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Determination of fluoxetine and norfluoxetine in human plasma by high-performance liquid chromatography with ultraviolet detection in psychiatric patients

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Abstract

A rapid high-performance liquid chromatographic method is described for the simultaneous determination of the widely used antidepressant drug, fluoxetine and its principal metabolite norfluoxetine in plasma. After liquid–liquid extraction the compounds were separated in a reversed-phase column and assayed by ultraviolet absorption at 226 nm. The analytical interference from psychoactive drugs and their metabolites was also studied. The extraction recoveries were 93 and 87% for norfluoxetine and fluoxetine, respectively. The limit of quantitation under the described conditions was 14 nmol/l for both compounds. The method was found to be reproducible with coefficients of variation less than 10%. A great variability in plasma concentrations of fluoxetine and norfluoxetine as well as in fluoxetine/norfluoxetine ratios was found among the 29 patients studied. This result suggests the implication of genetically polymorphic enzymes, presumably CYP2D6, CYP2C9 and CYP2C19 in the metabolism of fluoxetine to norfluoxetine. Therapeutic drug monitoring should thus be useful in patients treated with regular doses.

Keywords: Fluoxetine; Norfluoxetine

1. Introduction

Fluoxetine is a bicyclic derivate of phenylpropylamine, a drug which enhances serotonergic neurotransmission through potent and selective inhibition of the neuronal reuptake of serotonin. It is prescribed for a variety of psychopathological conditions including mood and eating disorders, obsessive–compulsive disorders, depression in the elderly and dysthymia [1]. According the wholesales data of fluoxetine in the Autonomous Community of Extremadura (1.1 million inhabitants), Spain, the number of inhabitants treated on average day doses increased from 0.06% (1990) to 0.75% by 2000, representing 23% of the all antidepressant use [2].

Fluoxetine is well absorbed from the gastrointesti-

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nal tract after oral administration, it is highly protein bound, and has a large volume of distribution (14–100 l/kg). The main metabolite of fluoxetine is norfluoxetine (*N*-demethylfluoxetine), which has a similar pharmacological activity compared to the parent compound. Both fluoxetine and norfluoxetine have elimination half-lives of several days (1–4 and 7–15 days respectively) [3]. The pharmacokinetics of fluoxetine has been shown to be non-linear in both healthy volunteers and patients with depression [1]. Fluoxetine displays a large interindividual variability in its pharmacokinetics. Cytochromes P450 CYP2C9, CYP2D6 and also CYP2C19 appear to be involved in fluoxetine metabolism [4–6]. Individuals who are poor metabolizers of fluoxetine have been shown to be poor metabolizers of dextromethorphan [7]. Furthermore, poor metabolizers of debrisoquine were also poor metabolizers of fluoxetine. These results suggest that fluoxetine pharmacokinetics is influenced by the polymorphic CYP2D6 activity [8]. High-performance liquid chromatography with fluorescence detection and gas chromatography with electron capture detection have been used to determined serum concentration of fluoxetine and norfluoxetine [9–14], however the routine equipment for determination of psychotropic drugs usually have ultraviolet detector [15–19].

The aims of the present study were to develop a simple, accurate method for the quantitative determination of fluoxetine and its *N*-demethyl metabolite in plasma using liquid–liquid extraction and reversed-phase high-performance liquid chromatography with ultraviolet detection, and to study the variability of fluoxetine/norfluoxetine ratio in patients during steady-state.

2. Experimental

2.1. Chemicals

Fluoxetine, norfluoxetine, and doxepine hydrochloride (internal standard, I.S.) were supplied by Research Biochemicals Inc. (Natick, MA, USA). Fluoxetine (200 μ*M*), norfluoxetine (200 μ*M*) and doxepine (633 μ*M*) stock solutions were prepared in hydrochloric acid (0.01 *M*) and stored under refrigeration. Acetonitrile, methanol, dimethyloctylamine (DMOA) and *n*-heptane were of ultraviolet grade from Merck (Darmstadt, Germany). Isoamyl alcohol, glacial acetic acid, hydrochloric acid and potassium hydroxide from Panreac (Barcelona, Spain) were of analytical reagent grade.

2.2. Instrumentation

The liquid chromatographic system consisted of a Beckman model 110B pump, and a Beckman 166 programmable detector module coupled to a 386 PC with Beckman Gold software V.7.11 (Beckman Instruments, Inc., Fullertone, CA, USA). The mobile phase was a mixture of acetonitrile (30%), water (67%), acetate buffer (3%) and 400 μl of DMOA. Before analysis, the mobile phase was filtered through a 0.22-μm filter (Millipore, USA), and then degassed ultrasonically for 15 min. The assay was run at room temperature. Separation was carried out using a BDS Hypersil C_{18} column (100×4.6 mm I.D., 3 μm). The flow-rate was set at 0.6 ml/min and detection wavelength at 226 nm.

2.3. Extraction

Sample preparation was carried out by liquid–liquid extraction. An aliquot of 500 μl of human plasma was pipetted into a 10-ml polypropylene tube and alkalinised with 500 μl of sodium hydroxide (0.5 *M*). The plasma was extracted with 1.5 ml of heptane–isoamyl alcohol (97:3 v/v) after addition of 50 μl of the internal standard working solution (doxepine 6.33 μ*M*, I.S.). The tubes were capped, shaken vertically for 10 min and then centrifuged for 10 min at 3000 rpm. Then, 1 ml of the organic layer was transferred into another tube for back-extraction with 75 μl of acetic acid (25 m*M*). The tubes were vortex-mixed for 5 min and centrifuged for 5 min at 3000 rpm. The upper organic layer was aspirated off and the remaining organic phase was evaporated under nitrogen flow for 5 min. Twenty microliters of the acidic solution were injected into the HPLC system for analysis.

2.4. Preparation of the calibration curve

A calibration curve based on peak-height ratios (fluoxetine/doxepine and norfluoxetine/doxepine)

was constructed for all the assays by adding known amounts of fluoxetine and norfluoxetine to drug-free human plasma. Concentrations of the two compounds equivalent to 100, 200, 400, 800 and 1600 nmol/l were assayed.

2.5. Study in patients

Plasma levels of fluoxetine and norfluoxetine were determined in 29 patients (nine males and 20 females, 26–83 years) who were receiving treatment with 20 mg/day of fluoxetine for at least 15 days. None of the patients were comedicated with CYP2D6 or CYP2C9 drugs. The samples were collected in the morning before the daily dose. They gave their prior written consent to participate in the study. The study was performed according to the Helsinki Declaration and was approved by the Ethical Committee of Extremadura University Hospital.

3. Results

3.1. Chromatography

A chromatogram of blank plasma from a healthy drug-free blood donor spiked with 200 nmol/l of fluoxetine and norfluoxetine is shown in Fig. 1A. The retention times for doxepine (I.S.), norfluoxetine and fluoxetine were 3.4, 7.7 and 8.9 min respectively. The three peaks were adequately resolved without any interference from endogenous compounds, as shown in Fig. 1A and B. The most clinically used, 38 selected psychoactive drugs, were tested for the possible interferences. These compounds are listed in Table 1 with the relative retention times respect to

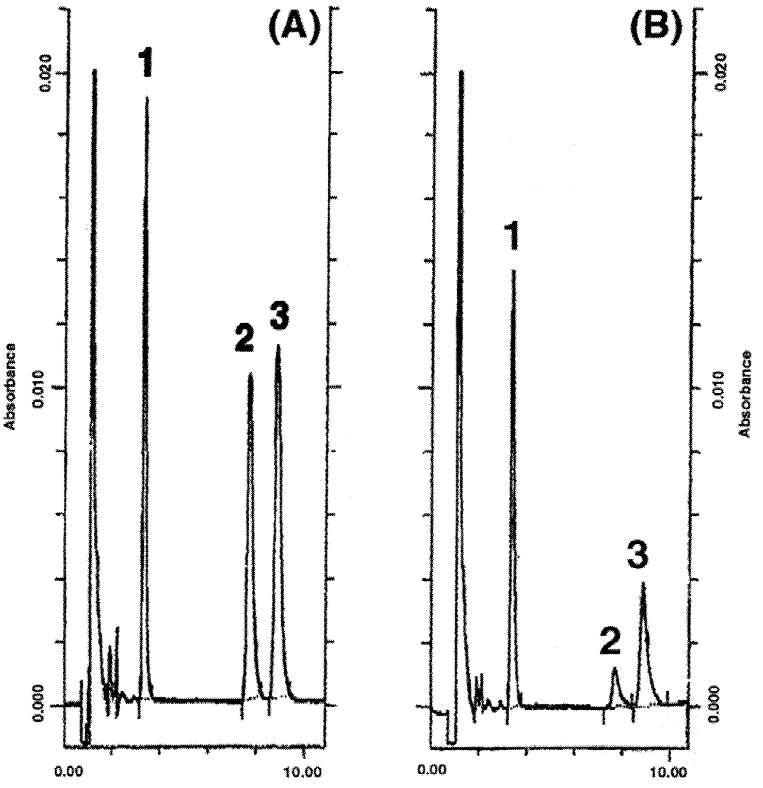

Fig. 1. (A) Blank plasma spiked with 200 nmol/l of norfluoxetine and fluoxetine. (B) Chromatograms of a patient plasma treated with 20 mg dose of fluoxetine. Peaks are: 1, doxepine; 2, norfluoxetine and 3, fluoxetine.

Table 1
Relative retention times (rRT) calculated as the difference in min between internal standard (I.S.) and the drug tested for interferences

Drug	rRT (min)	Drug	rRT (min)
thioproperazine	−2.79	carbamazepine	1.04
zopiclone	−2.19	pinazepam	1.14
sulpiride	−1.90	lithium	1.24
bentazepam	−1.14	phenytoin	1.65
tiapride	−1.10	imipramine	1.75
chlorazepate	−0.94	fluvoxamine	1.80
amineptine	−0.50	halazepam	2.00
valpromide	−0.35	maprotiline	2.26
bromazepam	−0.12	amitriptiline	2.69
doxepine (I.S.)	0.00	alprazolam	3.55
haloperidol	0.18	lorazepam	3.87
lormetazepam	0.19	triazolam	4.14
levomepromazine	0.61	norfluoxetine	4.33
lofepramine	0.62	fluoxetine	5.48
trazodone	0.63	clomipramine	6.54
clometiazol	0.69	trifluoperazine	6.88
chlorpromazine	0.77	midazolam	9.46
perphenazine	0.79	loprazolam	10.07
zolpidem	0.84	diazepam	10.62
biperiden	0.91	pimozide	16.80
thioridazine	1.01		

doxepine. None of these antidepressants, anxiolytic or neuroleptic drugs interfered with the fluoxetine or norfluoxetine peaks.

3.2. Linearity, recovery and limit of detection

Calibration curves were linear over the range 100–1600 nmol/l (r^2=0.999, n=6) for both fluoxetine and norfluoxetine. The extraction recovery calculated as the mean (n=6) for norfluoxetine and fluoxetine are shown in Table 2. The limits of detection (LOD) defined as three times the baseline noise were 10 nmol/l for both fluoxetine and norfluoxetine. The limits of quantitation (LOQ) were 14 nmol/l for both fluoxetine and norfluoxetine (n=4, C.V. less than 15%).

Table 2
Recovery of the analytical method (n=6)

Concentration (nmol/l)	%Recovery (mean±SD)	
	Fluoxetine	Norfluoxetine
100	86.1±3.3	92.5±7.1
200	87.1±3.3	92.5±8.8
400	87.1±3.3	92.7±8.5
800	86.7±3.1	92.7±8.6
1600	86.9±3.1	92.7±8.6

3.3. Accuracy, precision, and intra-assay reproducibility

Assays were performed over a 3-day period, using spiked human plasma samples (n=6). The results are listed in Table 3. The method was found to be reproducible, as indicated by the low values obtained for the coefficients of variation less than 10%.

3.4. Patient plasma concentrations of fluoxetine and norfluoxetine

The patient plasma concentrations of fluoxetine and norfluoxetine varied widely, for fluoxetine from 15 to 865 nmol/l, and norfluoxetine from 14 to 602 nmol/l among the 29 patients studied. Distribution of fluoxetine/norfluoxetine logarithm concentration ratios also showed a great interindividual variability (Fig. 2).

Table 3
Accuracy, intra-assay reproducibility and precision (n=6)

	Conc. added (nmol/l)	Accuracy (%)	C.V. intra-assay (%)	C.V. inter-day (%)
Fluoxetine	200	97.2	3.4	8.1
	400	99.7	2.3	7.2
	800	99.9	2.3	7.4
Norfluoxetine	200	98.0	0.7	5.9
	400	98.9	2.5	4.6
	800	99.9	3.3	6.6

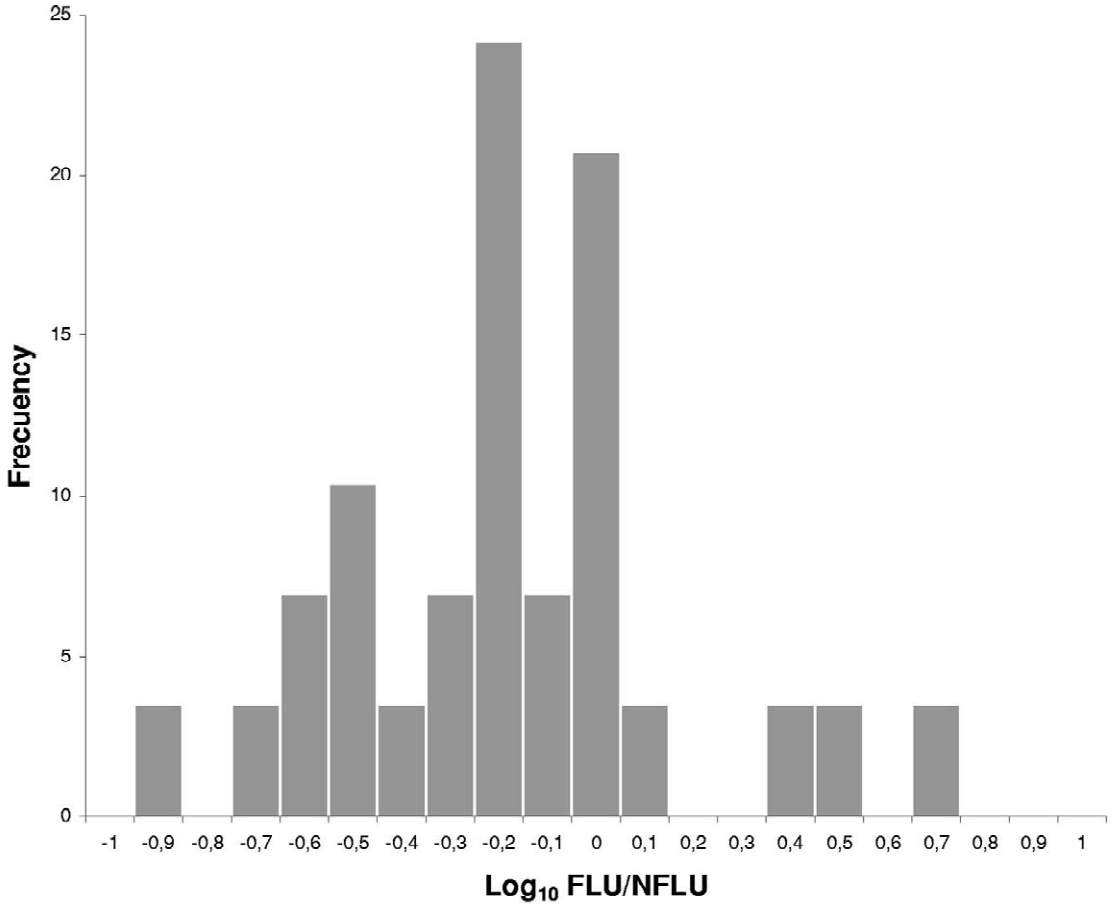

Fig. 2. Distribution, x axis: fluoxetine/norfluoxetine plasma concentration ratios in steady-state (logarithmic scale). y axis percentage of the 29 patients treated with fluoxetine 20 mg/day.

4. Discussion

The present study was aimed at developing an HPLC method suitable for simultaneous determination of fluoxetine and its major metabolite, norfluoxetine in human plasma samples. The method was developed to be rapid, simple, specific, easy to perform and inexpensive. Baseline separation could be obtained by HPLC for all compounds to be determined within less than 10 min, similar to previously published methods [15–19].

Fluoxetine and its metabolite can be determined by electron-capture gas chromatography [12–14] and fluorescence detection [9–11]. We developed a procedure for simultaneously quantifying fluoxetine and its active metabolite norfluoxetine in plasma by reversed-phase HPLC followed by ultraviolet detection, which are common equipments for routine for therapeutic drug monitoring in clinical laboratories. The most significant advantage of the present method is that the chromatography conditions are simple to adapt for the analysis of the most commonly used psychotropic drugs like risperidone [20], thioridazine [21], haloperidol [22], clozapine [23].

The intra-assay reproducibility (less than 10%), the LOQ and LOD (14 nmol/l and 10 nmol/l for both fluoxetine and norfluoxetine, respectively) were found to be similar or better than in previously published HPLC with ultraviolet detection methods [15–19].

The plasma concentrations of fluoxetine and norfluoxetine in the 29 patients were within the range reported in the literature [24,25], however, a great interindividual variability was found both in fluoxetine and norfluoxetine plasma concentration as well as in fluoxetine/norfluoxetine ratio (Fig. 2). A possible explanation for that finding could be the involvement of the polymorphic cytochrome enzymes CYP2D6, CYP2C9 and CYP2C19 in the metabolism of fluoxetine to norfluoxetine, as it has been previously suggested [4–6,26,27]. Thus, poor metabolizers might get higher plasma concentrations than extensive metabolizers when treated with the same dose regime. We have already shown that around 7% of Spaniards, as other Caucasian populations are poor metabolizers of CYP2D6. Furthermore, recently we have shown the variability of CYP2C9 activity and phenotype among Spaniards and also among Cubans [28,29]. Since these enzymes, particularly CYP2D6, are involved in the metabolism of several psychotropic drugs used in combinations in the clinical practice pharmacokinetic interactions may occur [30]. Furthermore, since the activity of CYP2D6 is polymorphic unexpected side-effects due to higher than expected plasma concentrations (among poor metabolizers) or therapeutic failures (among ultrarapids) may occur. In addition, since fluoxetine is a potent inhibitor of CYP2D6 and also metabolised by this enzyme [8,31,32], inter-individual variability of plasma concentration might be expected.

In the light of the potential variability of fluoxetine plasma concentration the therapeutic drug monitoring might be an useful tool for several aspects, including individual dose optimisation, detection of drug interactions and assessments of patients compliance [24,33]. The method described here is suitable and useful in a clinical laboratory for therapeutic drug monitoring.

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