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Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 351–356
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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A rapid HPLC-DAD method for the analysis of fluoxetine and norfluoxetine in plasma from overdose patients

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Received 19 May 2004; received in revised form 14 June 2004; accepted 15 June 2004
Available online 29 July 2004

Abstract

There is a need for fast, simple and reliable analytical methods for the analysis of fluoxetine and norfluoxetine in patients who voluntarily or involuntarily have taken an overdose of the drug. A new liquid chromatographic method with diode array detection is presented herein for the determination of fluoxetine and its main active metabolite in human plasma for toxicological purposes. A mobile phase composed of acetonitrile and aqueous tetramethylammonium perchlorate allows to obtain the complete separation of the analytes on a C18 reversed phase column. The fast and accurate sample pre-treatment step is carried out by means of solid-phase extraction using hydrophilic–lipophilic balance cartridges and loading 100 μL of plasma only. This procedure gives satisfactory extraction yield values, as well as good plasma sample purification from matrix interference. Linearity was obtained in the 150–3000 ng/mL range for both analytes. Selectivity with respect to other psychotropic drugs was satisfactory. The method seems to be suitable for the analysis of fluoxetine and its metabolite in human plasma for depressed patients in overdose.

Keywords: Fluoxetine; Norfluoxetine; Toxicological analysis; Human plasma; Liquid chromatography; Diode array detection

1. Introduction

Fluoxetine (FLU)(*d*,l-*N*-methyl-3-phenyl-3-[(α,α,α-trifluoro-*p*-tolyl)oxy]propyl-amine, Scheme 1a) is the parent drug of the SSRI (selective serotonin reuptake inhibitor) antidepressant class, which was introduced onto the drug market in the 1980s. In fact, FLU is a selective inhibitor of serotonin uptake and not of norepinephrine uptake, with little affinity for muscarinic, histaminic, serotonergic, or noradrenergic receptors [1]. FLU has been approved worldwide in the therapy of major depression [2], and has also demonstrated to be effective in the treatment of other syndromes, such as bulimia nervosa, panic fits and obsessive–compulsive disorders [3,4].

FLU has a clinical efficacy similar to that of tricyclic antidepressants, but usually causes less cardiovascular and anticholinergic side effects [5], even if episodic reports of dysrhythmia and syncope associated with FLU treatment and overdose have raised some concerns [6,7]. The most common adverse events associated with initiating FLU treatment are nausea, insomnia, nervousness and somnolence [8]. FLU is administered once daily as capsules or once weekly as an enteric-coated formulation; in the former case, the typical dose is 20 mg for the treatment of depression, anxiety disorder and obsessive–compulsive disorders [5,9]; when necessary, and in the case of bulimia, the dose can be increased up to 60 or 80 mg/day [5,10].

After administration, fluoxetine is subject to hepatic metabolism by cytochrome P450 enzymes, with a mean

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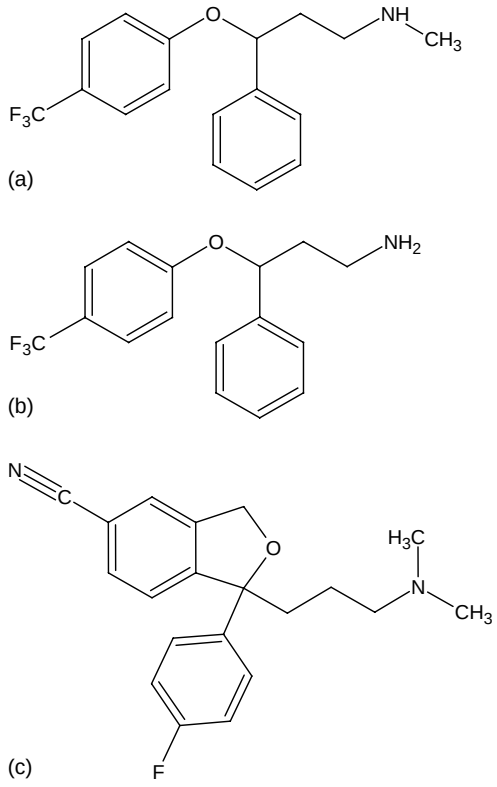
doi:10.1016/j.jpba.2004.06.008

half-life of 1–3 days [5]. The main product of metabolism is the demethylated metabolite norfluoxetine (NFLU, Scheme 1b) which has comparable pharmacological activity [11] and longer half-life (4–16 days) [5,12]. Since FLU plasma levels are usually about 80 ng/mL (when receiving 40 mg/day of drug) [13] and those of NFLU can be 100–130% of those of FLU, it can be concluded that NFLU contributes significantly to the therapeutic efficacy of fluoxetine [14].

FLU seems to be rather safe in overdose: very high amounts of the drug, when ingested alone, can cause seizures [15,16], sinus tachycardia, psychomotor agitation, dyskinesia [17], but the patients usually recover without sequelae if supportive care is provided. Reviews of hundreds of overdose cases, with FLU intake doses up to 1500 mg, report consequent FLU levels ranging from 232 to 1390 ng/mL, and none of the patients died [18,19]. However, a patient died of FLU poisoning with plasma levels equal to 5600 ng/g of the drug and 3300 ng/g of NFLU [20], and the association of FLU overdose and alcohol seems to be rather dangerous [21].

Several HPLC methods are present in the literature for the analysis of FLU and NFLU in human plasma, usually coupled with UV [22–28], fluorimetric [29–32] or mass spectrometry [33–35] detection; gas chromatography with mass spectrometry detection [21] and micellar electrokinetic chromatography [36] have also been used for the determination of FLU in biological fluids. Enantioselective analytical methods are growing in number and importance [37–39], since NFLU enantiomers have different potency and a formulation containing single enantiomer FLU is currently under development [40].

In the past few years, our research group has developed sensitive methods based on HPLC with fluorimetric detection [31,32] and different kinds of biological sample pre-treatment for the therapeutic drug monitoring of fluoxetine and norfluoxetine in human plasma. Recently, however, the need to determine very high fluoxetine levels in patients who took an overdose of the drug has led us to the development of a faster and more feasible analytical method, based on HPLC with diode array detection (DAD). The use of DAD instead of the normal UV detection allows to control the purity of chromatographic peaks, and thus to detect the presence of interference in each plasma sample.



Scheme 1. Chemical structures of (a) fluoxetine, (b) norfluoxetine and (c) the IS (citalopram).

2. Experimental

2.1. Chemicals and solutions

Eli Lilly Italia S.p.A. (Sesto Fiorentino, Italy) kindly provided fluoxetine hydrochloride (99.7% purity) and norfluoxetine hydrochloride (87.7% purity). Citalopram (used as the internal standard, IS, Scheme 1c) was kindly provided by H. Lundbeck A/S (Copenhagen, Denmark). Tetramethylammonium perchlorate (analytical grade) and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA).

Perchloric acid (65% m/m, analytical grade), acetonitrile and methanol (HPLC grade) were from Carlo Erba (Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a Millipore MilliQ apparatus (Milford, MA, USA).

The stock solutions (1 mg/mL) of FLU and NFLU were prepared by dissolving in 20 mL of methanol an amount of hydrochloride corresponding to 20 mg of free base. The different standard solutions were prepared by diluting suitable amounts of the stock solution with the mobile phase. Stock solutions of the analytes in methanol were stable for at least three months when stored at −20 °C, while the standard working solutions were prepared from the stock solutions immediately before the analysis.

2.2. Apparatus and chromatographic conditions

The chromatographic assays were performed on an Agilent (Waldbronn, Germany) 1100 Series apparatus, consisting of an isocratic pump (flow rate: 1.0 mL/min, loop 50 μL) and a diode array detector; for quantitative purposes the detector was set at 230 nm.

Compounds were separated on a Varian (Harbor City, CA, USA) Microsorb MV C18 reversed-phase column (150 mm × 4.6 mm i.d., 5 μm) connected to a Phenomenex (Torrance, California, USA) C18 cartridge precolumn.

The mobile phase was composed of acetonitrile and a pH 3.0, 17 mmol/L tetramethylammonium perchlorate solution

(50:50 (v/v)). Before use, the mobile phase was filtered through a 0.45 μm Varian nylon filter. The DAD detector was set in the 200–320 nm range and quantitative analysis was performed at 230 nm.

2.3. Plasma sample collection

Plasma samples were taken from depressed patients who took overdoses of Prozac® and put into vials containing EDTA as the anticoagulant. The blood was centrifuged for 20 min at 1400 × *g* and the supernatant plasma frozen and maintained at −20 °C until analysis which was usually carried out within one month.

The same procedure was used to separate plasma from the blood of healthy volunteers ("blank" plasma).

2.4. Extraction procedure

Patient plasma samples, or blank plasma to which a suitable amount of standard solution of FLU and NFLU was added, was subjected to an accurate solid-phase extraction (SPE) before HPLC analysis. For the SPE procedure Oasis HLB (hydrophilic–lipophilic balance) cartridges (30 mg, 1 mL) from Waters (Milford, Mass., USA) were used. The sorbent of these cartridges is a macroporous polymer made from two monomers, the lipophilic divinylbenzene and the hydrophilic *N*-vinylpyrrolidone.

The cartridges were activated with 1 mL of methanol two times and conditioned with 1 mL of ultrapure water two times. Aliquots of 100 μL of plasma diluted with 500 μL of water were then loaded onto the cartridges. After loading the plasma samples, the cartridges were washed twice with 1 mL of water and with 1 mL of a water/methanol (80/20 (v/v)) mixture. The elution of the analytes was carried out with 500 μL of methanol. The methanol extract was evaporated to dryness in a rotary evaporator at 37 °C and the residue redissolved in 250 μL of mobile phase.

2.5. Method validation

2.5.1. Linearity

Calibration curves were obtained in the 150–3000 ng/mL range of plasma concentration for both FLU and NFLU by spiking blank plasma with suitable amounts (e.g. 50 μL) of standard solution of the analytes, followed by the extraction procedure and analytical HPLC procedures described above.

The ratios between the area of analytes and that of the IS were plotted against the analyte concentrations added to blank plasma. The calibration curves were obtained by means of the least square method.

2.5.2. Precision

The blank plasma was spiked with FLU, NFLU at three different concentrations to give plasma concentrations of 150, 750 and 3000 ng/mL of each analyte (and the IS at a constant concentration of 1250 ng/mL). After thorough mixing, extraction and HPLC analysis were then performed. The procedure was repeated at least six times within the same day to obtain the repeatability and over different days to obtain the intermediate precision.

2.5.3. Extraction yield

Different samples of spiked blank plasma were extracted as previously described and injected into the HPLC system. The mean extraction yield values of the analytes were calculated by comparing the peak areas of blank plasma spiked with the analytes and subjected to the extraction procedure with the peak areas obtained from the corresponding standard solutions.

The precision and recovery assays were carried out according to USP XXV [41] guidelines.

2.5.4. Accuracy

The accuracy of the proposed method was evaluated by means of recovery studies. Appropriate amounts of the two analytes (namely 150, 750 and 1500 ng/mL for each analyte) were added to plasma samples containing known amounts of FLU and NFLU (i.e. previously analysed samples). The spiked samples were submitted to the extraction procedure described above and analysed by HPLC. Recoveries of the analytes added to spiked human plasma were calculated by interpolating the ratio peak areas on the calibration curves. The procedure was repeated six times in the same day to obtain repeatability values.

3. Results and discussion

3.1. Chromatographic conditions

Our previous papers on the HPLC determination of therapeutic levels of FLU and NFLU in human plasma [31,32] prompted us to develop another method for the analysis of the same compounds when present at higher concentrations, due either to overdose or metabolic anomalies.

For this purpose, the mobile phase was modified by increasing the pH value of the buffer (from 2 to 3) and a C18 reversed phase column was used instead of a C8 column.

Moreover, the fluorimetric detection used previously was substituted with DAD, for two main reasons: first, sensitivity is not an issue when dealing with overdoses; and second, DAD instrumentation is more widespread than fluorescence detectors. At the same time, DAD allows to control the purity of chromatographic peaks, thus highlighting possible interference in the analysis. FLU and NFLU have a main absorbance maximum at a wavelength of about 210 nm, however this wavelength is disadvantageous in terms of selectivity; for this reason, a detection wavelength of 230 nm was chosen as a reasonable compromise to carry out quantitative analysis. Under these working conditions the analytes are well separated and FLU has a retention time of 6.6 min, while NFLU has a retention time of 5.7 min and the IS (citalopram) of 3.9 min (Fig. 1).

3.2. Plasma sample pre-treatment

In order to speed up the plasma sample pre-treatment procedure, a new solid-phase extraction (SPE) procedure was developed, using Oasis HLB cartridges. These cartridges are very easily and rapidly imbibed, thus sharply reducing the activation and conditioning times. Furthermore, due to their high sorbent power, HLB cartridges allow to obtain very good extraction yield results even when loading very high amounts of the analytes.

A very simple SPE procedure has been developed, which comprises the loading with 100 μL of plasma and eluting the analytes with 500 μL of methanol. Initially, a washing with water alone was tried, however interference was detected, which hindered the determination of the analytes. For this reason, a further step with water/methanol was added, and the results have been satisfactory.

According to the expected FLU and NFLU concentrations the eluate can then be injected as such, or dried and redissolved in a lower volume of mobile phase. The most frequently used redissolution volume is 250 μL, as reported in the Experimental section, and all the values for the validation of the method are obtained in this way.

The chromatogram of a blank plasma sample subjected to the above described SPE procedure is reported in Fig. 2a. As can be seen, no significant interference from the plasma matrix is detected at the retention times of the analytes or the IS. This is confirmed by the injection of the same blank plasma sample spiked with 1250 ng/mL of FLU and NFLU and subjected to the same SPE procedure (Fig. 2b). The analyte peaks are still neat and well separated, and retention times are equal to those of the standard solution.

3.3. Method validation

Calibration curves were set up on blank plasma spiked with standard solutions; good linearity was found in the 150–3000 ng/mL plasma concentration range for both analytes.

The regression equation of FLU was $y = 1.008\ (\pm 0.027) + 0.012\ (\pm 3.1 \times 10^{-4})\ x$; the linear correlation coefficient was $r = 0.998$. The regression equation of NFLU was $y = 1.112\ (\pm 0.016) + 0.015\ (\pm 3.0 \times 10^{-4})\ x$; the linear correlation coefficient was $r = 0.997$. Both calibration curves were obtained by means of the least square method; in both equations y is the ratio between the area of FLU or NFLU and that of the IS, and x is the concentration of FLU or NFLU, expressed as ng/mL.

The limit of quantification (LOQ) and the limit of detection (LOD) for FLU and NFLU in plasma samples were 30 ng/mL and 15 ng/mL (corresponding to injected solution concentrations of 12 ng/mL and 6 ng/mL) respectively.

These values were calculated according to USP XXV Edition guidelines [41]; i.e. LOD as the concentrations which give a 3:1 signal:noise ratio and LOQ as the concentrations which give a 10:1 signal:noise ratio.

Absolute recovery and precision assays were also carried out. To 100 μL of blank plasma, three different concentrations of each analyte were added. The procedure was repeated six times for each concentration within the same day and over different days.

The results were very good: the mean absolute recovery (extraction yield) values range from 93 to 101% for both analytes, while the R.S.D. (%) values for precision range from 1.9 to 3.1% (repeatability) and from 2.1 to 3.2% (intermediate precision) (Table 1).

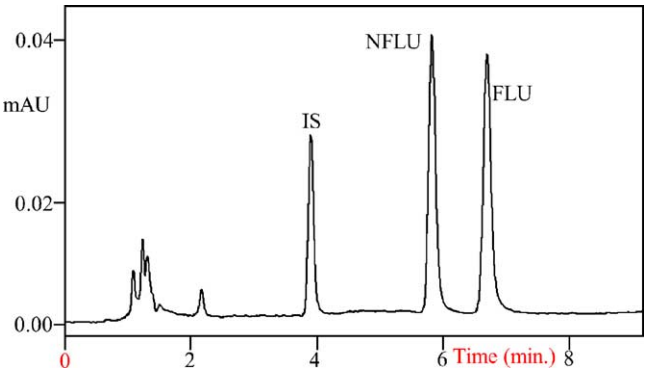


Fig. 1. Chromatogram of a standard solution containing 500 ng/mL of FLU and NFLU and 500 ng/mL of the IS.

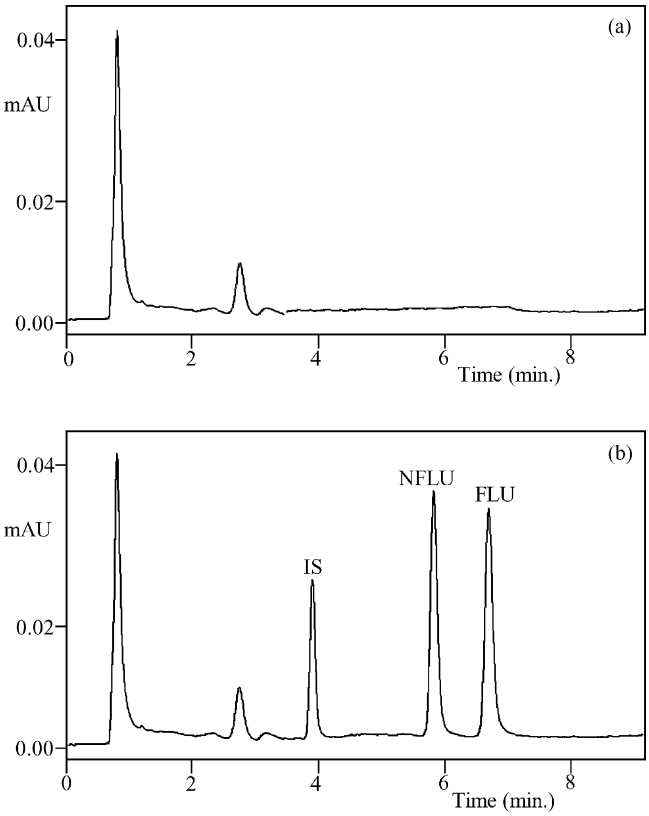


Fig. 2. Chromatogram of (a) a blank plasma sample after the SPE procedure and (b) the same plasma sample, after spiking with 1250 ng/mL of FLU and NFLU and 1250 ng/ml of IS.

Table 1
Extraction yield and precision data

Analyte and amount added (ng/mL)	Mean extraction yield (%)	Repeatability (R.S.D., %)[a]	Intermediate precision (R.S.D., %)[a]
FLU			
150	101	2.9	3.1
750	94	2.8	2.9
3000	93	1.9	2.1
NFLU			
150	95	3.1	3.2
750	93	2.6	2.9
3000	93	2.0	2.2

[a] $n = 6$.

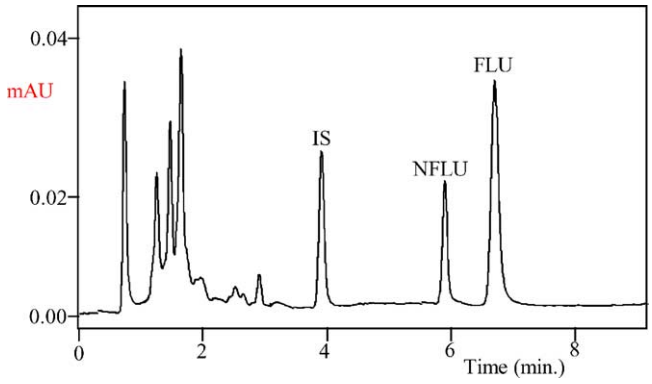


Fig. 3. Chromatogram of a plasma sample from a patient who took 120 mg/die of FLU, after the SPE procedure.

3.4. Analysis of patient plasma

The validated method was applied to plasma samples taken from depressed patients under oral chronic FLU therapy, who voluntarily or involuntarily took overdoses of the drug.

The chromatogram of a plasma sample from a patient who voluntarily took 120 mg/day of FLU for more than a week, taken 12 h after the last drug intake, is reported in Fig. 3. As can be seen, all analyte peaks are well separated and no interference is present, even if other peaks (probably due to endogenous compounds) are present in the chromatogram. The following concentrations of the analytes were found in this sample by interpolating on the calibration curves: 1140 ng/mL of FLU, 350 ng/mL of NFLU.

3.5. Selectivity

Depressed patients often undergo polypharmacy and, furthermore, patients who attempt suicide often do so with multiple drugs. For this reason, it is important to know the possible interference of other drug on the toxicological analysis of FLU.

Several common psychotropic drugs have been tested for interference (see Table 2). As can be noted, none of them did interfere with the determination of FLU and NFLU. On the contrary, since some of the tested drugs can be identified, their qualitative (and possibly semi-quantitative) analysis can be attempted with this method in case of poisoning with multiple substances.

Table 2
Compounds tested for interference

Compound	Retention time (min)
Fluoxetine	6.6
Norfluoxetine	5.7
Antiepileptics	
Carbamazepine	4.3
Ethosuximide	2.9
Phenobarbital	3.3
Oxcarbazepine	3.4
Primidone	2.7
Antidepressants	
Amoxapine	4.2
Clomipramine	7.9
Fluvoxamine	5.2
Sertraline	7.0
Citalopram (IS)	3.9
Antipsychotics	
Haloperidol	5.1
Chlorpromazine	7.5
Fluphenazine	7.2
Thioridazine	9.7
Clozapine	3.4
Olanzapine	2.7
Quetiapine	3.4
Risperidone	3.5
Levosulpiride	2.6
Anxiolytics	
Clonazepam	4.7
Flurazepam	4.4
Lorazepam	4.5

Thus, the method seems to be selective enough for the determination of FLU and NFLU for toxicological purposes, even when other drugs are coadministered.

3.6. Method accuracy

Method accuracy was evaluated by means of recovery studies. Known amounts of analyte standard solutions were added to plasma from patients whose analyte levels were

already known. The mixture was then analysed. The assay was repeated 3 times, and the mean recovery values resulted to be: 97% for FLU, 91% for NFLU.

4. Conclusion

The proposed chromatographic method with diode array detection has proven to be suitable for the analysis of FLU and NFLU levels in the plasma of depressed patients in overdose with Prozac capsules. The pre-treatment of biological samples, based on an accurate SPE procedure, needs only 100 μL of plasma and gives satisfactory extraction yield values. The method, that is more rapid and feasible than our two previous papers [31,32], shows a very good selectivity, superior to that reported in other papers, which describe interference from tricyclic antidepressant drugs [27,28]. Furthermore, the proposed method uses very low volumes (100 μL) of plasma, much lower than those used by other authors (e.g. 2 mL of whole blood [21]) and this is surely a great advantage.

Acknowledgements

This work was financially supported by the Italian Ministry for Instruction, University and Research (MIUR, ex-40% and ex-60% funds). Thanks are due to Eli Lilly Italia S.p.A. for providing pure drugs enabling the development of the assays.

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