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Short communication

High-performance liquid chromatography separation and quantitation of ofloxacin enantiomers in rat microsomes

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Abstract

A sensitive, simple and accurate method for determination of enantiomers of ofloxacin in microsomal incubates was developed by chiral ligand-exchange RP-HPLC with fluorescence detection to examine stereoselective metabolism of ofloxacin in the glucuronidation process. The C_{18} stationary phase was used as analytical column. The solution of chiral mobile phase additive was made up of 6 m*M* L-phenylalamine mixed with 3 m*M* $CuSO_4$ in water. Mobile phase consisted of the solution of chiral mobile phase additive–methanol (86:14).The fluorescence detector was operated at λ_{ex} 330 nm and λ_{em} 505 nm. The flow-rate of mobile phase was set at 1.0 ml/min. The achiral ODS column offers good separation of the two enantiomers in less than 25 min. The recovery of the assay was 97.9±6.1% (n=10) for *S*-ofloxacin and 99.6±6.0% (n=10) for *R*-ofloxacin. The method provides a high sensitivity and good precision (RSD<10%). The LOD was 0.6 μ*M* for both enantiomers and the LOQ was 5.70±0.45 μ*M* (n=8) for *S*-ofloxacin and 5.66±0.47 μ*M* (n=8) for *R*-ofloxacin. The standard curves showed excellent linearity over the concentration range 5.5–2078 μ*M* for *S*-(−)-ofloxacin and *R*-(+)-ofloxacin. The enantioselective method developed has been applied to determine the stereoselectivity of glucuronidation metabolism of ofloxacin optical isomers in rat liver microsomes.

Keywords: Enantiomer separation; Ofloxacin

1. Introduction

A large group of chiral drugs are the pyridone carboxylic acid derivatives which are widely used as bactericidal agents in clinical medicine. One drug in this group is ofloxacin (OFLX), 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. The carbon in position C_3 is chiral which results in two enantiomers, *S*-(−)-OFLX and *R*-(+)-OFLX. *S*-(−)-OFLX is 8–128-times more potent than *R*-(+)-OFLX with the different bacterial strains in vitro [1]. Studies on OFLX pharmacokinetics in humans have shown differences in the pharmacokinetic parameters and in the disposition of the *S*-(−)- and *R*-(+)-OFLX enantiomers [2,3]. OFLX is metabolized in different pathways in humans. One metabolic pathway is glucuronidation, with the carboxyl group present resulting in formation of the corresponding glucuronide conjugate [4].

Several analytical methods for determination of OFLX enantiomers in serum and urine have been reported using high-performance liquid chromatography (HPLC) [3,5,6]. These methods involve de-

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rivatization with chiral reagent, L-leucinamide after activation of the carboxyl group of OFLX with diphenylphosphonic chloride, the use of chiral elutes and chiral stationary phase with bovine serum albumin immobilized on silica gel. The aim of this study was the development of a semi-preparative reversed-phase (RP)-HPLC method to obtain a single enantiomer of OFLX with high optical purity and of enantioselective assay of *S*-(−)- and *R*-(+)-OFLX in rat liver microsomal incubates. The enantioselective assay established was used to examine possible enantiomeric difference in glucuronidation metabolism of OFLX and measure the stereoselective activity of glucuronyltransferase in rat liver microsome.

2. Experimental

2.1. Materials

Racemic ofloxacin and ciprofloxacin (internal standard, I.S.) standard references were kindly donated by the Zhejiang Provincial Institute for Drug Control (Hangzhou, China). Uridine 5-diphosphoglucuronic acid (UDPGA) and tris(hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and solvents were analytical reagent or chromatographic grade and obtained from common commercial sources.

2.2. Chiral ligand-exchange RP-HPLC system

The Shimadzu modular HPLC system employed comprised a LC-6A pump, RF-535 fluorescence monitor. A Rheodyne injector equipped with a 20-μl loop and C-R4A chromatographic data system with recorder were employed.

The analytical column used was packed with C_{18} stationary phase (CLC-ODS 15 cm×4.6 mm I.D., particle size 10 μm, Shimadzu, Kyoto, Japan). The solution of chiral mobile phase additive (CMPA) was made up of 6 m*M* L-phenylalamine mixed with 3 m*M* $CuSO_4$ in water. The mobile phase consisted of CMPA solution–methanol (86:14). The fluorescence detector was operated at λ_{ex} 330 nm and λ_{em} 505 nm. The flow-rate of mobile phase was set at 1.0 ml/min. Chromatographic assay was carried out at room temperature.

2.3. Separation of ofloxacin enantiomers by preparative chiral chromatography

The preparative chiral HPLC conditions were: column: Shim-pack PREP-ODS (25 cm×20 mm I.D., particle size 10 μm); mobile phase: 6 m*M* L-phenylalanine and 3 m*M* copper sulfate containing 14% methanol; flow-rate of mobile phase: 4.6 ml/min; detector: fluorescence (λ_{ex} 330 nm and λ_{em} 505 nm). One mg of racemic OFLX was applied each injection. The elutes containing ofloxacin enantiomers were collected separately. Methanol was removed from the elutes in a water bath at 45°C under vacuum and the residues were saturated with ammonium acetate. The enantiomer was extracted with methylene chloride (4×50 ml). The organic solvent was evaporated to a small volume at 45°C under vacuum. Anhydrous sodium sulfate was added and the liquid was then filtered. The filtrate was evaporated to dryness at 45°C under vacuum. The residue was further dried over phosphorus pentoxide in a vacuum desiccator. The optical activity of enantiomer was estimated by polarimetery (PE 241 polarimeter, Perkin-Elmer). The first peak eluted is *S*-(−)-ofloxacin and the second is *R*-(+)-ofloxacin.

2.4. Incubation and assay

The glucuronidation reaction was started by addition of UDPGA after microsomal incubates were preincubated for 5 min. The reaction was stopped by addition of 1.0 ml of methanol after 30 min at 37°C in a shaking water bath. The incubation mixtures were centrifuged at 1880 *g* for 15 min to remove protein after 50 μl I.S. (1 mg/ml) was added. A 20-μl aliquot of supernatant was injected into the chiral ligand-exchange RP-HPLC system.

3. Results and discussion

3.1. Selectivity and chromatography

To optimize the separation conditions for OFLX enantiomers by using chiral ligand-exchange RP-

HPLC, some effective factors including pH of mobile phase, concentration of organic modifier and concentration of ligands etc. were evaluated. The resolution (R_s) between *S*-(−)- and *R*-(+)-OFLX was 1.69–1.75 when the pH of mobile phase was adjusted to 3.4–4.0. R_s was less than 1.23 when the pH of mobile phase was adjusted to ≤3.1 and Cu^{2+} will be precipitated, which blocks the chromatographic system when the pH was adjusted to ≥4.5. R_s decreased when the concentration of methanol in the mobile phase increased, and increased when the concentration of methanol decreased. So, it is necessary to adjust the methanol concentration in mobile phase in order to get good chromatographic separation of *S*-(−)- and *R*-(+)-OFLX. There are no effects on R_s when 6 m*M* L-phenylalamine and 3 m*M* $CuSO_4$ or 8 m*M* L-phenylalamine and 4 m*M* $CuSO_4$ were used in the CMPA.

Fig. 1A shows the chromatographic separation between *S*-(−)- and *R*-(+)-OFLX using the preparative chiral system. The enantiomeric purity of *S*-(−)- and *R*-(+)-OFLX was 99.14% and 99.29%, respectively, determined by chiral ligand-exchange RP-HPLC (Fig. 1B).

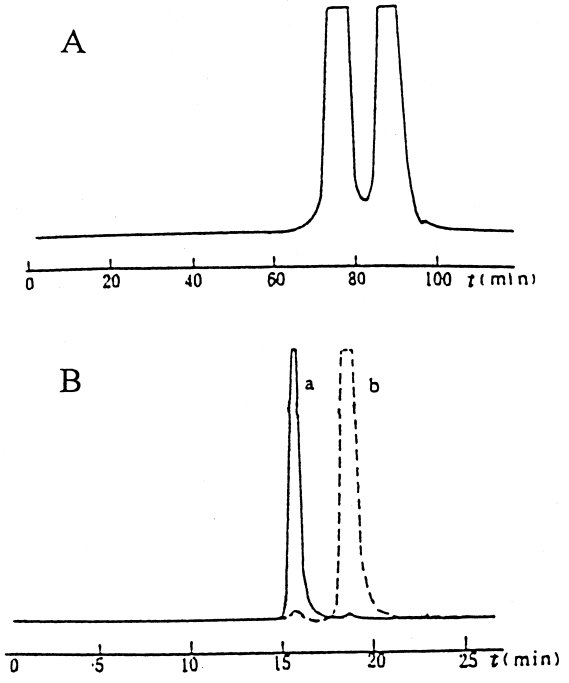

Fig. 1. (A) Chromatogram of (*RS*)-ofloxacin under preparative chiral HPLC conditions. (B) Typical HPLC chromatogram of *S*-(−)-ofloxacin (a) and *R*-(+)-ofloxacin (b).

The pure enantiomers of *S*- and *R*-OFLX were injected into the chiral RP-HPLC system under the same chromatographic conditions with the microsomal samples to identify the chromatographic peaks. *S*-(−)-OFLX eluted at about 13 min and *R*-(+)-OFLX eluted at 15.8 min. There were no interfering peaks found at the same retention time of *S*- and *R*-OFLX and I.S. in the chromatogram of blank microsomal incubate (Fig. 2).

3.2. Linearity of calibration

To assure the reliability of assay, quantitative determinations were carried out in the presence of the I.S. for blank microsomal samples in which different concentrations of enantiomers were spiked. Chiral RP-HPLC showed good linearity throughout the concentration range from 5.54 to 2078 μ*M* for both enantiomers. The concentration of OFLX enantiomer (x) was related to the ratio of peak height (y) as follows: $y=3.602x+0.052$ ($r=0.9992$, $n=12$) for *S*-(−)-OFLX; $y=3.421x+0.022$ ($r=0.9993$, $n=12$) for *R*-(+)-OFLX.

3.3. Recovery

The blank microsomal samples spiked with OFLX enantiomer were analyzed according to the method described in Section 2.4. The recovery of assay was calculated by comparison of peak heights in microsomal samples with those in corresponding standard solutions. The recoveries from microsomal samples at concentrations of 11.1 and 41.6 μ*M* ($n=5$) was 97.5±9.4% and 98.3±2.8% for *S*-OFLX and 99.4±8.5% and 99.8±3.5% for *R*-OFLX. The overall average recovery was 97.9±6.1% ($n=10$) for *S*-OFLX and 99.6±6.0% ($n=10$) for *R*-OFLX.

3.4. Limits of detection and quantitation

To determine the limit of detection (LOD) and the limit of quantitation (LOQ), blank microsomal samples were spiked with small amounts OFLX enantiomer and analyzed according to Section 2.4. The results indicate that the LOD was 0.6 μ*M* for both OFLX enantiomers and the LOQ calculated with a

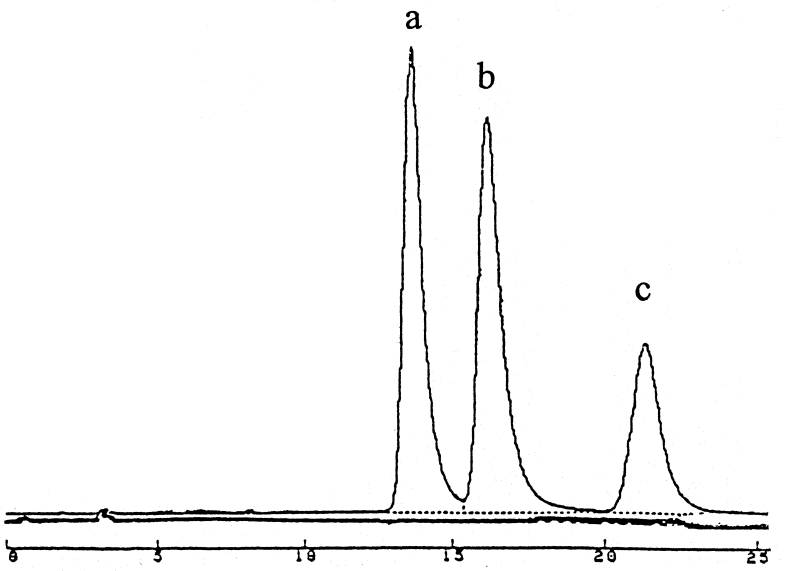

Fig. 2. Chromatograms of a blank microsomal sample (bottom) and a microsomal sample spiked with 41.6 μ*M* OFLX enantiomer (top). Peaks: a=*S*-(−)-OFLX, b=*R*-(+)-OFLX, c=ciprofloxacin (I.S.).

standard curve was 5.70±0.45 μ*M* (*n*=8) for *S*-OFLX and 5.66±0.47 μ*M* (*n*=8) for *R*-OFLX.

3.5. Repeatability and accuracy

The blank microsomal samples spiked with 5.54 and 277.0 μ*M* OFLX enantiomer were analyzed according to Section 2.4 along with calibration curves for each batch samples. The concentration of each sample was calculated from the calibration curve. The intra-assay relative standard deviations (*n*=4) were 9.4% and 2.6% for *S*-OFLX and 9.8% and 2.2% for *R*-OFLX. The inter-assay relative standard deviations (*n*=4) were 2.1% and 0.66% for *S*-OFLX and 2.9% and 1.1% for *R*-OFLX.

3.6. In vitro studies on the chiral glucuronidation metabolism of OFLX

The microsome induced by β-naphthoflavone were suspended in phosphate buffer (pH 7.8) as incubation media. Racemic OFLX was added into the incubation media as substrate at concentrations from 27.7 μ*M* to 969.5 μ*M* for each enantiomer. The sample of each concentration was duplicate. The enzymatic parameters were calculated from the Michaelis–Menten equation. V_{max} (μ*M*/min/mg) and K_m (m*M*) were 0.434±0.07 and 0.17±0.03 for *S*-(−)-OFLX and 0.39±0.06 and 0.16±0.03 for *R*-(+)-OFLX.

4. Conclusions

The method described in this paper can separate the enantiomers of OFLX at an analytical and preparative scale and the method is easy to carry out, allows direct separation of enantiomers and is economic. In conclusion, a sensitive, simple and accurate method for determination of enantiomers of OFLX in microsomal incubates was developed by chiral ligand-exchange RP-HPLC with fluorescence detection. The achiral ODS column offers good separation of the two enantiomers in less than 25 min. The chiral assay established provides good linearity and a wide range, high sensitivity, and has been applied to study possible stereoselectivity of OFLX glucuronidation in rat liver microsomes.

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