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Analysis of flurbiprofen, ketoprofen and etodolac enantiomers by pre-column derivatization RP-HPLC and application to drug–protein binding in human plasma

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Abstract

A stereoselective reversed-phase high-performance liquid chromatography (HPLC) assay to determine the enantiomers of flurbiprofen, ketoprofen and etodolac in human plasma was developed. Chiral drug enantiomers were extracted from human plasma with liquid–liquid extraction. Then flurbiprofen and ketoprofen enantiomers reacted with the acylation reagent thionyl chloride and pre-column chiral derivatization reagent (*S*)-(−)-α-(1-naphthyl)ethylamine (*S*-NEA), and etodolac enantiomers reacted with *S*-NEA using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT) as coupling agents. The derivatized products were separated on an Agilent Zorbax C_{18} (4.6 mm × 250 mm, 5 μm) column with a mixture of acetonitrile–0.01 mol·L^{-1} phosphate buffer (pH 4.5) (70:30, v/v) for flurbiprofen enantiomers, acetonitrile–0.01 mol·L^{-1} phosphate buffer (pH 4.5) (60:40, v/v) for ketoprofen enantiomers and methonal–0.01 mol·L^{-1} potassium dihydrogen phosphate buffer (pH 4.5) (88:12, v/v) for etodolac enantiomers as mobile phase. The flow of mobile phase was set at 0.8 mL·min^{-1} and the detection wavelength of UV detector was set at 250 nm for flurbiprofen and ketoprofen enantiomers and 278 nm for etodolac enantiomers. The assay was linear from 0.5 to 50 μg·mL^{-1} for each enantiomer. The inter- and intra-day precision (R.S.D.) was less than 10% and the average extraction recovery was more than 87% for each enantiomer. The limit of quantification for the method was 0.5 μg·mL^{-1} (R.S.D. < 10%, $n = 5$). The method developed was used to study the drug–protein binding of flurbiprofen, ketoprofen and etodolac enantiomers in human plasma. The results showed that the stereoselective binding of etodolac enantiomer was observed and flurbiprofen and ketoprofen enantiomers were not.

Keywords: Flurbiprofen; Ketoprofen; Etodolac; Enantiomer; RP-HPLC; Drug–protein binding

1. Introduction

Most non-steroidal anti-inflammatory drugs (NSAIDs), such as flurbiprofen [(±)-2-(2-fluorobiphenyl-4-yl)propionic acid], are potent used clinically as the racemate [1]. NSAIDs are non-selective inhibitor of prostaglandin biosynthesis in humans and indicated for the acute or long-term treatment of the signs and symptoms of rheumatoid arthritis and osteoarthritis. It was established that two enantiomers of NSAID exert different pharmacodynamic effects [1]. The binding of drugs to plasma proteins is an important factor, which determines the pharmacokinetics and pharmacological effects of drugs. A clear understanding of the plasma protein binding behavior of the enantiomers is therefore fundamental to their safe and rational use. Hence, it is necessary to stereoselectively detect and quantify each enantiomer in biological media [2]. Over the last two decades, several analytical methods have been reported for the enantioselective determination of NSAID enantiomers. Most of these methods involved direct separation using chiral high-performance liquid chromatographic (HPLC) columns and pre-column derivatization with optical pure chiral reagents. The direct separation of enantiomers has been accomplished on various types of columns, such as α_1-acid glycoprotein [3,4], vancomycin [5] and cellulose [6]. However, chiral HPLC columns are expensive and used for limited chiral drugs. The chiral derivatizing agents used in the separation of flurbiprofen enantiomers included L-leucinamide [7], *S*-(α)-methylbenzyamine [8], (*S*)-(−)-1-phenylethylamine [9], etc.,

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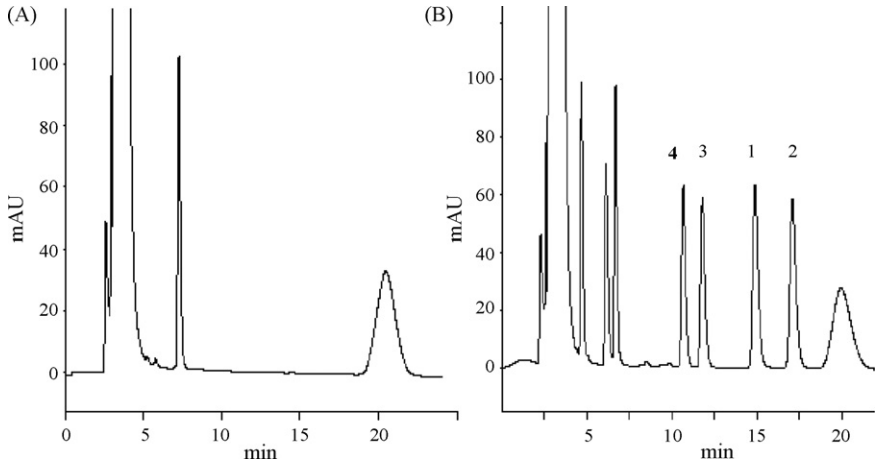

Fig. 1. HPLC chromatograms of flurbiprofen enantiomers. (A) Blank plasma and (B) blank plasma spiked with racemic flurbiprofen and ketoprofen. Peaks: (1) *S*-flurbiprofen; (2) *R*-flurbiprofen; (3) *R*-ketoprofen (I.S.); (4) *S*-ketoprofen.

but the most procedures were time-consuming for pre-column derivatization and sample preparation. It has been reported that (*S*)-(−)-α-(1-naphthyl)ethylamine (*S*-NEA) was a useful chiral derivatizing agent. Its amino group can react with the carboxylic group of NSAIDs forming the amides and the diastereoisomeric amides can be resolved on routine HPLC columns [10–12]. However, there is no report of the *S*-NEA to separate flurbiprofen enantiomers. In this paper, a chiral HPLC method for the resolution of flurbiprofen, ketoprofen and etodolac enantiomers using *S*-NEA as a pre-column derivatizing agent in human plasma was described. The derivatizing procedure was simplified and the derivatizing time was shortened.

2. Experimental

2.1. Materials

Racemic flurbiprofen, ketoprofen and etodolac (purity > 99.6%) were kindly provided by Jiu Zhou Pharmaceutical Limited Company (Zhejiang, China). *S*-(+)-Ketoprofen (purity > 99.8%) was obtained from Southwest Pharmaceutical Manufactory (China). (*S*)-(−)-α-(1-Naphthyl)ethylamine, purity is 99% and it is stable at 4 °C) were purchased from J&K Chemical (Tokyo, Japan). 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT) were obtained from Sigma (St. Louis, MO, USA). Triethylamine (TEA) was obtained from Shanghai Chemical Reagent Plant (Shanghai, China). Thionyl chloride was obtained from Mei Xing Chemical Co. Ltd. (Shanghai, China). Drug-free human plasma was supplied by Ningbo Blood Center (Ningbo, Zhejiang, China). All solvents used were HPLC grade and all chemicals were analytical grade.

2.2. Equipment and chromatographic conditions

Chromatographic analyses were carried out on an Agilent 1100 series liquid chromatograph (Agilent Technologies Corporation, USA) equipped with a UV detector. An Agilent Zorbax C_{18} column (4.6 mm × 250 mm, 5 μm) with an ODS guard column (10 μm, 10 mm × 5 mm I.D.) was utilized. For assaying flurbiprofen and ketoprofen eanatiomers, the mobile phase was composed of acetonitrile–0.01 mol·L^{-1} potassium

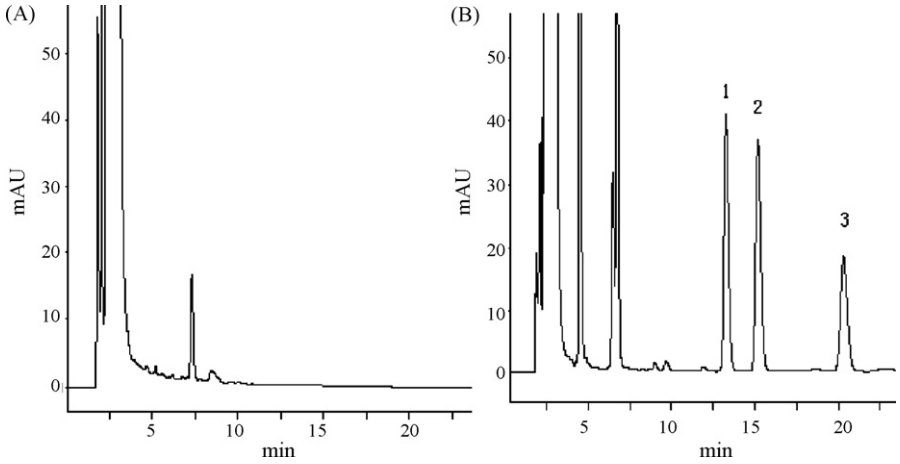

Fig. 2. HPLC chromatograms of ketoprofen enantiomers. (A) Blank plasma and (B) blank plasma spiked with racemic ketoprofen and flurbiprofen. Peaks: (1) *S*-ketoprofen; (2) *R*-ketoprofen; (3) *S*-flurbiprofen (I.S.).

Table 1
Calibration curves of assay

Drug	*S*-Enantiomer	*R*-Enantiomer
Flurbiprofen	$Y = 1.6261C + 0.0561$, $r = 0.999$	$Y = 1.6541C + 0.0321$, $r = 0.999$
Ketoprofen	$Y = 0.1214C + 0.0170$, $r = 0.999$	$Y = 0.1264C + 0.0156$, $r = 0.999$
Etodolac	$Y = 121.92C + 104.41$, $r = 0.999$	$Y = 116.60C + 117.97$, $r = 0.999$

r is the correlation coefficient.

dihydrogen phosphate buffer (pH 4.5) (70:30, v/v) for flurbiprofen eanatiomers and acetonitrile–0.01 mol·L^{-1} potassium dihydrogen phosphate buffer (pH 4.5) (60:40, v/v) for ketoprofen eanatiomers with the flow rate of 0.8 mL·min^{-1} and the detection wavelength of detector was set at 250 nm. For assaying etodolac eanatiomers, the mobile phase was composed of methonal–0.01 mol·L^{-1} potassium dihydrogen phosphate buffer (pH 4.5) (88:12, v/v) and the detection wavelength was set at 278 nm.

2.3. Preparation of spiked human plasma samples

Appropriate amounts of the 10, 50 and 100 μg·mL^{-1} standard solutions of flurbiprofen, ketoprofen and etodolac were taken, and evaporated to dryness under a gentle stream of air. Then the residues were reconstituted with drug-free human plasma to give final enantiomer concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 μg·mL^{-1} for each enantiomer of flurbiprofen, ketoprofen and etodolac. *R*-Ketoprofen was used as internal standard for assaying flurbiprofen enantiomers and *S*-flurbiprofen was used as internal standard for assaying ketoprofen enantiomers.

2.4. Extraction and derivatization

To 150 μL of plasma samples, 20 μL of the internal standard solution (100 μg·mL^{-1}) and 100 μL of 1 mol·L^{-1} sulfuric acid were added in a 10 mL tube. The mixture was extracted with 2 mL of dichloromethane. After vortexing for 3 min, the samples were centrifuged for 10 min at 3000 rpm. Then, the organic layer was transferred to another tube and evaporated to dryness under a gentle stream of nitrogen. For assaying flurbiprofen and ketoprofen eanatiomers, 100 μL of 1% TEA (in dichloromethane) and 100 μL of thionyl chloride (2% in dichloromethane) were added to the residue. The reaction was made at 30 °C for 30 min. The reaction mixture was evaporated to dryness under a gentle stream of nitrogen. An aliquot of 100 μL of 2.5 mg·mL^{-1} *S*-NEA (in dichloromethane) was added to the residue. The reaction was continued at 30 °C for 30 min. After chiral derivatization was completed, the formed product was evaporated to dryness under a gentle stream of nitrogen. For assaying etodolac enantiomers, 50 μL of 2.5 mg·mL^{-1} HOBT (in 1% pyridine), 100 μL of EDC (2.5 mg·mL^{-1} in dichloromethane) and 50 μL of *S*-NEA (5 mg·mL^{-1} in dichloromethane) were added to the residue. The reaction was carried out at 30 °C for 2 h. The reaction products were evaporated under vacuum. The residue was reconstituted with 100 μL of mobile phase. Finally, an aliquot of 20 μL of the resulting solution was injected into the HPLC system.

2.5. Enantiomers–protein binding in human plasma

The present assay has been used to quantify the concentration of each enantiomer of flurbiprofen, ketoprofen and etodolac in human plasma for drug–protein-binding study. The plasma samples were equilibrated at 37 °C for 15 min prior to ultrafiltration. The ultrafiltration was performed using an Amicon microcon and centricon centrifugal device with the filter membrane of 30,000 M_r cut-off. Samples of 0.5 mL volume were centrifuged at 10,000 × *g* (37 °C) for 5 min. The plasma samples and ultrafiltrates, approximately 150 μL, were then analyzed as described in Section 2.4.

3. Results and discussions

3.1. Validation study

3.1.1. Selectivity

Figs. 1–3 show the typical chromatograms of enantiomer derivatives flurbiprofen, ketoprofen and etodolac in blank human plasma and spiked samples. The *R* and *S* enantiomers and the internal standard were completely separated from the matrix compounds under the chromatographic conditions employed.

3.1.2. Linearity of calibration

Calibration curves were constructed by performing a regression linear analysis of the peak area ratios of the enantiomer to the internal standard or the peak area of the enantiomer (Y) versus the enantiomer concentrations (C). The calibration curves of the two enantiomers were linear over the concentration ranges studied (Table 1).

3.1.3. Precision and accuracy

The intra- and inter-day precision and accuracy were obtained by analyzing the spiked samples at three different concentrations of 1.0, 5.0 and 50.0 μg·mL^{-1} for the enantiomers in five replicates within 1 day and on 5 consecutive days, respectively. Accuracy of this assay was calculated from calibration curves. The extraction recoveries of the enantiomers were assessed at three concentration levels of 1.0, 5.0 and 50.0 μg·mL^{-1}. The extraction recoveries of the enantiomers were calculated in five replicates by comparing the enantiomeric peak area ratios from the spiked plasma samples with the peak area ratios obtained from a standard solution containing the same concentration of enantiomers. The results are shown in Table 2.

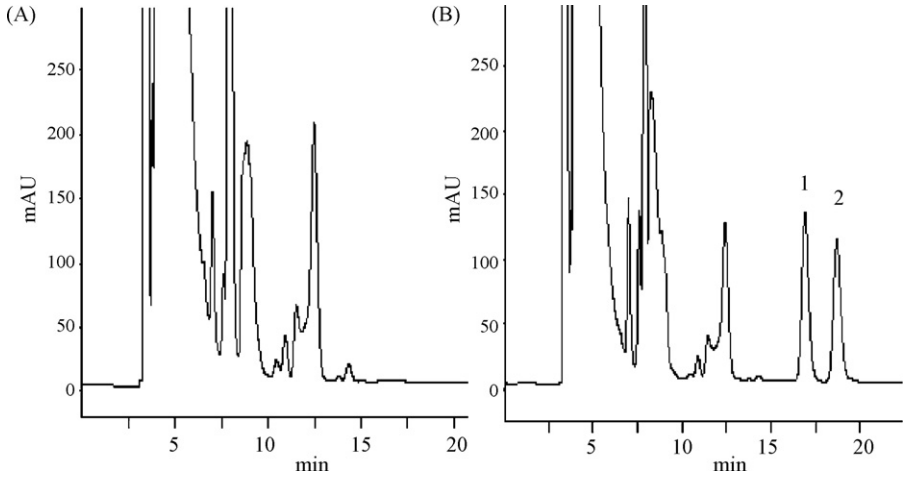

Fig. 3. HPLC chromatograms of e etodolac nantiomers. (A) Blank plasma and (B) blank plasma spiked with racemic etodolac. Peaks: (1) *S*-etodolac; (2) *R*-etodolac.

3.1.4. Sensitivity

The lower limit of detection, at a signal-to-noise ratio of 3, was about 0.15 $\mu g \cdot mL^{-1}$ in plasma for each enantiomer of flurbiprofen, ketoprofen and etodolac. And the lower limit of quantification, defined as the lowest enantiomeric concentration which can be quantitatively determined with suitable precision and accuracy (signal-to-noise ratio of 10) was 0.5 $\mu g \cdot mL^{-1}$ (R.S.D. < 10%, $n = 5$) in plasma for each enantiomer of flurbiprofen, ketoprofen and etodolac.

3.2. Optimization of derivatization

Separation of enantiomers using derivatizing agents is useful and economic method for analyzing chiral drug [13–15]. The carboxylic group of profen reacts with the amino group of *S*-NEA forming the amide. During the derivatization of profen enantiomers, the amounts of *S*-NEA and thionyl chloride, reaction time and reaction temperature for the conversion of enantiomers to their corresponding diastereomers were optimized. An excess of *S*-NEA and thionyl chloride was needed in derivatization in order to complete the reaction. The results (Fig. 4) showed that when the molar amount of *S*-NEA and thionyl chloride was increased to 5 and 50 times that of the flurbiprofen enantiomers, respectively, the yield of each enantiomer reached to a maximum; and that the formation of the diastereomers was completed within 30 min at 30 °C. It is difficult to separate etodolac enantiomers by using same method as flurbiprofen and ketoprofen so EDC and HOBT were used as coupling agent. And the amounts of *S*-NEA, EDC and HOBT for the conversion of etodolac enantiomers to their corresponding diastereoisomers were optimized. The results (Fig. 5) showed that when the molar amount of EDC and *S*-NEA was increased to five times that of the etodolac enantiomers, the yield of each enantiomer reached to a maximum. Additional amounts of *S*-NEA, thionyl chloride and EDC did not spoil the chromatogram.

3.3. Application to enantiomers–protein binding study

The binding of flurbiprofen, ketoprofen and etodolac enantiomers to Chinese plasma is shown in Figs. 6–8. The bound fraction of each enantiomer of flurbiprofen and ketoprofen was about 90% at the concentration of 3 $\mu g \cdot mL^{-1}$. The bound fraction decreased with the increasing of the enantiomeric concentration of flurbiprofen and ketoprofen. When the concentration of enantiomer was at 50 $\mu g \cdot mL^{-1}$, the bound fraction dropped to about 70%. Enantiomers of flurbiprofen and ketoprofen displayed concentration dependent and extensive binding

Table 2
Recovery and precision of assaying enantiomers in human plasma ($n = 5$, $\bar{x} \pm S$)

Drug	Spiked amount ($\mu g \cdot mL^{-1}$)	Extraction recovery (%)		Precision (%)			
		S	*R*	Intra-day		Inter-day	
				S	*R*	*S*	*R*
Flurbiprofen	1	89.8 ± 4.1	91.4 ± 4.1	4.0	3.1	5.2	5.3
	5.0	89.4 ± 3.0	90.3 ± 3.0	3.5	3.8	3.8	3.0
	50.0	87.4 ± 2.6	88.5 ± 2.8	1.0	1.4	1.7	2.0
Ketoprofen	1	88.0 ± 3.6	90.4 ± 4.6	4.0	4.7	4.7	4.6
	5.0	87.7 ± 4.1	88.4 ± 3.5	3.0	3.0	4.3	3.7
	50.0	88.7 ± 1.1	89.8 ± 1.9	0.87	0.79	1.5	1.6
Etodolac	1	93.2 ± 4.9	92.7 ± 4.5	7.2	6.5	8.1	7.9
	5.0	92.4 ± 3.7	91.8 ± 3.9	6.6	6.7	7.1	6.4
	50.0	90.3 ± 3.5	89.41 ± 4.0	2.8	2.8	5.7	4.1

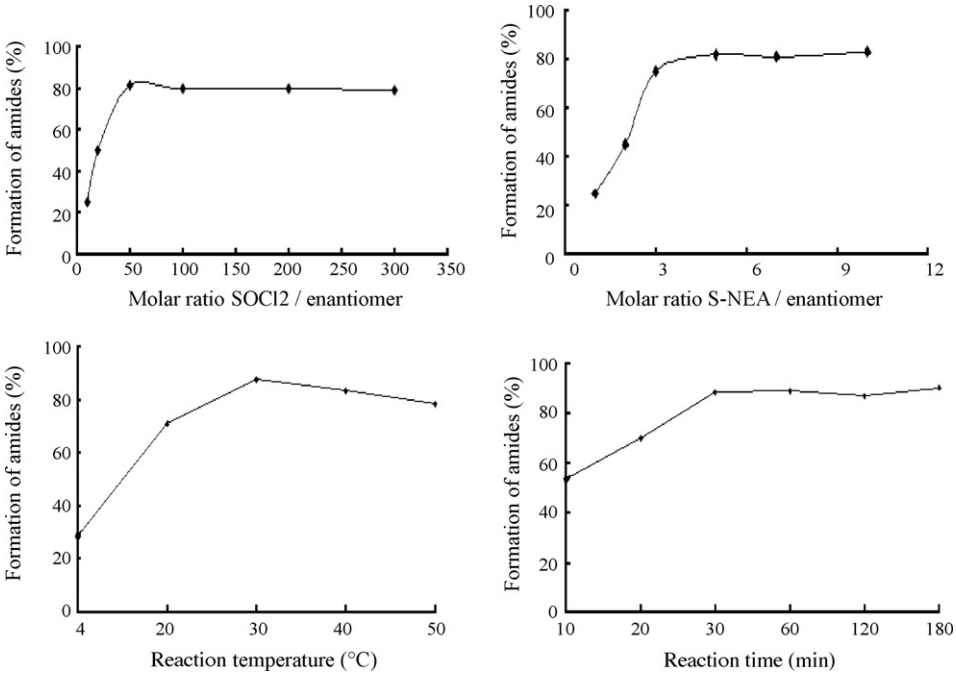

Fig. 4. Effect of reagent concentration, reaction temperature and reaction time on the formation of the diastereoisomeric amides formed by reaction of racemic flurbiprofen and *S*-NEA (100% of *y*-axis means complete reaction).

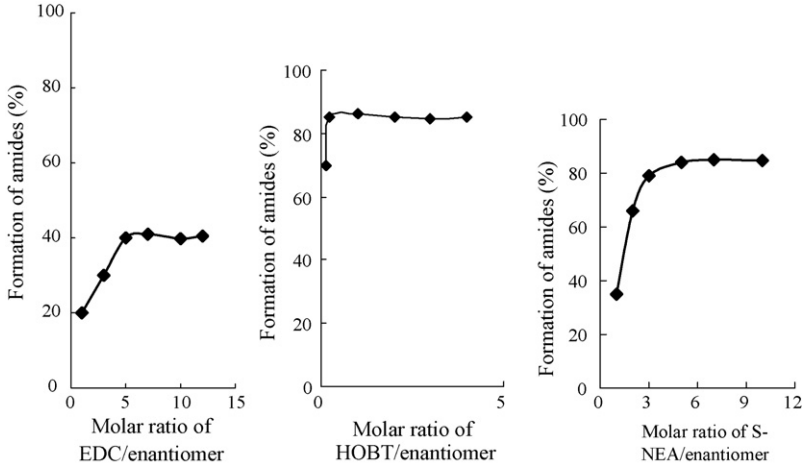

Fig. 5. Effect of reagent concentration on the formation of the diastereoisomeric asmides formed by reaction of racemic etodolac and *S*-NEA (100% of *y*-axis means complete reaction).

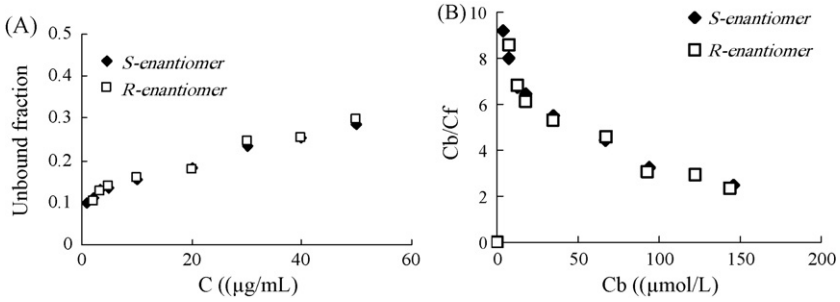

Fig. 6. The binding of flubiprofen enantiomers to human plasma. (A) Unbound fraction of flubiprofen enantiomers at various initial concentrations. (B) Scatchard plots for the protein binding of flubiprofen enantiomers.

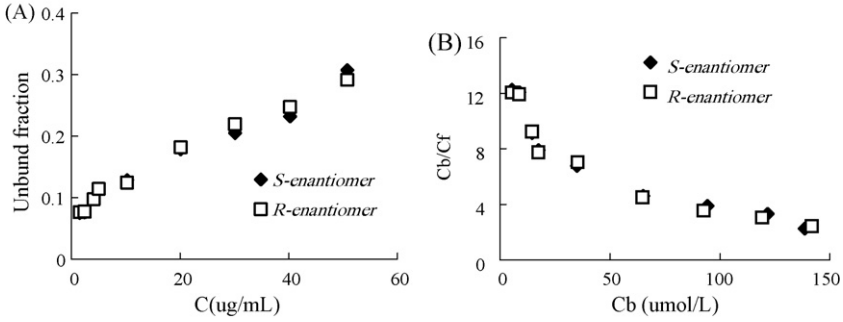

Fig. 7. The binding of ketoprofen enantiomers to human plasma. (A) Unbound fraction of ketoprofen enantiomers at various initial concentrations. (B) Scatchard plots for the protein binding of ketoprofen enantiomers.

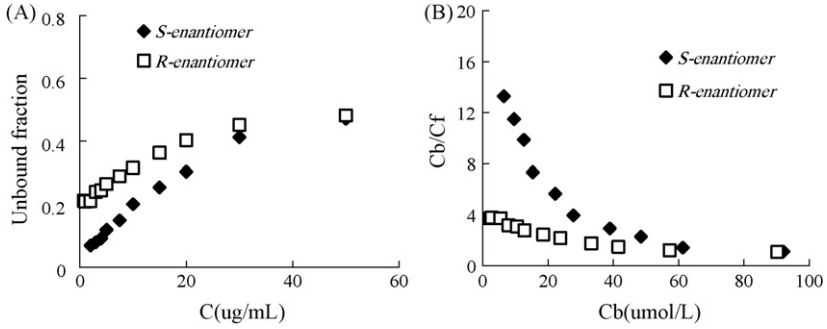

Fig. 8. The binding of etodolac enantiomers to human plasma. (A) Unbound fraction of etodolac enantiomers at various initial concentrations. (B) Scatchard plots for the protein binding of etodolac enantiomer.

to Chinese plasma. However, the unbound fraction ratio of *S*-/*R*-etodolac was from 0.30 to 0.98 with the enantiomeric concentration from 0.5 to 50 $\mu g \cdot mL^{-1}$.

Flurbiprofen displayed concentration dependent and extensive binding to human plasma [16,17]. Scatchard plots showed that there were two classes of binding sites for the enantiomers of flurbiprofen on human plasma: one with high-affinity and the other with low-affinity. Binding to the primary (high-) affinity sites occurs when the concentration of flurbiprofen is low as it can be seen from scatchard plots. With the increasing of the concentration of flurbiprofen, high-affinity binding sites become saturated and flurbiprofen primarily binds to the low-affinity binding sites. Ketoprofen and etodolac enantiomers showed similar characteristics with flurbiprofen enantiomers' binding to human plasma. The binding to high-affinity site was only slightly greater in the case of the *S*-flurbiprofen compared to the *R*-flurbiprofen. The binding of ketoprofen enantiomer was not stereoselective. The enantioselectivity was observed in etodolac enantiomers' binding to Chinese plasma and *S*-etodolac was greater than that of *R*-etodolac.

4. Conclusion

The stereoselective HPLC methods were developed and validated for the rapid quantitative determination of flurbiprofen, ketoprofen and etodolac enantiomers in human plasma. The method was reliable, accurate and suitable for determining concentration of the enantiomers of flurbiprofen, ketoprofen and etodolac in human plasma.

Acknowledgments

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