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Determination of fluoxetine and its metabolite norfluoxetine in serum and brain areas using high-performance liquid chromatography with ultraviolet detection

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Abstract

A high-performance liquid chromatography (HPLC) method using only 0.1 ml of serum or homogenate from brain areas has been developed for the determination of fluoxetine (FLU) and its metabolite, norfluoxetine (N-FLU), with ultraviolet detection at 227 nm. The small volume of sample required in this method allows studies in small animals, such as mouse. The method provides recoveries of up to 90% for both compounds. Acceptable coefficients of variation were found for both within-run and day-to-day assays. The limit of detection was 5.0 ng/ml. No interferences were found with tricyclic antidepressant drugs and benzodiazepines, which allows this method to be used in clinical studies. Pharmacokinetic parameters for the two compounds are reported in mouse serum, frontal cortex and caudate nucleus. We also report the values of FLU and N-FLU in serum from humans who were treated once daily with 20 mg of FLU, obtained after 1, 14 and 28 days of treatment. © 1998 Elsevier Science B.V.

Keywords: Fluoxetine; Norfluoxetine

1. Introduction

Fluoxetine (FLU) is an antidepressant drug that enhances serotoninergic neurotransmission through the selective inhibition of neuronal reuptake of serotonin [1]. Norfluoxetine (N-FLU), its N-demethylated metabolite, also inhibits serotonin reuptake. Chemical structures of fluoxetine and norfluoxetine are presented in Fig. 1.

Several studies have determined the concentrations of these compounds in plasma, serum or tissue (see Ref. [2] for review). Determination of FLU and N-FLU using gas chromatography with electron capture detection (GC–ECD) was originally described by Nash et al. [3]. In 1991, a more rapid, selective and sensitive method, using a solid-phase extraction column, was described by Dixit et al. [4]. More recently, a GC–mass spectrometric (GC–MS) method that allowed the simultaneous determination of the enantiomers of FLU and N-FLU was published [5]. However, high-performance liquid chromatography (HPLC) remains the most widely used analytical method [6]. Ultraviolet [7–13] or fluorescence detection [14–19] are the most commonly used. These chromatographic methods allow quantification of FLU and N-FLU after liquid- [3,5,7–

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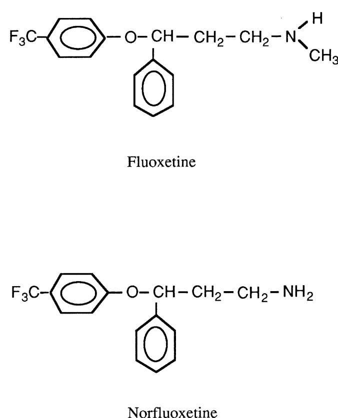

Fig. 1. Chemical structures of fluoxetine and norfluoxetine.

13,15–19] or solid-phase extraction [4,14]. Previous liquid chromatography extraction procedures reported in the literature require 1 or 2 ml of serum, with the exception of the method of Thomare et al. [10] who used 0.25 ml of plasma, and these methods are sometimes quite time-consuming. A number of them have recoveries of about 60–70%.

This report describes a rapid and simple method, using only 0.1 ml of serum, for quantifying FLU and N-FLU with an improved extraction recovery. The method was applied to the determination of the pharmacokinetic parameters of the two compounds in serum, frontal cortex and caudate nucleus from NMRI mice after a single intraperitoneal (i.p.) injection of FLU (10 mg/kg) and in clinical practice for therapeutic drug monitoring using the samples of the same volume.

2. Experimental

2.1. Chemicals

FLU and its metabolite, N-FLU, were supplied by Eli Lilly (Indianapolis, IN, USA).

Protriptyline (P) (used as an internal standard) and tetramethyl-ammonium perchlorate were purchased from Sigma (Paris, France). All other chemicals were from Merck (Darmstadt, Germany).

Stock solutions (1 mg/ml) of FLU, N-FLU and P were prepared in methanol and stored at −20°C. All stock solutions were further diluted with methanol to give working solutions of 5 μg/ml.

2.2. Chromatography

Chromatography was performed using a solvent delivery Varian 9002 pump with a 50-μl fixed volume injector (Varian, Les Ulis, France) coupled with a Kratos Spectroflow 773 spectrophotometer (Cunow, Cergy, France) and a Shimadzu CR-4A integrator (Touzart et Matignon, Vitry sur Seine, France). Chromatography was carried out on a C_8 endcapped column (125×4.0 mm I.D.; particle size, 4 μm) from Merck. The device was completed with a precolumn (C_8 endcapped, 5 μm, 4×4.0 mm I.D.; Merck).

The mobile phase was a mixture of an acidic aqueous solution (containing 0.1 ml of perchloric acid and 1.5 g of tetramethyl-ammonium perchlorate per liter) and acetonitrile (58:42, v/v). The filtered mobile phase was used at a flow-rate of 1.2 ml/min. The column effluent was monitored at 227 nm.

2.3. Sample preparation

The animals were killed by cervical dislocation. Blood samples were obtained from the retro-orbital sinus and withdrawn into tubes without additive. The serum was obtained from blood by centrifugation at 3000 *g* for 5 min. The brain areas were rapidly removed and weighed. For human serum, blood samples from the anterocubital vein, taken just before the morning fluoxetine dose at 8.00 a.m., were placed in tubes without additive. Serum was separated by centrifugation at 3000 *g* at 4°C for 5 min. A 1.0-ml volume of 0.6 *M* sodium carbonate–sodium bicarbonate buffer (pH 9.8) containing the internal standard P (100 ng/ml) was added either to 0.1 ml of serum (either from mice or from clinical patients) or to a weighed brain sample. For the brain area determinations, the samples were homogenized using a Econo-grind homogenizer (Radnoti Glass Technology, Inc., Morovia, CA, USA). After the addition of 7 ml of a mixture of ethyl acetate and *n*-heptane (20:80, v/v), the vials were capped and vigorously mixed for 1.5 min, then centrifuged at 3000 *g* for 10 min. The organic layer was transferred to another tube containing 0.2 ml of acidic phosphate buffer (0.025 *M* potassium dihydrogen phosphate adjusted to pH 2.3 with 85% phosphoric acid) then mixed for 1 min and centrifuged at 3000 *g* for 10 min. The

organic layer was discarded, and a 50-μl aliquot of the aqueous phase was injected for chromatographic separation.

2.4. Calibration curves

To prepare standard curves, appropriate amounts of FLU and N-FLU were added to 0.1 ml of blank serum to yield the following concentrations of each: 0, 10, 25, 50, 100, 250 and 500 ng/ml. These samples were then prepared according to the procedure described in Section 2.3. Quantification was performed by calculating the peak-height ratios of each compound to the internal standard.

3. Results and discussion

3.1. Selection of internal standard

Haloperidol [7], clomipramine [9], mefloquine [10], imipramine [11], doxepine [12] and P [8,14] have been used in previous reports with UV detection. After pilot investigations, we choose P as the internal standard because (1) it is no longer used in clinical practice in France, (2) it is well separated from FLU and N-FLU, (3) the percentage recovery of it from samples was similar to that of FLU and N-FLU (91–95%) under the conditions used and (4) no interfering peaks were detectable in blank brain and serum samples.

3.2. Chromatographic separation

The chromatographic separations obtained from serum spiked with P, N-FLU and FLU, and from mouse samples of serum and cortex, are shown in Fig. 2. Retention times for P, N-FLU and FLU are 8.1, 9.6 and 12.7 min, respectively. The three peaks are completely resolved without any interference from endogenous compounds. We tested to determine if there were possible interferences with other drugs commonly administered with fluoxetine in clinical practice. No interference was observed with amitriptyline, clomipramine, demethylclomipramine, imipramine, nortriptyline, bromazepam, clorazepate, diazepam, flunitrazepam, nitrazepam, nordiazepam, acepromazine, aceprometazine, chlorpromazine and ciamemazine.

3.3. Extraction recovery

The extraction recoveries determined by comparing peak height-ratios of the extracts with those obtained by direct injection of the same amount of compound ranged from 95 to 96% for FLU and 89 to 92% for N-FLU (Table 1). The previous liquid chromatography extraction procedures reported in the literature were usually liquid–liquid extraction and the recoveries of FLU ranged between 62 [13] and 86% [9] and between 55 [9] and 80% [3] for N-FLU, with the exception of the extraction method of Potts and Parli [18] (86–96% for FLU and 92–93% for N-FLU) who used an initial volume of 0.5 ml. The good recovery obtained with our method allowed us to use very small sample volumes. We used 0.1 ml of serum compared to previous methods, which usually required 0.5 to 2 ml of sample (except for the method of Thomare and al. [10], who used 0.25 ml of plasma). More recently, Clausing et al. [19] reported a method for the determination of FLU using 0.1 ml of serum, however, their method could not be used to determine N-FLU in serum.

3.4. Linearity and the limit of detection

A good linear relationship was obtained in the range assayed, i.e. 10–1000 ng/ml for FLU and N-FLU ($r=0.99997$ for FLU and $r=0.9997$ for N-FLU, $n=6$). The linear regression equations are $y=0.003x-0.005$ for FLU and $y=0.004x-0.014$ for N-FLU.

The limit of detection under the described conditions was 5.0 ng/ml for both compounds, with a signal-to-noise ratio of three. This sensitivity is comparable to or better than those reported previously [2], despite the small volume of sample used in our method.

3.5. Accuracy, precision and reproducibility

We investigated the precision of this method by calculating the coefficient of variation (C.V.) of the

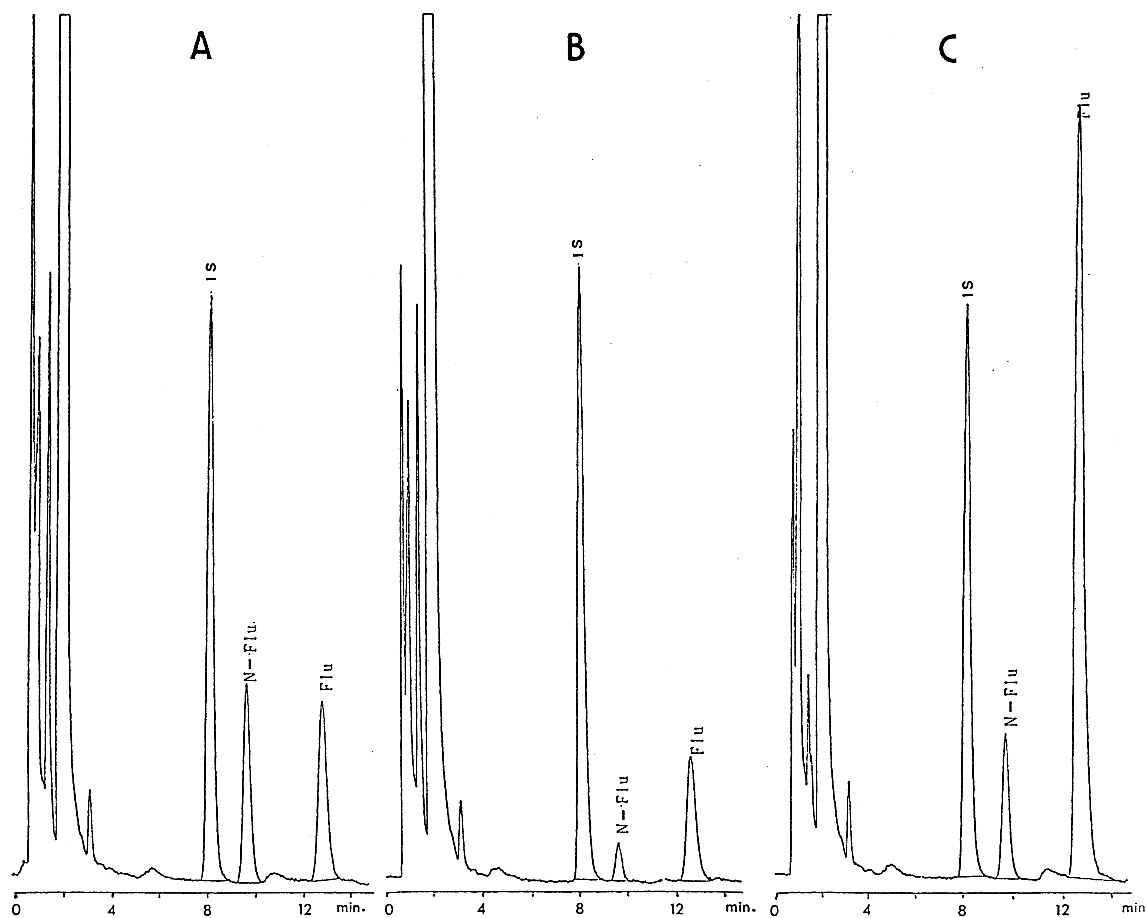

Fig. 2. Chromatogram of (A) an extract of 0.1 ml of a spiked serum sample containing 100 ng/ml of protriptyline (used as the internal standard; I.S.) and 75 ng/ml of both fluoxetine (FLU) and norfluoxetine (N-FLU), (B) an extract of 0.1 ml of serum with a measured concentration of 52 ng/ml of FLU and 12 ng/ml of N-FLU and (C) an extract of a mouse frontal cortex (weighing 32.8 mg), 1 h after a single i.p. injection of FLU (10 mg/kg), with a measured concentration of 11 000 ng/g of FLU and 1660 ng/g of N-FLU. (Attenuation=0.01 AUFS).

Table 1
Recovery of the analytical method

Sample (n=8)	Concentration (ng/ml)	Recovery (mean±S.D.) (%)
N-FLU	25	89.0±3.2
	150	90.4±4.5
	500	91.8±3.4
FLU	25	96.5±3.0
	150	95.4±3.3
	500	95.3±3.1

Spiked serum samples of fluoxetine (FLU) and norfluoxetine N-FLU) were used (n=6).

concentrations measured on different days (n=6, inter-run precision) and on the same day (n=10, intra-run precision) for drug-free serum samples supplemented with different amounts of both compounds. After the addition of 50 (for the low concentration determination) or 100 ng/ml of the internal standard, samples were processed according to the procedure described above. As shown in Table 2, the C.V. never exceeded 9.4% at any of the concentrations examined, indicating good assay precision.

Table 2
Intra- and inter-assay validation for fluoxetine (FLU) and norfluoxetine (N-FLU)

Concentration (ng/ml)	Intra-assay (%)	Inter-assay (%)
FLU		
25	7.3	7.5
50	4.9	4.9
150	1.7	4.2
500	1.3	0.9
N-FLU		
25	7.2	9.4
50	6.1	7.1
150	2.1	6.1
500	1.8	3.2

Drug-free serum samples were supplemented with different amounts of both compounds. ($n=10$ for intra-assay and $n=6$ for inter-assay determinations).

3.6. Stability

The serum aqueous acidic phase extract remained stable for seven days when stored at +4°C.

3.7. Applications

This HPLC–UV method was used to study the pharmacokinetic parameters of FLU and N-FLU in mouse serum, frontal cortex and caudate nucleus. Serum and brains were taken from a series of four Swiss NMRI mice (R. Janvier, Le Genest St-Isle, France), weighing 22±2 g, after an i.p. injection of 10 mg/kg of FLU. We used the dose of 10 mg/kg in this assay as the fluoxetine-induced effect on serotonin levels in rat and mouse brains have been shown to be maximal at this dose [20] and no data were available on the concentrations of FLU and N-FLU in brain at this dose. Sampling was performed as described above at various times after the injection: 0.25, 0.5, 1, 3, 5, 7, 9, 12, 15, 18, 24 and 48 h. The pharmacokinetic curves obtained for FLU and N-FLU in serum and frontal cortex are presented in Fig. 3. The results obtained in frontal cortex and caudate nucleus were similar. The calculated half-life was 4.0 h in serum and 5.0 h in the brain areas for FLU and 9.9 h in serum and 8.6 h in brain for N-FLU. The half-lives observed in NMRI mouse are twelve-fold lower for FLU and nineteen-fold lower for N-FLU than the values previously described for humans (48 h for FLU after a single dose and 96 h for N-FLU) [21]. The same phenomenon was found using clomipramine in the same strain of mice (a half-life of 50 min in NMRI mouse vs. 20–25 h in human) [22]. These data should be taken into account to standardize experimental pharmacological protocols in animals in which a true steady-state is achieved, thereby improving the predictive value of experimental work on antidepressants in animals.

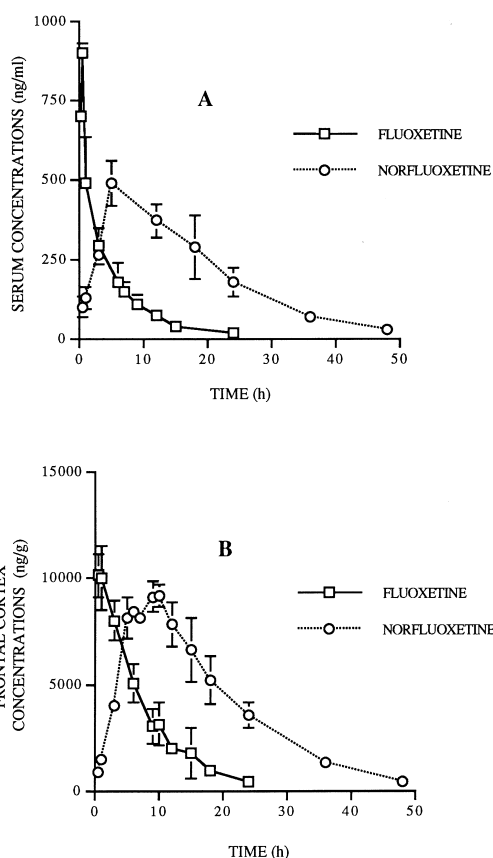

Fig. 3. Time-course of fluoxetine and norfluoxetine concentrations in serum (A) and frontal cortex (B) after a single i.p. injection of 10 mg/kg of fluoxetine in NMRI mice. (mean±S.D., $n=4$ at each time point).

We used the described assay to measure FLU and N-FLU in eight patients with depressive disorders

who were treated with FLU (20 mg daily). Our method is particularly good for patients from whom it is difficult to withdraw blood, since such a small sample volume is required. After 1, 14 or 28 days of treatment, the serum concentrations (mean±S.D., in ng/ml) found for FLU and N-FLU were 15.7±8.5 and 16.8±10.7; 60.6±32.6 and 94.4±36.3; 92.9±33.4 and 149.7±36.5, respectively. These data were in agreement with those reported in the literature [3,7,9]. We noted that, after fourteen days of treatment, steady-state levels were not achieved, which is in agreement with the long half-life of FLU in humans. We also found that after long-term administration, the levels of the metabolite, N-FLU, in serum were higher than those of FLU in six patients.

4. Conclusion

The HPLC–UV method described here enables the determination of FLU and its metabolite, N-FLU, with the same validation performance as that of previously published methods and improved extraction recoveries. The very small volume of serum required in this method allows pharmacokinetic studies of FLU to be performed in small animals, such as mouse. The method is suitable for therapeutic drug monitoring in clinical practice.

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