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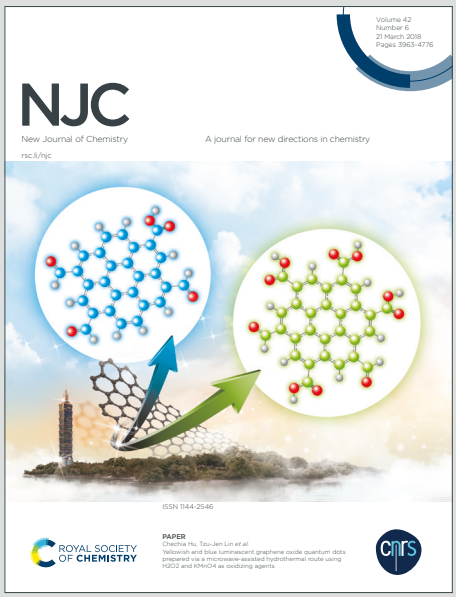

# Chiral liquid chromatography–mass spectrometry (LC–MS/MS) method development with β-cyclodextrin (β-CD) derivatized chiral stationary phase for the enhanced separation and determination of flurbiprofen enantiomers: Application to a stereoselective pharmacokinetic study

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**Abstract**

A method was developed and validated for the enantioselective determination of flurbiprofen in rat plasma using liquid chromatography/electrospray ionization-tandem mass spectrometry under reversed-phase elution mode. Two polysaccharide derivatized chiral stationary phases and a homemade β-cyclodextrin derivatized based chiral column were evaluated. The latter one per-4-chlorophenylcarbamate-β-cyclodextrin bonded chiral stationary phase which was synthesized in our lab enabled the highly sensitive detection and complete separation (resolution 2.0) of the flurbiprofen enantiomers. The assay was carried out after the solid-phase extraction procedure with C18 cartridges, and with *R*-(−)-ibuprofen used as the internal standard. The developed method has been validated for specificity, linearity, accuracy, precision, recovery, matrix effect, stability, carry over effect and dilution effect. The lower limit of quantification for *R*-flurbiprofen and *S*-flurbiprofen was 10 ng $mL^{-1}$ in rat plasma, respectively. Linearity was confirmed in the range of 10.0–20000.0 ng $mL^{-1}$ with a correlation coefficient ($r^2$) greater than 0.996. The established method was successfully applied to a stereoselective pharmacokinetic study of flurbiprofen enantiomers in rat plasma following oral administration.

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**Key words:** cyclodextrin derivatized chiral stationary phase, flurbiprofen enantiomers, LC–MS/MS, stereoselective pharmacokinetics

## 1. Introduction

The 2-arylpropionic acids (2-APAs) are an important group of nonsteroidal anti-inflammatory drugs (NSAIDs) which are widely used for the treatment of pain and inflammation in rheumatic and musculoskeletal disorders [1-2]. Epidemiological researches have demonstrated that long-term use of this class of drugs can reduce Alzheimer's disease risk development and delay its onset [3-5]. In the last few decades, 2-APAs analyses have been the focus of intensive pharmaceutical research, since these drugs possess a stereogenic center in the propionic acid moiety. Enantiomers of chiral 2-APAs show identical physicochemical properties but may differ in their biological properties such as pharmacology, pharmacokinetics, metabolism and toxicology [6]. One enantiomer may be effective, while the other could be inactive or responsible for side or antagonist effects, despite this, most chiral 2-APAs intended for either human or animal use are still administered as racemates [7].

Flurbiprofen (FBP), 2-(2-fluorobiphenyl-4-yl) propanoic acid, is a commercially available anti-inflammatory/analgesic of the chiral 2-APAs class. Clinically, it is used as a racemate for the management of pain and inflammation in patients with rheumatoid arthritis and osteoarthritis [8]. Enantiospecific differences of the two enantiomers in pharmacological properties have been reported after administration of FBP racemates, since the *S*-(+) form was much more effective in cyclooxygenase inhibition which was responsible for the anti-inflammatory activity and has been also related to a neuroprotective function, whereas the other form performed the very undesirable side effects, such as gastrointestinal irritation [9].

Several methods have been established for the determination of racemic FBP in plasma samples including high-performance thin-layer chromatography (HPTLC) [10], gas chromatography with mass spectrometry [11], high-performance liquid chromatography (HPLC) coupled with ultra-violet/visible (UV/Vis) [12-13],

fluorescence [14-16], and tandem mass spectrometric detection [8,17-18]. However, only a few HPLC methods have been reported for the stereoselective determination of FBP in plasma [19-22]. In these stereoselective assays, chiral separations were obtained by the use of pre-column derivatization or chiral stationary phases (CSPs). Since the reported methods [19,20,22] for the determination of FBP enantiomers in plasma needed a derivatization reaction with a tedious workup procedure, the HPLC method by CSP emerged as a powerful method for chiral separation of FBP. Teng et al. [21] have reported a stereospecific HPLC-UV analysis of FBP on a Chiralpak AD-RH column, but the sensitivity of this method was poor (50 ng $mL^{-1}$ for each enantiomer) and the analysis time was approximately 20 min. Moreover, the commercial CSP used for chromatographic separation sometimes requires specific elution mode, such as normal-phase mode and reversed-phase mode, and often it is very expensive. Thus, there is a need for new packing materials offering higher enantioselectivity with the simplified preparation process and shortened analysis time. By far, cyclodextrin (CD) derivatives have been used extensively in CSPs ascribing to their ability to accommodate guest molecules through the formation of inclusion complexes. Although there has emerged a series of CD CSPs developed via different ways, there still exist many challenges in this area. On one hand, the development of stable and functional linkages is relatively difficult. On the other hand, CD rims modification usually enhances CSP's enantioselectivity toward specific categories of enantiomers while diminishing its original recognition ability to some enantiomers. In other words, the drugs that can be chirally separated on each CSP is limited. Hence, it is a great challenge to explore novel approaches for the fabrication of more versatile CD CSPs. Recently, we reported a β-CD derivatized CSP by one step amino linkage immobilized to silica gel [23]. The column with per-4-chlorophenylcarbamate-β-CD bonded CSP (CPCDP) exhibited more excellent chiral recognition ability for azole antifungal agents, proton pump inhibitors and dihydropyridine calcium antagonists in normal phase mode than its analogy, commercial 3,5-dimethylphenyl carbamate-β-CD based CSP, due to different position of electro-withdrawing or electron-donating functionalities on phenylcarbamate moiety. The enhanced enantioselectivities were attributed to the enlarged difference in the formed temporary complexes aroused from multiple intermolecular interactions between CSP and solutes, which may include inclusion complexation, π–π stacking interaction, hydrogen-bonding, dipole–dipole interaction

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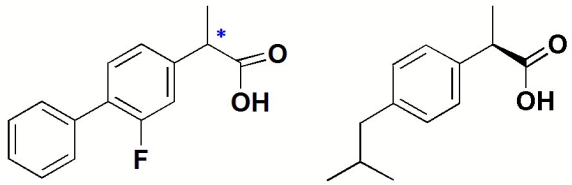
and steric hindrance.

The present study focused on the further exhibited enhanced enantioselective ability of the per-4-chlorophenylcarbamate-β-CD bonded CSP (CPCDP) towards FBP enantiomers. An efficiency chiral separation method with reversed-phase was developed and interfaced with a simple and reproducible LC-MS/MS method system. The relatively high-resolution value was obtained with the lower LLOQ (10 ng $mL^{-1}$ for each enantiomer) in a shorter analysis time (10 min), and the validated method was successfully applied to a stereoselective pharmacokinetic study in the rat after oral administration of racemic FBP.




(a) (b)

**Fig. 1.** The chemical structures of (a) FBP and (b) *R*-(−)-IBF (IS).

## 2. Materials and methods

### 2.1. Chemicals and consumables

Racemic flurbiprofen (FBP) was purchased from the National Institute for Food and Drug Control (Beijing, China). *R*-(−)-ibuprofen (IBF) was obtained from Sigma-Aldrich (St. Louis, USA). Their chemical structures were shown in Fig.1. Ultrapure water was purified by the Milli-Q academic water purification system (Millipore, MA, USA) and was used in all experiments. Methanol (MeOH) and acetonitrile (ACN) of HPLC grade were procured from Fisher Scientific (Fair Lawn, NJ, USA). Other HPLC grade solvents, n-hexane (n-hex), isopropanol (IPA), diethyl ether (DEE) and ethyl acetate (EtAc) were supplied by Concord Technology (Tianjing, China). Analytical-grade ammonium acetate and glacial acetic acid were provided by Sigma-Aldrich (St. Louis, USA). Formic acid (analytical grade) was purchased from Tianjin Heng Xing Chemical Reagent Co., Ltd. (Tianjin, China). The cartridge used for SPE, Sep-pak C18 (1 cc/50 mg), Sep-pak $NH_2$ (1 cc/50mg) and Oasis HLB (1 cc/30mg) were from Waters

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Corporation (Waters Co., Milford, MA, USA).

### 2.2. Preparation of standards, reagents and calibration solutions

A target amount of 25 mg racemic FBP was accurately weighed on an analytical balance and dissolved with MeOH in a 25 mL amber-colored volumetric flask to make a storage solution with a concentration of 1.00 mg $mL^{-1}$. A methanolic stock solution of *R*-(−)-IBF (internal standard, IS) was prepared similarly with the target concentration of 0.1 mg $mL^{-1}$. This solution was diluted with MeOH to make a working IS solution of 5 µg $mL^{-1}$. Calibration standards were obtained daily from the stock solution of FBP by sequential dilution with blank rat plasma, yielding a series of final concentrations namely, 0.01, 0.05, 0.10, 0.20, 1.00, 5.00, 10.0 and 20.0 µg $mL^{-1}$ for *R*-(−)- and *S*-(+)-FBP in plasma. Similarly, quality control (QC) samples were made from the stock solution of FBP at the concentrations of 0.02 (low quality control, LQC), 1.00 (medium quality control, MQC) and 16.0 µg $mL^{-1}$ (high quality control, HQC) for each enantiomer of FBP. These solutions were protected from light and stored at 4 °C before use.

### 2.3. Sample preparation

FBP was extracted from plasma samples using a solid-phase extraction method with Sep-pak $C_{18}$ cartridge (1 cc/50 mg). Briefly, previously frozen plasma samples were thawed and vortexed and 100 µL was transferred to a centrifuge tube then 10 µL aliquot of the IS working solution was added followed by 10 µL of 0.1% (v) formic acid. The tube was vortexed for 3 min. The cartridge was preconditioned with 3 mL of MeOH and then equilibrated with 3 mL of water. Then, the sample solution was loaded onto the SPE column. When the sample had eluted under gravity, the column was washed with 5% (v) MeOH in water (1 mL). Vacuum was applied to the cartridge for 2 min to completely dry the resin. Finally, elution was carried out with 1 mL MeOH into pre-labeled tubes, and the elute was evaporated to dryness under a gentle stream of nitrogen at the ambient temperature. The obtained residue was reconstituted with 100 µL mobile phase and vortexed for 3 min. After filtering through a 0.22 µm nylon syringe filter, an aliquot of 10 µL was used for injection in the chromatographic system.

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## 2.4 Chiral LC–MS/MS

All sample analysis was carried on an Agilent (Palo Alto, CA, USA) 1200 HPLC system coupled to an Agilent 6410 triple-quadrupole mass spectrometer with electrospray ionization. The enantioseparation of FBP was performed on the CDCDP column, which was prepared in the lab according to our previous report [23]. Another two columns for comparison, Chiralpak AD-RH and OJ-RH column (150 × 4.6 mm i.d., 5 μm), coupled to a guard column (10 × 4.6 mm i.d., 5 μm), were supplied by Daicel Chiral Technologies Co., Ltd (Tokyo, Japan). The column temperature was set at 20 °C. An isocratic mode was used to separate and elute targeted analytes from the column. The optimized enantiomeric separation was obtained with the mobile phase consisting of 5 mM ammonium acetate buffer at pH 5.0 and MeOH (30:70, v/v), with a flow rate of 0.5 mL $min^{-1}$. The autosampler temperature was set at 4°C and the injection volume was 10 μL. Detection was implemented using multiple reaction monitoring (MRM) mode in the ESI negative ion mode to monitor precursor-product ion pair transitions of m/z 243.0–199.1 for FBP enantiomers and m/z 205.0–161.1 for *R*-(−)-IBF, respectively. Other instrumental parameters were set as follows: capillary voltage 3.50 kV; source temperature 150 °C; drying gas temperature 500 °C; desolvation gas flow 15 L $min^{-1}$; nebulizer gas pressure 35 psi. Nitrogen was used as nebulizing and desolvation gas. Argon was applied as collision gas.

## 2.5 Method validation

The validation was performed based on "Guidance for Industry: Bioanalytical Method Validation" from the United States Food and Drug Administration [24].

### 2.5.1 Specificity

The assay selectivity was determined by analyzing extracts of multiple samples (n=6) of plasma from different batches to evaluate the possibility of endogenous interferences at the retention time of analytes and the IS. The peak area of the endogenous components co-eluted with the compound of interest should be less than

20% of the peak area of the lower limit of quantification (LLOQ) and 5% of the peak area of the IS.

### 2.5.2 Linearity and LLOQ

The linearity of the developed method was determined by fitting two calibration curves containing eight nonzero levels on three consecutive days. The calibration curves were originated individually by using the peak area ratio of each enantiomer to IS (y-axis) versus the corresponding concentration of enantiomer (x-axis) whereby a least-square $1/x^2$-weighted linear regression. The acceptance criterion was that the coefficient correlation ($r^2$) must be more than 0.99. The LLOQ is the concentration at which a method can discriminate with a signal-noise ratio greater than or equal to 5. The accuracy of each spiked LLOQ sample (n=6) should be within ±20% and its precision should not be greater than 20%.

### 2.5.3 Accuracy and precision

The precision and accuracy tests were assessed by investigating six replicates of QC samples at three levels (0.02, 1.00, 16.0 μg $mL^{-1}$) for each enantiomer on three consecutive days. The final concentration of the spiked sample was determined by the calibration curve. The accuracy was calculated through the ratio of the concentration obtained from the standard curve versus the nominal concentration spiked in the blank sample. The precision of intra- and inter-day were expressed as the relative standard deviation (RSD), while accuracy was reported as the relative error (RE). For successful method validation, the intra- and inter-day precision values were required not to exceed 15% and the accuracy was required to be within ±15%.

### 2.5.4 Extraction recovery and matrix effect

To estimate the extraction recovery of each enantiomer, the individual peak areas of analytes and IS obtained from QC samples (spiked before extraction) were contrasted with that achieved from unextracted samples (spiked after extraction) at three QC concentration levels in sextuplicate copies. The matrix effect was executed by a

comparison of peak area ratios of analytes to IS in the spiked post-extraction plasma samples (A) with those of neat standard solutions (B) at the equivalent concentration (six replicates each). The ratio (A/ B×100%) was defined as the IS-normalized matrix factor, and the RSD of the IS-normalized matrix factor should be less than 15%.

### 2.5.5 Stability

The stability of analytes in rat plasma was estimated by analyzing sextuplicate plasma samples at two QC concentration levels (LQC and HQC). The different storage and handling conditions were as follows: (a) bench-top stability at room temperature for 12h; (b) freeze–thaw stability after three freeze–thaw cycles at −80°C; (c) long-term stability at −80°C for 120 days; (d) autosampler tray stability at 4°C for 24 h. The analytes were considered to be stable when the accuracy biases were within ±15% of the freshly prepared concentrations.

### 2.5.6 Carry over effect

Carry over was performed in each analytical run by injecting two blank samples after the high concentration standard (20000.0 μg $L^{-1}$). The outcomes of the carry over effect should be less than or equal to 20% of the analytes and 5% for the IS.

### 2.5.7 Dilution effect

With the objective of appraising the dilution process, six replicates of dilution QC samples were determined, which were prepared at a concentration (100.0 μg $mL^{-1}$ for each enantiomer) above the upper limit of quantification (ULOQ) level and diluted 10-fold to a concentration (10000.0ng $mL^{-1}$ for each enantiomer) within the calibration curve range. The accuracy should be in the range of 85–115% of the nominal concentration, and the precision should less than 15%.

## 2.6 Application to a pharmacokinetic study

Six Sprague Dawley rats (male; weight, 220–240g) were purchased from Animal

Center of Shenyang Pharmaceutical University (license no. SCXK-liao-20150001). The rats were allowed to adapt for five days under controlled conditions (temperature: 25 °C, a standard diet, free access to water) and fasted 12 h before the experiment. Following the oral administration of 10 mg $kg^{-1}$ of racemic FBP to six Sprague Dawley rats, orbital blood samples were collected at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 24 h in heparinized tubes. The pharmacokinetic parameters of FBP enantiomers were determined using DAS 2.1.1 software and the entire data were expressed as mean ± standard deviation. The animal protocol was permitted by the Committee on the Ethics of Animal Experiments of Shenyang Pharmaceutical University (permit number, SYPU-IACUC-C2019-6-16-202).

## 3. Results and discussion

### 3.1 Optimization of chromatographic conditions

In the routine CSP enantioseparation, it seemed to be that organic eluents (normal phase elution mode) were frequently used. But in analyses of samples from biological fluids, the choice of reversed-phase mode was much more beneficial due to the low sample solubility of polar species in certain mobile phases and the lack of appropriate enantioselectivity in organic eluents. Besides, for LC–MS applications the reversed-phase mode has better compatibility and suitability. Thus, with the aim of achieving the desired stereospecific separation, the enantioseparation performances of three CSPs in reversed-phase mode were tested and compared: a homemade CDCDP column (immobilized with per-4-chlorophenylcarbamate-β-CD), a Chiralpak AD-RH column [coated with amylose tris(3,5-dimethylphenylcarbamate)] and a Chiralpak OJ-RH column [coated with cellulose tris (4-methylbenzoate)]. The latter two columns have been reported to exhibit chiral recognition ability for FBP extracted from different matrices [21, 25]. According to the reported results, the analysis time of FBP for complete chiral separation on the Chiralpak AD-RH column was about 20 min [21], while that on Chiralpak OJ-RH column was about 35 minutes [25]. The prolonged retention time was not conducive to the high-throughput biological sample analysis. However, on CDCDP column the analyte enantiomers were completely separated within 10 min (the resolution was about 2.0, Fig. 2a) after optimization of the conditions. Besides, at the similar retention time level, the resolution of FBP on Chiralpak AD-RH

and Chiralpak OJ-RH were less than 1.5 [1.0 for the AD-RH (Fig. 2b) and 0.8 for the OJ-RH column (Fig. 2c)]. Therefore, the CDCDP column was selected for the subsequent experiment due to its enhanced enantioseparation ability towards FBP.

The possible chiral separation mechanism might be based on the inclusion-complexation between the CSP and the analyte [26]. Chiralpak AD-RH and OJ-RH columns are polysaccharide derivatives. So, they both have the functional groups for chiral interactions: methyl, carboxyl and benzene ring [27-28]. However, the enantiomeric resolution of FBP on AD-RH and OJ-RH column was not the same, and FBP obtained better enantioseparation on AD-RH column. A reasonable explanation may be that the hydrogen-bonding effect might be an essential factor and be beneficial to the spatial recognition of CSP to the FBP enantiomers. By comparing the structure of CSP for AD-RH and OJ-RH columns, it can be known that there is one more amino group in the structure of AD-RH column. And FBP contains electronegative benzene ring group, which can form hydrogen bonds with polar groups such as amino groups on the CSP. The derivatized β-CD in CDCDP column increased the hydrogen bonding capability on the analyte and the analyte with bulky substituents near the chiral center gave the opportunity for steric interactions also, thus resulting in enhanced and better enantioseparation ability in a relatively short analysis time [24, 29].

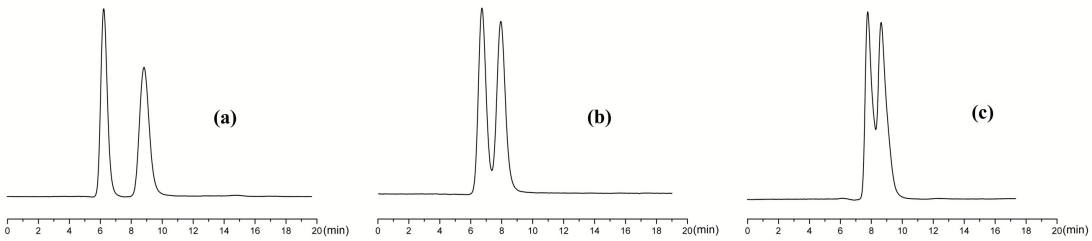


**Fig. 2.** The enantioseparation of FBP on (a) CDCDP, (b) AD-RH and (c) OJ-RH column.

Using CDCDP for the enantiomeric separation of *R*-(−)-FBP and *S*-(+)-FBP, the detailed mobile phase condition optimization was studied. Initially, MeOH/ACN-water without any additive in the mobile phase, the FBP enantiomers could not be separated. Then, the addition of acidic additives and buffer solutions were evaluated. The

experimental results showed that the composition of the mobile phase including MeOH (organic phase) and formic acid or ammonium acetate buffer solution (aqueous phase) significantly improved the separation of FBP. However, when formic acid was present in the mobile phase, the retention time was prolonged. Then, the different concentrations of ammonium acetate buffer solution were investigated to obtain a satisfactory chiral separation. With the increase of buffer concentration, there was no obvious difference in the resolution of FBP, however, the response of *R*-(−)-FBP and *S*-(+)-FBP two enantiomers was gradually increased with the concentration of ammonium acetate in the range of 2.0-10 mM. It should be noted that using MS detection, when no buffer salt was present in the mobile phase, the ESI-MS signal intensity of the analyte was poor. Conversely, too high a buffer concentration may cause the intensity of the analyte to be dramatically suppressed. The typical MRM chromatograms, which represented the effect of the mobile phase on the enantioseparation of *R*-(−)-FBP and *S*-(+)-FBP, were shown in the Supplementary Fig. S1. Although the enantiomeric resolution could be enhanced by further decreasing the amount of organic solvent, the retention times of two enantiomers were increased as well as their detection sensitivity. For the high throughput pharmacokinetic study of enantiomers, rapid analysis time and good sensitivity were mainly comprehensively considered. When 70% (v) MeOH and 30% (v) 5 mM ammonium acetate buffer (pH adjusted to 5.0 with glacial acetic acid) was used, the retention and resolution were optimal and an acceptable sensitivity was obtained.

### 3.2 Sample preparation

During sample pretreatment, the liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were compared in order to obtain optimum extraction and preconcentration of FBP enantiomers from plasma. For each procedure, the recoveries were calculated and then compared to the extraction efficiency results obtained from another one. Therefore, plasma samples containing each enantiomer of FBP at concentrations of 0.02, 1.00 and 16.0 μg $mL^{-1}$ were analyzed, respectively. For each concentration, three replicates were made.

In the LLE part, according to the literature [12,19,21] and the solubility of FBP in different solvent systems, n-hex-EtAc (60:40, v/v), n-hex-DEE (80:20, v/v) and

trimethylpentane-IPA (95:5, v/v) are effective in extracting the FBP from plasma with good recoveries (above 90% for these three extraction solvent systems). However, when using trimethylpentane as the extraction solvent, its vapor could form explosive mixture, which was in danger of combustion and explosion in case of heat source and open fire. Thus, trimethylpentane-IPA was abandoned. When using n-hex-DEE (80:20, v/v) as the extraction solvent, the recovery of FBP in plasma was lower than that of using n-hex-EtAc (60:40, v/v), and DEE is also costlier than ethyl acetate. Consequently, n-hex-EtAc (60:40, v/v) was selected as the final extraction solvent for LLE process. The overall extraction recovery results of FBP (mean ± SD) are summarized in Table 1.

Table 1

The recovery results for FBP after using various sample preparation procedures.

| Liquid-liquid extraction (LLE) | Recovery (%, mean ± SD) | | Solid-phase extraction (SPE) | Recovery (%, mean ± SD) | |
|---|---|---|---|---|---|
| | *R*-(−)-FBP | *S*-(+)-FBP | | *R*-(−)-FBP | *S*-(+)-FBP |
| N-hex-EtAc (60:40, v/v) | 91.4 ± 8.5 | 92.3 ± 8.7 | Sep-pak C18 | 92.8 ± 4.4 | 94.3 ± 3.5 |
| N-hex-DEE (80:20, v/v) | 88.5 ± 7.1 | 89.3 ± 7.8 | Oasis HLB | 74.1 ± 4.0 | 75.8 ± 3.2 |
| N-hex-IPA (95:5, v/v) | 66.9 ± 5.9 | 68.1 ± 5.4 | Sep-pak $NH_2$ | 50.2 ± 6.7 | 53.2 ±7.3 |

During the development of the SPE extraction procedure several different cartridges were also examined including the Sep-pak C18, Oasis HLB and Sep-pak $NH_2$. They were checked in the same experimental conditions: the biological sample (100 µL of plasma) with 10 µL aliquot of the IS working solution before SPE; 5% (v) MeOH in water as the washing agent (1mL) and MeOH used as an eluting solvent (1mL). After preliminary optimization, C18 columns offered slightly more effective FBP extraction than HLB and $NH_2$. Additionally, other modifications of the sample matrix, before SPE with C18 cartridges, were tested for checking whether the addition of organic solvent or decreasing the pH of the sample can improve the extraction

efficiency of FBP from plasma. Thus, 0.5 mL of the mixture of MeOH-water (10:90, v/v), 0.01% (v) formic acid, 0.05% (v) formic acid and 0.1% (v) formic acid, respectively, was added to the plasma samples, and next, the SPE procedure was carried out in the same experimental conditions as described above. As it is shown in Table 1, the highest recoveries of FBP from plasma was acquired after the acidification of biological matrix with 0.1% (v) formic acid. Summarizing, the SPE methodologies with C18 cartridges offered better extraction of FBP from plasma samples than the tested LLE procedures. The detailed description of the developed SPE method with C18 cartridges chosen as the most optimal for the preparation of rat plasma was presented in the section "Sample Preparation". It should be noted that the developed SPE procedure based on C18 cartridges allowed higher recoveries of FBP was the first report on the extraction of FBP from plasma and the results were comparable to the previous data [12,19,21] reported in the literature. In addition to the high extraction recovery and stable recovery results, the samples treated by the SPE process were also considered to be less interfered by endogenous substances, especially for the expensive chiral column, which can prolong its service life and reduce the analysis cost.

### 3.3 Method validation

#### 3.3.1 Specificity

Assay selectivity is demonstrated by the absence of interfering peaks at the retention times of *R*-(−)-FBP and *S*-(+)-FBP and IS in extracted blank plasma samples. Fig. 3 showed the typical chromatograms of a blank plasma sample, a blank plasma sample added to racemic FBP at the LLOQ level (10 ng $mL^{-1}$ per enantiomer), and an actual rat plasma sample obtained at 0.25h after oral administration of 10 mg $kg^{-1}$ FBP racemic. The retention times for *R*-(−)-FBP and *S*-(+)-FBP were about 6.5 min and 9.0 min respectively. These chromatograms demonstrate the absence of any chromatographic and/or mass spectrometric interference.

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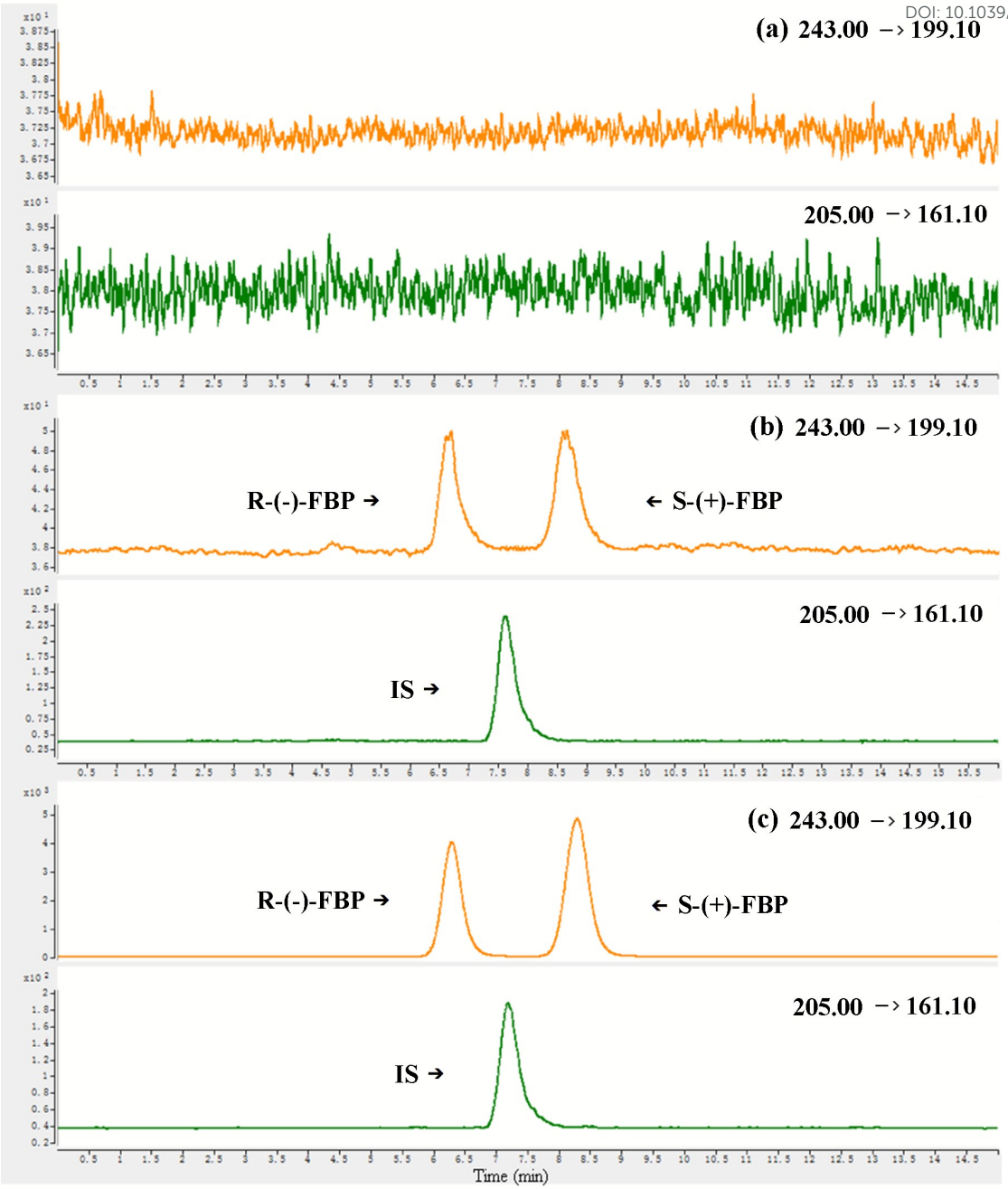


**Fig. 3.** Typical chromatograms of FBP enantiomers and *R*-(−)-IBF (IS) for (a) blank plasma sample, (b) extracted rat plasma at LLOQ level, (c) extracted rat plasma obtained at 0.25 h after oral administration of 10 mg $kg^{-1}$ racemic FBP.

### 3.3.2 Linearity and LLOQ

Calibration curves for both enantiomers were validated in the concentration range of 0.01–20.0 μg $mL^{-1}$ in rat plasma. The degree of linear correlation was calculated as the coefficient $R^2$ >0.996 for standard curves based on three replicates of an 8-point

calibration curve. With the stated assay conditions, this method offered an LLOQ of 10.0 ng $mL^{-1}$ for each enantiomer with an accuracy and precision of 5.4 and 8.9% for *R*-(−)-FBP, and 4.7 and 8.3% for *S*-(+)-FBP, respectively. The proposed method had much higher sensitivity than the limits of quantification 100 ng $mL^{-1}$[12] or 50 ng $mL^{-1}$ [19,21] reported in literatures and the results were sufficient for the research on pharmacokinetics in rat following oral administration of FBP.

### 3.3.3 Accuracy and precision

The outcomes of precision and accuracy for QC samples at three concentration levels (LQC, MQC and HQC) analyzed in six replicates on three consecutive days are presented in Table 2. The RSD values of intra- and inter-day precision for *R*-(−)-FBP were <6.3%, whereas the RE values of accuracy varied from 4.8 to 9.4%. For *S*-(+)-FBP, the RSD values of intra- and inter-day precision were within 7.3% while the RE values of accuracy ranged from 2.6 to 3.9 %. These data fulfilled the desired criteria, demonstrating that this method was feasible and reproducible for the measurement of FBP enantiomers in rat plasma.

Table 2

Intra- and inter-day accuracy and precision of *R*-(−)-FBP and *S*-(+)-FBP in plasma samples.

| Analyte | Nominal concentration (ng $mL^{-1}$) | Intra-day(n=6) | | | Inter-day(n=18) | | |
|---|---|---|---|---|---|---|---|
| | | Calculated concentration (ng $mL^{-1}$) | RE (%) | RSD (%) | Calculated concentration (ng $mL^{-1}$) | RE (%) | RSD (%) |
| *R*-(–)-FBP | 20.0 | 21.2 ± 0.98 | 5.8 | 4.6 | 21.2 ± 1.34 | 5.8 | 6.3 |
| | 1000.0 | 1094.8 ± 48.1 | 9.4 | 4.4 | 1092.7 ± 55.5 | 9.3 | 5.1 |
| | 16000.0 | 16886.0 ± 837.8 | 5.5 | 5.0 | 16760.9 ±907.4 | 4.8 | 5.4 |
| *S*-(+)-FBP | 20.0 | 20.6 ± 0.64 | 2.9 | 3.1 | 20.6 ± 1.19 | 3.1 | 5.8 |
| | 1000.0 | 1039.0 ± 72.1 | 3.9 | 6.9 | 1037.9 ± 76.2 | 3.8 | 7.3 |
| | 16000.0 | 16518.2 ± 865.5 | 3.2 | 5.2 | 16421.1 ±864.2 | 2.6 | 5.3 |

### 3.3.4 Extraction recovery and matrix effect

The recoveries of *R*-(−)-FBP and *S*-(+)-FBP changed from 88.9 to 101.2% and

from 89.9 to 100.5% at three distinct QC concentrations in rat plasma with all RSD values within 6.1%. The IS-normalized MFs from six lots of blank plasma were between 90.5 and 106.8% for the two enantiomers. These results confirm the validity of the extraction procedure for sample preparation before chromatography to obtain reproducible and reliable quantitative results for the analyte, without interference of matrix components.

### 3.3.5 Stability

The results of the stability studies are presented in Table 3. No significant degradation of analytes or IS was observed in all the conditions as described in Section 2.5.5. As per the stability conditions described above, the accuracy in plasma ranged from -7.3 % to 7.7 % and -7.7 % to 7.8% for *R*-(−)-FBP and *S*-(+)-FBP respectively.

Table 3

Stability results of *R*-(−)-FBP and *S*-(+)-FBP under various storage conditions (n = 6).

| Nominal concentration (ng $mL^{-1}$) | Sample conditions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Room temperature for 12 h | | Three freeze–thaw cycles | | −80 ˚C for 30 days | | On the autosampler tray 4 ˚C for 24 h | |
| | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) |
| *R*-(−)-FBP | | | | | | | | |
| 20.0 | 5.2 | 6.3 | 1.9 | 7.7 | 4.8 | 3.8 | 4.3 | 5.5 |
| 16000.0 | -2.9 | 4.5 | -7.3 | 6.3 | 3.3 | 3.7 | 2.1 | 4.1 |
| *S*-(+)-FBP | | | | | | | | |
| 20.0 | 4.9 | 6.1 | 2.5 | 7.8 | 3.7 | 3.2 | 3.6 | 4.5 |
| 16000.0 | -2.8 | 5.2 | -7.7 | 6.7 | 2.2 | 3.0 | 2.2 | 3.9 |

### 3.3.6 Carry over effect

No corresponding peak at the retention time of analytes and IS was found in blank samples injected immediately following the analysis of the high concentration standard sample, which indicated that the carry over effect in this proposed approach was absent.

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### 3.3.7 Dilution effect

It was shown that the accuracy values of 1/10 dilution specimens were 5.3% for *R*-(−)-FBP and 4.8% for *S*-(+)-FBP, while the precision results were 6.2% for *R*-(−)-FBP and 5.1% for *S*-(+)-FBP. All of the results were within the acceptance criteria.

## 3.4 Application to a pharmacokinetic study

The applicability of the method described in this study was demonstrated through the determination of *R*-(−)-FBP and *S*-(+)-FBP in rat plasma after oral administration of 10 mg $kg^{-1}$ of racemic FBP. The mean plasma concentration-time profiles of FBP enantiomers quantified until 24 h are presented in Fig. 4, and the corresponding pharmacokinetic parameters are summarized in Table 4.

As can be seen in Fig. 4, the concentrations of the two enantiomers both rapidly increased following oral absorption, and reached the maximum plasma concentration after 1.5h. The plasma concentration of *S*-(+)-FBP was consistently higher than that of *R*-(−)-FBP after oral administration of racemic FBP, which was in accordance with the literature data [21]. By comparing the drug concentration-time curves of the two enantiomers more precisely, we can find that the mean $C_{max}$ value of *S*-(+)-FBP was 1.4 times greater than that of *R*-(−)-FBP, and the area under the plasma concentration versus time curve ($AUC_{0-\infty}$) was 1.5-fold higher than that of *R*-(−)-FBP, which repeatedly corroborated that *S*-(+)-FBP presented the enhanced pharmacodynamic activity compared with *R*-(−)-FBP. Significant differences could be observed between enantiomers with relatively higher plasma concentration ($P < 0.05$) and AUC for *R*-(−)-FBP than that obtained for *S*-(−)-FBP. This significantly stereoselectivity data analyses were first analyzed and observed in our study. Moreover, the plasma concentration of *S*-(+)-FBP after oral administration had always been much higher than that of *R*-(−)-FBP, which might be beneficial for *S*-(+)-FBP to perform the anti-inflammatory action in vivo. Besides, this was also consistent with the previous literatures data of the analogue drug, ibuprofen [28] in which the *S*-isomer form of ibuprofen was almost entirely responsible for the anti-inflammatory action.

To some extent, conversion between the two enantiomers in vivo may contribute

to the result. However, according to the results in previously reported literature [19], there was no indication to prove the chiral inversion in the whole process. Therefore, according to the previous research, all these pharmacokinetic phenomena and results of *R*-(−)-FBP and *S*-(+)-FBP of the noticeable difference in plasma level could be mainly due to the stereoselectivity of protein binding [20]. Briefly, this study warranted a sound conclusion that the pharmacokinetics of FBP enantiomers after oral administration of racemic FBP was stereoselective.

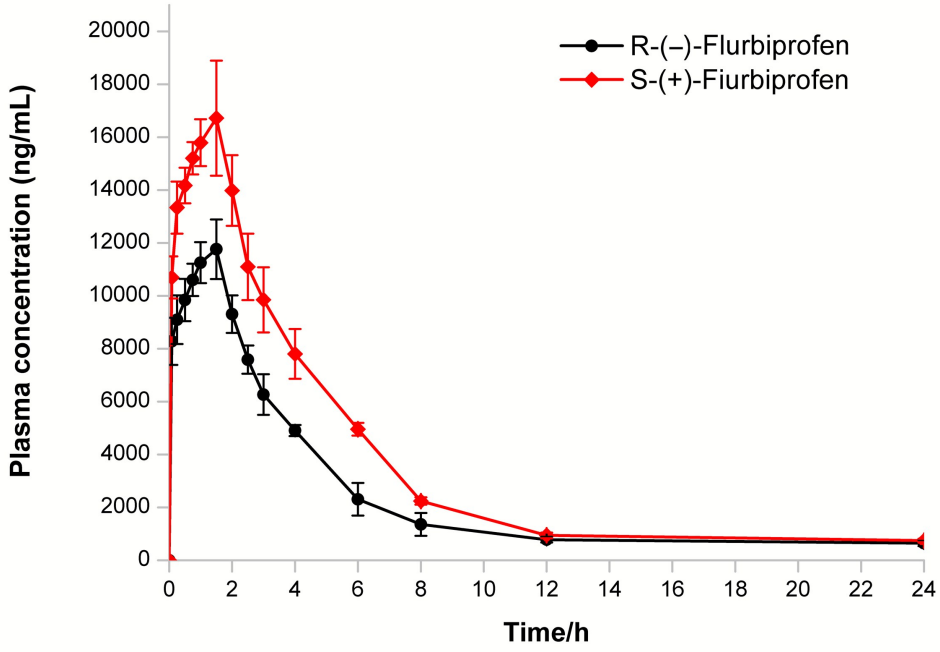


**Fig. 4.** Mean plasma concentration–time curve of *R*-(−)- and *S*- (+)-FBP after oral administration of 10 mg kg$^{-1}$ racemic FBP to SD rats (n = 6).

Table 4

Pharmacokinetic parameters of *R*-(−)-FBP and *S*-(+)-FBP after oral administration of 10 mg kg$^{-1}$ racemic FBP to SD rats (n = 6).

| Parameters | Racemic FBP | |
|---|---|---|
| | *R*-(−)-FBP | *S*-(+)-FBP |
| $C_{max}$ (μg mL$^{-1}$) | 11.78 ± 1.11 | 16.99 ± 1.82 |
| $T_{max}$ (h) | 1.50 ± 0.00 | 1.50 ± 0.00 |
| $t_{1/2}$ (h) | 3.39 ± 0.51 | 4.31 ± 0.74 |

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| | | |
|---|---|---|
| $AUC_{0-t}$ (μg h $mL^{-1}$) | 57.56 ± 4.28 | 86.01 ± 4.84 |
| $AUC_{0-\infty}$ (μg h $mL^{-1}$) | 57.89 ± 4.36 | 89.12 ± 2.91 |

## 4. Conclusion

A new, specific, accurate and precise LC-MS/MS method for the quantification of FBP enantiomers in rat plasma was developed and validated. The presented LC-MS/MS method, based on rapid and effective SPE with C18 cartridges, which reduced the sample volume and the time required for sample preparation and enantioseparation, can be considered as an interesting analytical tool in comparison to previously reported HPLC methods. This new LC-MS/MS method has been proved to be used for the application for quantifying the rat plasma concentration of each enantiomer in real samples successfully. The pharmacokinetic outcomes revealed that except for the same $T_{max}$ value of two enantiomers of FBP, *S*-(+)-FBP also displayed prominently higher $C_{max}$ and AUC values than *R*-(−)-enantiomer. There was no chiral inversion between *R*-(−)- and *S*-(+)-FBP during the whole experimental process. Thereby, a high level of stereoselectivity of FBP pharmacokinetics in rat plasma was preliminarily proved. According to the previous literature, this is probably due to the different protein binding rate.

## Declaration of Competing Interest

None.

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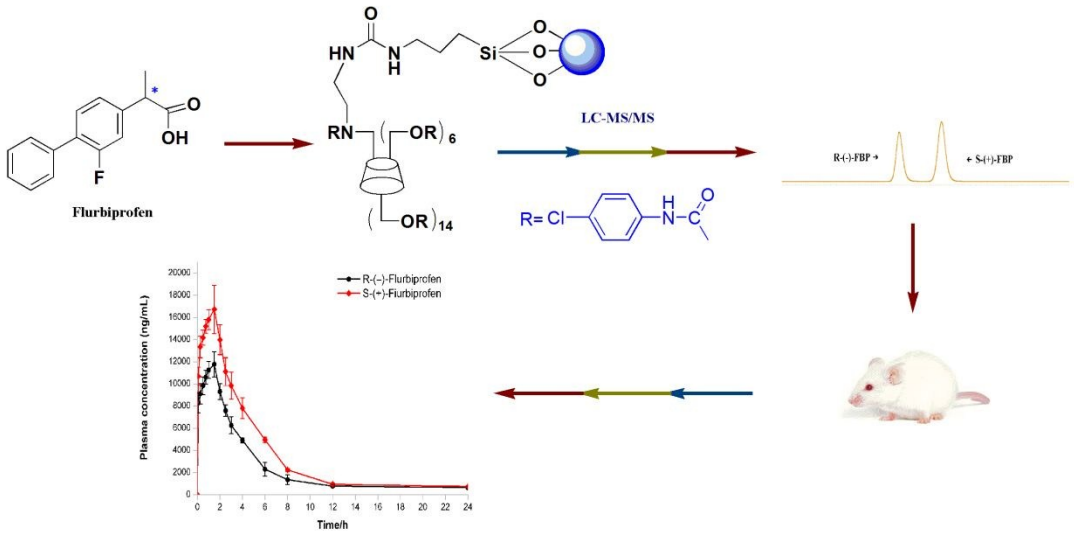


Home-made chiral stationary phase for enhanced enantioselective separation and determination of flurbiprofen by LC-MS/MS for the first time.