation were further verified by measuring the Minimum Inhibitory Concentration (MIC) values against *Escherichia coli* ATCC 25922. In addition, the binding selectivity of GFX enantiomers to protein receptor were evaluated by antibody using enzyme-linked immunosorbent assay (ELISA) for the first time.**

In the current study, GFX was found to be only slightly soluble in methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), and hexane (HE). Due to the limited solubility, GFX was not suitable for the cost-effective and rapid HPLC resolution. Therefore, GFX methyl ester (Figure 1) was synthesized to be subjected on the column in order to enhance the solubility and resolution efficiency, followed by the hydrolysis of the separated ester to obtain GFX enantiomers.

To achieve the best possible separation of the enantiomers of GFX methyl ester, 5 types of mobile phase were evaluated on four types of chiral stationary phases (CSPs). The resultant resolution factor ($R_S$), retention time ($t$), and enantioselectivity ($\alpha$) have been summarized in Table 1. It was found that the Chiralpak AS-H and the AD-H were the only columns that were capable of sufficiently resolving the enantiomers of GFX methyl ester. No separation signal was observed when the Chiralcel OD-H and the Chiralpak IA columns were tested with this multimodal elution system. Compared to the performance on the AD-H column, the AS-H column demonstrated higher chiral recognition ability toward the enantiomers of GFX methyl ester. The racemate could be baseline-separated ($R_S$≥1.39) on the AS-H column under both normal and polar organic mode, while only partially separated on the AD-H column under normal phase conditions. This result indicated that the enantioseparation of GFX methyl ester was significantly affected by the position and number of substituents introduced on the phenyl groups of the carbamate derivatives[3]. The AS-H column consisting of (s)-α-methyl group as substituent may provide better spatial structure to recognize GFX methyl ester than the AD-H column (3, 5-dimethyl group as substituent). From Table 1, It could be also noted that the relative short retention times of the two enantiomers occurred using ACN or hexane with 30% ethanol as mobile phase on the AS-H column ($R_S$≥1.80). The enantiomers of GFX methyl ester could be well resolved in 5 min under

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these two conditions. Although the resolution was better when ACN was used, the solubility of GFX methyl ester in hexane was higher than that in ACN. Furthermore, it has been reported that highly polar solvents could partially or totally dissolve the coated-type CSPs when applied to a continuous separation, and that this could lead to a reduction in the life time of the column[4]. Considering the separation time, the solubility of the racemate and the CSP stability in preparative application, the enantioselectivity ($\alpha$=1.36) and resolution ($R_S$=1.80) were acceptable when n-hexane was used. For this reason, the mobile phase of hexane with ethanol as organic modifier was selected for the additional optimization work on the AS-H column.

Next, the effect of the ethanol concentration in the mobile phase was evaluated. 15%, 20%, and 30% of ethanol were tested and the result showed that the increasing amount of ethanol in the mobile phase led to reduction in the retention times as well as the resolution of the two enantiomers. Considering the resolution efficiency, HE/EtOH/DEA (80:20:0.1, v:v:v) ginving a resolution of 2.59 at 7 min was finally selected as the optimized mobile phase for the preparative separation of GFX methyl ester enantiomers on AS-H column. The separation time per run in current study was relatively shorter than those of the published data (>10 min)[5].

The optimized method was used to resolve the racemic sample on the preparative scale. GFX methyl ester was injected as a 9.5 mg/mL sample onto a preparative HPLC column (AS-H) and the separation was performed at a flow rate of 10 mL/min using hexane with 20% ethanol as mobile phase. After a selection of different volumes of column loading, 13 mL of GFX methyl ester in hexane was found to be the best one considering retention, production rate and purity of enantiomers (data not shown). Through extracting and drying in vacuo, 79.2 mg of the first eluted enantiomer and 69.4 mg of the second eluted enantiomer were obtained from the collected elution in 1 h. Individual fractions of enantiomers isolated were re-injected onto the analytical chiral column to determine their enantiomeric purity. Both of the collected fractions provided an enantiomeric excess more than 99% (Figure 2). The collected enantiomers of GFX methyl ester were also hydrolyzed to afford the corresponding enantiomers of GFX. The absolute configurations of two hydrolyzed enantiomers were confirmed by a combination of NMR and polarimetry. The $^1$H NMR results for the two enantiomer hydrolysates are as follow. First eluted enantiomer hydrolysate: (600 MHz, DMSO-d6): 8.69 (1 H, s), 7.74 (1 H, d, $J_{HF}$=12.3 Hz), 4.22-4.11 (1 H, m), 3.75 (3 H, s), 3.14-3.29 (3 H, m), 2.80-3.00 (4 H, m), 0.99-1.12 (7 H, m);

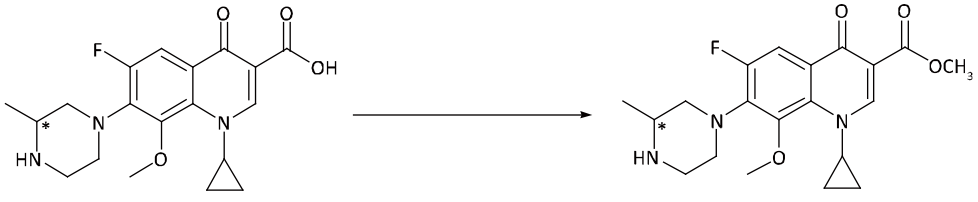


**Figure 1.** Esterification of GFX ratemate. *Represents the Chiral Center.

**Table 1.** Separation Performance of GFX Methyl Ester on AS-H and AD-H Columns

| Mobile Phase | CSPs | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AS-H | | | | AD-H | | | |
| | $T_R$ (min) | | $\alpha$ | $R_S$ | $T_R$ (min) | | $\alpha$ | $R_S$ |
| | P1 | P2 | | | P1 | P2 | | |
| MeOH/DEA (100/0.1) | 2.63 | 2.97 | 1.54 | 1.45 | 2.69 | - | - | - |
| EtOH/DEA (100/0.1) | 5.37 | 6.19 | 1.24 | 1.39 | 5.65 | - | - | - |
| ACN/DEA (100/0.1) | 3.67 | 4.34 | 1.40 | 2.85 | 10.3 | - | - | - |
| HE/EtOH/DEA (70/30/0.1) | 3.56 | 4.11 | 1.36 | 1.80 | 7.51 | 8.23 | 1.13 | 1.97 |
| HE/IPA/DEA (70/30/0.1) | 6.72 | 8.66 | 1.41 | 2.59 | 6.42 | 6.75 | 1.08 | 0.78 |

***Note*.** Flow rate: 1.0 mL/min; column temperature: 35 °C; UV: 254 nm; injection: 2.0 μL; '–' means unretained.

Second eluted enantiomer hydrolysate: (600 MHz, DMSO-d6): 8.69 (1 H, s), 7.72 (1 H, d, $J_{HF}$=12.3 Hz), 4.22-4.11 (1 H, m), 3.75 (3 H, s), 3.13-3.29 (3 H, m), 2.74-2.95 (4 H, m), 0.99-1.10 (7 H, m). The NMR results above were consistent with those reported elsewhere in the literature for GFX[6]. The specific rotation values of the two hydrolyzed enantiomers were as follow. $[\alpha]^{25}_{D}$=-0.094 (*c* 0.001; 70% $H_2O$: DMF=7:3) for the first eluted enantiomer hydrolysate; $[\alpha]^{25}_{D}$=+0.104 (*c* 0.001; 70% $H_2O$: DMF=7: 3) for the second enantiomer hydrolysate. According to previous studies, the *S*-GFX is a levorotatory enantiomer and the *R*-GFX is a dextrorotatory enantiomer[7-8]. Therefore, it is possible to confirm the following absolute configuration and the enantiomeric elution order: *S*-(−)-GFX was the first eluted enantiomer hydrolysate and *R*-(+)-GFX was the second eluted enantiomer hydrolysate.

In order to evaluate the biological selectivity of GFX enantiomers, *Escherichia coli* ATCC 25922 and antibody were used to test the antibacterial activities and the binding affinity of the enantiomers to protein receptor, respectively. The MIC values of *S*-(−)- and *R*-(+)-GFX against *Escherichia coli* ATCC 25922 were both 0.002-0.004 μg/mL, indicating that *S*-(−)- and *R*-(+)-GFX enantiomers had no significant difference on the antibacterial activity as reported by

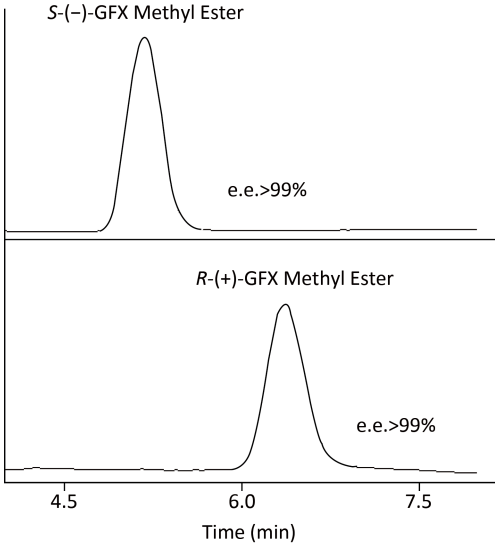


**Figure 2.** Chromatograms of the isolated enantiomers of GFX methyl ester following their reinjection onto the Chiralpak AS-H column. Mobile phase, HE/EtOH/DEA (80/20/0.1, v/v/v); flow rate: 1 mL/min; column temperature: 35 °C; UV: 254 nm; injection: 5 μL.

Breen J[9]. Two antibodies raised by *S*-(−)-GFX (S-antibody) and *R*-(+)-GFX (R-antibody) were used as model binding protein receptor for the enantiomers of GFX. ELISA results showed that the 50% inhibitory concentration ($IC_{50}$) of *S*-(−)-GFX for S-antibody (0.76 ng/mL) was 30 times lower than that of *R*-(+)-GFX (22.3 ng/mL). On the contrary, the $IC_{50}$ of *S*-(−)-GFX for R-antibody (40.9 ng/mL) was 20 times higher than that of *R*-(+)-GFX (2.7 ng/mL). This indicated that both of the two antibodies exhibited higher affinity to their immunizing hapten than to their opposite isomers. It was presumed that the steric hindrance and the molecular interactions between GFX and antibody may contribute to the distinct binding activities of GFX enantiomers to antibody[10]. Although further evaluations are needed to clarify the mechanism of GFX chirality on antibody, the unequal inhibitory activities of GFX enantiomers to antibody in this study has provided the first example that the two enantiomers of GFX are able to bind a specific protein receptor enantioselectively, and this is significantly different with their identical antibacterial activities. Moreover, the generation of enantioselective antibody in current study also provides a prospective to construct an immunoassay method for the rapid analysis of GFX enantiomers in the future. There is no yet any literature about the antibody or immunoassay of GFX enantiomers so far.

In conclusion, a preparative HPLC method of Chiralpak AS-H for the rapid and efficient separation of GFX enantiomers has been developed by using a pre-column esterification strategy. Using HE/EtOH/DEA (80/20/0.1, v/v/v) as the mobile phase, 79.2 mg of the *S*-isomer and 69.4 mg of the *R*-isomer could be well separated within 1 h. And the purity of the two collected enantiomers were more than 99%. After a bioactivity evaluation, it was found that the inhibitory activities of GFX enantiomers to antibody were enantioselective. It is the first time to report the biological chiral selectivity of GFX enantiomers. This will be critically significant to develop an immunoassay for the analysis of GFX at chiral level.

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