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Determination of fluoxetine, norfluoxetine and their enantiomers in rat plasma and brain samples by liquid chromatography with fluorescence detection

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Abstract

Fluoxetine (FLX) and norfluoxetine (NFLX) racemic mixtures were determined by reversed-phase liquid chromatography with fluorescence detection (λ_{exc} = 227 nm, λ_{em} = 305 nm). The calibration curves prepared from drug-free plasma and brain were linear in the range of 5–1000 ng ml^{-1} and 100–40,000 ng g^{-1} for doped samples, with detection limits of 3.2 and 2.1 ng ml^{-1} in plasma and 31.5 and 26.1 ng g^{-1} in brain tissue for FLX and NFLX, respectively. Enantiomer determination was carried out through normal phase HPLC-FD (λ_{exc} = 224 nm, λ_{em} = 336 nm) after precolumn chiral derivatization with *R*-1-(1-naphthyl)ethyl isocyanate. Standard curves also prepared in a drug-free matrix were linear for each enantiomer over the range of 2–1000 ng ml^{-1} and 20–7000 ng g^{-1} with detection limits for the four compounds ranging between 0.2 and 0.5 ng ml^{-1} in plasma and between 3.0 and 8.2 ng g^{-1} in brain tissue. In both methods the analytes were isolated from the biological matrix by a new solid-phase extraction procedure with recovery in plasma and brain over 90 and 87%, respectively. The repeatability of this extraction procedure was satisfactory within-day and between-day with CV < 9.1%. This study also offered the opportunity to obtain an assessment of the potential relationships between the concentration of individual enantiomers of FLX and NFLX in plasma and brain tissue after chronic treatment with racemic FLX at a dose intended to mimic the human plasma concentration of FLX in standard clinical conditions, and therefore should make for more reliable extrapolation of neurochemical findings in other species.

Keywords: Fluoxetine and norfluoxetine enantiomers; HPLC-FD; Chiral derivatization; Chronic treatment with fluoxetine; Rat plasma and brain levels

1. Introduction

Fluoxetine (FLX) (*N*-methyl-3-phenyl-3-[(α,α,α-trifluoro-*p*-tolyl)oxy]-propylamine), one of the most frequently prescribed drugs for the management of depression, and its active *N*-demethylated metabolite norfluoxetine (NFLX) (Fig. 1) share the property of blocking the serotonin (5-HT) transporter. Although FLX is one of the most researched selective serotonin reuptake inhibitors (SSRIs) the factors affecting the therapeutic efficacy have not been fully characterized. Previous clinical studies have failed to identify a clear cut correlation between clinical response and plasma levels of the parent drug and its active demethylated metabolite NFLX [1,2]. In fact, in patients treated with standard clinical doses (20 mg day^{-1}) of racemic FLX, plasma FLX concentration increased steadily from 0.08 μM on day 3 to 0.16 μM by day 14 [3], and it has been suggested that plasma concentrations of FLX + NFLX above 0.3 μM are associated with a lower therapeutic efficacy [2]. To some extent, difficulties in identifying useful correlations may be ascribed to the fact that conventional methods for the determination of FLX and NFLX plasma levels do not discriminate between their respective enantiomers. It should be borne in mind that FLX is administered as a racemate of the *R* and *S* enantiomers, although it has been found that the chiral forms of FLX and NFLX differ considerably in their pharmacological properties. While the two enantiomers of FLX are approximately equipotent in blocking 5-HT reuptake, the enantiomers of NFLX show marked differences in pharmacological activity, with the *S*-enantiomer being

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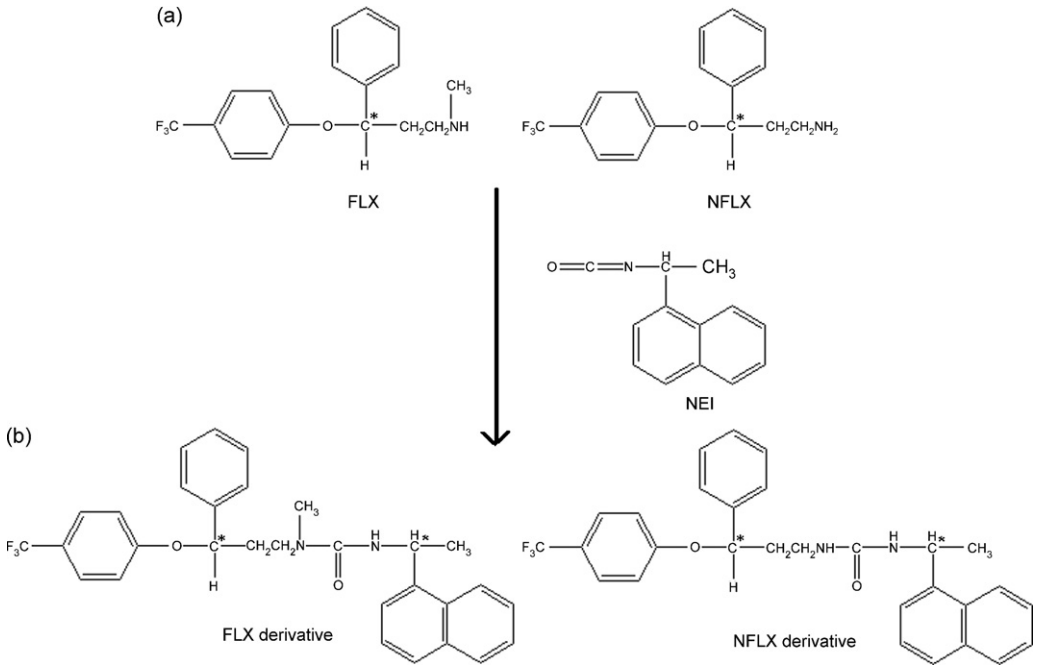

Fig. 1. (a) Chemical structures of fluoxetine and norfluoxetine. The asterisk indicates chiral centers of the molecules. (b) Diastereoisomers obtained after derivatization of fluoxetine and norfluoxetine enantiomers with NEI chiral reagent in dry hexane.

approximately 20 times more potent than the *R*-enantiomer as a 5-HT-reuptake inhibitor both in vitro and in vivo [4,5]. Based on these findings, it is clear that an adequate understanding of the role of pharmacokinetic variability in explaining intraindividual and interindividual differences in clinical response must take into account the concentration of individual enantiomers. Furthermore, this is especially critical in view of evidence from in vitro [6,7] and in vivo [8] studies indicating that the metabolism of FLX and NFLX by cytochromes CYP2D6 and possibly CYP2C9 is stereoselective [9].

From a neurochemical point of view, the antidepressant effect of SSRIs is thought to derive from the property of blocking the 5-HT transporter. Yet, blockade of the 5-HT reuptake process takes place within a few hours, whereas the onset of their therapeutic effect ranges from 2 to 3 weeks of treatment. This suggests that adaptative changes in brain 5-HT neurotransmission account for the antidepressant activity of SSRIs. In animal models (i.e. rodent brains), the prolonged administration of SSRIs has been reported to desensitize raphe 5-HT_{1A} autoreceptors, as assessed by single unit recordings and microdialysis [10,11]. However, other studies have failed to observe such effects with regard to the ability of these agents to desensitize and down-regulate the density of raphe 5-HT_{1A} autoreceptors after chronic (2–3 weeks) treatments even using large doses of SSRIs [12–14]. In spite of its widespread pharmacological use, the physiological disposition of FLX and NFLX enantiomers in animals have not been thoroughly investigated yet in chronic studies. Due to the fact that disposition studies of FLX and NFLX enantiomers in animals may help to understand its action mechanism and facilitate extrapolation of biochemical findings (mostly the neurochemical adaptation of 5-HT neurotransmission) to man, we determined the dose-proportion after long-term intraperitoneal administration to rats (steady-state conditions), a specie widely used in neurochemical studies of this drug. The aim of this investigation was to see which of these doses result in plasma levels of the drug itself and of its metabolite comparable to those found in depressed patients under standard clinical treatment. This study also offers the opportunity to obtain an assessment of potential relationships between the concentration of individual enantiomers of FLX and NFLX in plasma and brain tissue after chronic treatment with racemic FLX at a dose intended to mimic the human plasma concentration of FLX in standard clinical conditions, and therefore should make for more reliable extrapolation of neurochemical findings across species.

Although it was in 1988 when fluoxetine was introduced commercially, it was in 1976 when the first clinical trials were carried out along with the development of the first analytical methodology. With regard to the analytical methods subsequently developed the techniques used are basically gas chromatography with electronic capture (ECD), mass spectrometry (MS) and nitrogen–phosphorus detections (NPD) [15–17] and HPLC with ultraviolet (DAD), fluorescence (FD) and MS detections [18–21]. Many of the proposed methods for the determination of fluoxetine and norfluoxetine in plasma did not discriminate between their respective enantiomers. Nevertheless, over recent years, separation by HPLC has been developed enormously with stationary chiral phases, which has enabled the separation of the fluoxetine enantiomers [22] and the monitoring of the *R* and *S* enantiomers of fluoxetine and norfluoxetine in plasma following treatment with the racemic drug [23]. With regard to the separation methods based on derivatization with chiral agents – indirect method – GC–MS [24] or GC-ECD [25] employing (*S*)-(−)-*N*-trifluoroacetylpropile chloride as a derivatizing agent, were used more often while in the case of HPLC-FD, previous derivatization with (1-naphtyl)ethyl isocyanate [26] was fundamentally used.

Finally, the majority of previously reported methods suggest liquid–liquid extraction of this antidepressant from the biological matrix [20,23]. These procedures involved multiple steps and are labour intensive, time consuming and require relatively large quantities of organic solvents. In this regard, a sample clean-up step using solid-phase extraction technique allows faster, more robust analytical procedure and lower detection limits [27–29].

The purpose of this work was to develop a simple and sensitive method for the simultaneous separation of FLX and NFLX enantiomers by normal-phase HPLC-FD in order to investigate any potential source of variability in the plasma and brain tissue samples concentrations of the *R* and *S* enantiomers of FLX and NFLX in a large population of rats receiving chronic FLX treatment. In the same way a new method for plasma and brain tissue sample preparation was accurately implemented. The method involves four steps extraction based on SPE and improves the extraction recovery leading to higher sensitivity.

2. Experimental

2.1. Chemical and solutions

All reagents were analytical grade of the highest purity available. Racemic fluoxetine and norfluoxetine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Optically pure *R* and *S* enantiomers of FLX and NFLX were kindly donated by Professor Pierre Baumann (Prilly-Lausanne, Switzerland). Serotonin (5-HT), 5-hydroxyindolacetic acid (5-HIAA) and *R*-1-(1-naphthyl)ethyl isocyanate were supplied by Sigma (St. Louis). HPLC grade solvents hexane, isooctane and tetrahydrofuran were purchased from Romil (Cambridge, UK). Hydrochloric acid, ammonium hydroxide, tetramethyl ammonium chloride, methanol, sodium bicarbonate, sodium citrate, acetonitrile, dichloromethane and isopropyl alcohol were obtained from Merck (Darmstadt, Germany).

Stock solutions were prepared in water to contain 1 mg ml^{-1} of each racemic compound or each enantiomer. Aqueous reference solution containing both FLX and NFLX or the mixture of the enantiomers to a final concentration 10 mg l^{-1} was prepared from the standard stock solution of each compound. Working standard solutions were prepared by diluting the appropriate volume of the 10 mg l^{-1} reference solution up to 10 ml with water.

The derivatization reagent was a solution 200 μM of *R*-1-(1-naphthyl)ethyl isocyanate (NEI) in hexane prepared daily from pure reagent stored at −80 °C.

2.2. Apparatus and chromatographic conditions

Chromatographic experiments were performed using a Hewlett Packard (Palo Alto, CA, USA) HPLC system Model HP 1100 with a fluorescence detector provided with an autosampler.

Chromatographic separation of (±)-FLX and (±)-NFLX was achieved using a column Extrasil CN (15 cm × 0.40 mm) with a particle size of 5 μm (Tracer Anal., Barcelona, Spain) and the mobile phase was 75% tetramethyl ammonium chloride in acetonitrile with a flow rate of 1 ml min^{-1}. The detection conditions were determined from observations made from excitation and emission spectrum. The excitation and emission wavelengths of (±)-FLX and (±)-NFLX were set at 227 and 305 nm, respectively.

Enantiomeric separation of the fluorescent derivatives of FLX and NFLX with NEI was achieved using a 25 cm × 0.46 mm Apex Silica column with a particle size of 5 μm from Jones Chromatography (Littleton, USA). The mobile phase consisted of a mixture of tetrahydrofuran and isooctane (40:60, v/v) while the flow rate was 1 ml min^{-1}. The excitation and emission wavelengths for the derivatives were set at 224 and 336 nm, respectively.

2.3. Sample collection

Experiments were performed on male Sprague–Dawley rats (Harlan Ibérica, Barcelona, Spain) weighing 225–250 g. Animals were group-housed under standard laboratory conditions (22 ± 1 °C, 60–65% relative humidity, 12-h light:12-h dark alternate cycles with lights on at 07:00 a.m., food and water ad libitum) and were acclimatized to the research facility for 1 week before the study. All the procedures involving animals and their care were conducted in conformity with the European Communities Council Directive on "Protection of Animals Used in Experimental and Other Scientific Purposes" (86/609/EEC).

Racemic FLX hydrochloride was dissolved in saline solution (0.9% NaCl) in doses of 1, 3, 5 and 10 mg kg^{-1}, and injected intraperitoneally (i.p.) at 09:30 a.m. for 21 days. Control rats received only the vehicle (0.9% NaCl, i.p. daily). These conditions were chosen in order to make a comparison to previous studies where similar doses (1–10 mg kg^{-1}) of FLX were also administered daily for 2–3 weeks in order to observe adaptative changes in 5-HT neurotransmission of rat brain [14,30–33].

Rats were anesthetised with ether and killed by decapitation during the light phase, 3 h after the last injection (i.e. 12:30 a.m.), and the brains were removed and dissected on ice for the preparation of frontal cortices and then stored at −80 °C until analysis. Blood samples were drawn by cardiac puncture and collected into tubes containing 0.5 ml of sodium citrate 0.129 M. The plasma was obtained by centrifugation at 1375 × *g* for 15 min and was stored at −80 °C until analysis.

2.4. Extraction procedure

The solid-phase extraction (SPE) of (±) FLX, (±) NFLX and their enantiomers *R* and *S*, were optimized using C18 cartridges (Waters, Milford, USA) (1 cm^3, 100 mg). The whole process was controlled with a Visiprep DL vacuum system from Supelco (Bellefonte, PA, USA).

The conditioning of the SPE cartridges was effected by passing 1 ml HCl 1 M, 1 ml of methanol, 1 ml of water and 1 ml $NaHCO_3$ 1% through the cartridge to establish a slightly basic pH. Thereafter it was loaded with 0.5 ml plasma previously diluted with 0.5 ml of water and washed sequentially with 1 ml of water and 1 ml of acetonitrile. The retained compounds were eluted with 1 ml of dichloromethane:isopropyl alcohol:ammonia solution (25%) (78:20:2) and evaporated to dryness under nitrogen. The residue was reconstituted with 0.5 ml of hexane.

Brain tissue sample (50 mg) was put onto a 1.5 ml eppendorf microtube and homogeneized with an ultrasonic cell disruptor (Model Labsonic, B. Braun Mesulgen AG, Leinfelden, Germany) in 0.5 ml of water. After centrifugation at 13,000 rpm for 10 min at 4 °C in a refrigerated centrifuge (Model Sorval RMC 14, Sorval Instruments Inc., Lansdale, USA), the supernatant was loaded onto the previously conditioned SPE cartridge following the above mentioned process.

2.5. Derivatization procedure

The determination of the enantiomers of FLX and NFLX required the derivatization of the amine moiety to form diasterisomers, which were able to be separated using normal phase chromatography. For the preparation of these derived diasterisomers the chiral agent *R*-1-(1-naphthyl)ethyl isocyanate (NEI) was selected. This way 100 μl of NEI solution 200 μM were added to the extract obtained after SPE. Following evaporation and dissolution of residue in 0.25 ml of mobile phase, the resulting sample was quantitated by HPLC.

2.6. Analysis of serotonin (5-HT) and 5-hydroxyindol acetic acid (5-HIAA)

Quantitation of the 5-HT and 5-HIAA content of the frontal cortex extracts was performed by reversed phase liquid chromatography with fluorescence detection. Brain tissue was homogenised with an ultrasonic cell disruptor (Model Labsonic, B. Braun Mesulgen AG, Leinfelden) and centrifuged at 13,000 rpm for 10 min at 4 °C in a refrigerated centrifuge (Model Sorval RMC 14, Sorval Instruments Inc.). The supernatant was filtered through a 0.22 μm Waters Durapore filter (Milford, MA, USA) and 100 μl were injected into the HPLC system.

Chromatographic separation was carried out at room temperature using a Spherisorb ODS2 (15 cm × 0.4 cm, 5 μm) column with a Tracer ODS (1 cm × 0.4 cm) guard column cartridge both from Teknokroma (Barcelona, Spain). Optimum separation was achieved with a binary mobile phase where solution A was acetonitrile and solution B was tetramethylammonium chloride (0.3%, pH 2.8). The gradient program was: 1–9 min, 1:99 (A:B); 9–10 min, from 1:99 (A:B) to 5:95 (A:B); 10–22 min, 5:95 (A:B); 22–23 min, return to initial conditions. The flow rate was kept at 1 ml min^{-1}. The excitation and emission wavelength were set at 227 and 338 nm, respectively. The detection limits were 1.2 ng g^{-1} for 5-HT and 5.8 ng g^{-1} for 5-HIAA.

2.7. Statistics

Results are expressed as arithmetic mean ± S.E.M. values obtained from the indicated number of rats. Statistical analysis of absolute neurotransmitter and drug levels were carried out by one-way analysis of variance (ANOVA). Significant differences between groups were evaluated using post hoc Bonferroni's test after significant ANOVA. A probability value of $p < 0.05$ was considered significant. One-way ANOVA with Bonferroni's post-test was performed using Graph Pad Prism Version 4.00 for Windows (Graph Pad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Optimization of derivatization procedure

The separation proposed for the enantiomers requires prior derivatization of secondary and primary amines of FLX and NFLX, respectively. Among the possible chiral agents which could have been used for the derivatization of amines, NEI was chosen in this study as it permits the joint derivatization of primary and secondary amines and, furthermore, the diasteroisomers formed (Fig. 1) are stable.

In order to increase the efficiency of derivatization, some parameters such as NEI amount, temperature and incubation time were studied.

The added NEI amount was adjusted to values ranging from 5×10^{-9} to 4×10^{-8} mol. Fig. 2 shows a significant increase in the chromatographic response until 1.5×10^{-8} mol of NEI were added and from this concentration on the response was maintained. With the purpose of assuring the total derivatization of all the analytes in real samples, an excess of NEI, 2×10^{-8} mol, was added.

The effect of the derivatization temperature was investigated by incubating at 20, 35, 45, 55 and 65 °C for 30 min, but no variation was observed.

The time of derivatization was studied by measuring the peak area of each substance at different incubation times from 0 to 180 min, but no significant change was observed during this time so the incubation step was removed. In addition, it could also be observed that the diasteroisomers were stable for a minimum of 3 h, long enough to avoid the possible limitation that this aspect may have introduced into the method.

3.2. Chromatographic separation

Some parameters such as chromatographic column, mobile-phase composition and flow gradient were studied in order

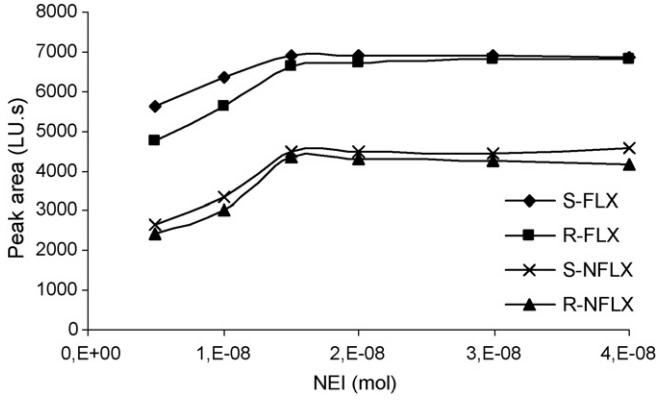

Fig. 2. Effect of added NEI amount on the derivatization efficiency. Chromatographic conditions: column, Apex Silica (5 μm, 25 cm × 0.46 mm); mobile phase, tetrahydrofuran:isooctane (40:60, v/v); flow rate, 1 ml min^{-1}; injected volume, 100 μl; sample concentration, 1 μg ml^{-1}; detection wavelength, λ_{exc} 224 nm, λ_{em} 336 nm.

to increase the efficiency of the chromatographic separation.

3.2.1. Chromatographic separation of fluoxetine and norfluoxetine racemics

Due to the high solubility in water of FLX and its metabolite NFLX, separation was carried out by reversed phase chromatography. Several kinds of chromatographic columns were used to optimize the peak resolution, including Spherisorb ODS2 (5 μm, 15 cm × 0.4 cm i.d.), Spherisorb ODS2 (5 μm, 25 cm × 0.4 cm i.d.), Spherisorb ODS2 (3 μm, 25 cm × 0.4 cm i.d.) and Extrasil CN (5 μm, 15 cm × 0.4 cm i.d.). Although the Spherisorb ODS2 column type permitted a good separation between the compounds for a reasonable analysis time, in the processing of the real samples we were able to observe the presence of an interfering signal close to the chromatographic peak of the NFLX, the influence of which could not be avoided despite working with a longer column and a smaller sized particle. Nevertheless, the column Extrasil CN (5 μm, 15 cm × 0.4 cm i.d.) enabled a correct separation of the chromatographic peaks of interest.

For the mobile phases, mixtures of water–acetonitrile were assayed, to which tetramethyl ammonium chloride (TMACl) was added which is capable of forming an ionic par with analytes and influencing in the retention times. In the absence of TMACl the chromatographic separation factors α and R_s were adequate, however, when this was added and the concentration was increased the chromatographic peaks became sharper at the same time as retention times decreased. A concentration 0.03 M of TMACl was most suitable for achieving a better resolution in a shorter analysis time.

The acid–base properties of the FLX and NFLX mean that the pH of the mobile phase could influence the chromatographic separation. The range studied was between 3 and 7 and no significant differences were observed with regard to the chromatographic resolution. In addition, it is known that maximum excitation and emission may vary with the pH, so at the same time the influence of this variable on the intensity of the signal was studied. A progressive reduction could be observed from pH 3 and therefore this was the value selected as optimum in the study. Furthermore, the influence of the presence of the organic acetonitrile modifier on the chromatographic separation was studied. Acetonitrile percentages below 20% and analysis times above 10 min were required, while for values above 40% the co-elution of compounds took place. The most suitable percentage for a good separation in a reasonable analysis time was 25% (Table 1).

3.2.2. Enantioselective chromatographic separation of fluoxetine and norfluoxetine

After the derivatization in hexane of *R* (−) and *S* (+) enantiomers of FLX and NFLX, the separation of the diasteroisomers obtained was carried out by chromatography in a normal phase using an Apex Silica column (5 μm, 25 cm × 0.46 cm) from Jones Chromatography (Littleton).

With regard to the mobile phase, work was carried out with apolar solvents such as hexane, tetrahydrofurane, chloroform and isooctane. From the results obtained it was deduced that hexane provoked a considerable increase in retention times and chloroform did not make an improvement in the resolution of the chromatographic peaks. Work was therefore carried out with variable compositions of tetrahydrofurane and isooctane and the chromatographic parameters of retention, separation and resolution were taken into account. It can be seen in Table 2 that with the exception of the mobile phase made up of tetrahydrofurane:isooctane (45:55), all the others obtained acceptable values for the separation parameters. In addition, for THF percentages below 40% the analysis time was longer than 18 min, for that reason the most suitable mobile phase was considered to be constituted by tetrahyrofuran:isooctane (40:60).

Table 1
Capacity factor (k), separation factor (α) and resolution (R_{ij}) for racemic FLX and NFLX obtained varying the acetonitrile (AcN) percentage in the mobile phase composition (tetramethylamonnium chloride 0.03 M:AcN)

AcN (%)	Compound	t_r (min)	K	α	R_{ij}
20	NFLX	7.46	2.73	1.17	9.50
	FLX	8.40	3.20		1.33
25	NFLX	4.82	2.21	1.28	7.38
	FLX	5.74	2.83		1.66
30	NFLX	3.8	2.17	1.13	7.54
	FLX	4.15	2.46		0.90
40	NFLX	2.89	1.89	1.12	6.75
	FLX	3.11	2.11		0.77

Chromatographic conditions: column, Extrasil CN (5 μm, 15 cm × 0.46 mm); flow rate, 1 ml min^{-1}; injected volume, 100 μl; sample concentration, 1 μg ml^{-1}; detection wavelength, λ_{exc} 227 nm, λ_{em} 305 nm.

Table 2
Capacity factor (k), separation factor (α) and resolution (R_{ij}) for FLX and NFLX enantiomers obtained with different mobile phases

Mobile phase	Compound	t_r (min)	K	α	R_{ij}
THF:IOT (25:75)	*S*-FLX	13.41	1.68	1.21	10.01
	R-FLX	15.15	2.03	2.21	1.53
	S-NFLX	27.46	4.49	1.20	6.50
	R-NFLX	32.03	5.41		1.72
THF:IOT (30:70)	*S*-FLX	11.41	1.54	1.17	10.01
	R-FLX	12.59	1.80	2.06	1.36
	S-NFLX	21.20	3.71	1.26	6.00
	R-NFLX	25.60	4.69		2.00
THF:IOT (35:65)	*S*-FLX	9.13	1.28	1.15	9.50
	R-FLX	9.90	1.48	1.90	1.14
	S-NFLX	15.21	2.80	1.21	5.18
	R-NFLX	17.52	3.38		1.64
THF:IOT (40:60)	*S*-FLX	7.44	1.15	1.19	9.15
	R-FLX	8.18	1.36	1.64	1.63
	S-NFLX	11.21	2.24	1.18	4.39
	R-NFLX	12.63	2.65		1.59
THF:IOT (45:55)	*S*-FLX	5.62	0.87	1.16	7.49
	R-FLX	6.05	1.02	1.42	1.12
	S-NFLX	7.33	1.44	1.28	2.88
	R-NFLX	8.55	1.85		2.37

Chromatographic conditions: column, Apex Silica (5 μm, 25 cm × 0.46 mm); flow rate, 1 ml min^{-1}; injected volume, 100 μl; sample concentration, 1 μg ml^{-1}; detection wavelength, λ_{exc} 224 nm, λ_{em} 336 nm.

Table 3
Linearity and detection limits in plasma and brain tissue of racemic fluoxetine and norfluoxetine using the experimental conditions for the racemic determination[a] and of fluoxetine and norfluoxetine enantiomers using the experimental conditions for the enantiomers determination outlined in the text[b]

Compound	Slope ± S.D. (LU s ng^{-1} ml)	Intercept ± S.D. (LU s)	Correlation coefficient	LOD (ng ml^{-1})
Plasma				
FLX[a]	0.244 ± 0.007	3.884 ± 1.546	0.998	3.2
NFLX[a]	0.298 ± 0.007	2.968 ± 1.367	0.999	2.1
S-FLX[b]	9.256 ± 0.220	7.617 ± 20.449	0.999	0.2
R-FLX[b]	9.567 ± 0.271	7.089 ± 25.137	0.998	0.2
S-NFLX[b]	7.467 ± 0.241	−2.679 ± 22.251	0.997	0.5
R-NFLX[b]	7.409 ± 0.176	−7.651 ± 16.295	0.999	0.5
Brain tissue				
FLX[a]	0.025 ± 0.001	2.560 ± 3.124	0.999	31.5
NFLX[a]	0.026 ± 0.001	9.983 ± 5.972	0.996	26.1
S-FLX[b]	0.844 ± 0.020	−5.140 ± 47.336	0.997	3.0
R-FLX[b]	0.846 ± 0.028	−11.004 ± 62.414	0.995	3.5
S-NFLX[b]	0.826 ± 0.028	−1.560 ± 56.748	0.995	7.5
R-NFLX[b]	0.780 ± 0.030	−13.082 ± 70.552	0.993	8.2

Next, the effect of the column temperature was investigated between 20 and 55 °C (oven Model Gekno 2000, Essex, UK) and a decline in the chromatographic response was noticed as the temperature increased. Consequently this was maintained at 20 °C.

The experimental conditions indicated above allowed an effective chromatographic separation to be achieved with good resolution coefficients.

3.3. Assay validation

For the analytical assessment of the method, both in the case of the determination of racemic FLX and its metabolite NFLX and for the case of the separation of their enantiomers, a calibration was carried out in doped plasma and cerebral cortex samples of control rats. The calibration curves of the racemic compounds exhibited linearity in the range of 5–1000 ng ml^{-1} for spiked plasma samples and from 100 to 40,000 ng g^{-1} for the brain tissue.

The linear regression equations (10 concentration points) obtained using the least-squares method showed that the correlation coefficients were higher than 0.996. The calculation of the detection limits was based on a 2*N*/*m* ratio, where "*N*" is the noise and "*m*" is the sensitivity or slope of the respective calibration equation (Table 3).

The repeatability of the method was estimated by making 10 repetitive extractions at 2 different levels of concentration under the selected condition. The samples were also analyzed at intervals over a 2 week period ($n = 10$) in order to determine the interday R.S.D.s, the results in plasma and brain tissue were less than 9%.

The average recoveries from 10 separated batch assays (close to detection limits) were 95.5% for FLX and 92.1% for NFLX in plasma sample and 93.1 and 94.2% for FLX and NFLX in brain tissue (Table 4).

The calibration curves of each enantiomer ($n = 10$) were linear over the range of 2–1000 ng ml^{-1} for plasma samples and from 20 to 7000 ng g^{-1} for the brain tissue, with adjusted coefficients between 0.993 and 0.999. Furthermore, the detection limits were calculated for each enantiomer obtaining values which ranged between 0.2 and 0.5 ng ml^{-1} in plasma and 3.0 and 8.2 ng g^{-1} in the cerebral cortex, respectively. The detectability achieved with the method proposed for the separation of the *R* and *S* enantiomers of FLX and NFLX was an improvement on the results obtained by other authors for both assay matrices [26].

The recovery percentages studied at two concentration levels for all the enantiomers were higher than 90% in the case of the plasma matrix and 87% for the samples of cerebral cortex. The coefficients of in the day and interday variations varied between 2.6 and 9.1% (Table 4).

These methods were applied to plasma and brain tissue samples of rats treated with racemic fluoxetine (Figs. 3 and 4).

3.4. Plasma and brain fluoxetine and norfluoxetine concentrations and neurochemical effects

The described methods were used primarily to study steady-state plasma and brain FLX and NFLX levels in rats during chronic FLX administration and associated neurochemical changes. Although many studies have shown neurochemical and behavioural effects of chronic FLX treatment in rats, much less attention has been given to steady-state plasma and brain concentration of the drug required to produce such effects. Previous studies on the regulation of 5-HT$_{1A}$ receptors after chronic antidepressant treatment have produced controversial results. Depending on the dose administered, the duration and route of administration, as well as the brain area analysed, different authors have reported no changes, up- or down-regulation of 5-HT$_{1A}$ receptors [11,14,33]. The reasons for the choice of a particular protocol were rarely given. Some of these modifications of either behavioural, physiological or biochemical nature have been considered to be related to the antidepressant action of the drug. It is, however, surprising that these studies do not present data on the distribution of the drug and its metabolite in the plasma or brain of the treated animals. Here, the doses tested (1–10 mg kg^{-1}) are well within the range used for neu-

Table 4
Recoveries and intraday and interday repeatability ($n = 10$) of the analysis of racemic FLX and NFLX and *R* and *S* enantiomers at two concentation levels in plasma and brain tissue

Compound	Plasma (10 ng ml^{-1})			Plasma (500 ng ml^{-1})		
	Mean recovery (%)	R.S.D. (%) intraday	R.S.D. (%) interday	Mean recovery (%)	R.S.D. (%) intraday	R.S.D. (%) interday
FLX	95.5	5.0	6.3	96.3	5.5	4.0
NFLX	92.1	7.0	5.9	90.2	7.4	9.0
	Plasma (2.5 ng ml^{-1})			Plasma (500 ng ml^{-1})		
S-FLX	92.3	8.7	9.1	95.1	4.5	5.6
R-FLX	94.0	4.2	4.3	94.6	4.6	4.4
S-NFLX	91.2	2.6	4.7	90.8	4.4	4.8
R-NFLX	92.9	3.6	5.5	93.4	3.4	5.8
	Brain tissue (100 ng g^{-1})			Brain tissue (10 μg g^{-1})		
FLX	93.1	7.8	8.8	89.1	3.5	6.5
NLX	94.2	6.1	7.4	88.7	4.5	6.9
	Brain tissue (25 ng g^{-1})			Brain tissue (5 μg g^{-1})		
S-FLX	88.3	4.1	7.6	88.4	5.4	8.7
R-FLX	89.0	4.3	6.1	87.0	5.1	8.8
S-NFLX	91.2	8.4	9.0	94.5	8.1	9.1
R-NFLX	88.4	6.2	6.9	94.7	8.2	7.4

rochemical studies with racemic FLX. Furthermore, the dose range of 1–10 mg kg^{-1} was selected to encompass the plasma therapeutic range of FLX with regard to standard clinical use in the treatment of depression.

Both FLX and NFLX plasma concentrations increased linearly with the dose. The mean metabolite-to-parent drug concentration ratio ranged from about 1.8 to 0.91 mg kg^{-1} over the range investigated (Table 5), being statistically significantly lower between the 5 and 10 mg kg^{-1} doses. Evidence of capacity-limited elimination of the drug and/or its active metabolite emerged from previous single-dose studies of FLX administration in rats [34]. In humans chronic administration of FLX apparently raises the drug elimination $t_{1/2}$ compared with a single dose [35], further suggesting metabolism self-inhibition and/or saturation of microsomal enzymes. FLX and NFLX both concentrated in rat brain achieving levels 40–100 times those in plasma (Table 5). Results supported by previous tissue distribution studies [26,34], in which oral or intraperitoneally

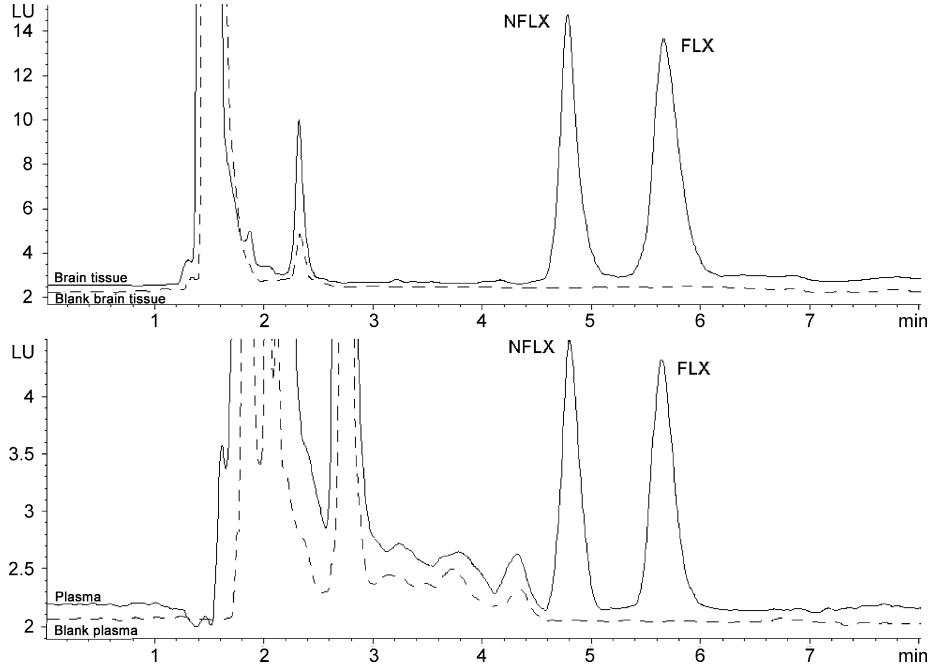

Fig. 3. Chromatogram of the fluoxetine and norfluoxetine in plasma and brain tissue of rat treated with 5 mg kg^{-1} under optimal experimental conditions. Blanks in broken line. Plasma: FLX, 160.6 ng ml^{-1}; NFLX, 118.4 ng ml^{-1}. Brain tissue: FLX, 7901.6 ng g^{-1}; NFLX, 6861.7 ng g^{-1}.

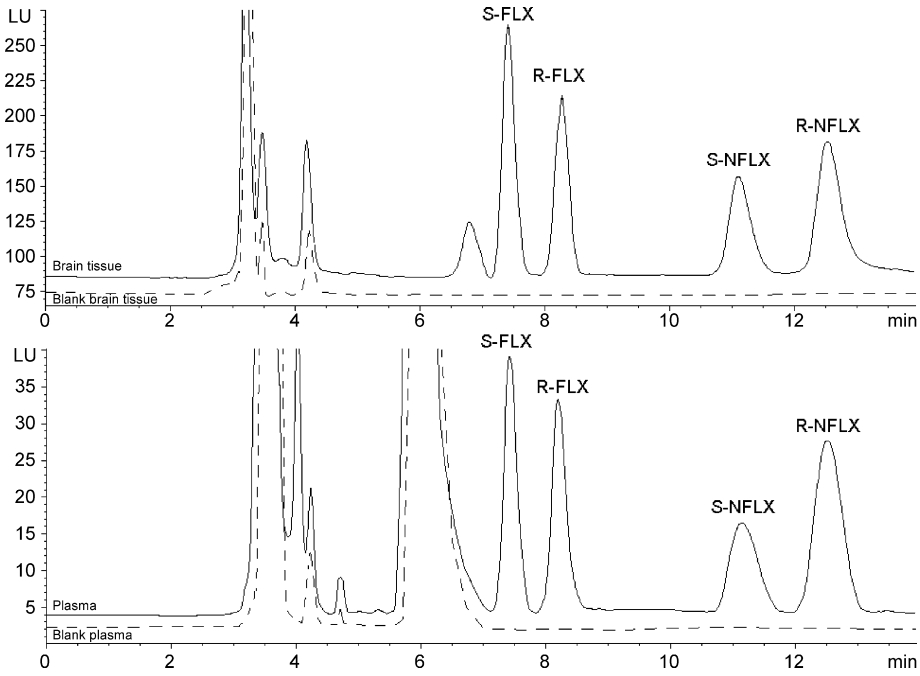

Fig. 4. Chromatogram of the fluoxetine and norfluoxetine enantiomers in plasma and brain tissue of rat treated with 5 mg kg^{-1} under optimal experimental conditions. Blanks in broken line. Plasma: *S*-FLX, 50.7 ng ml^{-1}; *R*-FLX, 51.4 ng ml^{-1}; *S*-NFLX, 38.7 ng ml^{-1}; *R*-NFLX, 101.0 ng ml^{-1}. Brain tissue: *S*-FLX, 3285.4 ng g^{-1}; *R*-FLX, 2324.7 ng g^{-1}; *S*-NFLX, 2219.8 ng g^{-1}; *R*-NFLX, 3819.7 ng g^{-1}.

Table 5
Mean plasma and brain concentrations of FLX and NFLX after intraperitoneal administration of different doses of racemic FLX during a period of 21 days

Compound	1 mg kg^{-1}	3 mg kg^{-1}	5 mg kg^{-1}	10 mg kg^{-1}
Plasma levels (ng ml^{-1})				
FLX	14.3 ± 2.9	45.9 ± 9.3	137.1 ± 13.9	310.1 ± 10.3
NFLX	25.5 ± 7.8	75.8 ± 8.0	124.4 ± 12.6	316.2 ± 16.5
Brain tissue levels (ng g^{-1})				
FLX	689.6 ± 42.6	3085.7 ± 472.5	7377.1 ± 891.5	27653.0 ± 3080.7
NFLX	775.4 ± 73.2	5445.5 ± 398.3	7354.7 ± 552.5	34494.9 ± 3346.8

Each value is the mean ± S.E.M. of six rats.

administered FLX rapidly attained higher concentrations in most organs of the rat (particularly liver, lung and brain) than in the blood. However, it must be emphasized that the brain to plasma ratio of FLX did not increase linearly with the dose (see Table 5).

Studies performed to date on the kinetic variability of FLX in humans and the potential relationship between plasma FLX levels and clinical response have described a broad range of racemic plasma levels of 40–500 ng ml^{-1} [1,3,8,36–39], and indeed no therapeutic window has been found for FLX [1]. However, it has been suggested that plasma concentrations of FLX + NFLX above 500 ng ml^{-1} are associated with a lower therapeutic efficacy [39]. Thus, our results indicate that doses of FLX above 5 mg kg^{-1} administered chronically will not mimic the chronic effects of FLX in depressed patients.

Levels of 5-HT and 5-HIAA concentrations as well as the 5-HIAA/5-HT ratio following 21-day administration of FLX at doses of 1, 3, 5 and 10 mg kg^{-1} are presented in Table 6. As shown in Table 6, 5-HT and 5-HIAA levels in the rat brain frontal cortex were affected by the long-term treatment with FLX. Chronic FLX treatment caused a dose-dependent decrease in the brain frontal cortex of 5-HT levels (by between 40 and 56% depending on the dose examined) ($F_{4,29} = 14.45$, $p < 0.0001$). In fact, the former effect reached post hoc significance in the 3 mg kg^{-1} FLX-treated rats, and was similar to that elicited by higher (5–10 mg kg^{-1}) FLX doses. Tissue 5-HIAA levels

Table 6
Dose–response of chronic FLX-treatment (21 days) on the levels of 5-HT, 5-HIAA and the 5-HIAA/5-HT ratio in rat frontal cortex

Treatment (mg kg^{-1}, i.p.)	5-HT (ng g^{-1})	5-HIAA (ng g^{-1})	5-HIAA/5-HT
Saline	77.3 ± 7.6	119.5 ± 9.6	1.57 ± 0.11
FLX 1	76.9 ± 7.7	119.8 ± 9.8	1.58 ± 0.12
FLX 3	47.0 ± 2.9**	52.9 ± 7.4***	1.19 ± 0.20
FLX 5	41.0 ± 3.8***	35.2 ± 5.6***	0.85 ± 0.10**
FLX 10	34.2 ± 2.2***	25.9 ± 2.0***	0.79 ± 0.10**

Results are the mean ± S.E.M. of data obtained from six rats per group. Data were analyzed by one-way ANOVA followed by Bonferroni's test.
** $p < 0.01$.
*** $p < 0.001$ compared with saline-treated rats.

Table 7
Mean plasma and brain concentrations of FLX and NFLX enantiomers after intraperitoneal administration of a 5 mg kg^{-1} day^{-1} dose of racemic FLX during a period of 21 days

Compound	Plasma levels (ng ml^{-1})	Brain tissue levels (ng g^{-1})
S-FLX	47.0 ± 2.3	3323.5 ± 217.2
R-FLX	47.8 ± 2.3	3499.1 ± 181.9
S-NFLX	51.0 ± 4.3	3200.0 ± 371.0
R-NFLX	92.0 ± 5.2*	4804.6 ± 342.3*

Each value is the mean ± S.E.M. of 36 rats.
* $p < 0.05$ vs. the *S*-enantiomer.

decreased more markedly (by between 56 and 78% depending on the dose examined) than those of 5-HT ($F_{4,29} = 37.66$, $p < 0.0001$). Again, the former effect reached post hoc significance in the 3 mg kg^{-1} FLX-treated rats where 5-HIAA levels were reduced compared to saline-treated rats, and again to a level similar to that elicited by higher (5–10 mg kg^{-1}) FLX doses. Consequently, these effects were associated with a lower 5-HT metabolism index (estimated by the [5-HIAA]/[5-HT] ratio) in the FLX-treated groups as indicated by one-way ANOVA ($F_{4,29} = 8.41$, $p = 0.0002$) (Table 6). In this case, the effect was significant at doses of 5 and 10 mg kg^{-1} of FLX. Previous studies have demonstrated that repeated administration of FLX decreases 5-HIAA, and, to a lesser extent, the 5-HT levels in the rat brain frontal cortex [30–32,40], changes which reflect limited access of 5-HT to intra-neuronal monoamine oxidase A [30,32,40], and FLX-elicited inhibition of 5-HT synthesis [31]. Moreover, the lack of effect of the higher doses of FLX (5–10 mg kg^{-1}) on 5-HIAA and/or 5-HT levels reported by others, are probably related to the duration of the wash out period (several days) which allowed full recovery of serotonergic neurons of the FLX-pretreated rats [30–32]. Therefore, these results confirm that the doses of FLX administered were adequate in altering 5-HT uptake and metabolism, and allow an inadequacy of the protocol used for the administration of FLX to be ruled out. Moreover, the results confirm that doses below 5 mg kg^{-1} of FLX will be sufficient to produce maximal neurochemical effects.

In the second series of experiments, the levels of the individual enantiomers of FLX and NFLX were examined under steady-state conditions at the dose (5 mg kg^{-1}) intended to mimic the standard clinical situation. In the analyzed real cases a stereoselective metabolism was recognized due to a nonracemic presence of NFLX enantiomers. The plasma levels of the (*R*)-enantiomer of NFLX were considerably increased in comparison to the *S*-enantiomer. In plasma, the ratio of the *R*-to-*S*-FLX was 1.02 compared to 1.05 for the cerebral cortex. In contrast, the ratio of *R*-to-*S*-NFLX was 1.81 for plasma versus 1.50 for cerebral cortex (Table 7). Furthermore, the low ratio of NFLX versus FLX of the *S*-enantiomers may indicate a poor metabolizer status of cytochrome CYP2D6 of the rats. Although the molecular basis for the enantioselective kinetics of FLX remains uncertain, recent work with human liver enzymes suggests that preferential demethylation of *R*-FLX can be traced back largely to the fraction of FLX metabolism which is catalyzed by cytochrome CYP2C9 [6,7], particularly during long-term dosing when the contribution of CYP2D6 is diminished due to the inhibitory action of FLX and NFLX enantiomers on the latter enzyme [7]. These data suggest that isomer dependent differences may exist in the metabolism and/or disposition of FLX and NFLX in Sprague–Dawley rats.

3.5. Conclusions

The analytical methods developed enable the quantification of racemic FLX and NFLX and their corresponding *R* and *S* enantiomers in a simple, rapid and sensitive way. A new method is proposed for the pre-treatment of samples of plasma and cerebral cortex by solid-phase extraction with recoveries of above 87% for all cases. These methods have been applied to the quantification of FLX, NFLX or the corresponding enantiomers in samples of plasma and cerebral cortex of a population of rats treated with racemic FLX, which made it possible to establish the dose under consideration in the animal treatment model in order to extrapolate the data to humans being treated for serious depression. In addition it has been possible to discover the behaviour of each enantiomer with regard to the relationship between their plasma concentration and their accumulation in the cerebral cortex.

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