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Sensitive micromethod for column liquid chromatographic determination of fluoxetine and norfluoxetine in human plasma

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ABSTRACT

A rapid, selective and sensitive micromethod has been developed for the determination of fluoxetine (FLU) and its demethylated metabolite norfluoxetine (N-FLU) using a 250-μl plasma sample and column liquid chromatography with ultraviolet detection at 226 nm. The limit of detection is 2.0 ng/ml for both FLU and N-FLU. Peak-height ratios are linear over a concentration range of 10–800 and 10–1000 ng/ml for FLU and N-FLU, respectively. Acceptable coefficients of variation are demonstrated for both within-run and day-to-day assays. Selected drugs were checked for interference. The method, which requires a very small volume of plasma, is sensitive enough for pharmacokinetic studies in animals, clinical pharmacology studies and drug monitoring in children or adult patients.

INTRODUCTION

Fluoxetine, N-methyl-8-[4-(trifluoromethyl)-phenoxy]benzenepropanamine (FLU), is a non-tricyclic antidepressant that exibits selective inhibition of serotonin uptake in presynaptic neurons [1]. It is widely prescribed in depressed outpatients with a diagnosis of major depressive disorder (DSM_{III} classification). The recommended initial dose of FLU is 20 mg per day [2]. Higher doses appear to be effective for the treatment of obsessive–compulsive disorder [3], while lower doses may be effective for the treatment of panic attacks [4]. FLU is mainly demethylated by the liver to norfluoxetine (N-FLU), also a potent inhibitor or serotonin reuptake, and other minor metabolites.

So, as for tricyclic antidepressants, FLU drug monitoring and pharmacokinetic studies require sensitive and specific analytical methods. FLU and N-FLU have been quantified by gas chromatography (GC) with electron-capture detection of the pentafluoropropionic anhydride derivative after liquid [5] or solid-phase extraction [6,7]. These macromethods (1 ml of plasma or serum required), even if sensitive and selective, are too time-consuming (extractions, purification, derivatization) to be widely used in clinical laboratories and do not fit adequately to routine analysis criteria. The previous liquid chromatography (LC) procedures are macro- or semimicromethods (0.1, 1 or 2 ml of plasma required) that use either a reversed-phase LC analysis (phenyl, cyano, C_{18} columns) and UV detection [8–11] or fluorescence detection [12,13]. They involve multi-

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step extractions and/or the utilization of drugs that can be co-administered to depressed patients (protriptyline, clomipramine, haloperidol) as internal standard (I.S.).

This report describes a rapid, simple, specific and sensitive micromethod using a reversed-phase LC procedure for separating and quantifying FLU and N-FLU in plasma, after a single extraction step using mefloquine as an I.S. The chromatographic system enables the determination of FLU and N-FLU with the same specificity as previously published methods using UV detection and an improved limit of detection. The method has been applied to the analysis of the two compounds in plasma samples from depressed patients.

EXPERIMENTAL

Chemicals

Fluoxetine hydrochloride and norfluoxetine maleate were kindly supplied by Eli Lilly (Saint-Cloud, France). Mefloquine base used as I.S. was obtained from Roche (Basle, Switzerland). Stock solutions containing 1 mg/ml as base of FLU, N-FLU and I.S. were prepared in methanol.

Acetonitrile, methanol (Merck, Darmstadt, Germany) and *n*-hexane (Carlo Erba, Milan, Italy) were UV grade.

Phosphate buffer (Fluka, Buchs, Switzerland), orthophosphoric acid (Carlo Erba), isoamyl alcohol (UCB, Leuven, Belgium) and sodium carbonate monohydrate (Sigma, St. Louis, MO, USA) were analytical-reagent grade.

Apparatus and chromatographic conditions

The LC system consisted of a Beckman Model 114A pump (Beckman, San Ramon, CA, USA) connected to a Wisp 712 B autosampler (Waters Millipore, Milford, MA, USA), a Shimadzu SPD-6A spectrophotometric detector (Touzart et Matignon, Vitry, France) set at 226 nm (0.01 a.u.f.s.) and a Shimadzu C-R6A integrator (Touzart et Matignon).

The eluent was a mixture of 0.067 *M* potassium dihydrogenphosphate buffer and acetonitrile (70:30, v/v) (the buffer was adjusted to pH 3.0 with orthophosphoric acid). Before analysis, the mobile phase was filtered through a 0.22-μm filter (Durapore GVWP 047, Bedford, MA, USA) with a Pyrex filter holder (Millipore, Bedford, MA, USA), then degassed ultrasonically. Separation was achieved at room temperature using a reversed-phase C_8 Ultrapore RPMC column (7.5 cm × 4.6 mm I.D.; particle size 5 μm) purchased from Beckman. The flow-rate was set at 2.0 ml/min with an average operating pressure of 70 bar.

At the end of each chromatographic session the column was washed with 60 ml of acetonitrile–deionized water (50:50, v/v).

Extraction procedure

In a 15-ml centrifuge tube, 250 μl of plasma (from human heparinized blood) were alkalinized with 125 μl of 2 *M* sodium carbonate. The 250 μl of plasma were then extracted with 10 ml of *n*-hexane–isoamyl alcohol (98:2, v/v) after addition of 70 μl of the working solution of I.S. The tube was capped, shaken horizontally for 20 min, then centrifuged for 10 min at 3000 *g*. The organic layer was transferred into another tube for back-extraction with 150 μl of 0.05% diluted phosphoric acid. The tubes were vortex-mixed for 1 min and centrifuged for 10 min. The upper organic layer was discarded and 50 μl of the acidic solution were injected into the LC system.

Preparation of the calibration curve

A calibration curve based on peak-height ratio (FLU or N-FLU) was constructed for each assay by adding known amounts of FLU or N-FLU to 1 ml of drug-free human plasma. Concentrations of the two drugs equivalent to 5, 10, 25, 50, 100, 200 and 300 ng/ml as base were assayed. An aliquot (70 μl) of I.S. methanolic solution (1.25 μg/ml) was added to each spiked plasma.

RESULTS AND DISCUSSION

Quantification, separation and plasma interferences

The micromethod described here enables the determination of FLU and its demethylated metabolite with the same specificity as previously

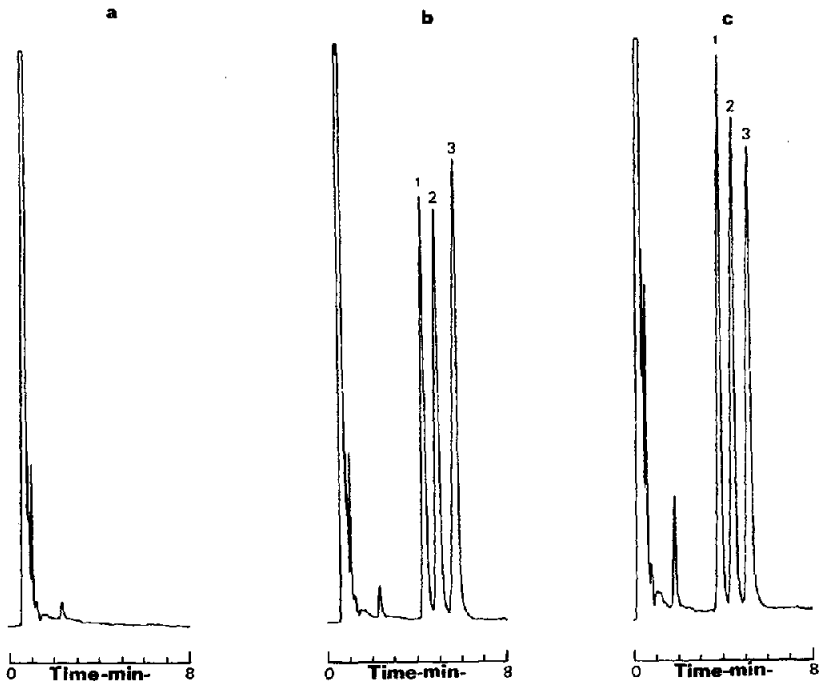

Fig. 1. Chromatograms of (a) an extract of drug-free human plasma, (b) a plasma patient sample containing 141 ng/ml fluoxetine (FLU) and 128 ng/ml norfluoxetine (N-FLU) and (c) a spiked human plasma with 200 ng/ml each of FLU and N-FLU. Peak 1 is N-FLU (retention time, 4.28 min), peak 2 is FLU (retention time, 4.89 min) and peak 3 is internal standard (retention time, 5.61 min). Plotter attenuation: 1.

published methods using UV detection and an improved limit of detection. Chromatograms obtained from drug-free human plasma and plasma spiked with FLU, N-FLU and I.S. and from a patient sample are shown in Fig. 1. The retention times were *ca.* 4.28, 4.89 and 5.61 min for N-FLU, FLU and I.S., respectively. The three peaks were adequately resolved without any interference from endogenous compounds. Capacity factors (k') were found to be 5.49, 6.41 and 7.50 for N-FLU, FLU and I.S., respectively.

The capacity factors of selected drugs checked for potential interference and which could be co-administered with FLU are listed in Table I. This shows that numerous antidepressants, anxiolytics and neuroleptics usually co-prescribed do not interfere with FLU or N-FLU dosage. As far as the metabolite of clomipramine (desmethylclomipramine) is concerned, there might be some interference, but this will only occur after long-term use of the chromatographic system (*i.e.*, when markedly decreased column efficiency occurs).

Linearity, recovery and limit of detection

Calibration curves were linear over the range 10–800 ng/ml ($r^2 = 0.997$, $n = 4$) for FLU and 10–1000 ng/ml ($r^2 = 0.998$, $n = 4$) for N-FLU.

TABLE I

CAPACITY FACTORS (k') OF SOME DRUGS TESTED FOR INTERFERENCE

Drug	k'	Drug	k'
Oxazepam	0.86	Levomepromazine	3.67
Lorazepam	0.92	Clorazepate dipotassium	3.70
Chlordiazepoxide	1.06	Maprotiline	3.85
Doxepine	1.26	Amitriptyline	4.02
Amineptine	1.74	Norfluoxetine	5.49
Loxapine	2.07	Desmethylclomipramine	6.04
Desipramine	2.80	Fluoxetine	6.41
Fluvoxamine	3.07	Clomipramine	6.78
Imipramine	3.09	Mefloquine	7.50
Clonazepam	3.20	Diazepam	7.87
Flunitrazepam	3.44	Chlorpromazine	7.99
Nortriptyline	3.46	Thioridazine	8.02

TABLE II

ACCURACY, PRECISION AND DAY-TO-DAY REPRODUCIBILITY

Day-to-day assays were performed over a six-week period using spiked human plasma samples (n = 6).

Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)
Fluoxetine		
5	4.4	9.4
10	10.2	5.0
50	51.0	6.2
300	298.0	4.6
Norfluoxetine		
5	5.0	10.8
10	9.0	5.6
50	51.0	3.8
300	299.0	4.4

The method was found to be reproducible, as indicated by the low values obtained for the coefficients of variation (C.V.) (Tables II and III). The extraction recoveries determined by comparing peak-heigt ratios of the extracts with those obtained by direct injection of the same amount of drug ranged from 72 to 73% for N-FLU and from 79 to 84% for FLU (Table IV).

TABLE III

ACCURACY, PRECISION, INTRA-ASSAY REPRODUCIBILITY

Spiked human plasma samples were used (n = 8).

Concentration added (ng/ml)	Concentration found (ng/ml)	C.V. (%)
Fluoxetine		
5	4.9	5.0
10	10.6	2.3
50	50.0	3.0
300	304.0	2.1
Norfluoxetine		
5	5.0	2.2
10	9.8	2.0
50	51.0	3.2
300	297.0	0.4

TABLE IV

RECOVERY OF ANALYTICAL METHOD

Sample (n = 8)	Concentration (ng/ml)	Recovery (mean ± S.D.) (%)
Fluoxetine	5	84.1 ± 4.9
	50	78.8 ± 7.6
	300	83.0 ± 2.8
Norfluoxetine	5	72.0 ± 7.3
	50	73.4 ± 5.9
	300	73.4 ± 5.4

The limit of detection under the described conditions was 2.0 ng/ml for N-FLU and FLU at a signal-to-noise ratio of 3. The integrator plotter (Shimadzu Model C-R6A) set at 1 and connected to the absorbance detector (Shimadzu Model SPD-6A) set at 0.001 a.u.f.s. allows this very low limit of detection. In spite of the native fluorescence shown by FLU and N-FLU at 310 nm (excitation, 235 nm) a higher plotter attenuation is required to maintain a smooth baseline [12]. The signal-to-noise ratio is therefore improved with the absorbance detector as compared with the fluorescence detector. On the other hand, the Ultrapore C_8 column used not only affords high sensitivity of detection with a fast LC analysis, but also ensures the specificity of the method with hydrophobic interaction.

Stability

FLU was stable in human plasma controls (50 and 200 ng/ml) for more than one month at −22°C. Spiked plasma samples extracted following the described procedure then stored at 4°C remain stable for at least 24 h without significant degradation.

Applications

The relationship between the plasma concentration of FLU and its effect remains unclear. This is possibly because early studies used high doses that were therefore in the horizontal upper part of the dose–response curve [2]. Some authors have reported the prescription of 20 mg ev-

ery two or three days for treatment of depression, and as little as 5 mg per day is said to be effective and well tolerated in one report [14].

As far as drug interactions are concerned, pharmacokinetic drug interactions between FLU and tricyclic antidepressants or antipsychotic drugs need further studies, mainly because FLU inhibits their metabolism and may increase their toxicity [15–17]. The proposed LC micromethod is sensitive and selective enough to study accurately the relationship between plasma concentrations of FLU and its effect using low doses and to explore pharmacokinetic drug interactions, either in animals or in patients.

CONCLUSION

The LC micromethod described here is simple, rapid, selective, reproducible, sensitive and allows simultaneous determination of FLU and its demethylated metabolite, N-FLU, in plasma. The very small volume of plasma required allows pharmacokinetic studies of the drug in animals, clinical pharmacology studies and drug monitoring in children or adult patients.

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