Influence of Gasoline Inhalation on the Enantioselective Pharmacokinetics of Fluoxetine in Rats

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ABSTRACT Fluoxetine is used clinically as a racemic mixture of (+)-(*S*) and (–)-(*R*) enantiomers for the treatment of depression. CYP2D6 catalyzes the metabolism of both fluoxetine enantiomers. We aimed to evaluate whether exposure to gasoline results in CYP2D inhibition. Male Wistar rats exposed to filtered air ($n = 36$; control group) or to 600 ppm of gasoline ($n = 36$) in a nose-only inhalation exposure chamber for 6 weeks (6 h/day, 5 days/week) received a single oral 10-mg/kg dose of racemic fluoxetine. Fluoxetine enantiomers in plasma samples were analyzed by a validated analytical method using LC-MS/MS. The separation of fluoxetine enantiomers was performed in a Chirobiotic V column using as the mobile phase a mixture of ethanol:ammonium acetate 15 mM. Higher plasma concentrations of the (+)-(*S*)-fluoxetine enantiomer were found in the control group (enantiomeric ratio $AUC_{(+)\text{-}(S)/(-)\text{-}(R)} = 1.68$). In animals exposed to gasoline, we observed an increase in $AUC_{0\text{-}\infty}$ for both enantiomers, with a sharper increase seen for the (–)-(*R*)-fluoxetine enantiomer (enantiomeric ratio $AUC_{(+)\text{-}(S)/(-)\text{-}(R)} = 1.07$), resulting in a loss of enantioselectivity. Exposure to gasoline was found to result in the loss of enantioselectivity of fluoxetine, with the predominant reduction occurring in the clearance of the (–)-(*R*)-fluoxetine enantiomer (55% vs. 30%). *Chirality 25:206–210, 2013.*

KEY WORDS: fluoxetine; enantiomers; LC-MS/MS; pharmacokinetics; gasoline inhalation exposure chamber

INTRODUCTION

Fluoxetine is used clinically as a racemic mixture of (+)-(*S*) and (–)-(*R*) enantiomers for the treatment of depression. Wong et al.[1] showed that the (+)-(*S*) isomer was slightly more potent than the (–)-(*R*) isomer as a serotonin-reuptake inhibitor in rat cortical synaptosomes. The pharmacokinetics of fluoxetine is enantioselective in rats (higher plasma concentrations of the (+)-(*S*) enantiomer),[2] pregnant sheep (enantiomeric ratio AUC(+)-(*S*)/(–)-(*R*) of 1.73),[3] and pregnant women (enantiomeric ratio (+)-(*S*)/(–)-(*R*) in maternal plasma of 2.91).[4]

In human liver microsomes, CYP2D6 and CYP2C9 contribute to the formation of the *N*-demethylated (–)-(*R*)-norfluoxetine metabolite, whereas only CYP2D6 is responsible for the formation of (+)-(*S*)-norfluoxetine.[5] The clearance values for the (–)-(*R*)-fluoxetine and (+)-(*S*)-fluoxetine enantiomers are 36 and 40 l/h, respectively, in extensive CYP2D6 metabolizers and 3 and 17 l/h, respectively, in poor CYP2D6 metabolizers.[6] In studies using human liver microsomes, the (+)-(*S*)-fluoxetine enantiomer is five to six times more potent as an inhibitor of CYP2D6 than the corresponding (–)-(*R*)-fluoxetine.[7]

Among environmental and occupationally important substances, gasoline is important because of the quantities used and the many opportunities for human exposure. The typical composition of gasoline is 80% paraffin, 14% aromatics, and 6% olefins. Threshold limit values—that is, time-weighted average (TLV-TWA) of 300 ppm (890 mg/m^3) and short-term exposure limit (TLV-STEL) of 500 ppm (1480 mg/m^3)—are established for occupational exposure during bulk handling of gasoline. The TLV-TWA is intended to minimize the potential for eye, mucous membrane, and upper respiratory tract irritation, whereas the TLV-STEL is intended to minimize the potential for acute depression of the central nervous system.[8]

The effects of gasoline exposure on the activity of CYP enzymes involved in drug metabolism are still not fully known. Intraperitoneal administration of gasoline to rats (at doses of 1 and 5 ml/kg) was shown to result in the induction of CYP2B (pentoxyresorufin *O*-dealkylase).[9] Ida et al.[10] reported a reduction in the activity of CYP enzymes (aminopyrine *N*-demethylase and aniline-*p*-hydroxylase) and no change in the activity of UDP-glucuronosyltransferase (UGT) enzymes (bilirubin glucuronidase) in rat liver microsomes exposed to gasoline for 6 min in a closed exposure chamber at concentrations of 5–10%.

The most sensitive and selective methods for the analysis of fluoxetine enantiomers in plasma have been described by Shen et al.[11] and Chow et al.[12] who used LC-MS/MS coupled with chiral stationary phases columns. However, the required volume of plasma (200–300 μl) and the limit of quantification (1–2 ng of each enantiomer per milliliter of plasma) do not allow the method to be applied to enantioselective pharmacokinetic studies in rats.

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In the present study, we developed and validated a more sensitive method (0.5 ng of each enantiomer per milliliter of plasma) to quantify the enantiomers of fluoxetine in volumes of only 200 μl of plasma. The method was applied to study the effect of gasoline exposure on the kinetic disposition of fluoxetine enantiomers in rats, an animal model in which the amount of sample available is particularly limiting. The method allowed for the quantification of fluoxetine enantiomers in the plasma of rats up to 12 h after a single 10-mg/kg oral dose (gavage) of the racemic drug was administered. This study was designed to evaluate whether exposure to gasoline (600 ppm in a nose-only inhalation exposure chamber for 6 h/day, 5 days/week for 6 weeks) results in the inhibition of CYP2D.

MATERIALS AND METHODS

Chemical and Reagents

FIuoxetine hydrochloride (98% TRC, Toronto, Canada) and metoprolol tartrate (97%; Sigma, St. Louis, MO, USA) were obtained as racemic mixtures. Chromatography-grade ethanol (Tedia, Fairfield, OH, USA), hexane (Acros Organics, NJ, USA), isoamyl alcohol (Fisher Scientific), and methanol (Merck, Darmstadt, Germany) solvents were purchased. Analytical-grade ammonium acetate and sodium hydroxide (JT Baker, Xalostoc, Mexico) were also obtained.

Standard Working Solutions

A stock solution of fluoxetine was prepared at a concentration of 1 mg of each enantiomer per milliliter of methanol and later diluted at concentrations of 4, 8, 40, 80, 200, 400, 800, 2000, and 4000 ng of each enantiomer per milliliter of methanol. A solution of metoprolol, which was used as an internal standard, was prepared at a concentration of 20 μg/ml of methanol and later diluted at a concentration of 0.4 μg/ml of methanol. All stock solutions were stored at –20 °C.

Chromatographic Analysis

The HPLC system consisted of a Shimadzu chromatograph (Kyoto, Japan) consisting of an LC-10 AS model pump and a Quattro Micromass spectrometer detector (Micromass, Manchester, UK) operating at 3.0 kV capillary voltage, 30 V cone voltage, 120 °C source temperature, and 200 °C desolvation temperature. Nitrogen and argon were used as the nebulizer (at a flow rate of 350 l/h) and collision gases (at a pressure of 2.05×10^{-3} mbar), respectively. The MassLynx version 3.5 program (Micromass, Manchester, UK) was used to register and integrate the peaks.

The separation of fluoxetine enantiomers was performed in Astec Chirobiotic® V 25 cm × 4.6 mm columns (Supelco, Torrence, CA, USA), with a CN Lichospher® 100 4 × 4 mm pre-column (Merck, Darmstadt, Germany) and 5-μm particles. The column was maintained at 23 °C in a Shimadzu CTO-10 ASVP model oven (Kyoto, Japan). The mobile phase consisted of ethanol:ammonium acetate 15 mM (85:15% v/v), eluting at a flow rate of 1 ml/min. Electrospray interfacing was employed. The molecule ionization method was positive with the equipment operating in selective ion-monitoring mode. We analyzed the following mass/charge (m/z) transitions: $310 > 44$ for fluoxetine and $268 > 116$ for the internal standard metoprolol.

Sample Preparation

We develop and validated a new liquid–liquid extraction method that was performed in plastic tubes using 200 μl of plasma, 25μl of a solution of metoprolol (0.4 μg/ml; internal standard), 200 μl of aqueous 2M NaOH, and 4 ml of hexane:isoamyl alcohol (99:1, v/v). After 30 min of horizontal stirring (± 250 cycles/min) and centrifuging for 10 min at 2000 g, the organic extracts were transferred to conical tubes and concentrated to dryness. The residue was dissolved in 150 μl of ethanol:15 mM ammonium acetate (85:15 v/v), of which a volume of 120 μl was subjected to chromatographic analysis.

Determination of the Matrix Effect

The matrix effect was evaluated by direct comparison of the peak heights of fluoxetine and metoprolol (internal standard) injected directly into the mobile phase, with the peak heights obtained for the standard solutions added to blank plasma extracts derived from a pool of plasma from Wistar rats.

Method Validation

Method validation was performed according to US FDA industry guidelines on bioanalytical method validation.[13] The calibration curves were constructed using 200-μl samples of drug-free rat plasma spiked with 25 μl of each diluted standard solution of fluoxetine and 25 μl of the internal standard solution, and submitted to the extraction procedure described above. The linear regression equations and the correlation coefficients were obtained from the peak height ratios (analyte/internal standard) plotted against the respective concentrations (0.5–500 ng of each enantiomer per milliliter of rat plasma).

Recovery of fluoxetine was evaluated in five replicates of three different concentrations in plasma samples (1, 200, and 400 ng of each enantiomer/ml), by direct comparison of the results obtained for samples extracted according to the analytical procedure with those obtained for the standard solutions added to the blank plasma extracts, corresponding to 100% recovery.

The quantitation limit was defined as the lowest concentration analyzed with a coefficient of variation and a percentage of inaccuracy of less than 20%. Thus, replicates were analyzed ($n = 10$) at a concentration of 0.5 ng of each enantiomer per milliliter of plasma.

Precision and accuracy were determined by analyzing fluoxetine in plasma samples spiked with three different concentrations (1, 200, and 400 ng of each enantiomer per milliliter of plasma). For intra-assay evaluation, aliquots of each spiked rat plasma sample were analyzed in quintuplicate using a calibration curve. For inter-assay evaluation, aliquots of each spiked rat plasma sample were analyzed in quintuplicate over five consecutive days.

The stability of 3 freezing (–20 °C) and thawing (25 °C) cycles of 12 h, as well as the postprocessing (24 h at 5 °C) and short-term stability (4 h at room temperature) were determined. Stability was evaluated by quintuplicate analysis of drug-free rat plasma spiked with fluoxetine at concentrations of 1, 200, and 400 ng of each enantiomer per milliliter. The results of the stability tests are reported as accuracy in relation to freshly prepared samples.

Experimental Study

This method was applied to study the kinetic disposition of fluoxetine enantiomers in control rats and rats exposed to gasoline that were treated with a single dose of the racemic drug. The experimental study was approved by the Ethics Committee for the Use of Animals of the Faculdade de Ciências Farmacêuticas de Araraquara, Universidade Estadual Paulista (Araraquara, SP, Brazil) and the animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Brazilian Association for Laboratory Animal Science.

Male Wistar rats ($n = 72$) weighing approximately 250 g were used for the study. The animal room was maintained at a temperature of 20 ± 1 °C and 60% ± 20% relative humidity, with a 12-h light–dark cycle. Rat chow and tap water were available ad libitum, except 12 h before fluoxetine administration.

The animals were divided into two groups: exposed to filtered air (control group, $n = 36$) or exposed to gasoline (600 ppm) in a nose-only inhalation exposure chamber for 6 h/day, 5 days/week for 6 weeks.[14] On the last day of exposure, the animals received a single dose of 10-mg/kg racemic fluoxetine, dissolved in polyethylene glycol:saline (7:3, v/v) by oral gavage. The animals were kept in the exposure chamber for 6 h after drug administration, and then transferred to their usual housing with access to food and water. At 12 h, they were again immobilized in the exposure chamber long enough to perform the final blood collection. Serial blood samples (0.5 ml) were collected by caudal incision at 0, 15, and 30 min, and at 1, 2, 3, 4, 6, and 12 h after fluoxetine administration.[15,16] Heparin (Liquemine®, Produtos Roche Químicos e Farmacêuticos, São

Paulo, Brazil) was used as an anticoagulant. For each animal, two samples were randomly collected at different time points ($n = 8$ samples/time point). Blood samples were centrifuged and plasma aliquots were separated and immediately frozen at –20 °C until analyzed.

Pharmacokinetics

The area under the plasma concentration versus time curve was estimated from 0 to infinity ($AUC_{0-\infty}$) using the Gaussian quadrature as an integration method[17], and apparent total clearance was calculated using $Cl = dose/AUC_{0-\infty}$.

For comparison between the $AUC_{0-\infty}$ values, a hypothesis test was established for the differences between the AUCs of each enantiomer in each group, exposed and control, and between the groups. The variances were estimated considering that the estimator used for AUC is a linear combination of the mean concentrations at each sampling time and after a F test to verify the homogeneity of the variances[18].

RESULTS AND DISCUSSION

The method developed and validated in this study allows for the direct resolution of the enantiomers of fluoxetine in a chiral-phase column, and uses only 200 μl of plasma. The resolution of the enantiomers of fluoxetine in a chiral phase Chirobiotic V column was described previously by Bakhtiar and Tse[19] and Borges et al.[20] The order of elution of the fluoxetine enantiomers in the sequence (+)-(*S*)-fluoxetine and (–)-(*R*)-fluoxetine was established according to the study by Guo et al.[2], which reports higher plasma concentrations of the (+)-(*S*)-fluoxetine enantiomer (Fig. 1).

No significant matrix effect (Table 1) was observed on the analysis of both fluoxetine enantiomers and the internal standard metoprolol in rat plasma. Values of approximately 85% were obtained. However, we emphasize that the analysis of plasma samples with hemolysis should be avoided due to a significant matrix effect.

Recoveries for both enantiomers were greater than 80% and were independent of the concentration (Table 2). Gatti et al.[21] reported a recovery of 72–77% when human plasma samples were precipitated with acetonitrile, extracted with hexane:isopropanol (97:3, v/v), and subjected to a clean-up procedure with 20 mM phosphoric acid. Unceta et al.[22] reported 92–95% recovery in the analysis of fluoxetine enantiomers in rat plasma using CN solid-phase extraction columns. Chow et al.[12] showed recoveries of 107–122% for extraction from sheep plasma in basic medium with methyl *tert*-butyl ether.

The quantitation limit of 0.5 ng of each fluoxetine enantiomer per milliliter of plasma (Table 2) suggests that the method developed in this study is more sensitive than the method described by Chow et al.[12], which also employed a chiral-phase column and detection by LC-MS/MS, and inferred a limit of quantification of each enantiomer of 1 ng/ml of plasma from the extraction of 300-μl aliquots of sheep plasma. The study by Shen et al.[11], using LC-MS/MS and extracting 200-μl aliquots of plasma, reported a quantification limit of each enantiomer of 2 ng/ml plasma. The quantitation limit of 0.5 ng of each enantiomer of fluoxetine/ml of plasma led to the analysis of plasma samples collected from rats up to 12 h after the administration of a single oral dose of 10 mg/kg of racemic fluoxetine.

Our method displayed linearity in the range of 0.5–500 ng of each enantiomer per milliliter of rat plasma, with a correlation coefficient of greater than 0.99 (Table 2). In studies of inter- and intra-assay precision and accuracy, the results were within the acceptable error range of 15%, demonstrating that the method is precise and accurate (Table 2).

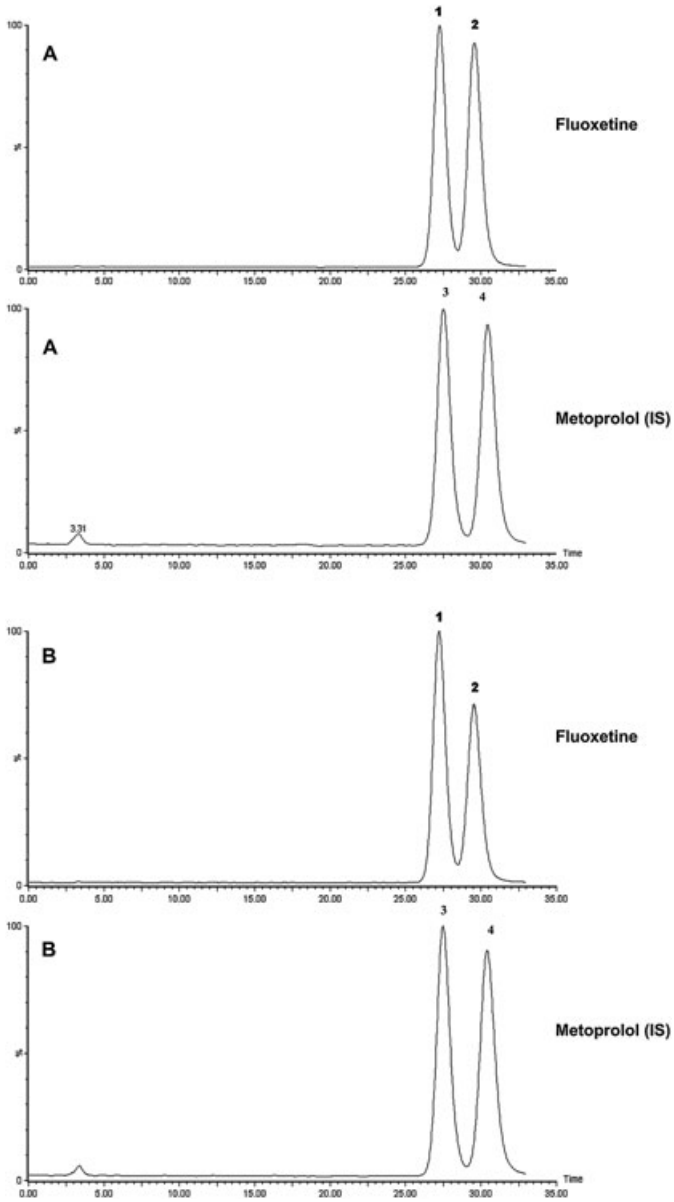

Fig. 1. Analysis of the enantiomers of fluoxetine in plasma. (**A**) Chromatograms of a sample of plasma spiked with 50 ng/ml fluoxetine. Peak 1: (+)-(S)-fluoxetine; peak 2: (-)-(R)-fluoxetine; peaks 3 and 4: metoprolol (IS). (**B**) Chromatograms of a plasma sample from a rat treated with racemic fluoxetine. Peak 1: (+)-(S)-fluoxetine; peak 2: (-)-(R)-fluoxetine; peaks 3 and 4: metoprolol (IS).

TABLE 1. Matrix effect for fluoxetine and internal standard (IS) in a pool of rat plasma

	Matrix effect (%)		
Concentration, ng/ml	(+)-(*S*)-fluoxetine	(–)-(*R*)-fluoxetine	IS
1	81.20	80.78	94.71
200	86.39	90.33	
400	81.89	89.05	

Fluoxetine was found to be stable in rat plasma during three cycles of freezing and thawing, for 4 h at room temperature, and for 24 h in the auto-injector at 5 °C, given that deviations of less than 15% were measured compared with newly prepared samples (Table 3).

TABLE 2. Validation parameters of the analysis method of fluoxetine enantiomers in rat plasma

	(+)-(*S*)-fluoxetine	(–)-(*R*)-fluoxetine
Absolute Recovery (%)		
1 ng/ml	87.60	81.81
200 ng/ml	84.33	79.67
400 ng/ml	81.40	80.20
Linearity (ng/ml)	0.5–500	0.5–500
Straight line equation	0.0771619X + 0.0208118	0.0782715X + 0.0276739
R^2	0.997	0.997
Limit of Quantification (ng/ml)	0.5	0.5
Precision (CV %, $n = 10$)	9.21	9.73
Accuracy (Inaccuracy%)	−7.62	−5.35
Inter-assay Precision (CV %)		
1 ng/ml ($n = 5$)	5.98	5.83
200 ng/ml ($n = 5$)	5.66	6.78
400 ng/ml ($n = 5$)	6.28	7.43
Intra-assay Precision (CV %)		
1 ng/ml ($n = 5$)	5.51	5.00
200 ng/ml ($n = 5$)	7.57	7.18
400 ng/ml ($n = 5$)	3.18	3.19
Inter-assay Accuracy (error %)		
1 ng/ml ($n = 5$)	−0.99	−2.87
200 ng/ml ($n = 5$)	−6.28	−5.94
400 ng/ml ($n = 5$)	−1.70	−0.98
Intra-assay Accuracy (error %)		
1 ng/ml ($n = 5$)	7.58	−8.74
200 ng/ml ($n = 5$)	−11.71	−12.78
400 ng/ml ($n = 5$)	−9.53	−10.74

CV = coefficient of variation [(standard deviation/mean) × 100]; r = linear correlation coefficient.
Error % = $[(C_{obs} - C_{added})/C_{added}] \times 100$.

TABLE 3. Stability of fluoxetine enantiomers in rat plasma evaluated as % deviation compared with freshly prepared samples

Concentration	Short term (4 h)	Freezing/thawing (3 cycles)	Postprocessing (24 h)
1 ng/ml			
(+)-(*S*)-fluoxetine	−5.54	−12.73	2.30
(–)-(*R*)-fluoxetine	9.43	1.13	5.75
400 ng/ml			
(+)-(*S*)-fluoxetine	5.6	−6.00	6.36
(–)-(*R*)-fluoxetine	9.95	9.49	6.89

TABLE 4. Pharmacokinetic parameters of fluoxetine enantiomers after the administration of 10 mg/kg to Wistar rats exposed to gasoline and to control rats ($n = 8$ at each time point)

	Parameter	(+)-(*S*)-fluoxetine	(–)-(*R*)-fluoxetine
Control group	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	650.91 ± 182.54	386.48 ± 130.90
	Cl/f ($liter \cdot h^{-1} \cdot kg^{-1}$)	7.68 ± 4.64	12.93 ± 4.38
	$AUC_{(+)}/AUC_{(-)}$	1.68 **	
Gas group	$AUC_{0-\infty}$ ($ng \cdot h \cdot ml^{-1}$)	923.32 ± 201.70*	857.71 ± 196.96*
	Cl/f ($liter \cdot h^{-1} \cdot kg^{-1}$)	$5.41 \pm 1{,}40$*	5.82 ± 1.34*
	$AUC_{(+)}/AUC_{(-)}$	1.07	

*Different from controls ($P < 0.05$).
**AUC are different for enantiomers ($P < 0.05$). Data are presented as means ± SD.

This method was applied to study the effect of gasoline exposure on the pharmacokinetics of fluoxetine enantiomers in rats treated with a single dose (by oral gavage) of 10 mg/kg of the racemic drug. The current study is the first to report the AUC and clearance values of the individual enantiomers of fluoxetine in rats. The data presented in Table 4 show higher plasma concentrations of the enantiomer (+)-(*S*)-fluoxetine in the control group, with an enantiomeric ratio $AUC_{(+)\text{-}(S)/(-)\text{-}(R)}$ of 1.68 (Fig. 2). The plasma accumulation of the enantiomer (+)-(*S*)-fluoxetine is consistent with the findings of Guo et al.[2], although in the present study, the animals were subjected to stress as a result of prolonged restraint in the exposure chamber (6 h/day, 5 days/week for 6 weeks). In animals exposed to gasoline at concentrations of 600 ppm, which is twice the TLV-TWA of 300 ppm, an increase was observed in the $AUC_{0-\infty}$ for both enantiomers. However, a steeper increase was noted for the (–)-(*R*)-fluoxetine enantiomer, resulting in an enantiomeric ratio $AUC_{(+)\text{-}(S)/(-)\text{-}(R)}$ of 1.07, and thus, a loss of enantioselectivity (Fig. 2). The data presented in Table 4 also shows that exposure to gasoline reduces the clearance of the (–)-(*R*)-fluoxetine enantiomer by approximately 55%, whereas that of the (+)-(*S*)-fluoxetine enantiomer is reduced by only 30%. Considering that in human liver microsomes CYP2D6 and CYP2C9 are involved in the metabolism of (–)-(*R*)-fluoxetine and only CYP2D6 is involved in the metabolism of (+)-(*S*)-fluoxetine[5], the enantiomeric ratio $AUC_{(+)\text{-}(S)/(-)\text{-}(R)}$ reduction in animals exposed

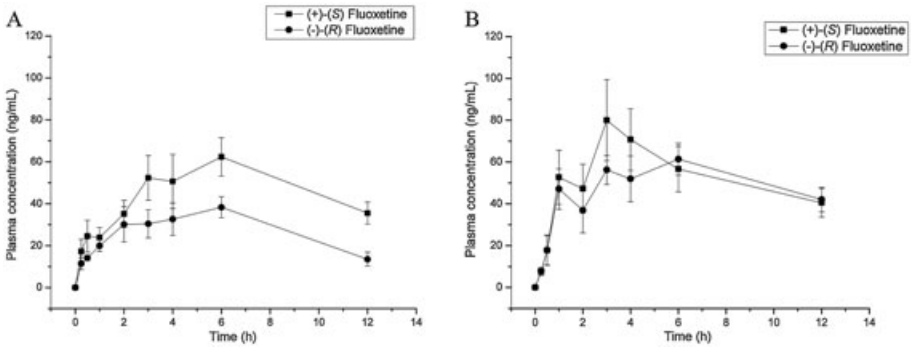

Fig. 2. Pharmacokinetic profile of (+)-(*S*) and (–)-(*R*) fluoxetine enantiomers represented by the mean plasma concentrations observed and their respective standard errors of the mean after the administration of 10 mg/kg fluoxetine to Wistar rats ($n = 8$ at each time point). **A**, control group; **B**, gasoline-exposed group.

to gasoline suggests that the CYP isoforms involved in the metabolism of the (–)-(*R*)-fluoxetine enantiomer are inhibited predominantly.

CONCLUSION

In conclusion, exposure to gasoline (6 h/day, 5 days/week for 6 weeks) at concentrations of 600 ppm, which is equivalent to twice the TLV-TWA of 300 ppm, results in the loss of enantioselectivity in the pharmacokinetics of fluoxetine, reducing predominantly the clearance of the enantiomer (–)-(*R*)-fluoxetine in rats treated with a single oral dose (by gavage) of the racemic drug.

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