Analysis of Fluoxetine and Norfluoxetine Enantiomers in Human Plasma and Amniotic Fluid by LC-MS/MS and Its Application to Clinical Pharmacokinetics in Pregnant Women

Abstract
Fluoxetine (FLX) is the drug most frequently used for the treatment of depressive states during pregnancy and is available for clinical use as a racemic mixture of (+)-(S)- and (-)-(R)-FLX. FLX N-demethylation produces its active metabolite norfluoxetine (NorFLX). The present study describes the development and validation of an enantioselective method for sequential FLX and NorFLX analysis in plasma and amniotic fluid by LC-MS/MS coupled to a Chirobiotic V column. The quantitation limits were 0.04 and 0.1 ng/ml plasma, 0.005 and 0.05 ng/ml amniotic fluid, respectively for FLX and NorFLX enantiomers. The plasma concentration range were 0.04-20 ng/ml for FLX and 0.1-20 ng/ml for NorFLX and amniotic fluid were 0.005-10 ng/ml for FLX and 0.05-10 ng/ml for NorFLX. The validated method was applied to a study of FLX kinetic disposition in plasma and amniotic fluid samples collected up to 672 hours after a single 20 mg dose of FLX to one healthy pregnant woman. The methods for analysis of FLX and NorFLX enantiomers in plasma and amniotic fluid were compatible to application to clinical pharmacokinetics study involving the administration of FLX single dose to pregnant woman. The present study describes for the first time the development and validation of a method for the quantitation of FLX enantiomers in amniotic fluid using LC-MS/MS in human.

Keywords: Fluoxetine; Norfluoxetine; Enantiomers; LC-MS/MS; Pregnancy; Pharmacokinetics; Amniotic fluid

Daniela Miarelli Carvalho1, Gabriela Campos de Oliveira Filgueira1, Maria Paula Marques2, Juciene Aparecida Caris2, Geraldo Duarte1, Ricardo Carvalho Cavalli1, Vera Lucia Lanchote2 and Elaine Christine Dantas Moisés1*

1 Departament of Obstetrics and Gynecolog Ribeirão Preto Medical School University of São Paulo Avenida Bandeirantes, 3900, 14049-900 Ribeirão Preto – SP – Brazil
2 Department of Clinical, Toxicology and Bromatologic Analyses School of Pharmaceutical Sciences of Riberão Preto University of São Paulo Avenida do Café, s/n, 14040-903, Ribeirão Preto – SP - Brazil

*Corresponding author: Christine Dantas Moisés
E-mail: elainemoises@fmrp.usp.br (E.C.D.M)
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Introduction

Fluoxetine (FLX) is an antidepressant of the amine type chemically designated as N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propane-1-amine (Figure 1). FLX increases the activity of serotonin in the brain because of its highly selective characteristic action in the inhibition of serotonin (5-HT) reuptake [1,2]. FLX is an antidepressant of low toxicity widely prescribed to the general population and to pregnant women because of its high efficacy and low reaction frequency compared to tricyclic antidepressant classes and monoamine oxidase inhibitors [3].

FLX is clinically available as a mixture of (S) and (R) enantiomers. The FLX enantiomers have a similar potency in blocking 5-HT reuptake [4-6], while their active metabolite (+)-(S)-norfluoxetine (NorFLX) is 20 times more potent than (-)-(R)-norfluoxetine both in vitro and in vivo [7,8]. The wide interindividual variability in the clinical response to FLX may be related to differences in the plasma concentrations of each enantiomer of both FLX and the active metabolite NorFLX [9-11].

The methods reported for the analysis of FLX and NorFLX enantiomers in human or rat plasma employ derivatization with the enantiomerically pure reagent R-1-(1-naphthyl)ethyl isocyanate [11-18] or chiral stationary-phase columns such as Chiralcel OD-R,[19-22] Chirobiotic V [23,24] or Chiral AGR [25]. FLX and NorFLX enantiomers are analyzed by GC-MS systems [26,27] or HPLC with a UV detector [18-21,23], HPLC with fluorescence detection [22] or LC-MS/MS in human plasma [26] or sheep plasma [25]. Kim et al. [26] reported the analysis of FLX and NorFLX enantiomers in plasma using LC-MS/MS, with limits of quantitation (LOQ) of 0.1 ng/ml and 0.5 ng/ml, respectively, for the two enantiomers of FLX and NorFLX. The analysis of FLX and NorFLX enantiomers in amniotic fluid (AF) was previously described only in pregnant sheep using GC-MS [27].

Antidepressants including FLX can cross the placental barrier and reach the AF, which constitutes one main fetal exposure route [28,29]. The AF composition undergoes alterations during pregnancy, according to the feto-maternal exchanges. Early in the pregnancy this fluid is basically a maternal serum ultrafiltrate and during the course of gestation fetal urine becomes the main component of the AF. Around the 16th week of gestation the fetus swallows about 7 ml/day of fluid, increasing this volume over the weeks of gestation, reaching up to delivery, a maximum of 210-760 ml/day leading to a constant exposure to the drug present in the AF [29].

The present study describes the development and validation of enantioselective methods for the sequential analysis of FLX and NorFLX in plasma and amniotic fluid using LC-MS/MS coupled to a Chirobiotic V chiral stationary phase column. LOQ values were compatible with application to the study of clinical pharmacokinetics and distribution in amniotic fluid of the FLX and NorFLX enantiomers in a pregnant woman treated with a single oral dose of the racemic drug.

Material and Methods

Chemicals and Reagents

Fluoxetine hydrochloride (98%), norfluoxetine as a racemic mixture and the pure enantiomers (+)-(S)-fluoxetine and (+)-(S)- norfluoxetine were obtained from TRC, Toronto, Canada. Metoprolol tartrate (97%) as a racemic mixture was obtained from Sigma, St. Louis, MO, USA. The solvents (ethanol, hexane and methanol), obtained from Panteac, Barcelona, Spain, were HPLC grade. Isoamyl alcohol (Fisher Scientific), ammonium acetate and sodium hydroxide (JT Baker, Xalostoc, Mexico) were analytical grade. Water was obtained from the Milli-Q Plus purification system (Millipore, Belford, MA, USA). Vacuum evaporation (Christ RVC 2-25 CD and Christ CT 04-50 SR, Osterode am Harz, Germany).

LC-MS/MS conditions

The enantiomers of FLX and NorFLX were resolved through a 4.6 mm × 25 cm Astec Chirobiotic V column packed with 5 µm particles (Supelco, Bellefonte, PA) with a 4 × 4 mm CN Lichospher 100 precolumn packed with 5 µm particles (Merck, Darmstadt, Germany) and a mobile phase consisting of a mixture of ethanol: 15 mM ammonium acetate (85:15 v/v) at a flow rate of 0.8 ml/min. The column was maintained at 23°C.

The mass spectrometry detection system (MS/MS) consisted of a Waters 1525 µ binary gradient pump, an automatic injector 2777, a TCM/CHM column heater and a triple-quadrupole XEVO TQ-S mass spectrometer, all from Waters (Milford, USA), equipped with an ionization interface by electronbuelization operating in the positive ion mode. The source and desolvation temperatures were kept at 159 and 350°C, respectively. Nitrogen was used as the nebulizing gas at a flow rate of 900 l/h. Argon was used as the collision gas at a pressure of approximately 1.68 × 10⁻³ mbar. The voltage of the cone was maintained at 25 V for the enantiomers of FLX and NorFLX and for IS. The collision energy was 7 eV for the FLX enantiomers and 3 eV for the NorFLX enantiomers. Optimal MS/MS conditions were obtained by direct infusion of the standard FLX and NorFLX solution at a concentration of 1 ng of each enantiomer/ml mobile phase at a flow rate of 10 ml/min.

The determinations were carried out in the multiple reaction-monitoring (MRM) modes. The protonated ions [M + H]⁺ and their respective ion products were monitored in the 310→44 transitions for the FLX enantiomer, the 296→134 transitions for the NorFLX enantiomers, and the 268→116 transitions for IS.
Data were acquired and quantitated using the MassLynx software version 4.1 (Micromass, Manchester, United Kingdom), which was also used to record and integrate the peaks.

**Sample preparation**

A stock solution of FLX and NorFLX was prepared at the concentration of 1 mg of each enantiomer/ml and later diluted to obtain concentrations of 0.2, 0.4, 0.8, 2, 4, 8, 20, 80, 200 and 400 ng of each FLX and NorFLX enantiomer/ml methanol. The methopropol solution, used as the internal standard (IS), was prepared at the concentration of 0.4 µg/ml in methanol and later diluted to the concentration of 8 ng/ml in methanol. All solutions were stored at -20°C. Twenty-five µl of the IS solution, 200 µl of a 2 M NaOH aqueous solution and 6 ml hexane: isoamyl alcohol (99:1 v/v) were added to 500 µl plasma aliquots and 1000 µl amniotic fluid aliquots. After shaking for 30 min in a horizontal shaker (± 300 cycles/min), the tubes were centrifuged at 2800 g for 20 min. The extracts were then transferred to plastic tubes and concentrated to dryness by the vacuum evaporation method. The residues were reconstituted in 90 µl ethanol: 15 mM ammonium acetate (85:15 v/v) and 50 µl were injected into the chromatography column.

**Elution order of fluoxetine and norfluoxetine enantiomers**

The elution order was determined by the individual analysis of the pure enantiomers (+)-(S)-FLX and (+)-(S)-NorFLX under the conditions described in item sample preparation (Figure 3).
Method validation

The method was validated for pregnant women human plasma and amniotic fluid according to the recommendations of the current U.S. FDA Guidance for Industry: Bioanalytical Method Validation [30]. The following parameters were assessed and validated: selectivity, residual effect, matrix effect, LOQ, linearity, precision, accuracy, and stability.

The matrix effect was assessed using plasma samples from eight different volunteers, four samples being normal, two lipemic and two hemolyzed. For amniotic fluid, six samples from different parturients were assessed. The matrix effect was assessed by comparing the areas of the peaks referring to the FLX, NorFLX and IS standards, directly injected with and without the matrix. Blank samples were processed according to the procedure described in section sample preparation and IS, FLX and NorFLX were then added at the same concentrations as the samples for the quality control of low concentrations (QCL). 1.00 and 5.00 ng/mL of each enantiomer were used for FLX and NorFLX, respectively. The coefficient of variation (CV) of the NMFs concerning the QCL and QCH sample must be lower than 15%.

The linearity of each method for the analysis of FLX and NorFLX in human plasma and amniotic fluid was determined by the analysis of three analytical curves, including the analysis of the blank sample (containing no analyte or internal standard) and

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\text{Figure 3: Chromatograms related to the elution order of FLX and NorFLX enantiomers (50 ng/mL methanol). Racemic mixture the enantiomers of FLX (A), standard of (+)-(S)-FLX (B), racemic mixture the enantiomers of NorFLX (C), standard of (+)-(S)-NorFLX (D).}
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of the zero sample (containing only the internal standard). The curves were constructed using 500 µl aliquots of blank plasma or 1000 µl aliquots of blank amniotic fluid enriched with 25 µl of each standard solution of FLX and NorFLX. The samples were extracted and analyzed as described in sections sample preparation and LC MS/MS conditions.

The analytical curves for FLX were constructed at concentrations of 0.04, 0.1, 0.2, 0.4, 1, 4, 10 and 20 ng of each enantiomer/ml plasma and 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 2, 5 and 10 ng of each enantiomer/ml amniotic fluid. The analytical curves for NorFLX were constructed at concentrations of 0.1, 0.2, 0.4, 1, 4, 10, and 20 ng of each enantiomer/ml plasma and 0.05, 0.1, 0.2, 0.5, 2, 5 and 10 ng of each enantiomer/ml amniotic fluid. The analytical curves were approved when the quality controls showed deviation of 15% at most and of up to 20% for the lower limit of quantitation (LLQ).

The precision and accuracy of the method were determined by intra- and inter-assay studies at LLQ: 0.04 and 0.1 ng of each enantiomer/ml plasma and 0.005 and 0.05 ng of each enantiomer/ml amniotic fluid; QCL: 0.1 and 0.2 ng of each enantiomer/ml plasma and 0.02 and 0.1 ng of each enantiomer/ml amniotic fluid; quality control of mean concentration (QCM): 5 ng of each enantiomer/ml plasma and 4 ng of each enantiomer/ml amniotic fluid; QCH: 16 ng of each enantiomer/ml plasma and 8 ng of each enantiomer/ml amniotic fluid, and quality control for dilution (QCD): 80 ng of each enantiomer/ml plasma (1:5) and 20 ng of each enantiomer/ml amniotic fluid (1:20). All solutions were aliquoted and stored at -20°C until the time for analysis, with the first day of analysis coinciding with the day of preparation of the solutions.

To assess intra-assay precision and accuracy five replicates of these solutions were analyzed in a single analytical run and to assess inter-assay precision and accuracy five replicates of each solution were analyzed during three different runs.

Intra- and inter-assay precision was determined by calculating the CV of the results obtained, and accuracy was expressed as relative standard error. For the method to be considered precise, the CV should be 15% or less, with values up to 20% being admitted for the LLQ.

To assess stability, plasma and amniotic fluid samples were prepared and spiked with FLX and NorFLX at QCL and QCH concentrations. To determine stability after freezing and thawing cycles, the spiked samples were frozen to -70°C for 24 h and then thawed and frozen again for 24 h, with this process being repeated up to the third thawing cycle, when they were extracted and analyzed. For the determination of post-processing stability, the extracted samples were kept in the auto-injector at 12°C for 24 h, and then injected for chromatographic analysis. Short-term stability was assessed with the samples kept at room temperature (23°C) for 2 h and then extracted and analyzed. All results were compared to those obtained with newly prepared samples and were expressed as percent deviation.

**Pharmacokinetic application**

The study was approved by the Research Ethics Committee of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo. A healthy pregnant woman selected in the Obstetrics sector of the institution during the prenatal care period was investigated after giving written informed consent to participate in the study. The patient was admitted to the Clinical Research Unit in her 32th week of gestation and received a single oral dose of 20 mg racemic fluoxetine hydrochloride (Prozac®, Eli Lilly). Blood samples (4 ml) were collected into tubes containing EDTA at times zero, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24, 48, 72, 96, 168, 336, 504 and 672 h after administration of the drug. At the time of delivery, the patient received an additional 20 mg dose of racemic fluoxetine hydrochloride by the oral route for the collection of maternal blood samples and of umbilical vessel and intervillous space blood samples and amniotic fluid samples. Blood samples were centrifuged at 2800 g for 10 min at 4°C. Plasma and amniotic fluid samples were stored at -70°C until the time for analysis.

The pharmacokinetic parameters were calculated using the PhoenixTM WinNonlin® software, version 6.3.0.393 (Pharsight, Certara L.P.). The area under the plasma concentration versus time curve (AUC0-∞), total apparent clearance (CL/F), apparent volume of distribution (Vd/F), maximum plasma concentration (Cmax) and time needed to reach it (tmax) were determined directly from the values obtained from the plasma concentrations. These data were determined considering a bicompartamental model for FLX and a monocompartamental model for NorFLX.

**Results and Discussion**

The present study describes for the first time the development and validation of a method for the analysis of FLX and NorFLX enantiomers in amniotic fluid using LC-MS/MS with application to clinical pharmacokinetic studies. The study also reports the development and validation of a method for the sequential analysis of FLX and NorFLX enantiomers in plasma using LC-MS/MS.

The order of elution in the sequence (+)-(S)-NorFLX, (+)-(S)-FLX, (-)-(R)-NorFLX, (-)-(R)-FLX was determined using the pure enantiomers (+)-(S)-FLX and (+)-(S)-NorFLX (Figure 3).

The method for the analysis of the FLX and NorFLX enantiomers in plasma and amniotic fluid using LC-MS/MS was validated with the demonstration of the absence of the matrix effect and of the residual effect (Table 1).

The enantiomers of FLX and NorFLX and the IS were extracted from human plasma and amniotic fluid at basic pH using an isoamyl alcohol: hexane mixture (1:99, v/v) as the extraction solvent. Linearity in plasma was observed in the 0.04-20 ng/ml intervals for the FLX enantiomers and in the 0.1-20 ng/ml intervals for the NorFLX enantiomers. Linearity in amniotic fluid was 0.005-10 ng/ml for FLX enantiomers and 0.05-10 ng/ml for the NorFLX enantiomers (Tables 2 and 3).

The methods for the analysis of the FLX and NorFLX enantiomers in plasma and amniotic fluid showed CV and relative standard error values lower than 15% in the intra- and inter-assay studies of precision and accuracy (Tables 2 and 3). The plasma and amniotic fluid samples remained stable in the freezing and thawing process after processing and a short duration interval (Table 4).

The lower quantitation limits were 0.04 ng of each FLX enantiomer.
enantiomer/ml of plasma and 0.1 ng of each NorFLX enantiomer/ml of plasma (Table 2). The method is more sensitive than that described by Kim et al. [26] who reported LOQ values of 0.1 ng of each FLX enantiomer/ml of plasma and 0.5 ng of each NorFLX enantiomer/ml of plasma. It should be pointed out that the cited authors also used LC-MS/MS, although they did not report the stationary phase of the chiral column employed in the process of enantiomer resolution.

The low LOQ values estimated in the present study permitted the analysis of the FLX enantiomers in plasma samples collected up to 168 h, or approximately six elimination half-lives, after the administration of a single oral dose of 20 mg racemic FLX to a patient in the 32nd week of pregnancy. Regarding the active NorFLX metabolite, plasma samples were analyzed up to 672 h, or approximately three elimination half-lives after the administration of a single oral dose of FLX (Figure 4).

The low concentrations of the drugs in amniotic fluid require the development of more sensitive analytical methods than those reported for plasma. In the present study, the method developed and validated for the analysis of FLX and NorFLX in amniotic fluid showed LOQ values of 0.005 ng of each FLX enantiomer/ml and of 0.05 ng of each NorFLX enantiomer/ml amniotic fluid (Figure 5 and Table 6. The ratio of the amniotic fluid/maternal plasma concentrations was approximately 11% for both FLX and NorFLX enantiomers in amniotic fluid and plasma samples collected simultaneously 160 min after the administration of...
a single oral dose of 20 mg racemic FLX (Table 5). The data suggest that there was distribution of FLX and NorFLX in amniotic fluid.

The pharmacokinetic parameters (Table 5) showed greater proportions of the (-)-(R)-FLX and (+)-(S)-NorFLX enantiomers in plasma after the administration of racemic fluoxetine.

The values of the area under the curve (AUC) of plasma concentration vs time were higher for the (-)-(R)-fluoxetine and (+)-(S)-norfluoxetine enantiomers, suggesting that the (+)-(S)-fluoxetine enantiomer is preferentially metabolized to NorFLX (Figure 6 and Table 5). However, Kim et al. [26] have reported plasma (+)-(S)-fluoxetine concentrations approximately twice higher than (-)-(R)-fluoxetine concentrations in pregnant women treated with a daily oral dose of 20 mg racemic fluoxetine for at least 3 weeks. Considering that the metabolism of (+)-(S)-fluoxetine depends on CYP2D6, while the metabolism of (-)-(R)-fluoxetine depends on CYP2D6 and on CYP2C9 and that fluoxetine and norfluoxetine are potent and reversible inhibitors time-dependent on CYP2D6 [31], the plasma accumulation of the (+)-(S)-fluoxetine enantiomers may occur after multiple doses but not after a single dose as administered in the present study.
racemic FLX.

pregnant woman after the administration of a single oral dose of 20 mg racemic fluoxetine.

Figure 6: Plasma concentrations of FLX and NorFLX enantiomers in a healthy pregnant woman after the administration of a single oral dose of 20 mg racemic fluoxetine.

References


Conclusion

The methods developed and validated here using LC-MS/MS coupled to a Chirobiotic V chiral phase column are precise and accurate and show sensitivity for the analysis of the FLX and NorFLX enantiomers in plasma and amniotic fluid, with applicability to clinical pharmacokinetics. The methods were applied to the investigation of stereoselectivity in the pharmacokinetics of FLX and NorFLX in a pregnant patient treated with a single oral dose of 20 mg of racemic fluoxetine.

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