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Simultaneous determination of fluoxetine and norfluoxetine enantiomers using isotope discrimination mass spectroscopy solution method and its application in the CYP2C9-mediated stereoselective interactions

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ABSTRACT

In this study, we developed an LC–MS/MS method based on an isotope discrimination mass spectroscopy solution (IDMSS) technology to simultaneously quantify enantiomers of fluoxetine (FLX) and norfluoxetine (NFLX) in a CYP2C9 incubation mixture. *S*-FLX and *S*-NFLX were labeled to form *S*-FLX-d5 and *S*-NFLX-d5. The method has several advantages over conventional chiral separation methods, in terms of the analysis period, resolution, and lower limit of quantification. The primary advantage of the method is that the two enantiomers can always be simultaneously determined by mass spectroscopy regardless if they are separated on column or not, owing to which it has high throughput and high sensitivity. The lower limit of quantification (amount on column) is 12.5 and 1.25 pg for FLX and NFLX, respectively. The retention time of FLX, NFLX, and the internal standard is only 1.9 min. The calibration curves were linear over the concentration range of 0.1–100 ng/ml for NFLX and 1–1000 ng/ml for FLX with an accepted reproducible (RSD < 10%) and accurate (CV < 10%). No significant kinetic isotope effect was found in the metabolism of *S*-FLX-d5 catalyzed by CYP2C9*1 and CYP2C9*2. The half-maximal inhibitory concentration values between *R*-FLX and *S*-FLX catalyzed by CYP2C9*1 and CYP2C9*2 were determined in this study. The inhibitory effects of *R*- to *S*-FLX were stronger than those of *S*- to *R*-FLX in both CYP2C9*1 and CYP2C9*2. The IDMSS technology is useful for stereoselective study of chiral compound in vitro.

1. Introduction

Fluoxetine (FLX), having the chemical formula (±)-N-methyl-3-phenyl-3-[(α,α,α-trifluoro-p-tolyl)oxy] propylamine, is one of the most widely prescribed selective serotonin reuptake inhibitors used for the treatment of depression and obsessive compulsive disorder [1]. So far it is available as racemate in clinic, while its two individual enantiomers do not have identical activity [2]. There are some obvious pharmacokinetic differences between these two enantiomers; for example, the rate of elimination of *S*-FLX is lower than that of *R*-FLX. In human liver microsomes, CYP2C9, CYP2D6, and CYP3A4 are the dominant contributors to the N-demethylation of FLX to norfluoxetine (NFLX) [3]. Therefore, CYP2C9 polymorphism contributes to the interindividual variability in the stereoselective pharmacokinetics of the FLX enantiomers at steady state ($R \gg S$) [4]. It is well known that the administration of racemic mixtures often results in far more complex pharmacokinetics than when a single enantiomer is administered. CYP2C9 displays high gene polymorphism [5,6], which may further increase the complexity of FLX metabolism and lead to differences in metabolic activity among individuals and ethnic groups. Therefore, it is desirable to investigate the metabolism of FLX enantiomers catalyzed by the most common genotype of CYP2C9 to enable individualized administration and thus enable safer use of FLX.

It has been reported that the metabolism of FLX in live human microsomes clearly shows substrate inhibition [7]. Therefore, a sensitive chiral separation method with high throughput that can detect FLX and NFLX at trace level is needed to study the enzyme kinetic profiles of the FLX enantiomers. Several methods have been successfully employed to separate the enantiomers of FLX and/or NFLX, such as capillary electrophoresis [8–11], gas chromatography [12,13], thin-layer chromatography [14], high-performance liquid chromatography (HPLC) (including chiral stationary phase (CSP) [15–20], chiral mobile phase additive (CMPA) [21,22], and chiral derivation reagent (CDR) [23–26]) methods. However, none of these methods are completely efficient; this is either because their resolution is unacceptable or because they are time consuming (some of the methods have tedious sample pretreatment or derivation processes, and some need long analysis times, typically more than 20 min per injection), with limited sensitivity (≫1 ng/ml).

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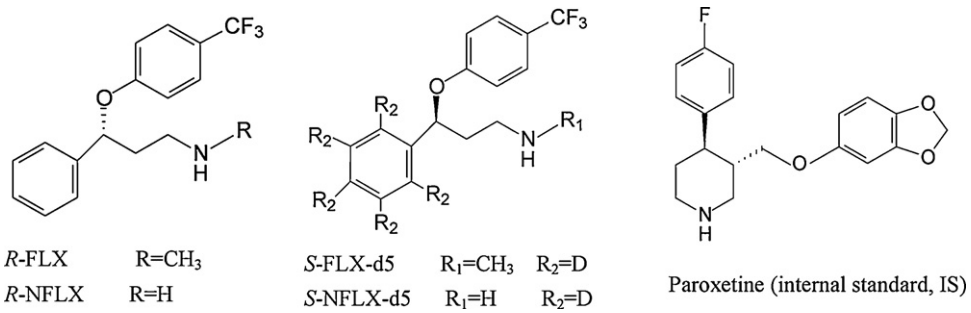

Fig. 1. The structures of FLX, NFLX, and IS.

Stable isotope labeled compounds are popularly used as internal standards in liquid chromatography–mass spectroscopy (LC–MS), because they possess the same properties as unlabeled compounds in LC and can be discriminated via MS on the basis of their different molecular weights. Therefore, enantiomers can also be discriminated by mass spectroscopy if one of the enantiomers is labeled by the isotope regardless if they are separated on column or not. Unfortunately, few studies attempted using this method for chiral separation.

The objectives of the present study were as follows: (a) to develop a sensitive and high-throughput LC–MS/MS method coupled with a stable isotope labeling technology (isotope discrimination MS solution, IDMSS) for the simultaneous determination of FLX and NFLX enantiomers (Fig. 1) in a CYP2C9 incubation mixture and (b) to study the metabolic interactions between FLX enantiomers catalyzed by CYP2C9 using this developed method.

2. Experimental

2.1. Reagents and chemicals

R-FLX hydrochloride, *R*-NFLX, *S*-FLX hydrochloride, *S*-NFLX, tolbutamide, β-NAPDH, β-NAPD, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma–Aldrich (St. Louis, MO, USA). *S*-FLX-d5 hydrochloride and *S*-NFLX-d5 (chemical purity: 98%, ee ≥ 99%, isotopic purity: 99.5%) were synthesized by Toronto Research Chemicals Inc. (North York, Canada). Paroxetine hydrochloride was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Formic acid (for MS) was purchased from Fluka (Steinheim, Germany) and was diluted to the desired concentration using deionized distilled water. The deionized water was purified by the Purelab ultra option-S7 water system (ELGA, UK). HPLC-grade methanol and acetonitrile were purchased from Tedia Company (OH, USA). Bovine serum albumin (BSA) and Tris hydrochloride were supplied by Shanghai Sangon Biotechnology (Shanghai, China).

2.2. Instrumentation

An Agilent 1290 infinity LC system equipped with a G4220A binary pump, G4226A autosampler, and G1330B 1290 thermostat coupled with a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Germany) with an electrospray ionization (ESI) source was used for the LC–MS/MS analysis.

2.3. Preparation of recombinant enzyme homogenates expressed in sf9 cells with the recombinant baculovirus

Homogenates of the recombinant CYP2C9*1 were prepared as previously described [27]. In brief, sf9 cells infected with recombinant baculovirus were harvested 72 h after the infection. The cells were then subjected to three cycles of freezing and thawing, then resuspended in PBS (1×) (pH 7.4), and sonicated in 5 s bursts; they were kept for at least 1 min on ice between bursts. Blank S9 homogenates used for the validation of the developed method were obtained from blank sf9 cells without adding the virus. The protein concentration of the homogenates was measured by the bicinchoninic acid (BCA) method with BSA as standard. CYP2C9*2 was purchased from BD Gentest (Woburn, MA, USA). The activities of CYP2C9 were observed using tolbutamide as a substrate.

2.4. Standard sample and quality control (QC) sample preparation

Standard stock solutions containing 1 mg/ml of *S*-FLX-d5 hydrochloride, *S*-FLX hydrochloride, *S*-NFLX, *S*-NFLX-d5, *R*-FLX hydrochloride and *R*-NFLX were prepared in dimethyl sulfoxide. Working solutions with concentrations of 0.1–100 μg/ml for *S*-FLX-d5 hydrochloride, *S*-FLX hydrochloride, and *R*-FLX hydrochloride and 0.01–10 μg/ml for *S*-NFLX-d5, *S*-NFLX and *R*-NFLX were prepared by serial dilutions with dimethyl sulfoxide. Paroxetine hydrochloride used as an internal standard (IS) was prepared in acetonitrile with a concentration of 400 ng/ml. All solutions were stored at −20 °C until use.

Standard samples for calibration curves were prepared by spiking the incubation mixtures (100 μl, blank S9 homogenates instead of CYP2C9 homogenates) with 0.4 μl of the appropriate standard working solution to yield the following concentrations: 1, 2, 5, 10, 50, 200, 500, 800, and 1000 ng/ml for *S*-FLX-d5 hydrochloride, *S*-FLX hydrochloride and *R*-FLX hydrochloride; and 0.1, 0.2, 0.5, 1, 5, 20, 50, 80, and 100 ng/ml for *S*-NFLX-d5, *S*-NFLX and *R*-NFLX.

QC samples for calibration curves were prepared identically to the standard samples, with concentrations of 0.2, 5, and 80 ng/ml for *S*-NFLX-d5 and *R*-NFLX and 2, 50, and 800 ng/ml for *S*-FLX-d5 hydrochloride and *R*-NFLX hydrochloride. Samples with the lowest and highest concentrations for each of the calibration curves were taken as the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), respectively. Each batch of the standard samples was freshly prepared.

Prior to the LC–MS/MS analysis, the sample (200 μl) was mixed with 200 μl of precooled (4 °C) IS solution. The mixture was subsequently vortexed for 90 s and then centrifuged at 15,700 × *g* for 15 min, and a 5 μl aliquot of the supernatant was injected into the LC–MS system for analysis.

2.5. LC–MS/MS assay procedure

Chromatographic separation of the sample was carried out using a Zobax RRHD Eclipse plus C_{18} column (2.1 mm × 50 mm, 1.8 μm, Agilent, Germany) with an infinity in-line filter operating at 30 °C. The mobile phase composition used was 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B) delivered at a constant flow rate of 0.3 ml/min at a

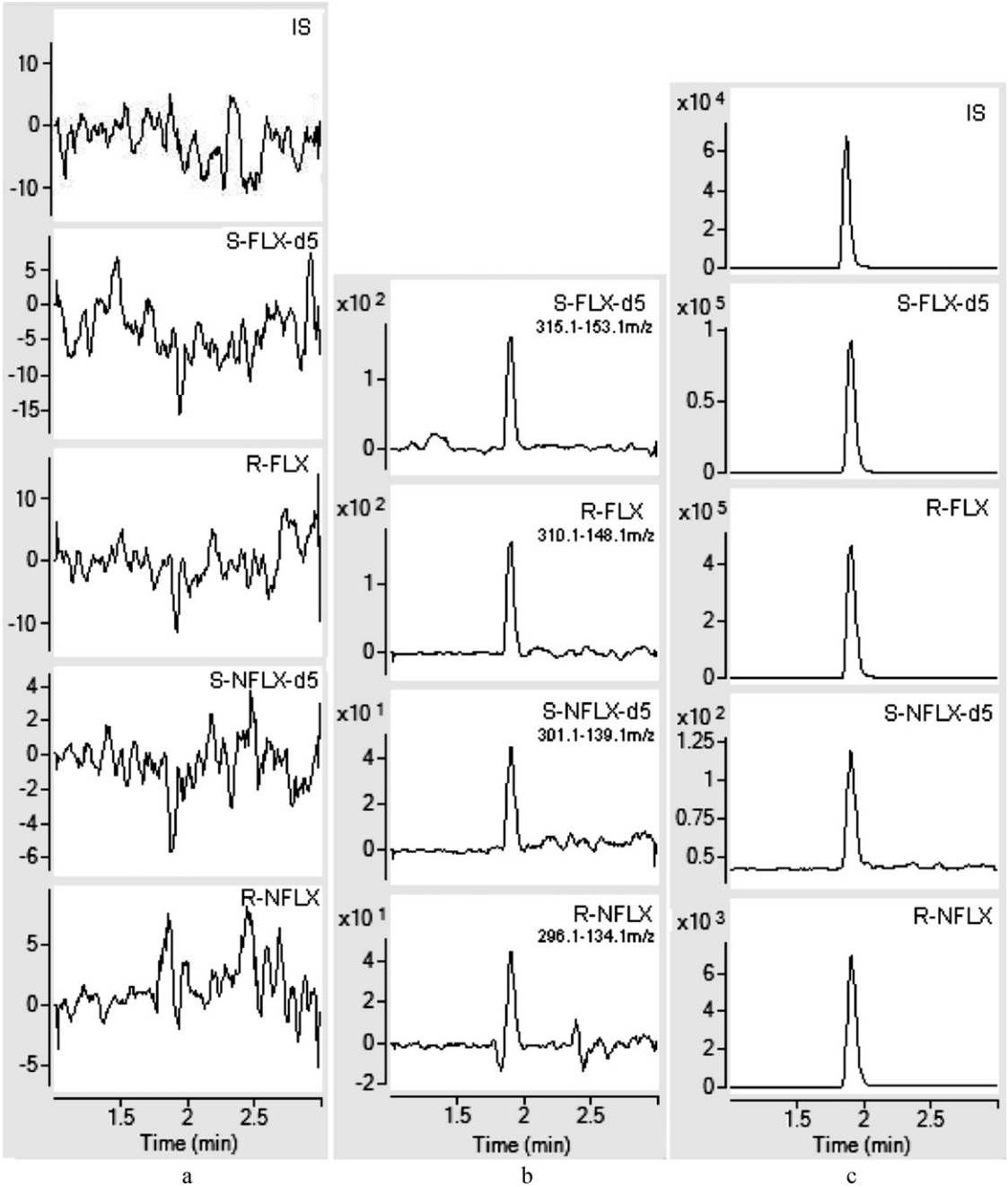

Fig. 2. The chromatograms of FLX, NFLX, and IS. (a) Blank matrix sample; (b) LLOQ sample; (c) sample of inhibition of *R*-FLX (28.5 μM) to *S*-FLX-d5 (5.8 μM) catalyzed by CYP2C9*1.

nonlinear gradient from 30% B for 0.9 min, then swiftly increased to 80% within 0.1 min and maintained till 2.4 min, and then return to the original conditions immediately. The total run time was 3 min. Autosampler parameters included an injection volume of 5 μl and a sample tray temperature of 4 °C. The mass spectrometer was operated in the positive ion mode using ESI under the following general conditions: capillary voltage, 2800 V; nebulization pressure, 45 psi; temperature of drying gas, 340 °C; drying gas flow, 8 L/min; temperature of sheath gas, 370 °C; sheath gas flow, 11 L/min; accelerator voltage, 4 V; collision energy, 1 eV for FLX and NFLX and 15 eV for IS; fragment voltage, 90 V for FLX and NFLX and 115 V for IS. Data was acquired using Mass Hunter software (Agilent) in the multiple reaction monitoring (MRM) mode by recording ion currents for the following transitions: 315.1 *m*/*z* to 153.1 *m*/*z* for *S*-FLX-d5, 310.1 *m*/*z* to 148.1 *m*/*z* for *R*-FLX, 301.1 *m*/*z* to 139.1 *m*/*z* for *S*-NFLX-d5, 296.1 *m*/*z* to 134.1 *m*/*z* for *R*-NFLX and *S*-NFLX, and 330.1 *m*/*z* to 192.2 *m*/*z* for IS. To protect the mass spectrometer from contamination from the samples, the mobile phase flow was diverted to the waste 1 min before each run.

2.6. Method validation

The developed method was validated for recovery, LLOQ, ULOQ, linearity, range, accuracy, precision, selectivity, stability, matrix effect, and carry-over in the spiked samples according to the US Food Drug Administration (FDA), Guidance for Industry: Bioanalytical Method Validation [28].

Recovery of the analyte and the IS was determined by comparing the responses of the analytes from the QC samples ($n = 6$) with those of analytes spiked in a post-extraction blank incubation mixture with similar concentrations. The recovery of the analytes was determined at QC-low, QC-med, and QC-high concentration levels, and the recovery of the IS was determined at a single concentration of 400 ng/ml.

Experiments to evaluate matrix effects were conducted according to the protocol proposed by Matuszewski et al. [29]. MS/MS peak areas corresponding to known amounts of standard solutions of the analytes were compared with those measured from the same amount of analytes spiked in an incubation mixture extract, just after the completion of the extraction process. Matrix effect (%ME) was calculated as follows: %ME = (peak area of post-extraction spiked analyte/peak area of standard analyte) × 100%. This equation means that no matrix effect is observed when %ME is equal to 100%. Values greater than 100% indicate a signal enhancement, whereas values lower than 100% indicate a trend in ionization suppression.

Carry-over was evaluated by preparing an aliquot of a QC sample and initially making three injections and subsequently three more injections of a blank incubation sample [30]. Carry-over was expressed as the percentage difference between the mean analyte-to-IS area ratios for the blank incubation sample and QC sample. The percentage carry-over was calculated as follows: %Carry-over = (BL/QC) × 100, where BL was the mean analyte-to-IS area ratio in the blank incubation sample and QC was the corresponding ratio in the QC sample. Carry-over was taken to be insignificant if less than 5% [31].

Calibration curves were constructed by plotting the concentrations of FLX and NFLX on the *X*-axis versus the chromatographic peak area ratios of FLX and NFLX to IS on the *Y*-axis. The calibration curves were calculated by least-squares linear regression using a $1/x$ weighting factor. The precision and accuracy of the developed method were established by analyzing the QC samples. Six replicates of each concentration point were analyzed in three analytical runs. Relative standard deviation (RSD%) and coefficient of variation (CV%) measured from the QC samples provided the precision and accuracy of the method, respectively.

The stability of analytes in the incubation mixture was estimated by analyzing the QC samples at three concentration levels, using six replicates for each concentration level. The experiments to evaluate the stability of analytes included evaluation of (1) stability of analytes in the blank incubation mixture at 37 °C for 1 h; (2) post-preparative stability of analytes in the auto-sampler at 4 °C for 10 h; (3) enantiomer racemization in the blank incubation mixture at 37 °C for 1 h.

2.7. Kinetic isotope effect to the CYP2C9-mediated metabolism of S-FLX-d5

Enzyme kinetic constants and the effect of incubation time were determined in CYP2C9-mediated metabolism of *S*-FLX and *S*-FLX-d5. The incubation mixture included 0.1 M Tris hydroxychloride buffer solution at pH 7.4, 3.3 mM $MgCl_2$, 3.3 mM glucose 6-phosphate, 1.0 U/ml glucose-6-phosphate dehydrogenase and 0.5 mg/ml protein CYP2C9*1 homogenates (or 50 pmol/ml CYP2C9*2 homogenates). To determine the enzyme kinetic constants, the concentrations of *S*-FLX and *S*-FLX-d5 both ranged from 0.5 to 360 μM, and to evaluate the effect of incubation time, the concentrations of *S*-FLX and *S*-FLX-d5 were both 30 μM, respectively. The resulting mixtures were preincubated at 37 °C for 5 min. Then, the reaction was initiated by adding 2 μl of β-NADP/β-NADPH solution (1.3 mM in 10% $NaHCO_3$ solution) and the reaction mixtures (final volume of 200 μl) were incubated in a shaking water bath at 37 °C for 30 min for the enzyme kinetic constants study and for 10, 30, and 60 min for the effect of incubation time study, respectively. The reaction was terminated by adding an equal amount of pre-cooled IS solution. The mixtures were subsequently vortexed for 90 s and then centrifuged at 15,700 × *g* for 15 min. A 5 μl aliquot of the supernatant was injected into LC–MS system for analysis. The enzyme kinetic constants were determined by nonlinear regression analysis of the data (Graphpad primer 5, GraphPad Software Inc., San Diego, CA). The following equation was applied, assuming a Michaelis–Menten equation: $v = V_{max} \times S/(K_m + S)$, where v was the rate of reaction, V_{max} was the maximum velocity, K_m was the substrate concentration at which the rate of metabolism was 50% of V_{max}, and S was the substrate concentration. The two-sided Student's test was used for statistical comparison. A value of $P < 0.05$ was considered to be statistically significant.

2.8. CYP2C9-mediated metabolic interaction between FLX enantiomers

A single FLX enantiomer (5.7 μM) and an incubation mixture (including 0.1 M Tris hydroxychloride buffer solution at pH 7.4, 3.3 mM $MgCl_2$, 3.3 mM glucose 6-phosphate, and 1.0 U/ml glucose-6-phosphate dehydrogenase) with different concentrations of its antipode were added to 0.5 mg protein/ml for CYP2C9*1 homogenates and 50 pmol/ml for CYP2C9*2, respectively. To evaluate the half-maximal inhibitory concentration (IC_{50}) of *R*- to *S*-FLX, the concentration of *R*-FLX was changed from 0 to 57 μM, and to evaluate the IC_{50} of *S*- to *R*-FLX, the concentration of *S*-FLX was also modified from 0 to 57 μM. The resulting mixtures were preincubated at 37 °C for 5 min. Then, the reaction was initiated by adding 2 μl of β-NADP/β-NADPH solution (1.3 mM in 10% $NaHCO_3$ solution) and the reaction mixtures (final volume of 200 μl) were incubated in a shaking water bath at 37 °C for 30 min. The reaction was terminated by adding an equal amount of precooled IS solution. The mixtures were subsequently vortexed for 90 s and then centrifuged at 15,700 × *g* for 15 min. A 5 μl aliquot of the supernatant was injected into LC–MS system for analysis. The apparent IC_{50} values were determined by nonlinear regression analysis of the data (Graphpad primer 5, GraphPad Software Inc., San Diego, CA). The fitting formulation was as follows: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{((\log IC_{50} - X) \times \text{HillSlope})})$, where X was the log of dose of concentration; Y was the response, decreasing as X increases; Top and Bottom was the plateaus in same units as Y; $\log IC_{50}$ was the same log units as X; HillSlope was the slope factor.

3. Results and discussion

3.1. Selectivity

It can be observed from LC–MS/MS chromatograms of the blank incubation samples and LLOQ samples that *S*-FLX-d5, *R*-FLX, *S*-NFLX-d5, and *R*-NFLX were well separated by MRM extraction and that their retention times were the same at 1.9 min. No interference peaks of endogenous constituents from the matrix were observed at the retention times of FLX, NFLX, and IS; this absence of peaks indicated the high specificity of the developed method (Fig. 2). In this study, sample preparation was carried out by direct precipitation of protein with acetonitrile. Thus, this method proved to be simple and valid, and it was a promising candidate for extraction of FLX and NFLX from biological matrices. The mean absolute recovery of QC samples in three concentrations ranged from 103.2% to 106.2% for *S*-FLX-d5, 102.3% to 108.0% for *R*-FLX, 92.0% to 105.1% for *S*-NFLX-d5, and 99.4% to 105.8% for *R*-FLX, respectively. The absolute recovery of IS at 400 ng/ml was 103.2%.

3.2. Matrix effect

The matrix effect (%ME) was evaluated by analyzing QC-low, QC-medium, and QC-high samples. Although the mean values of the matrix effect were −31.1% ± 3.1%, −28.7% ± 2.4%, −25.4% ± 2.0%, and −24.6% ± 4.6% for *S*-FLX-d5, *R*-FLX, *S*-NFLX-d5, and *R*-NFLX, respectively, the relative standard deviation values were stable, and all were lower than 5%. Therefore, the matrix effect for the developed method was satisfactory. Mean carry-overs between injections of the QC and blank matrix samples for the analytes and IS were as follows: QC-low samples < 5%; QC-mid samples < 1%; QC-high samples < 0.2%; IS in all these samples < 0.1%. The results indicated that all analytes and internal standards met the acceptable limits of ≤5%.

3.3. Sensitivity, linearity and range

FLX and NFLX provided an excellent response in the MS detector. Under the LC–MS/MS conditions employed in this study, the limit of detection (signal-to-noise ratio of 3:1) was 0.02 ng/ml for *S*-FLX-d5, *R*-FLX, *S*-NFLX-d5, and *R*-NFLX. The LLOQ was 0.1 ng/ml for *S*-NFLX-d5 and *R*-NFLX (signal-to-noise ratio > 10), and 1 ng/ml for *S*-FLX-d5 and *R*-FLX (signal-to-noise ratio ≫ 10) in incubation mixtures (Fig. 2). Both the deviation of accuracy and the RSD of precision of the LLOQ samples were lower than 10% ($n = 6$, Table 1). Because concentrations of FLX were high in the metabolic interaction study, the lowest concentration of FLX on the calibration curve was 10 times that of NFLX. The calibration curves were linear over the concentration range of 0.1–100 ng/ml for NFLX and 1–1000 ng/ml for FLX with correlation coefficients (r^2) > 0.999 (Table 2).

3.4. Accuracy and precision

Accuracy and precision data for intra- and inter-day QC samples are listed in Table 1. The intra-day accuracy (CV%) ranged from −3.1% to 9.2% and the inter-day accuracy (CV%) ranged from −2.9% to 6.0% for all QC and LOQ samples of FLX and NFLX. The intra-day precision (RSD%) ranged from 0.2% to 8.8% and the inter-day precision (RSD%) ranged from 0.9% to 6.0% for all QC and LOQ samples of FLX and NFLX. The assay values for both intra- and inter-day were found to be within the acceptable limits.

3.5. Stability

The stability of the developed method was evaluated using QC samples at three different concentration levels. The results indicated that FLX and NFLX were stable in the auto-sampler at 4 °C for 10 h and in the incubator at 37 °C for 1 h (Table 3). In case of chiral compounds, enantiomer racemization may occur under conditions such as heating, illumining, or being in a biological matrix. In the current study, enantiomer racemization was studied by adding FLX and NFLX (1 and 50 ng/ml, respectively) to the blank incubation mixture at 37 °C for 1 h. Unfortunately, the enantiomer transformation could not be confirmed by the isotope discrimination MS method. For example, the *m*/*z* value did not change when *S*-FLX-d5 transformed to *R*-FLX-d5. Therefore, we evaluated stability using the method reported by Gatti et al. [32]. The separation of the enantiomers was achieved using a Chiralcel OD-RH column (2.0 mm × 150 mm, 5 μm, Daicel Chemical Industries, Ltd., France) and a mobile phase consisting of potassium hexafluorophosphate/acetonitrile. The enantiomers were detected through ultraviolet absorbance at 227 nm. The results indicated that no racemation of FLX and NFLX enantiomers occurred when they were incubated in the blank incubation mixture at 37 °C for 1 h (data not shown).

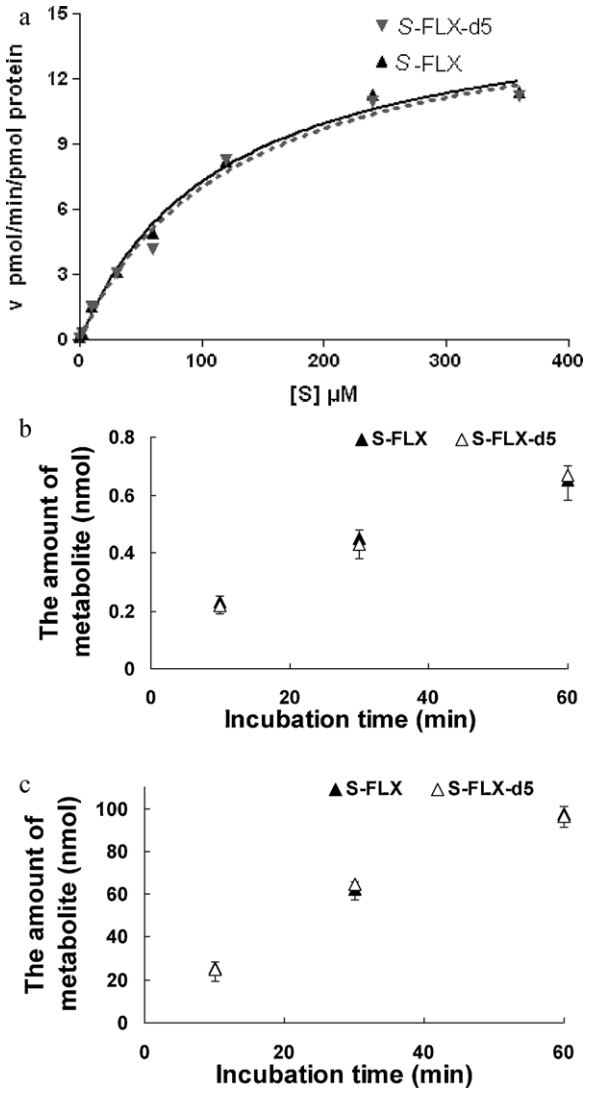

Fig. 3. Kinetic isotope effect of the metabolism of *S*-FLX-d5 catalyzed by CYP2C9*1 and CYP2C9*2. (a) Kinetics of *S*-FLX and *S*-FLX-d5 in CYP2C9*2. The concentrations of *S*-FLX and *S*-FLX-d5 were both from 0.5 to 360 μM. (b) Production rates of metabolites of *S*-FLX and *S*-FLX-d5 catalyzed by CYP2C9*2. The concentrations of *S*-FLX and *S*-FLX-d5 were both 30 μM. (c) Production rates of metabolites between *S*-FLX and *S*-FLX-d5 catalyzed by CYP2C9*1. The concentrations of *S*-FLX and *S*-FLX-d5 were both 30 μM.

3.6. Kinetic isotope effect to the metabolism of S-FLX-d5

Since the deuterium-labeling leads to different vibrational energy states and different Van-der-Waals binding energies, kinetic isotope effect may be present. This possible effect to the metabolism of *S*-FLX-d5 was investigated in this study. As shown in Fig. 3a, there were no dramatic differences in the kinetic constants between *S*-FLX and *S*-FLX-d5 in CYP2C9*2-mediated metabolism ($P \gg 0.05$). The K_m and V_{max} values of *S*-FLX and *S*-FLX-d5 were 15.76 ± 1.43 μM, 116.0 ± 18.5 pmol/min/pmol protein, and 15.83 ± 1.69 μM, 125.4 ± 21.2 pmol/min/pmol protein, respectively. There were also no dramatic differences in the production rates of metabolites mediated by CYP2C9*2 and CYP2C9*1 between *S*-FLX and *S*-FLX-d5 at the incubation time of 10, 30, and 60 min ($P \gg 0.05$, shown in Fig. 3b and c). The results indicated that deuterium substitution in the benzene ring of FLX

Table 1
Intra- and inter-day accuracy and precision for FLX and NFLX in incubation mixtures.

	Sample style	Concentration (ng/ml)	Intra-day (n = 6)		Inter-day (n = 18)	
			Accuracy CV%	Precision RSD%	Accuracy CV%	Precision RSD%
	LLOQ	1	7.4	5.5	4.2	3.4
	QC-low	2	6.8	2.2	3.7	3.5
S-FLX-d_5	QC-mid	50	−2.8	1.7	−2.2	2.2
	QC-high	800	−0.8	2.3	0.7	1.4
	ULOQ	1000	1.9	0.4	0.9	2.0
	LLOQ	1	9.2	6.1	6.0	3.5
	QC-low	2	6.3	4.1	2.8	3.4
R-FLX	QC-mid	50	−3.1	9.1	−2.9	1.6
	QC-high	800	0.1	2.3	0.2	0.4
	ULOQ	1000	1.2	0.5	1.0	0.7
	LLOQ	0.1	8.0	6.5	4.4	3.0
	QC-low	0.2	0.7	8.8	0.7	0.9
S-NFLX-d5	QC-mid	5	−1.0	1.0	−1.6	1.0
	QC-high	80	−2.2	2.2	−0.4	1.6
	ULOQ	100	1.5	0.2	0.4	1.4
	LLOQ	0.1	4.1	7.6	−1.8	6.0
	QC-low	0.2	0.5	1.8	1.2	0.9
R-NFLX	QC-mid	5	−0.8	0.7	−0.8	2.1
	QC-high	80	−1.0	3.0	0.0	1.0
	ULOQ	100	0.8	1.4	0.1	1.8

Table 2
Linear correlation parameters of FLX and NFLX.

	Slope ($\times 10^{-3}$)	Intercept ($\times 10^{-3}$)	Concentration range (ng/ml)	r^2
S-FLX-d5	1.797	0.016	1–1000	0.9993
R-FLX	1.955	1.438	1–1000	0.9993
S-NFLX-d5	3.427	0.147	0.1–100	0.9996
R-NFLX	3.422	0.593	0.1–100	0.9992

played negligible effect in the N-demethylation metabolism of FLX catalyzed by CYP2C9*1 and CYP2C9*2. Therefore, the kinetic isotope effect could be ignored in the metabolism of *S*-FLX-d5 catalyzed by CYP2C9*1 and CYP2C9*2.

3.7. Interactions between R-FLX and S-FLX mediated by CYP2C9

The IC_{50} values between *R*-FLX and *S*-FLX catalyzed by CYP2C9*1 and CYP2C9*2 were determined in this study. Considering the accuracy of determination of IC_{50} in the metabolic interaction study, the amount of NFLX produced was used to calculate the IC_{50} value. As shown in Fig. 4, the inhibitory effects of *R*- to *S*-FLX were both stronger than those of *S*- to *R*-FLX in CYP2C9*1 and CYP2C9*2. We anticipate that these results will enable individualized clinical administration of FLX to a certain extent.

3.8. Advantage and disadvantage of the method

Although the racemation of enantiomers cannot be detected by the isotope discrimination MS method, this method contributes to the clarification of the enzyme-catalyzing drug metabolism interaction study. For example, we first suppose that some of *R*-NFLX can easily transform to *S*-NFLX. Subsequently, we determine the IC_{50} of *R*-FLX to *S*-FLX mediated by CYP2C9. If the IC_{50} value is calculated using the metabolite of *S*-NFLX, it will not be accurate when CSP, CMPA, or CDR is used as the chiral separation method. This is because these conventional chiral separation methods cannot differentiate between the *S*-NFLX derived from the metabolite of *S*-FLX and the *S*-NFLX derived from the chiral transform of *R*-NFLX. Additionally, the concentration of the metabolite of *R*-FLX, *R*-NFLX, is high in this incubation mixture, especially at a high inhibitor concentration. However, the isotope discrimination MS method is

Table 3
Stability of FLX and NFLX in autosampler manager and incubation mixture at 37 °C (n = 6).

	Concentration (ng/ml)	10 h in autosampler at 4 °C			1 h incubated at 37 °C		
		Before (B)	After (A)	$\Delta_{A-B/B}$ (%)	Before (B)	After (A)	$\Delta_{A-B/B}$ (%)
	2	2.1	2.1	2.1	2.1	1.9	−7.1
S-FLX-d_5	50	48.0	48.6	1.3	57.2	56.9	−0.6
	800	818.6	793.7	−3.0	787.5	800.7	1.7
	2	2.1	2.1	3.3	2.0	1.9	−2.4
R-FLX	50	47.8	48.5	1.3	58.2	56.9	−2.4
	800	808.0	800.6	−0.9	785.9	794.5	1.1
	0.2	0.2	0.2	−0.9	0.2	0.2	0.7
S-NFLX-d_5	5	5.0	4.9	−0.1	5.9	5.9	0.0
	80	80.4	78.2	−2.7	78.5	80.4	2.4
	0.2	0.2	0.2	−0.3	0.2	0.2	4.9
R-NFLX	5	5.1	5.0	−2.1	5.9	5.9	−0.4
	80	79.7	79.2	−0.6	78.5	80.7	2.9

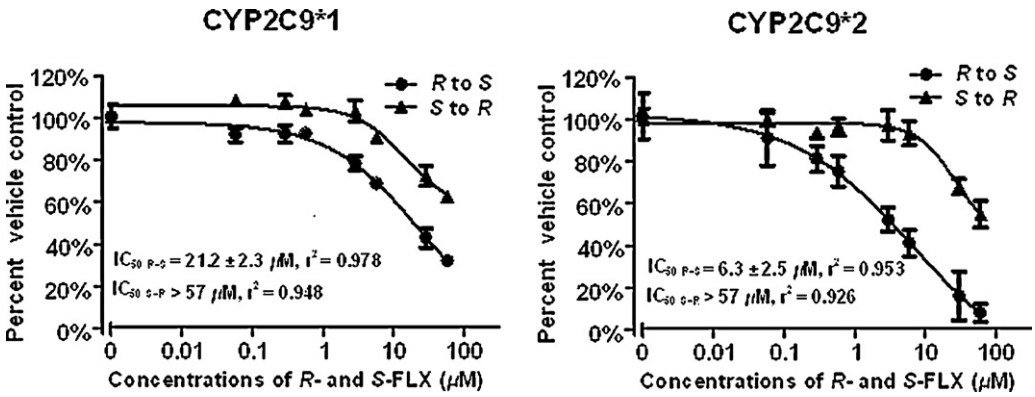

Fig. 4. Representative plots of IC_{50} curves for *R*- and *S*-FLX interaction catalyzed by CYP2C9*1 and CYP2C9*2. Data points represented the mean ± SD from triplicate incubations. Round symbols (●) represent data generated from *S*-FLX-d5 (5.7 μM) and *R*-FLX (0–57 μM) incubated with CYP2C9*1 and CYP2C9*2 (0.5 mg/ml for CYP2C9*1 and 50 pmol/ml for CYP2C9*2) for 30 min at 37 °C, while triangular symbols (▲) represent data generated from *R*-FLX (5.7 μM) and *S*-FLX-d5 (0–57 μM) incubated with CYP2C9*1 and CYP2C9*2 (0.5 mg/ml for CYP2C9*1 and 50 pmol/ml for CYP2C9*2) for 30 min at 37 °C.

Table 4
The comparison of chiral separation method for FLX and NFLX in biological samples.

Method	Retention time of the latter peak of the enantiomers (min)		Resolution		LLOQ (amount on the column, pg)		Expense	Reference
	FLX	NFLX	FLX	NFLX	FLX	NFLX		
CSP	20.6	16.7	nbs	nbs	2000	3000	Expensive	[16]
CSP	21.3	18.7	nbs	nbs	2000	2000	Expensive	[17]
CSP	7.5	8.2	bs	bs	60	60	Expensive	[18]
CSP	17.6	15.2	nbs	nbs	3750	3750	Expensive	[19]
CMPA	14.0	17.0	bs	bs	100	200	Inexpensive	[22]
CDR	28.5	19.8	bs	bs	125	–	Expensive	[25]
CDR	9.5	13.0	bs	bs	325	325	Inexpensive	[26]
IDMSS	1.9	1.9	sd	sd	12.5	1.25	Expensive	Current study

Notes: bs: baseline separation; nbs: not baseline separation; sd: simultaneous determination regardless if the enantiomers were separated or not on column.

used, the racemation does not affect the determination of the IC_{50} value. Although some amount of *R*-NFLX can easily transform to *S*-NFLX, the value of IC_{50} is calculated using the change in the amount of *S*-NFLX-d5.

Table 4 lists the parameters of the reported methods for determination of chirality of FLX and NFLX in biological samples. It can be clearly seen that the IDMSS method is advantageous in terms of the analysis period, resolution, and LOQ over the CSP, CMPA, and CDR methods. The primary advantage of the IDMSS method is that the two enantiomers can always be simultaneously determined by mass spectroscopy regardless if they are separated on column or not, which leads to the high throughput and high sensitivity of the method. Therefore, it is also a recommended method for the stereoselective pharmacokinetic study of racemic compound whose concentrations are low in biological specimen. Certainly, it is very important that the labeled site of a molecule should be stable in biological mediator. At the same time, kinetic isotope effect should also be considered.

The high expense of isotope label, however, may make the method limited. Additionally, the IDMSS method is unsuitable for the analysis of enantiomer impurity.

4. Conclusion

In this study, we developed an LC–MS/MS method that can simultaneously quantify the enantiomers of FLX and NFLX in a CYP2C9 incubation mixture. This method is based on an isotope discrimination MS solution technology. This method is sensitive (limit of detection = 0.02 ng/ml), reproducible (RSD < 10%), and accurate (CV < 10%), with sufficient throughput to enable its use in the analysis of a large number of samples. The present method has several obvious advantages in terms of the analysis period, resolution, and LLOQ over the conventional CSP, CMPA, and CDR methods. The developed method is useful in research on chiral compounds in vitro.

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