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Simultaneous determination of fluoxetine and norfluoxetine enantiomers in biological samples by gas chromatography with electron-capture detection

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ABSTRACT

An electron-capture gas chromatographic procedure was developed for the simultaneous analysis of the enantiomers of fluoxetine and norfluoxetine. The assay involves basic extraction of these enantiomers from the biological samples, followed by their conversion to diastereoisomers using the chiral derivatizing reagent (*S*)-(−)-N-trifluoroacetylprolyl chloride. The method was utilized to detect and measure the quantity of these enantiomers in plasma and urine of patients and in liver and brain tissue of rats treated with (*R,S*)-fluoxetine.

INTRODUCTION

Fluoxetine (FLU, Fig. 1), a selective 5-hydroxytryptamine (5-HT) uptake inhibitor [1,2] is now one of the most frequently prescribed antidepressant drugs [3]. Although the pharmacology of FLU has been studied extensively and it is known to be metabolized to the selective 5-HT uptake inhibitor N-desmethylfluoxetine (norfluoxetine, N-FLU), much is still unknown about the metabolism and elimination of FLU and its metabolites [4]. Tyrer *et al.* [5] suggested that differences in the metabolism of FLU to N-FLU may account for differences found in the response of patients to FLU treatment. This study, however, did not investigate whether the observed individual differences in the response to FLU could be due to differences in the elimination of the FLU or N-FLU stereoisomers. Clinically, FLU is administered as a racemic compound (mixture of two stereoisomers). With other drugs administered as racemates, differences in the elimination rate and metabolism of the enantiomers have been observed [6,7]. These differences can influence the therapeutic efficacy of a drug since the pharmacological properties of the two stereoisomers are often different. In the case of FLU it has been noted that, although both stereoisomers inhibit 5-HT uptake, their time

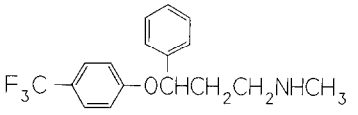

Fig. 1. Structure of fluoxetine [N-methyl-3-phenyl-3-(4-trifluoromethylphenoxy)propylamine].

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courses of 5-HT uptake inhibition are significantly different [8]; these experiments were conducted by administering (*R*)- and (*S*)-enantiomers separately and studying 5-HT uptake at a variety of time intervals [8]. (*S*)-FLU has also been reported to be more potent than (*R*)-FLU at inhibiting 5-HT uptake in cortical synaptosomes, at inhibiting [^{3}H]-FLU binding (indicative of binding to the 5-HT carrier) in cortical membranes, at inhibiting 5-HT uptake *ex vivo* in synaptosomes from brain stem and cortex, and at antagonizing *p*-chloroamphetamine-induced depletion of brain 5-HT [9]. Similarly, (*S*)-N-FLU is more potent than (*R*)-N-FLU at inhibiting uptake of 5-HT in rat brain synaptosomes *in vitro* and at antagonizing *p*-chloroamphetamine-induced depletion of rat brain 5-HT, but the differences in potency are more pronounced than those observed between (*R*)-FLU and (*S*)-FLU [9].

Several procedures exist for the analysis of FLU and N-FLU (*e.g.* refs. 10–13) and two recent HPLC assays provide for simultaneous analysis of the individual stereoisomers of FLU and N-FLU [14,15], but, to our knowledge, no such gas chromatographic assay was available. Described in this manuscript is a useful analytical method developed for the separation and quantitation of FLU and N-FLU stereoisomers in tissue and biological fluids. This procedure was used to analyse these compounds in brains and livers of rats treated with (*R,S*)-FLU and in plasma and urine samples of patients receiving (*R,S*)-FLU (Prozac).

EXPERIMENTAL

Chemicals

Pure standards of (*R,S*)-FLU · HCl, (*R,S*)-N-FLU maleate and of the individual enantiomers were provided by Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). Reagents were purchased from various sources: glass-distilled toluene from BDH (Toronto, Canada); potassium carbonate from Fisher Scientific (Ottawa, Canada); 0.9% sodium chloride (saline) solution from Fisher Diagnostics (Ottawa, Canada); the internal standard L-1-(2-allylphenoxy)-3-(isopropylamino)-propan-2-ol tartrate (*l*-alprenolol) from Sigma (St. Louis, MO, USA); and the chiral derivatizing reagent (*S*)-(−)-trifluoroacetylprolyl chloride from Aldrich (Milwaukee, WI, USA).

Animals

Male Sprague–Dawley rats (Ellerslie Biosciences, Edmonton, Canada), 200–300 g, were housed in pairs in an environmentally controlled room using a 12 h light–12 h dark cycle. At the appropriate time animals were injected intraperitoneally with either saline or (*R,S*)-FLU · HCl (10 mg/kg; dissolved in saline). At times 1, 3, 5, 10, 15 and 24 h post-injection groups of animals ($n = 5$) were sacrificed by decapitation and the brains and livers were removed and immediately frozen in dry ice-cooled isopentane or dry ice, respectively.

Human samples

Plasma and/or 24-h urine samples were obtained from male ($n = 1$) and female ($n = 9$) subjects being treated for major depression with (*R,S*)-FLU (Prozac). These participants received 20 mg of (*R,S*)-FLU once daily, usually taken between 7:00 and 8:00 a.m. Blood and urine samples were taken before FLU treatment began and after three weeks of treatment. A blood sample (25–30 ml) was collected between 9:00 and 10:00 a.m. and centrifuged at 1000 *g* for 10 min to obtain plasma. Urine was collected for the previous 24 h, ending at 8:00 a.m. the day of blood collection. The plasma and urine samples were frozen at −20°C until analysed.

Sample preparation

Rat brain and liver tissue were weighed and homogenized in five volumes of ice-cold 0.1 *M* perchloric acid. An aliquot (300–600 μl) was removed from the homogenized sample and used in the analytical procedure. Appropriate calibration standards of FLU and N-FLU enantiomers were also prepared along with the samples by diluting standard solutions of (*R,S*)-FLU and (*R,S*)-N-FLU in control tissue homogenate prepared from drug-naive rats or in plasma or urine from

drug-naive patients. The final calibration concentrations ranged from 10 ng (0.032, 0.034 nmol) to 1.0 μg (3.2, 3.4 nmol) per volume of tissue homogenate or body fluid for each stereoisomer of FLU and N-FLU. In all studies, the volumes of tissue homogenate or biological fluids used in the calibration curves were the same as those of the samples taken from the (*R,S*)-FLU-treated subjects; these calibration curves were included with each assay run.

Sample extraction

An aliquot (300–600 μl) of rat tissue homogenate, plasma (1–2 ml) or urine (3 ml) were placed in a screw-cap culture tube (Fisher Scientific) and diluted, if required, to a final volume of 2 ml with distilled water. To this solution was added 100 μl of the internal standard solution (*l*-alprenolol; 0.223 mg/ml dissolved in methanol). The samples were then basified by adding solid potassium carbonate (400 mg). The basic solution was then vortex-mixed briefly, and 4 ml of glass-distilled toluene were added. The two immiscible phases were mixed for 15 min in an Ika Vibrex VXR vortex-mixer (Janke and Kunkel, Staufen, Germany) and centrifuged for 15 min at 1000 *g* in a benchtop centrifuge (Sorvall GLC-2B general laboratory centrifuge, Du Pont, Wilmington, DE, USA). The toluene layer was transferred to a second 160 mm × 15 mm screw-cap culture tube to which were added 2 ml of 0.5 *M* HCl. The toluene–HCl mixture was vortex-mixed for 10 min and centrifuged for 10 min at 1000 *g*. The toluene layer was discarded and the remaining aqueous layer was basified with solid potassium carbonate (400 mg). Toluene (4 ml) was again added to the basic solution and the two immiscible liquid phases vortex-mixed for 10 min and centrifuged for 10 min at 1000 *g*. The toluene layer was retained and transferred to a 100 mm × 13 mm screw-cap culture tube. The organic solvent was evaporated under a stream of nitrogen. To the dry residue were added 100 μl of the derivatizing solution (40 μl of (*S*)-(−)-trifluoroacetylprolyl chloride per milliliter of glass-distilled toluene). The tubes were briefly vortex-mixed and the derivatization reaction was allowed to proceed at 60°C for 60 min. Following completion of the reaction the samples were evaporated to dryness under a stream of nitrogen and then redissolved in 100 μl of glass-distilled toluene. Of this solution 2 μl were used for gas chromatographic (GC) analysis.

Instrumental analysis

All samples were analysed using an HP 5880 gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a ^{63}Ni electron-capture detector. The chromatographic column installed was a 15 m × 0.25 mm I.D. DB-5 cross-linked fused-silica capillary (0.22 μm thickness; J & W Scientific, Palo Alto, CA, USA). The conditions for separation were as follows. Initial oven temperature 145°C, held for 0.5 min, followed by an increase to 207°C at a rate of 30°C/min. The oven temperature was held at 207°C for 4 min followed by an increase to 225°C at a rate of 1°C/min.

Once the oven temperature reached 225°C the rate of temperature increase was changed to 25°C/min and the temperature allowed to rise to 300°C. This final oven temperature of 300°C was maintained for 2 min. The detector and injection port temperatures were held constant at 325 and 270°C, respectively. All injections of sample were carried out using the splitless mode of injection with a purge off time of 0.5 min. The ultra-pure helium (Union Carbide, Edmonton, Canada) carrier gas flow-rate and 10% methane–argon (Union Carbide) make-up gas flow-rate were 1 and 30 ml/min, respectively.

Mass spectra (for confirmation of structures of the derivatives) were recorded in the electron-impact mode using a VG 7070E mass spectrometer (VG Instruments, Manchester, UK), linked to a Varian Vista gas chromatograph (Varian Instruments, Sunnyvale, CA, USA) containing a 30 m × 0.25 mm I.D. DB-5 (0.22 μm film thickness; J & W Scientific) fused-silica capillary column. The temperature program used for separation was the same used for GC–electron-capture detection analysis of these stereoisomers.

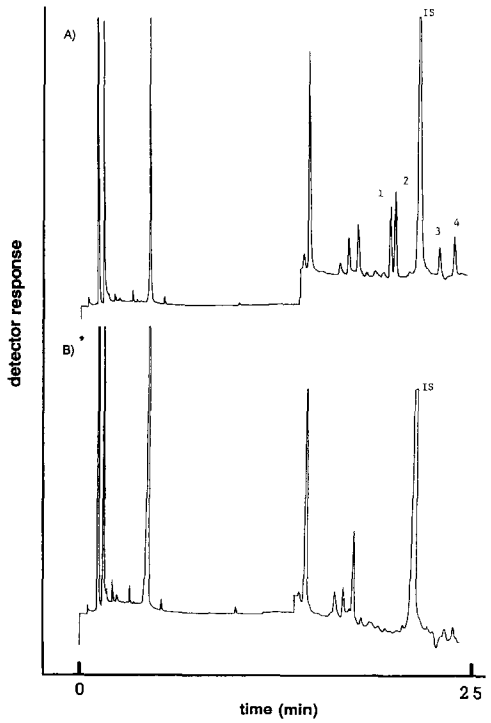

Fig. 2. Derivatized extract of rat brain from (A) fluoxetine · HCl-treated (10 mg/kg) animals sacrificed 3 h post-dose and (B) drug-naive rats injected with saline. The GC peaks of the stereoisomers are identified as follows: 1 = (*S*)-norfluoxetine; 2 = (*R*)-norfluoxetine; IS = internal standard; 3 = (*R*)-fluoxetine; 4 = (*S*)-fluoxetine. The GC retention times of these peaks were 19.7, 20.1, 21.7, 22.9 and 23.9 min, respectively.

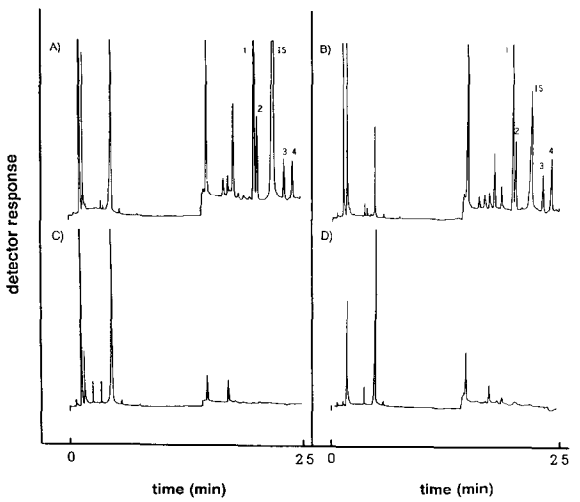

Fig. 3. Derivatized extract of (A) plasma and (B) urine obtained from depressed patients treated with fluoxetine (Prozac; 20 mg once daily) for three weeks or (C) plasma and (D) urine from drug-naive individuals. The GC peaks of the stereoisomers are identified as follows: 1 = (*S*)-norfluoxetine; 2 = (*R*)-norfluoxetine; IS = internal standard; 3 = (*R*)-fluoxetine; 4 = (*S*)-fluoxetine.

RESULTS AND DISCUSSION

The (*R*)- and (*S*)-isomers of FLU and N-FLU were separated chromatographically following their conversion to diastereoisomers using the chiral derivatizing reagent (*S*)-(−)-trifluoroacetylprolyl chloride. Fig. 2 is a representative chromatogram of the derivatized extract of a brain tissue homogenate obtained from (*R,S*)-FLU-treated rats. Similarly, Fig. 3 shows typical chromatograms of derivatized extracts of patient plasma or urine obtained following the ingestion of (*R,S*)-FLU by a patient. Extracts of liver and brain tissue homogenates obtained from drug-naive rats and plasma or urine samples collected from untreated patients showed that no chromatographic peaks interfered with the analysis of the enantiomers of FLU or N-FLU.

The identity of the chromatographic peaks representing the derivatized enantiomers of FLU and N-FLU were confirmed by comparing their GC retention times and mass spectra to authentic standards of (*R*)- and (*S*)-FLU and (*R*)- and (*S*)-N-FLU, similarly derivatized. The mass spectra and proposed fragmentation patterns of the derivatized enantiomers of FLU and N-FLU are depicted in Fig. 4.

Calibration graphs were obtained by analyzing standards prepared in parallel with the samples for each assay run. Eight-point calibration graphs were generated over the concentration range of 10 ng to 1.0 μg (0.032–3.2 nmol of FLU; 0.034–3.4 nmol of N-FLU) of each enantiomer per volume of tissue homogenate, plasma or urine extracted. Regression analysis of the relationship between standard concentration and the chromatographic peak-height ratio of the enantiomer/internal standard yielded a linear relationship over the concentration range analyzed, with a typical r^2 value of >0.99.

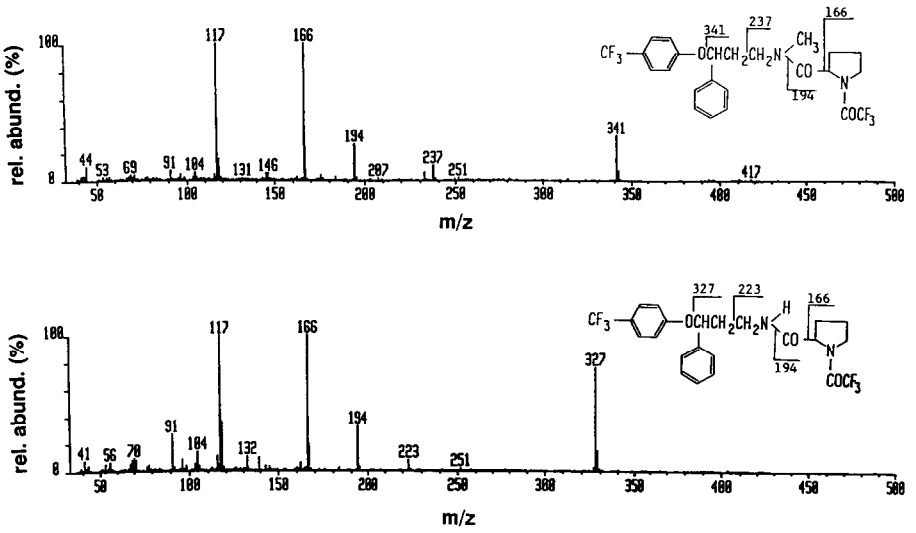

Fig. 4. Electron-impact mass spectra of fluoxetine (upper) and norfluoxetine (lower) after derivatization with (*S*)-(−)-trifluoroacetylprolyl chloride. Mass spectra were recorded using a VG 7070E mass spectrometer linked to a Varian Vista gas chromatograph. The fragment ion *m/z* 117 ($Ph\text{-}\dot{C}H\text{-}CH=CH_2$) is produced by elimination of the radical ion CF_3-Ph-O and rearrangement of the propylamine side-chain with the loss of a molecule of 1-(trifluoroacetyl)pyrrolidine-2-carboxamide or 1-(trifluoroacetyl)pyrrolidine-2-(N-methyl)carboxamide in the spectra of N-FLU and FLU, respectively. The identities of the other diagnostic ions are described above.

Calibration curves generated on different days were reproducible. Mean slopes of the curves for the derivatives of (*R*)- and (*S*)-FLU and of (*R*) and (*S*)-N-FLU were 0.69, 0.70, 1.27 and 1.19, respectively (coefficients of variation ranged from 3.0 to 5.5%). The mean *y*-intercepts were 0.024, 0.040, 0.028 and 0.017, respectively. To verify that the analyses of samples were reproducible, plasma was spiked with 0.118 μg/ml of each N-FLU enantiomer and 0.100 μg/ml of each FLU enantiomer. The spiked samples were analyzed in quadruplicate. The mean concentrations determined experimentally for these samples were 0.110 and 0.106 for (*S*)- and (*R*)-N-FLU, respectively, and 0.092 and 0.093 μg/ml of plasma for (*S*)- and (*R*)-FLU, respectively (coefficients of variation ranged from 5.3% for (*R*)-N-FLU to 8.2% for (*S*)-FLU in these studies). These results indicate that the developed analytical procedure is both sensitive and reproducible.

The tissue concentration–time curves determined for (*R*)- and (*S*)-FLU and (*R*)- and (*S*)-N-FLU in rat brain and liver tissue indicate that both enantiomers of FLU (Fig. 5) were rapidly cleared from both tissues and were no longer detected after 10 h. The N-FLU stereoisomers persisted in the tissues for the 24-h study period. Peak brain levels of the N-FLU enantiomers occurred at approximately 9 h. The time course of elimination observed and total tissue levels (concentration of both stereoisomers combined) of FLU and N-FLU were comparable to previously reported levels in rats receiving the same dose of FLU [12]. Little difference was observed in the tissue levels and time course of elimination for the individual enantiomers of FLU and N-FLU; a similar pattern (*i.e.* levels of (*R*)- and (*S*)-N-FLU were higher than those of (*R*)- and (*S*)-FLU, but there was little difference between levels of the (*R*)- and (*S*)-enantiomers in each case) was observed by Fuller *et al.* [9] after administration of (*R*)- or (*S*)-FLU. In a recently published paper, using a dose of racemic FLU twice that reported in the present paper, Potts and Parli [15] also found a similar pattern of elimination of the enantiomers of FLU and N-FLU in rat brain, although the differences in levels of (*R*)-FLU and (*S*)-FLU appear to be slightly greater than reported here.

In the present study, levels of the enantiomers

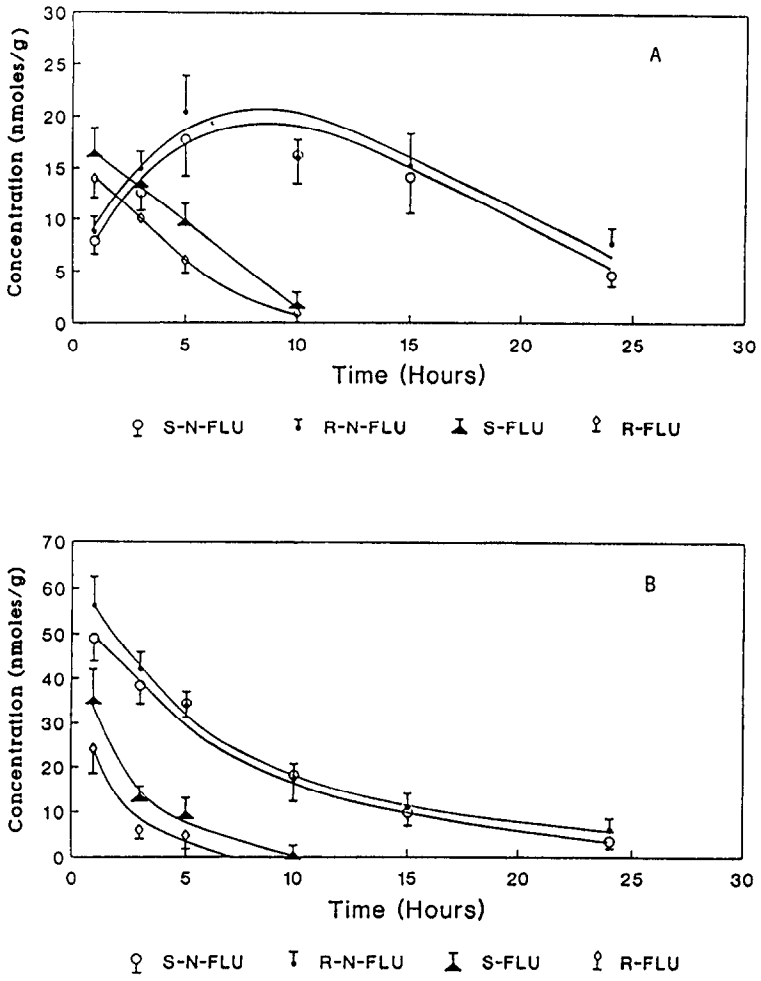

Fig. 5. Tissue concentration–time profile of (*S*)-norfluoxetine, (*R*)-norfluoxetine. (*S*)-fluoxetine and (*R*)-fluoxetine detected in (A) rat brain and (B) rat liver obtained from fluoxetine-treated (10 mg/kg) rats. Groups (five animals per group) were dosed with either fluoxetine · HCl or saline and sacrificed at 1, 3, 5, 10, 15 or 24 h after injection. Results are expressed as nmol/g and represent means ± S.E.M.

of FLU and N-FLU were also determined in the plasma and urine of patients who had received a three-week course of oral (*R,S*)-FLU (20 mg once daily). The mean plasma levels of (*S*)-FLU, (*R*)-FLU, (*S*)-N-FLU and (*R*)-N-FLU were determined to be 44.9, 23.2, 66.5, and 31.3 ng/ml, respectively (Table I). The total (*R,S*)-FLU and (*R,S*)-N-FLU (total concentration of both enantiomers) plasma levels were consistent with previously reported levels (*e.g.* ref. 4) in patients on similar doses. Pronounced differences were observed in the plasma and urine concentrations of the individual (*S*)- and (*R*)-enantiomers of N-FLU and FLU. The concentration of the (*S*)-enantiomer was higher than the concentration of the (*R*)-enantiomer in the plasma and urine of these patients (Tables I and II). Peyton *et al.* [14] investigated levels of the enantiomers in plasma samples of a single patient receiving a 60-mg oral dose of racemic FLU and also reported that levels of the (*S*)-enantiomer were higher than those of the (*R*)-enantiomer for both FLU and N-FLU.

A high degree of correlation ($r = 0.91$) was

TABLE I

PATIENT PLASMA CONCENTRATION OF (*S*)-N-FLU, (*R*)-N-FLU, (*S*)-FLU AND (*R*)-FLU

Blood samples were drawn between the hours of 9:00 and 10:00 a.m. The patients had been treated for three weeks with fluoxetine · HCl (Prozac, 20 mg once daily) prior to sample collection.

Patient	Plasma concentration (ng/ml)				Ratio	
	(*S*)-N-FLU	(*R*)-N-FLU	(*S*)-FLU	(*R*)-FLU	(*S*)-N-FLU/(*R*)-N-FLU	(*S*)-FLU/(*R*)-FLU
FOO1B	86.8	55.0	76.4	21.9	1.58	3.49
FOO2B	90.0	27.1	47.1	18.3	3.32	2.57
FOO3B	42.6	22.2	60.3	16.6	1.92	3.63
FOO6B	16.7	7.5	14.9	10.0	2.23	1.49
FOO7B	77.5	31.1	45.9	24.2	2.49	1.90
FOO9B	52.6	32.8	38.6	41.4	1.60	0.93
FOO10B	99.1	41.7	31.1	29.9	2.38	1.04
Mean	66.5	31.1[a]	44.9	23.2[b]	2.22	2.15
S.E.M.	11.3	5.6	7.5	3.8	0.23	0.42

[a] $p < 0.05$, compared to values for (*S*)-N-FLU.

[b] $p < 0.05$ compared to S-FLU, one-tailed *post-hoc* protected Tukey's *t*-test, after ANOVA with repeated measures.

observed when the plasma and urinary concentration ratios of (*S*)- and (*R*)-N-FLU were compared (Fig. 6). A similar correlation ($r = 0.90$) was noted when plasma and urine concentration ratios of (*S*)- and (*R*)-FLU were compared. No correlation could be shown between the plasma concentration ratio of (*S*)- and (*R*)-N-FLU and the plasma concentration ratio of (*S*)- and (*R*)-

TABLE II

URINARY EXCRETION OF FREE (*R*)- AND (*S*)-N-FLU AND (*R*)- AND (*S*)-FLU IN PATIENTS TAKING (*R,S*)-FLUOXETINE · HCl (PROZAC, 20 mg ONCE DAILY) FOR THREE WEEKS

Patient	Drug excretion (μg/24 h)				Ratio	
	(*S*)-N-FLU	(*R*)-N-FLU	(*S*)-FLU	(*R*)-FLU	(*S*)-N-FLU/(*R*)-N-FLU	(*S*)-FLU/(*R*)-FLU
FOO1B	160	93	100	39	1.72	2.56
FOO2B	132	36	47	39	3.67	1.21
FOO3B	930	354	563	215	2.63	2.62
FOO5B	1959	1260	1153	513	1.55	2.25
FOO6B	153	57	29	21	2.68	1.38
FOO7B	150	44	50	38	3.41	1.32
FOO8B	395	111	142	123	3.56	1.15
FOO9B	921	556	383	334	1.66	1.15
FOO10B	496	209	135	138	2.37	0.98
FOO12B	1706	1181	511	406	1.44	1.26
Mean	700	390[a]	311	187[b]	2.47	1.59
S.E.M.	212	147	112	55	0.27	0.20

[a] $p < 0.05$, compared to values for (*S*)-N-FLU.

[b] $p < 0.05$, compared to (*S*)-FLU, one-tailed *post-hoc* protected Tukey's *t*-test, after ANOVA with repeated measures.

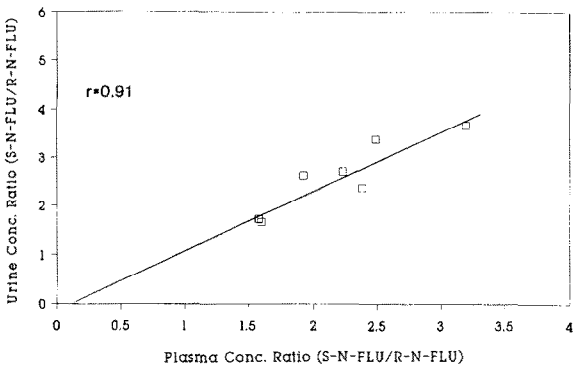

Fig. 6. Comparison of the (*S*)-norfluoxetine/(*R*)-norfluoxetine plasma concentration ratio to (*S*)-norfluoxetine/(*R*)-norfluoxetine urine concentration ratio. Samples of plasma and urine were obtained from depressed patients following three weeks of oral fluoxetine (Prozac, 20 mg once daily) treatment.

FLU. Marked inter-individual differences in the plasma and urinary concentrations of the individual enantiomers were also observed in these patients (Table II); other authors who studied plasma and urine levels of FLU and N-FLU but did not separate the enantiomers have also reported marked inter-individual differences in levels of FLU and N-FLU [5,10,11,16]. The amount of the free N-FLU and FLU enantiomers excreted at steady state over the 24-h period ranged from 1.3 to 24.4% of the administered daily dose for the patients studied.

The procedure described here is a useful method for the simultaneous analysis of (*R*)- and (*S*)-FLU and of (*R*)- and (*S*)-N-FLU. The method is valid for the separation, detection and quantitation of these stereoisomers in biological fluids and tissues. In the rat, there were marked differences in the clearance of FLU and N-FLU from the tissues studied, but no marked differences were observed in the clearance of the individual enantiomers of FLU or N-FLU. In contrast, pronounced differences in the plasma and urinary levels of the enantiomers of FLU and N-FLU were observed among the patients studied. Little is known about the stereospecific metabolism of (*R,S*)-FLU. The present study suggests that, since levels of (*S*)- and (*R*)-enantiomers of FLU and N-FLU were markedly different from one another in both plasma and urine, either the N-demethylation of FLU or some other route of FLU metabolism is stereospecific. It has been determined that approximately 70% of FLU metabolism in humans has yet to be accounted for [4], and it will be of great interest to determine the importance of stereospecificity in future studies on that metabolism.

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