The Stereospecific Determination of Fluoxetine and Norfluoxetine Enantiomers in Human Plasma by High-Pressure Liquid Chromatography (HPLC) with Fluorescence Detection

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A quantitative method for the simultaneous HPLC resolution and detection of the enantiomers of (R,S) fluoxetine (F) and their metabolites (R,S) norfluoxetine (N) in human plasma has been developed. F is a serotonin uptake inhibitor used in the treatment of depression and is administered as a racemate. After liquid–liquid extraction and derivatization with (R) napthyl ethyl isocyanate (NEI), the separation and detection of the resultant diasteriomers were achieved using normal phase HPLC and fluorescence. The four NEI diastereomers and the internal standard [(−)-*N*-methyl-γ-(2-methylphenoxy) benzenepropanamine hydrochloride], representing the enantiomers S-F, R-F, S-N, and R-N were resolved within 15 min. The assay for each analyte was linear using two concentration ranges of 1–10 and 10–500 ng/ml of human plasma. The precision and accuracy are reported as the coefficient of variation (%CV) and relative error (%RE). The sum of the chiral HPLC results from plasma samples were compared to the achiral gas chromatographic/electron capture (GC/EC) results. The correlation between these two methods, for total F and N, resulted in r^2 values of 0.98 and 0.89, respectively. The chiral HPLC method is currently being applied to clinical studies for the evaluation of the enantiomeric disposition of F.

KEY WORDS: high-performance liquid chromatography (HPLC); fluoxetine; enantiomers; naphthyl ethyl isocyanate derivatization; fluorescence detection; norfluoxetine.

INTRODUCTION

Fluoxetine hydrochloride, *dl-N*-methyl-3-phenyl-3-[(α,α,α,-trifluoro-*p*-tolyl)oxy]propylamine, is a serotonin reuptake inhibitor used in the treatment of depression and is currently marketed under the name Prozac (1,2). Fluoxetine (Fig. 1) is rapidly metabolized to its desmethyl metabolite, norfluoxetine (Fig. 1), which has similar pharmacological activity. Fluoxetine is efficacious at daily oral doses of 20–80 mg. Daily oral doses of 60 mg produce plasma concentrations at steady state between 200 and 500 ng/ml for fluoxetine and 180 and 450 ng/ml for norfluoxetine. Fluoxetine and norfluoxetine have been determined by a gas chromatographic method with electron-capture detection (3) and a high-pressure liquid chromatographic (HPLC) method with UV detection (4). Fluoxetine is a chiral molecule and is administered as a racemate. In order to study the disposition of the enantiomers of fluoxetine, chiral gas chromatographic (GC) and HPLC analyses were developed. The GC analysis was sensitive enough for single-dose studies but lacked the ability to quantitate the enantiomers of the metabolite, norfluoxetine (5). The chiral HPLC analysis was developed for the determination of the enantiomers of fluoxetine and norfluoxetine in high-dose, preclinical studies (6). The chiral HPLC analysis was modified for clinical studies and has a lower limit of quantitation for all four enantiomers. We describe a sensitive and precise method for the determination of the enantiomers of fluoxetine and norfluoxetine in human plasma. The method required the derivatization of the amine moiety of fluoxetine and norfluoxetine to form diasteriomers, which were separated using normal phase chromatography and fluorescence detection. Plasma samples were analyzed by the chiral HPLC assay and compared to the results obtained by the GC/electron capture (EC) assay.

MATERIALS AND METHODS

Chemicals

The enantiomers of fluoxetine and norfluoxetine and the internal standard (IS) (−)-*N*-γ-(2-methylphenoxy) benzenepropanamine hydrochloride (Fig. 1) were synthesized at Eli Lilly and Company (Indianapolis, IN). The enantiomers of fluoxetine and norfluoxetine had optical purities in excess of 99%. *R*-1-(1-Naphthyl)ethyl isocyanate (NEI) was obtained from Aldrich Chemical Company (Milwaukee, WI). Isooctane, tetrahydrofuran (THF), and hexane were HPLC grade and purchased from Burdick and Jackson (McGraw Park, IL). Sodium hydroxide, 1 *N* certified reagent, was purchased from Fisher Scientific (Fair Lawn, NJ). Prepurified argon in a lecture bottle was bought from Air Products and Chemicals (Shelbyville, IN). Deionized water was filtered through a Gelman Water I system (Ann Arbor, MI).

Chromatography

The HPLC system consisted of a Beckman System Gold 126 Programmable Solvent Module (San Ramon, CA) and a Waters WISP 712 autoinjector (Milford, MA). The detector, a Perkin-Elmer LS 4 fluorescence detector (Beaconsfield, Buckinghamshire, UK), was set with an excitation wavelength of 285 nm (slit width, 15 nm) and an emission wavelength of 313 nm (slit width, 20 nm). The separation of the components was done by pumping mobile phase (70:30 isooctane:THF, v:v) at a flow rate of 1.0 ml/min through a Jones Apex Silica column (4.6 × 15 cm, 5-μm packing material) (Jones Chromatography, Littleton, CO, USA), which was maintained at 35°C (Fiatron TempControl System, Oconomowoc, WI). The signal from the detector was sent to a local Hewlett-Packard HP3393A integrator (Avondale, PA) and to a PE/Nelson Access*Chrom system (Cupertino, CA).

Standard Solutions

The enantiomers of fluoxetine and norfluoxetine were dissolved in deionized water to prepare stock solutions of 500 μg/ml. One milliliter from each of these stock solutions

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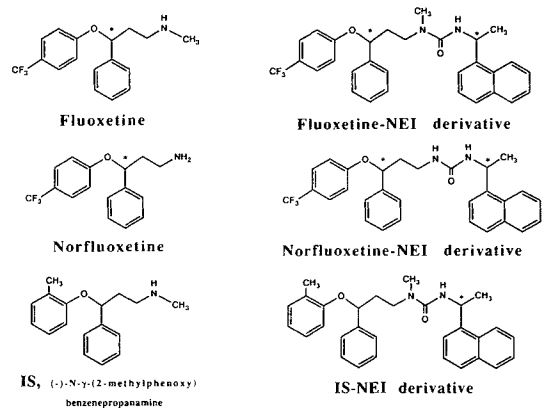

Fig. 1. Chemical structures of fluoxetine, norfluoxetine, the internal standard, and their NEI derivatives. The asterisk indicates chiral centers of the molecules.

was pipetted into a single 5-ml volumetric flask and filled to the mark with deionized water to produce a stock solution of 100 μg/ml for each enantiomer. This solution was diluted 1:10 with deionized water to produce a 10-μg/ml stock solution. Into nine separate 25-ml volumetric flasks 2.5, 6.25, 12.5, 25, 62.5, and 125 μl of the 10-μg/ml stock solution and 25, 62.5, and 125 μl of the 100-μg/ml stock solution were pipetted. The volumetric flasks were filled to the mark with drug-free plasma and the standards contain final concentrations of 1, 2.5, 5, 10, 25, 50, 100, 250, and 500 ng/ml. A 2-μg/ml internal standard solution was prepared in water. NEI (100 μ*M*) solutions were prepared in hexane.

Sample Preparation

A 1.0-ml volume of plasma was diluted with 0.5 ml of water in a 15-ml siliconized glass disposable test tube. A 0.025-ml aliquot of the 2-μg/ml IS solution and a 0.1-ml volume of 1 *N* sodium hydroxide were added to the sample. Following vortex mixing the sample was extracted with 5 ml of hexane by shaking on a reciprocal shaker (Eberbach Ann Arbor, MI) for 30 min. After centrifugation at 4°C for 10 min at 3000 rpm, the organic layer was transferred into a clean siliconized 15-ml screw-capped test tube. The sample was derivatized by the addition of 50 μl of the 100 μ*M* NEI solution. The sample was capped and derivatized for 30 min at 60°C in a multiblock heater (Lab-Line Instruments Inc., Melrose Park, IL). The organic layer was evaporated to dryness (Savant Speed Vac Concentrator/Evaporator; Hicksville, NY) and the residue reconstituted in 200 μl of mobile phase (70:30 isooctane:THF, v:v). Five to sixty-five microliters of the solution was injected onto the HPLC. Samples with concentrations of 1, 2.5, 5, and 10 ng/ml had injection volumes of 65 μl. The 25-, 50-, 100-, 250-, and 500-ng/ml samples had injection volumes of 50, 25, 25, 10, and 5 μl, respectively.

Validation: Accuracy, Precision, and Recovery

The validation of the HPLC method was designed to test for the accuracy, precision, linearity, and reproducibility of the method. To achieve this, a standard curve and five validation samples at five concentrations were assayed on 3

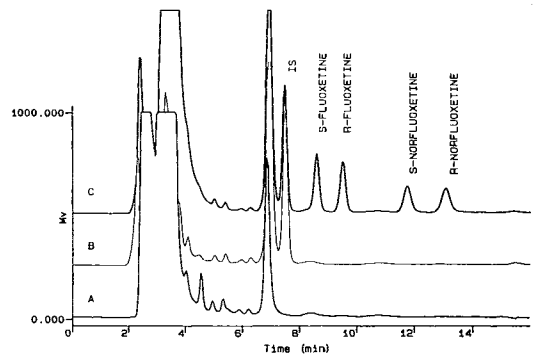

Fig. 2. Chromatograms of (A) blank plasma without IS, (B) blank plasma with 50 ng IS, and (C) plasma containing 25 ng/ml of each enantiomer.

consecutive days. The standard curves were generated by a linear least-squares regression analysis of the peak height ratio of each of the four enantiomers to the IS versus added concentration to drug-free plasma. The standard curves were evaluated for linearity by visual inspection as well as the correlation coefficient. The accuracy was based on the calculation of the relative error (%RE) of the mean concentration of the five determinations as compared to the added concentration. The precision was based on the calculation of the coefficient of variation (%CV). The recovery of fluoxetine was evaluated by radioactivity.

RESULTS AND DISCUSSION

The determination of the enantiomers of fluoxetine and norfluoxetine required the derivatization of the amine functionality with NEI (Fig. 1). The resultant diasteriomers were resolved by normal phase chromatography on a silica column with fluorescence detection. The analytical silica columns were screened for their ability to resolve a test mixture which consisted of the synthetically derived diasteriomers formed from the reaction of NEI with fluoxetine and norfluoxetine. The synthetically derived diasteriomers were identified by nuclear magnetic resonance (NMR), mass spectrometry (MS), and HPLC with UV detection. The resolu-

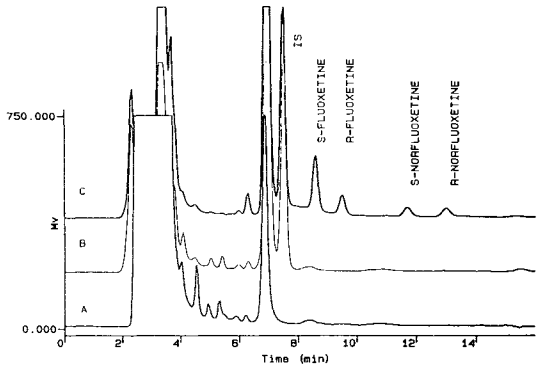

Fig. 3. Chromatograms of (A) a predose plasma sample without IS, (B) a predose plasma sample with 50 ng IS, and (C) a plasma sample taken from a healthy subject 24 hr after oral administration of a single 60-mg dose of racemic fluoxetine. The concentrations of the enantiomers are as follows: SF, 14.8 ng/ml; RF, 6.2 ng/ml; SN, 6.9 ng/ml; RN, 6.4 ng/ml; IS, 50 ng.

Table I. Mean Values from Calibration Curves for Each Enantiomer Assayed on 3 Separate Days

Enantiomer	Slope (mean ± SD)	Correlation coefficient (mean ± SD)	y intercept
1–10 ng/ml			
SF	0.0129 ± 0.0001	0.9985 ± 0.0007	0
RF	0.0132 ± 0.0004	0.9963 ± 0.0055	0
SN	0.0069 ± 0.0014	0.9894 ± 0.0117	0
RN	0.0069 ± 0.0013	0.9866 ± 0.0136	0
10–500 ng/ml			
SF	0.0136 ± 0.0003	0.9999 ± 0.0001	−0.0181 ± 0.0155
RF	0.0130 ± 0.0003	0.9999 ± 0.0001	−0.0163 ± 0.0003
SN	0.0102 ± 0.0003	0.9999 ± 0.0001	−0.0532 ± 0.0044
RN	0.0092 ± 0.0003	0.9999 ± 0.0001	−0.0436 ± 0.0067

tion of the compounds was column dependent. Jones Apex silica columns (Jones Chromatography, Littleton, CO) were identified as the most consistent source of reliable columns for the resolution of the diasteriomers. Typical chromatograms for the analysis of the enantiomers of fluoxetine and norfluoxetine in plasma can be seen in Figs. 2 and 3. The retention times of the NEI derivatives of *S*-fluoxetine (SF), *R*-fluoxetine (RF), *S*-norfluoxetine (SN), *R*-norfluoxetine (RN), and the IS are 8.6, 9.5, 11.6, 13.0, and 7.5 min, respectively. The derivatization conditions were optimized for both reaction time and completeness of the reaction by analyzing aliquots of three samples over a 4-hr time period and observing the time needed to obtain the maximum fluorescent signal. The complete conversion of the enantiomers of fluoxetine, norfluoxetine, and the IS was accomplished in 30 min. The extraction efficiency of fluoxetine from plasma, over the concentration range of 30 to 1500 ng/ml, was determined to be 73% (SD, 3.8%; $n = 15$) based on the radiolabeled enantiomers of fluoxetine. The recovery, expressed as a percentage, was determined by the comparison of the known radioactivity spiked into a plasma matrix to that recovered after the extraction procedure. The recovery of *S*-norfluoxetine from plasma was the same over the concentration range of 25 to 200 ng/ml. The absolute recovery of the IS is unknown, however, its peak height was constant for the entire validation study and therefore did not influence the linearity of the method. Racemization of the enantiomers in plasma and during sample preparation was not observed.

The assay was validated with a low calibration curve range of 1–10 ng/ml and a high calibration curve range of 10–500 ng/ml. The assay was linear over these concentration ranges. A statistical analysis of the low calibration curves (3 days for each enantiomer) showed the intercept not to be significantly different from zero ($P = 0.05$); therefore, the linear regression analysis was forced through the origin. The calibration curves from 1–10 and 10–500 ng/ml, for each of the enantiomers assayed on 3 separate days, gave the mean values for the slope, correlation coefficient, and *y* intercept shown in Table I.

The assay was accurate and reproducible as indicated by the interday and intraday statistics for percentage CV and percentage RE (Tables II and III, respectively). At the 1-ng/ml concentration the interday variation and relative error were less than 20.5 and 16.6%, respectively. On days 2 and 3 of the validation there was a small interference in the blank at the retention time of RF. The reported values were corrected for by the subtraction of the interference found in the blank. The interference on day 2 and day 3 was 0.48 and 0.17 ng/ml, respectively. Generally the quantitation of the fluoxetine enantiomers resulted in more accurate and precise determinations in comparison to the norfluoxetine enantiomers.

The following tricyclic antidepressants and metabolites were screened by this methodology: 2-hydroxydesipramine, 2-hydroxyimipramine, amitriptylene, clomipramine, desipramine, doxepin, imipramine, nortriptylene, and protriptylene. Imipramine, nortriptylene, and protriptylene had retention times similar to the internal standard and their presence would interfere with the analysis. Desipramine had a retention time similar to SF and would interfere with the analysis of SF. Amitriptylene, 2-hydroxydesipramine, 2-hydroxyimipramine, clomipramine, and doxepin had retention times similar to the times of the NEI-related peak adjacent to the internal standard. These compounds should not interfere with the analysis of the enantiomers of fluoxetine and norfluoxetine.

Table II. Interday Accuracy and Precision

Enantiomer Conc. (ng/ml)	SF			RF		SN		RN	
	N	%CV	%RE	%CV	%RE	%CV	%RE	%CV	%RE
1.0	14	19.2	−8.2	20.5	13.8	16.6	−7.5	11.2	7.5
2.5	14	6.5	−1.4	7.6	5.3	14.6	−5.0	13.7	−3.3
5.0	15	4.1	3.6	3.1	2.7	8.0	5.4	8.2	4.5
25.0	15	1.6	0.7	1.2	1.5	5.8	2.2	6.3	1.9
250.0	15	2.0	1.7	2.0	2.0	1.8	2.1	1.8	2.7

Table III. Intraday Accuracy and Precision

	S-Fluoxetine			*R*-Fluoxetine[a]		
	Mean ± SD (n = 5)	%CV	%RE	Mean ± SD (n = 5)	%CV	%RE
1.0 ng/ml						
Day 1	0.9 ± 0.1	9.8	−13.8	1.1 ± 0.1	8.7	7.0
Day 2	1.1 ± 0.1	12.7	9.6	1.2 ± 0.3	26.0	18.6
Day 3	0.8 ± 0.1	12.7	−23.5	1.2 ± 0.3	24.9	16.3
2.5 ng/ml						
Day 1	2.4 ± 0.2	6.3	−2.2	2.7 ± 0.1	3.6	7.0
Day 2	2.6 ± 0.1	3.6	5.1	2.7 ± 0.3	12.3	8.3
Day 3	2.3 ± 0.1	2.2	−6.8	2.5 ± 0.1	3.6	0.1
5.0 ng/ml						
Day 1	4.9 ± 0.1	2.5	−2.3	5.1 ± 0.1	1.2	2.0
Day 2	5.2 ± 0.1	1.6	4.8	4.9 ± 0.1	2.3	−1.6
Day 3	4.9 ± 0.1	3.0	−2.4	4.9 ± 0.2	3.7	−2.7
25.0 ng/ml						
Day 1	26.1 ± 0.4	1.4	4.2	26.3 ± 0.3	1.2	5.2
Day 2	26.6 ± 0.2	0.8	6.5	26.5 ± 0.2	0.6	6.0
Day 3	26.1 ± 0.4	1.5	4.1	26.6 ± 0.4	1.5	6.3
250.0 ng/ml						
Day 1	252.1 ± 5.8	2.3	0.8	253.7 ± 5.1	2.0	1.5
Day 2	248.2 ± 4.9	2.0	−0.7	250.3 ± 3.3	1.3	0.1
Day 3	247.3 ± 3.4	1.4	−1.1	245.8 ± 3.3	1.3	−1.7
	S-Norfluoxetine			*R*-Norfluoxetine		
	Mean ± SD (n = 5)	%CV	%RE	Mean ± SD (n = 5)	%CV	%RE
1.0 ng/ml						
Day 1	0.9 ± 0.1	9.4	−6.6	1.1 ± 0.1	5.6	4.6
Day 2	1.0 ± 0.1	13.4	2.6	1.1 ± 0.2	14.8	11.8
Day 3	0.8 ± 0.2	19.7	−21.3	1.1 ± 0.1	12.3	5.8
2.5 ng/ml						
Day 1	2.2 ± 0.4	16.6	−12.3	2.2 ± 0.3	13.6	−11.0
Day 2	2.5 ± 0.4	15.1	1.2	2.6 ± 0.4	13.7	4.9
Day 3	2.4 ± 0.3	14.6	−5.0	2.4 ± 0.3	11.7	−3.3
5.0 ng/ml						
Day 1	5.2 ± 0.4	7.9	4.7	5.2 ± 0.4	8.0	4.6
Day 2	5.1 ± 0.4	6.8	2.0	5.1 ± 0.3	6.2	2.2
Day 3	4.9 ± 0.5	8.0	5.4	4.8 ± 0.5	9.3	−4.1
25.0 ng/ml						
Day 1	25.3 ± 1.1	4.3	1.2	25.2 ± 1.1	4.4	0.8
Day 2	26.5 ± 0.9	3.4	5.9	26.2 ± 1.1	4.1	4.8
Day 3	28.1 ± 1.1	3.8	12.6	28.2 ± 1.1	4.0	13.0
250.0 ng/ml						
Day 1	252.5 ± 4.9	2.6	1.3	253.1 ± 6.5	2.6	1.3
Day 2	248.2 ± 3.8	1.5	−0.7	250.7 ± 3.6	1.4	0.3
Day 3	250.1 ± 4.3	1.7	0.1	251.0 ± 3.6	1.4	0.4

[a] On days 2 and 3 there was a small interference in the blank at the retention time of RF. The blank value, 0.48 and 0.17 ng/ml, respectively, was subtracted from the reported concentrations. Day 2, n = 4 for the 2.5-ng/ml sample. Day 3, n = 4 for the 1-ng/ml sample and the calibration curve was from 1 to 5 ng/ml due to the loss of the 10-ng/ml standard.

A representative plasma concentration–time profile of the isomers of fluoxetine and norfluoxetine is shown in Fig. 4. The S isomer of fluoxetine and norfluoxetine are present at higher concentrations than the R isomers, even though the R and S isomers exhibit similar concentration–time profiles.

The results of the chiral HPLC analysis for the isomers of fluoxetine and norfluoxetine were compared to those obtained with the nonchiral GC/EC assay. The concentration of the R and S isomers of fluoxetine and norfluoxetine were added together to determine the total fluoxetine and norfluoxetine by HPLC. These concentrations were compared to the results of the GC/EC assay. The comparison of the HPLC and GC plasma concentration–time profiles of fluoxetine and norfluoxetine is shown in Fig. 5 and indicated a good correlation between the two methods. Figure 6 shows the correlation between the HPLC and the GC assays for concentrations of fluoxetine and norfluoxetine with R^2 values of 0.98 and 0.89, respectively. The reanalysis of selected

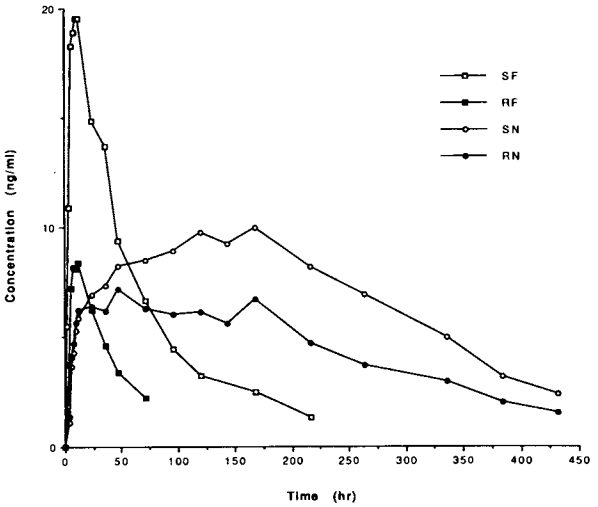

Fig. 4. Plasma concentration–time profiles of the enantiomers of fluoxetine and norfluoxetine in a subject after receiving a 60-mg oral dose of racemic fluoxetine.

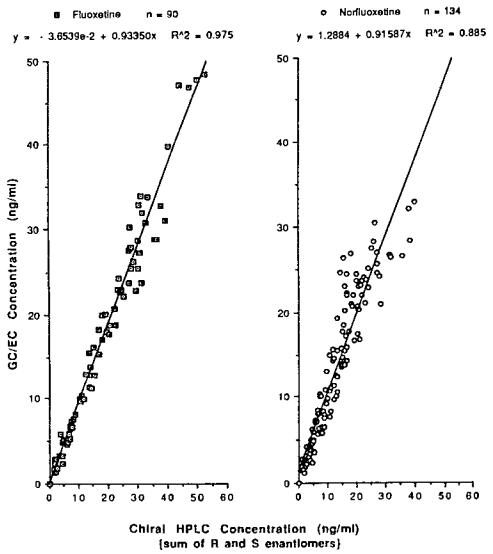

Fig. 6. The correlation between the HPLC and GC assays for fluoxetine (n = 90) and norfluoxetine (n = 134).

plasma samples, after storage at −4°C, for 1 year and 7 months indicated that both fluoxetine and norfluoxetine were stable.

Since the development and validation of the method, two improvements have been implemented. A scavenger silica column has been added to the chromatography system in order to increase the life of the analytical column. To guard against incomplete derivatization the NEI reagent was protected from air by layering argon over the hexane extracts during derivatization. This modification appears to have eliminated samples that were incompletely derivatized.

In conclusion, we have described a sensitive and precise method for the determination of the enantiomers of fluoxetine and norfluoxetine in plasma. The method required the derivatization of the amine moiety of fluoxetine and norfluoxetine with NEI to form diasteriomers, which were separated by normal phase chromatography and detected by fluorescence. This assay methodology will be used to study the disposition of the enantiomers of fluoxetine and norfluoxetine after the oral administration of racemic fluoxetine.

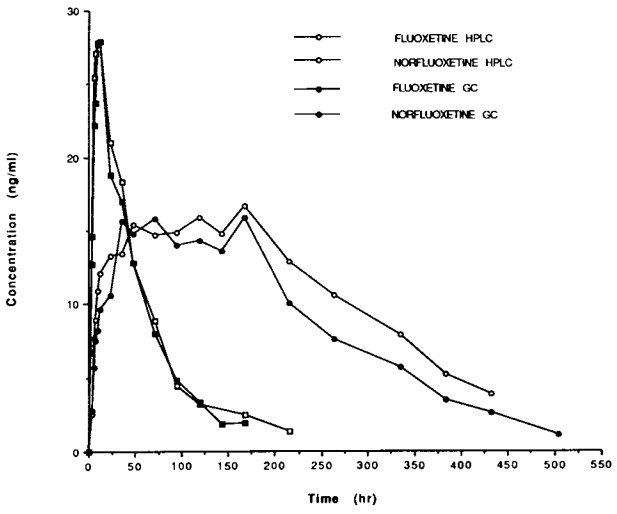

Fig. 5. Plasma concentration–time profile of fluoxetine and norfluoxetine by HPLC and GC.

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