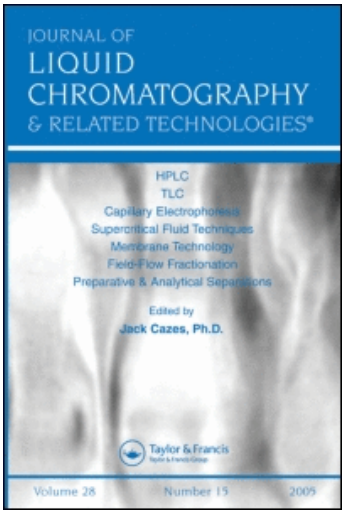

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ANALYSIS OF THE ENANTIOMERS OF FLUOXETINE AND NORFLUOXETINE IN PLASMA AND TISSUE USING CHIRAL DERIVATIZATION AND NORMAL-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

A stereospecific HPLC assay has been developed for the determination of the enantiomers of fluoxetine and an active metabolite, norfluoxetine, in plasma and tissue. Following derivatization with R-1-(1-napthyl)ethyl isocyanate, the diasteriomeric derivatives are resolved on a 5 μm silica column using a iso-octane/tetrahydrofuran mobile phase with fluorescence detection. The assay has a sensitivity of 5 ng/ml in plasma and 25 ng/gm in tissue. The calibration curves are linear over the range of 5-1000 ng/ml. The assay appears to be readily applicable to the study of enantioselective fluoxetine pharmacokinetics in animals and humans.

INTRODUCTION

Fluoxetine (*Prozac* ®, Eli Lilly and Company), (±)-N-methyl-3-phenyl-3-[(α, α, α-trifluoro-p-tolyl)-oxy]-propylamine (Fig. 1) is an important new

antidepressant currently marketed for the treatment of unipolar mental depression in the United States and many other countries (1,2). Fluoxetine has also been reported to be effective in the treatment of bulimia, obsessive-compulsive disorder, and cataplexy (3,4,5,6). Both fluoxetine and it's N-desmethylated metabolite, norfluoxetine (Fig. 1), enhance serotoninergic neurotransmission through selective inhibition of pre-synaptic serotonin re-uptake (7,8) Although fluoxetine and norfluoxetine are potent inhibitors of serotonin uptake, they are virtually devoid of an effect on the uptake of norepinephrine and dopamine (7,9) and have very low affinity for serotonin, muscarinic, and histamine receptors (10). Fluoxetine exhibits similar therapeutic efficacy to imipramine, amitriptyline, and doxepin in the treatment of depression (1,11,12). However, fluoxetine lacks the serious anticholinergic side effects and direct cardiac symptoms common among the tricyclic antidepressants (10,13,14). Following oral administration to man, fluoxetine is well absorbed from the gastrointestinal tract (12). The plasma half-lives of fluoxetine and norfluoxetine are 1-3 days and 7-15 days, respectively (15).

Since fluoxetine is marketed as a racemic mixture, the pharmacology, metabolism, and pharmacokinetics of the isomers of fluoxetine and its metabolites have important clinical implications. The enantiomers of fluoxetine are similarly effective in the in vivo and in vitro inhibition of serotonin uptake in rats (16). However, S-fluoxetine (SF) has a three-fold longer duration of action than R-fluoxetine (RF) (17). Since further studies where rats were dosed with SF or RF showed that the brain concentrations of the parent drugs were similar, as were the concentrations of their respective metabolites (16), there was no apparent reason for the longer duration of action of SF. Upon synthesis and testing of the optically pure isomers of norfluoxetine, the serotonin uptake activity of SNF was shown to be 16 fold greater in vitro and 20 fold greater in vivo than the activity of RNF (16,17). Therefore, the difference in the duration of action between the isomers of fluoxetine can be partially explained on the basis of conversion of RF to the relatively inactive RNF.

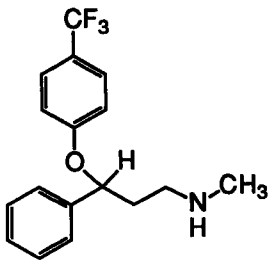
Although fluoxetine is marketed as a racemate, the human pharmacokinetics and metabolism of its enantiomers have not been reported. The enantiomers of a racemic drug often differ in pharmacologic, metabolic and/or pharmacokinetic properties such that the properties of the racemate do not reflect those of the active enantiomers (18,19,20,21). To perform thorough pharmacokinetic investigations with fluoxetine, the concentrations of the individual isomers of the parent drug and the metabolite should be quantitated because of the long half-life of norfluoxetine and the differences in the activities of its enantiomers.

A limited number of analytical methods have been reported for the assay of fluoxetine and norfluoxetine in biological fluids (22,23,24). None of these assays were capable of separating and quantitating the isomers of fluoxetine and norfluoxetine. In the present report, a simple, rapid, and sensitive analytical method for the quantitation of the enantiomers of fluoxetine and norfluoxetine in human and animal plasma and tissue is described.

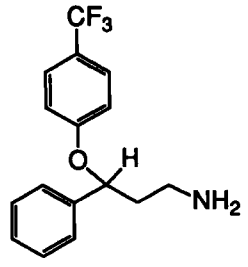
MATERIALS AND METHODS

Reagents and Materials

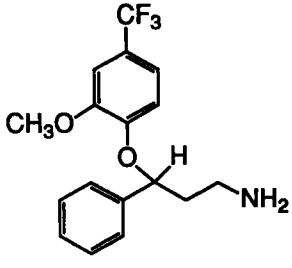
All reagents were analytical grade or higher quality. Hexane, 1-butanol, iso-octane, and tetrahydrofuran (Burdick and Jackson, UV Grade) were obtained from American Scientific Products. Water was purified with a Millipore Milli-Q water purification system. R-(-)-1-(1-Napthyl)ethyl isocyanate (NEI) was obtained from Aldrich Chemical Company. Sodium hydroxide 1N certified solution (Fischer SS266-1) was purchased from Fischer Scientific. Fluoxetine (racemic), S-fluoxetine (SF), R-fluoxetine (RF), S-norfluoxetine (SNF), R-norfluoxetine (RNF), and S-nornisoxetine (internal standard, Fig. 1) were synthesized at The Lilly Research Laboratories. Fluoxetine and its isomers were hydrochloride salts, while the norfluoxetine isomers and the internal standard were maleate salts. Round -bottom disposable glass culture tubes (15 x 125 mm) with teflon-lined screw caps were obtained from



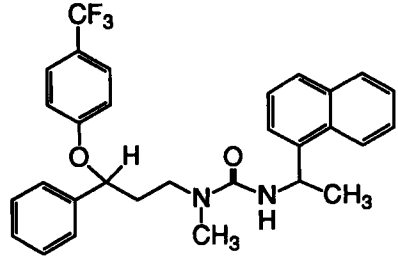
Fluoxetine



Norfluoxetine



Internal Standard



Fluoxetine-NEI Derivative

FIGURE 1. Chemical structures of fluoxetine, norfluoxetine, the internal standard, and the NEI derivative of fluoxetine.

Curtin Matheson Scientific . All glassware was silylated according to the procedure by Fenimore, et al.(25).

Standards and Solutions

Stock solutions of SF, RF, SNF, RNF, and S-nornisoxetine (internal standard) were prepared to a concentration of 100 µg/ml in water. A working standards solution was prepared by adding the appropriate amounts of SF, RF, SNF, and RNF stock solutions to water to obtain a concentration of 10 µg/ml. A working internal standard solution was prepared by diluting the stock solution of S-nornisoxetine with water

to obtain a concentration of 2 μg/ml. All standard solutions were prepared to reflect the concentration of the free base.

Aliquots of a 25 mM stock solution of NEI were prepared by adding 6.6 μl of NEI to 1.5 ml of hexane in a glass vial. Argon gas was layered onto the head-space of each vial and the vials capped tightly and stored at -70°C. A 200 μM working solution of NEI was prepared daily by warming an unused vial of the stock solution to room temperature, diluting with hexane and gently swirling to mix. The unopened aliquots of the stock solution can be stored frozen at -70°C for up to 2 months.

Chromatography

A Jones Chromatography Apex silica column (5 μm, 250 x 4.6 mm, Jones 4M25300), maintained at 35°C using a Fiatron CH-30 column heater, was used for the chromatographic separation of the NEI derivatives of SF, RF, SNF, RNF, and S-nornisoxetine. Silica columns from other manufacturers were tested with this system, however the Jones Chromatography silica column provided the best resolution. The mobile phase of 30% tetrahydrofuran / 70% iso-octane was filtered through a 5 μm pore size Millipore polytetrafluoroethylene membrane filter before use. A flow rate of 1.0 ml/minute was maintained using a Varian Model 9010 pump. The samples (75 μl) were injected into the system using a Varian Model 9095 autosampler. Detection was accomplished using a Perkin Elmer Model LS40 fluorescence detector set at an excitation wavelength of 218 nm and an emission wavelength of 333 nm. The detector output was captured by an Hewlett Packard HP1000 series computer for peak analysis.

Plasma and Tissue Extraction and Derivatization

Tissue samples were homogenized in a Brinkman Polytron homogenizer with 5-10 ml of water per gram of tissue. Plasma samples or tissue homogenates (0.5 ml) were placed in 15 x 125 mm silylated glass tubes. Water (0.5 ml), 50 μl of the working internal standard solution, and 100 μl of 1N sodium hydroxide solution were added, and

the samples were vortex-mixed gently. Five ml of 0.3% butanol in hexane (v/v) was added to the tubes, and the tubes were capped. The samples were extracted for a period of 30 minutes using an Eberbach reciprocal shaker at 125-150 cycles per minute. Following centrifugation for 15 minutes at 2000 x G, the organic layer was transferred to a clean tube. The working solution of NEI (100 µl) was added to the tubes and the samples were slowly taken to dryness in a nitrogen evaporator (N-Evap, Organomation Associates) at 50-55°C over a period of 20-30 minutes. To insure efficient removal of excess derivatizing agent, the samples were dried more vigorously for a period of ten minutes after it appeared that all the hexane was gone. The residue was reconstituted in 200 µL of mobile phase, placed in a glass limited volume insert (Sun Brokers Inc) in an autosampler vial , capped, and 75 µL injected for chromatographic analysis. Standard curve samples were constructed by adding the working standards solution to drug-free plasma or brain homogenate and serially diluting to give final concentrations of 5-1000 ng/ml.

Optimization Studies

The effect of column temperature was investigated by extracting and derivatizing a plasma sample spiked with 1000 ng/ml of SF, RF, SNF, and RNF and injecting aliquots of the resulting sample into the chromatographic system while maintaining the column temperature at 25, 30, 35, and 40°C.

The use of silylated versus non-silylated glassware was examined by preparing plasma samples containing 20, 200, or 1000 ng/ml of SF, RF, SNF, and RNF and incubating a portion of each sample for one hour at room temperature in both a silylated and a non-silylated glass vial. The samples which had been incubated in silylated glass were extracted and derivatized in triplicate in silylated tubes, while the samples from the non-silylated vials were processed identically in non-silylated tubes. The samples were quantitated by peak height.

A large volume of hexane/butanol extract was prepared, for the purpose of optimizing NEI concentration, by extracting multiple plasma

samples spiked with 1000 ng/ml of SF, RF, SNF and RNF and pooling the hexane/butanol extracts. Aliquots of the extract (5 ml) were placed in tubes to which 100 µl of NEI solutions (20, 50, 100, 200, 500, 1000, and 2000 µM) were added. Following evaporation and dissolution of the residue in mobile phase, the resulting samples were injected into the HPLC system and the peak heights quantitated.

The effect of derivatization time was investigated by preparing a 1000 ng/ml extract, as outlined above, and incubating 5 ml aliquots with 100 µl of 200µM NEI at 60°C for 0, 15, 30, or 60 minutes. The samples were evaporated, the residue dissolved in mobile phase, and the resulting samples quantitated by HPLC.

Assay Validation

The intra-assay and inter-assay accuracy and precision of the assay were determined by analyzing five replicates from low, medium, and high validation samples (10, 200, 1000 ng/ml in human plasma, and 10, 100, 1000 ng/ml in dog plasma) on three separate days. Calibration standard curve samples were prepared on each day of analysis using the plasma of the species being evaluated.

The extraction efficiency of fluoxetine and norfluoxetine from plasma was determined by extracting triplicate 0.5 ml aliquots of 1000 and 200 ng/ml solutions of ^{14}C-fluoxetine and ^{14}C-norfluoxetine and comparing the radioactivity in the unextracted plasma sample to the hexane/butanol extract.

Calculations

Standard curves were constructed for each component by plotting the concentration versus the peak height ratio (component peak height / internal standard peak height) and fitting a least squares linear regression analysis line to the data. Concentrations of SF, RF, SNF, and RNF in the samples were determined by extrapolating the peak height ratio to the least squares line of the standard curve.

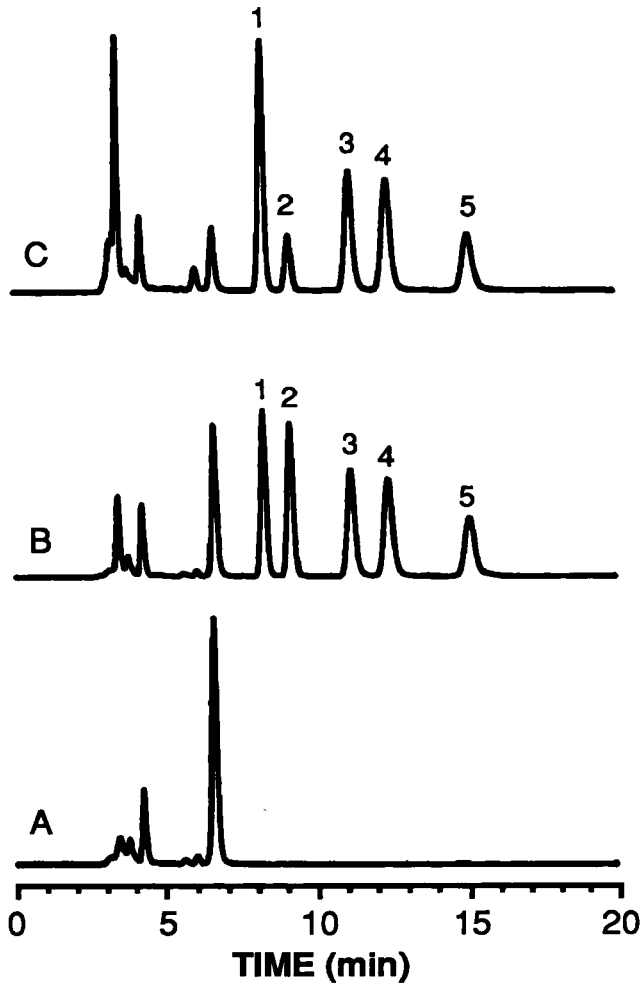


FIGURE 2. Typical chromatograms of (A) extract of a blank dog plasma sample, (B) extract of a 100 ng/ml standard in dog plasma and (C) extract of an 8 hour plasma sample from a dog after a 5 mg/kg oral dose of racemic fluoxetine. Peaks: 1-SF, 2-RF, 3-SNF, 4-RNF, 5-Internal Standard.

RESULTS AND DISCUSSION

Figure 2 depicts representative chromatograms of drug-free plasma, plasma spiked with 100 ng/ml of SF, RF, SNF, RNF, and a plasma sample taken from a dog 8 hr after oral administration of a 5 mg/kg dose of racemic fluoxetine. Under the conditions outlined here, the retention times for the diastereomeric derivatives of SF and RF were 8.3 and 9.3 min while those of SNF and RNF were 11.2 and 12.5 minutes, respectively. There were no interfering peaks from the normal components of plasma.

The separation of the diastereomeric derivatives of the enantiomers of fluoxetine and norfluoxetine at temperatures of 25-40°C are summarized in table 1. The diastereomeric derivatives of each compound were completely resolved, as indicated by the values of the peak resolution (R>2). Although the resolution values (R) decreased slightly as the column temperature was increased, the retention times decreased and the peak heights increased. A column temperature of 35°C provided shorter retention times and greater sensitivity, yet did not significantly decrease the resolution, as evidenced by the intermediate capacity (k') values and high resolution (R) values. Temperature did not significantly change selectivity (α).

The effects of incubating and extracting spiked plasma samples in silylated and non-silylated glassware are shown in table 2. All the samples prepared in non-silylated glassware had lower peak heights than the corresponding samples prepared in silylated glassware. The primary amines, SNF and RNF, showed a greater reduction in peak height upon use of non-silylated glassware, as compared to the secondary amines, SF and RF. The reduction in peak height with use of non-silylated glassware was significant at all levels tested, with the greatest percent reduction at 20 ng/ml.

As shown in figure 3, the maximum peak height response for all the assay components was seen at an NEI concentration of 100 µM. In the final conditions, the NEI concentration was doubled to 200 µM to provide an adequate excess of reagent. Figure 4 shows that a separate incubation of the derivatization mixture, aside from the dry-down step, does not increase the efficiency of the derivatization. The slow

TABLE 1
The Effect of Column Temperature on the K', R, and α Values for the NEI Derivatives of SF, RF, SNF, and RNF

Temp. °C	K' SF	K' RF	K' SNF	K' RNF	R SF RF	R SNF RNF	α SF RF	α SNF RNF
25	1.94	2.31	3.00	3.47	2.64	2.35	1.19	1.16
30	1.86	2.20	2.89	3.34	2.57	2.34	1.18	1.15
35	1.77	2.08	2.78	3.20	2.39	2.21	1.17	1.15
40	1.69	1.98	2.68	3.07	2.24	2.17	1.17	1.14

TABLE 2
The Effects of Incubating and Extracting Spiked Plasma Samples in Silylated and Non-Silylated Glassware

Plasma Conc. (ng/ml)	Glass Type[1]	Mean Peak Height[2] (% Reduction)[3] SF		RF		SNF		RNF	
1000	S	5526		4620		3411		3543	
1000	NS	5390	(2)	4497	(3)	2514	(26)	2594	(27)
200	S	3039		2547		1901		1991	
200	NS	2768	(9)	2289	(10)	940	(51)	957	(52)
20	S	160		131		88		89	
20	NS	140	(12)	101	(23)	28	(68)	22	(75)

1 S = Glassware silylated as outlined in methods
NS = Glassware not silylated

2 Peak heights between the three concentrations can not be directly compared because the attenuation of the chromatography system was changed to minimize the error within each concentration.

3 Percent reduction of mean peak height - Non-Silylated vs Silylated

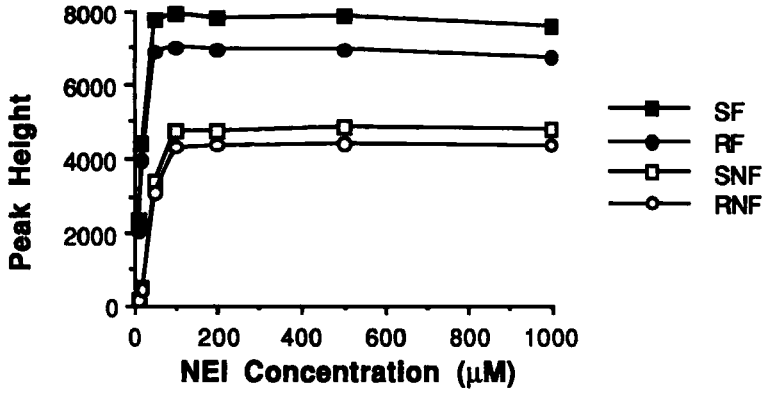


FIGURE 3. The effect of NEI concentration on the peak heights of the derivatives of SF, RF, SNF and RNF.

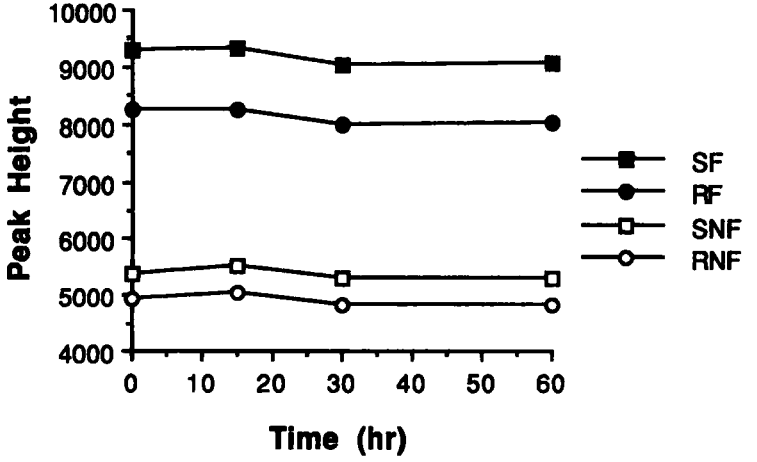


FIGURE 4. The effects of derivatization time (in addition to the dry-down step) on the peak heights of the derivatives of SF, RF, SNF and RNF.

TABLE 3
Plasma Extraction Efficiency of Fluoxetine and Norfluoxetine

Component	Concentration (ng/ml)	Extraction Efficiency (mean ± SD)	CV (%)
Fluoxetine	1000	86.3 ± 3.1	3.6
Fluoxetine	200	96.3 ± 6.8	7.1
Norfluoxetine	1000	91.5 ± 0.9	1.0
Norfluoxetine	200	92.5 ± 4.9	5.3

evaporation of the derivatization mixture at 50-55°C was adequate to complete the derivatization process.

The extraction efficiencies of fluoxetine and norfluoxetine, as measured by extracting 14C-fluoxetine or 14C-norfluoxetine from plasma, were 86.3-96.3% and 91.5-92.5%, respectively (Table 3). The extraction efficiency of the compounds was consistent, with the coefficient of variation values ranging from 1.0 to 7.1%.

The values determined for the precision and accuracy of the assay in human and dog plasma are presented in table 4. The accuracy of the assay was good, and the reproducibility was within acceptable limits. The standard curves for all four components gave a linear response with correlation coefficients greater than 0.999.

The utility of the assay was demonstrated in species other than dogs and humans by analyzing plasma and brain samples from rats dosed with racemic fluoxetine (Figure 5). The plasma terminal half-lives of SF, RF, SNF, and RNF were 3.4, 2.4, 16.4, and 12.4 hours, respectively. The longer half-lives of SNF and RNF, in conjunction with the rapid biotransformation of SF and RF, resulted in area under the curve (AUC) values for SNF and RNF which were 5-12 times higher than their corresponding fluoxetine isomers. The brain AUC values for SF, RF,

TABLE 4
Interassay Precision and Accuracy Determinations[1]

Plasma Type	Conc. (ng/ml)	Component	Detected Conc. (ng/ml)	Accuracy (%)	Precision (C.V.)
Dog	10	SF	11.3	113	8.2
		RF	11.3	113	7.9
		SNF	11.2	112	8.1
		RNF	10.7	107	8.5
	100	SF	103.3	103	3.9
		RF	102.8	103	4.2
		SNF	100.6	101	4.9
		RNF	100.1	100	5.1
	1000	SF	1014	101	3.0
		RF	1009	101	2.6
		SNF	1033	103	2.5
		RNF	1036	104	3.2
Human	10	SF	11.7	117	4.5
		RF	11.8	118	1.5
		SNF	11.9	119	14.8
		RNF	12.3	123	13.1
	200	SF	204.5	102	1.5
		RF	206.5	103	1.5
		SNF	205.1	103	2.0
		RNF	211.2	106	3.6
	1000	SF	1020	102	0.9
		RF	1024	102	1.0
		SNF	1018	102	1.6
		RNF	1038	104	2.6

The interassay precision and accuracy values were calculated from the intraassay precision and accuracy values from three days of validation (5 replicates/plasma concentration).

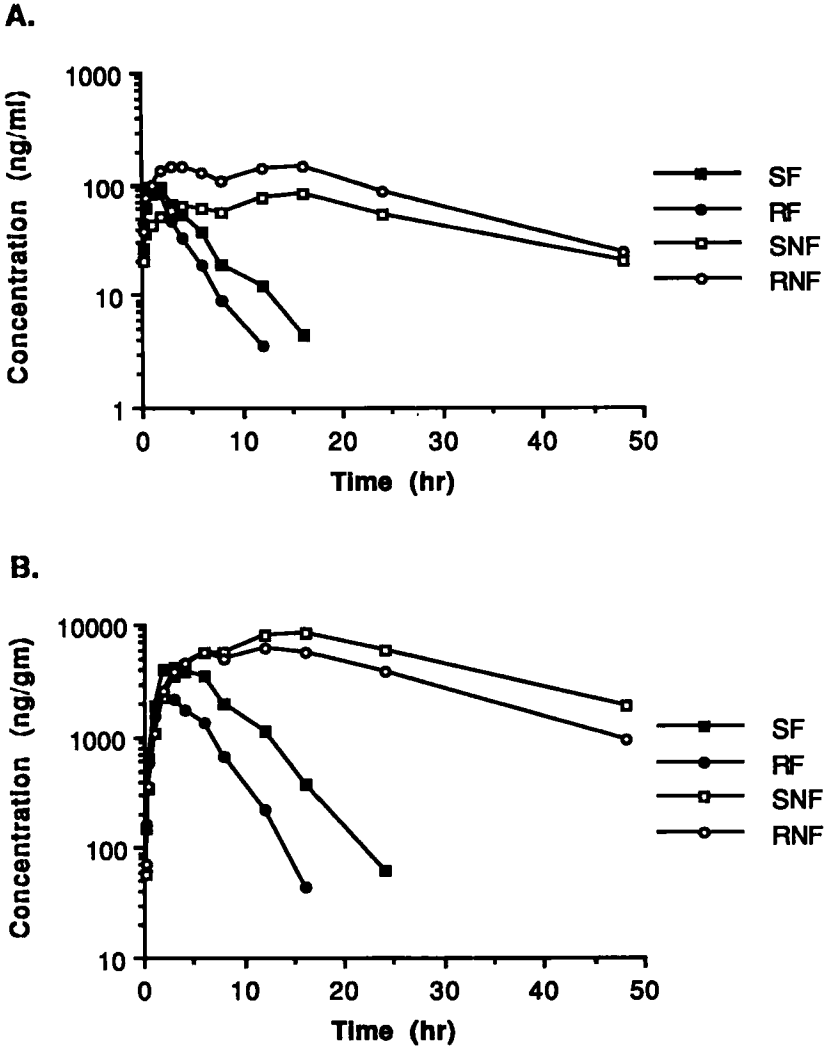


FIGURE 5. Mean (A) plasma and (B) brain concentrations of the enantiomers of fluoxetine and norfluoxetine in rats following a single 20 mg/kg oral dose of racemic fluoxetine.

SNF, and RNF greatly exceeded the corresponding plasma AUC values, indicating a potential for greater exposure of both drug and metabolite in the brain. The brain terminal half-lives were similar to those in the plasma. The plasma and brain AUC values for SF were greater than those for RF. In plasma, the AUC values for RNF are greater than those of SNF, while in the brain the reverse is true. These data suggest that isomer dependent differences may exist in the metabolism and/or disposition of fluoxetine and norfluoxetine.

In summary, the liquid chromatographic procedure described here for the separation and quantitation of the isomers of fluoxetine and norfluoxetine possesses several advantages: only commercially available and inexpensive reagents and chromatographic columns are required; the extraction and derivatization procedures are simple and rapid; good separation and low quantitation limits of the diasteriomeric derivatives are obtained. This assay has been used successfully to quantitate the isomers of fluoxetine and norfluoxetine in the plasma and tissues of rats, mice, dogs, and humans.

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