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Determination of fluoxetine and norfluoxetine in human plasma by high-pressure liquid chromatography with fluorescence detection[1]

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Abstract

Fluoxetine is an atypical antidepressant drug, which selectively inhibits the neuronal reuptake of serotonin, and is widely used in the treatment of depressive disorders. The aim of this research is the development of an HPLC method with fluorescence detection for the monitoring of fluoxetine plasma levels. The determination requires no more than 250 μl of plasma, which undergo solid phase extraction (SPE), then are injected in the HPLC. For the analytical separation a reversed phase C8 column (150 × 4.6 mm I.D.) was used, while the mobile phase was a mixture of acetonitrile and water containing perchloric acid and tetramethylammonium perchlorate (flow rate: 1 ml min^{-1}). The very low levels of analytes in plasma required the employment of a fluorescence detector ($\lambda_{exc} = 230$ nm, $\lambda_{em} = 290$ nm), which also granted a good selectivity. Fluoxetine is revealed as a single peak at a retention time of 9.7 min, while norfluoxetine, the main metabolite of fluoxetine, is revealed at a retention time of 8.1 min. Linearity was obtained over the concentration range 8–200 ng ml^{-1} for both substances. The method seems suitable, in accuracy and precision, for the determination of fluoxetine plasma levels of patients; furthermore, it is rapid and sensitive.

Keywords: Fluoxetine; Norfluoxetine; Liquid chromatography; Fluorescence detection; Solid-phase extraction

1. Introduction

Fluoxetine, (d,l-*N*-methyl-3-phenyl-3-[(α,α,α-trifluoro-p-tolyl)oxy]propylamine) (FLU, Fig. 1),

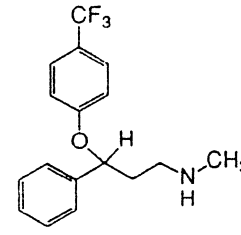

Fig. 1. Chemical structure of fluoxetine, *N*-methyl-3-phenyl-3-[(α,α,α-trifluoro-*p*-tolyl)oxy]propylamine.

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is an atypical antidepressant drug which strongly and selectively inhibits serotonin reuptake in presynaptic neurons [1,2]. It is widely prescribed to patients with serious depressive disorders. The usual suggested dose is 20 mg day^{-1}, which is very low, if compared to usual administered doses of traditional antidepressants such as imipramine, amitriptiline and doxepine for which the daily dose is about 150 mg day^{-1} [1]. Higher doses of fluoxetine seem to be suitable for the treatment of obsessive–compulsive disorders, while lower doses can be used to treat panic fits [3]. In vivo, fluoxetine is mainly demethylated in the liver to norfluoxetine (N-FLU), which is also a potent inhibitor of serotonin reuptake, as are several minor metabolites [1,2]. Clinical trials have demonstrated that fluoxetine has a therapeutical efficacy comparable to that of tricyclic antidepressants, but without their anticholinergic or cardiovascolar side effects [4]. The demand for fluoxetine assay is increasing with the increased use of the drug in order to monitor both compliance and unexpected toxic concentrations after a chronic use of the drug [5]. Thus, it is clear that reliable, accurate and sensitive analytical procedures are needed to determine the drug levels in plasma. In addition it should be emphasized that FLU drug monitoring and pharmacokinetic studies require much more sensitive and selective analytical procedures than those required for tricyclic antidepressants, because the FLU daily dosage is much lower.

Several methods for fluoxetine determination in biological fluids have been reported in the literature; these methods employ gas-chromatography [6] and high-performance liquid chromatography (HPLC) both in direct [7] and reverse [8,9] phase, also with pre-column derivation [10]. Detection is performed by different means including UV–VIS detectors [3,8,11–13], photodiode array detectors [14] and fluorimetric detectors [5,7,10,15]. Unfortunately, these methods require time consuming and complicated analytical procedures, particularly due to the laborious pretreatments of the plasma sample. The pretreatments are not only time consuming, but give low analyte extraction yields, a factor which influences the sensitivity of the method.

The goal of this study is to develop a simple and sensitive analytical method suitable for the clinical assay of fluoxetine and its metabolite levels in plasma. Therefore it must be highly sensitive, selective and rapid. This work is the first part of a broader study in collaboration with the Psychiatry Clinic, in order to find if a correlation between dosage of fluoxetine administered to patients and levels of FLU and N-FLU found in plasma exists and, moreover, if a correlation is present between plasma drug levels and therapeutic effects.

This method is based on the analysis of a plasma sample by means of high performance liquid chromatography with fluorescence detector, after a careful solid-phase extraction (SPE) step.

The choice of use of a fluorescence detector instead of a UV detector derives from the evaluation that fluoxetines are naturally fluorescent and from the necessity to dispose of an HPLC method of higher sensitivity and selectivity.

2. Experimental

2.1. Chemicals

Fluoxetine hydrochloride (99% purity) and norfluoxetine hydrochloride (87% purity) were kindly donated by Eli Lilly Italia S.p.A. (Sesto Fiorentino, Florence). Methanol and acetonitrile, perchloric acid, NaH_2PO_4, H_3PO_4 and NaOH (analytical grade) were produced by Carlo Erba (Milan). Tetramethylammonium perchlorate was purchased from Sigma (St. Louis, MO) Ultrapure water was obtained by means of a MilliQ apparatus by Millipore (Milford, Mass).

2.2. Apparatus and chromatographic conditions

The chromatographic system for HPLC analysis was composed of a model 9001 chromatographic pump (Varian, Walnut Creek) and a Varian 9075 fluorescence detector.

Separations were obtained on a reversed phase column (Res Elut, C8 150×4.6 mm, i.d. 5 μm, Varian). The injection was effected through a 20 μl loop. The mobile phase was composed of a

mixture of acetonitrile–tetramethylammonium perchlorate aqueous solution (pH 2.5, 0.017 M). The mobile phase was filtered through a Phenomenex membrane filter (47 mm membrane, 0.2 μm, NY) and degassed by an ultrasonic apparatus. The flow rate was maintained at 1 ml min^{-1}. The fluorescence intensity was monitored at 290 nm (excitation at 230 nm). Data processing was handled by a model 745 integrator (Waters).

Solid-phase extractions (SPE) were performed on Bond Elut C8 cartridges (1 cc 100 mg^{-1}) (Varian). The apparatus for vacuum elution was a Vac Elut (Varian). The column was maintained at room temperature.

A MicropH 2000 Crison (Barcelona) pHmeter and a ALC 4225 (Milan) centrifuge were used.

A Jasco UVIDEC-610 (Tokyo) double-beam spectrophotometer and a Perkin Elmer LS3 (Beaconsfield, UK) spectrofluorimeter were used for preliminary studies.

2.3. Solutions

Fluoxetine and norfluoxetine stock solutions (1 mg ml^{-1}) were prepared by dissolving 22.4 mg of fluoxetine hydrochloride and 22.5 mg of norfluoxetine hydrochloride in 20 ml of methanol; fluoxetine and norfluoxetine standard solutions were obtained by diluting the stock solution at first with ultrapure water and then with methanol.

The phosphate buffered solution (pH 7.5, 3.67×10^{-2} M) was prepared by dissolving 0.5 g of NaH_2PO_4 in 100 ml of ultrapure water and titrating with 4% H_3PO_4 solution.

The phosphate buffered solution (pH 3, 4.3×10^{-2} M) was prepared by dissolving 0.5 g of 85% H_3PO_4 in 100 ml of ultrapure water and titrating with 1 M NaOH solution.

2.4. Sample collection

The blood samples were collected from five patients, ~2 months after the initiation of fluoxetine therapy of daily doses of 20–60 mg, administered orally as Prozac® capsules. The blood sample was withdrawn 12 h after the last drug administration. Blood collection was stored in glass tubes containing EDTA. The blood samples were centrifuged (within 2 h of sample collection) at 2500 rpm for 25 min, the supernatant (plasma) was then transferred to a polypropylene tube and stored frozen at −20°C until HPLC analysis.

2.5. Sample extraction using the SPE procedure

A total of 250 μl of plasma were diluted with 1 ml of ultrapure water and transferred onto a previously conditioned C8 cartridge. Conditioning was performed by passing 1 ml of methanol through the column twice, followed by 1 ml of ultrapure water and 1 ml of pH 7.5 phosphate buffered five times, each time aspirating the liquid with a Vac Elut device. The solution collected from the cartridge during sample loading was discarded. The column was then washed with 1 ml of pH 3.0 phosphate buffer, 1 ml of pH 7.5 phosphate buffer and with 1 ml of ultrapure water. The cartridge was subsequently eluted with 1 ml of MeOH. The eluate was injected into the HPLC system through a 20 μl loop.

2.6. Calibration curves

Several concentrations of fluoxetine and norfluoxetine standard solutions were added to 250 μl aliquots of drug-free human plasma, obtained from healthy volunteers, and subjected to the extraction (SPE) procedure. Areas of the peaks at retention time (t_{ret}) = 9.7 min and t_{ret} = 8.1 min for FLU and N-FLU, respectively, obtained by injecting methanolic eluates in the column, were plotted against the concentration of the analytes added, in the concentration range of 8–200 ng ml^{-1}.

2.7. Absolute recovery

Analytical recoveries were performed at three different concentrations (30, 50, 100 ng ml^{-1}, for both substances). The recovery of the extraction procedure was determined by comparing the values of peak areas obtained with those of standard solutions of equivalent concentrations of both the analytes.

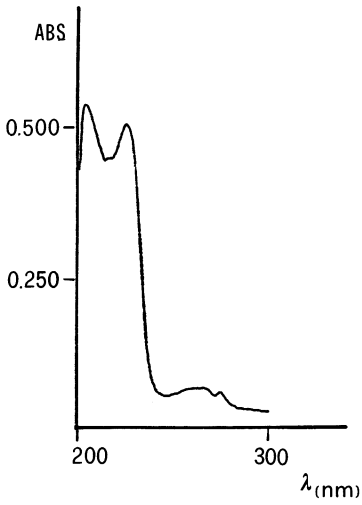

Fig. 2. Absorbance spectrum of a 10 μg ml^{-1} fluoxetine standard solution in methanol. ABS: absorbance.

3. Results and discussion

3.1. Development of the analytical method

The interesting paper by Gupta and coworkers [5] prompted us to develop our HPLC method for the dosage of FLU and N-FLU. Both methods are based on the employment of an HPLC apparatus with fluorimetric detector, but experimental conditions have been extensively changed in order to increase the sensitivity, selectivity and rapidity of the procedure, and to render it suitable for assay of very low levels of fluoxetines in human plasma. Preliminary studies were performed on fluoxetine and norfluoxetine standard solutions in methanol by means of spectrophotometric and fluorimetric measurements. The typical UV spectrum of FLU (conc. = 10 ppm), which contains two large bands at $\lambda = 208$ and 226 nm and two small ones at $\lambda = 268$ and 275 nm, is shown in Fig. 2. This spectrum is nearly superimposable with that of N-FLU. From fluorescence studies, it was found that FLU methanolic standard solutions showed a higher emission intensity when exciting at 230 nm (instead of 236 nm) [5]. The fluorescence spectrum for FLU (Fig. 3) contains an intense emission band with a maximum at 290 nm; a similar fluorescence spectrum was obtained for N-FLU. For this reason HPLC analyses were performed at 290 nm (instead of 310 nm) with an excitation wavelength of 230 nm.

Furthermore, two significant parameters, namely mobile phase composition, and flow rate were examined and modified accordingly. The composition of the mobile phase, composed of CH_3CN and ultrapure water (containing perchlorate and perchloric acid) in the ratio 37.5:62.5 (v/v) was changed to the ratio 50:50 (v/v), to obtain a better separation and shorter retention times of analytes. The new flow rate was 1 ml min^{-1}, instead of 2 ml min^{-1} [5].

Under these experimental conditions, fluoxetine had a retention time of 9.7 min, while norfluoxetine had a retention time of 8.1 min (Fig. 4). The peaks at $t_{ret} = 4.5$ and 5.2 min were due to methanol. It should be noted that, using the λ values reported above, an increase of peak area values for fluoxetines of $\sim 30\%$ with respect to those obtained following literature conditions [5] was obtained. Calibration curves for FLU and N-FLU, obtained plotting peak areas against drug concentrations, showed a good linearity over the range 8–200 ng ml^{-1}, where the least-square fitted line was $y = 2776\ x - 193$; $Rc = 0.9999$ for FLU and $y = 2640\ x + 708$; $Rc = 0.9994$ for N-

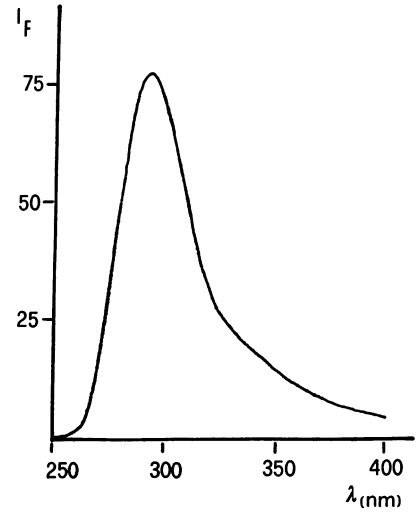

Fig. 3. Emission spectrum of a 500 ng ml^{-1} fluoxetine standard solution in methanol ($\lambda_{exc} = 230$ nm). I_F: fluorescence intensity.

Fig. 4. Chromatogram of a 100 ng ml^{-1} fluoxetine and norfluoxetine standard solution in methanol ($\lambda_{exc} = 230$ nm, $\lambda_{em} = 290$ nm).

FLU. When intermediate precision was evaluated for a 100 ng ml^{-1} concentration of FLU and N-FLU, the relative standard deviation (RSD%) was 2.1 and 1.3, respectively ($n = 6$).

3.2. Solid phase extraction and plasma calibration curves

Focusing the attention on biological material there was the need to drastically eliminate the matrix effect. For this purpose a solid-phase extraction method was utilized, using C8 cartridges (1 cc 100 mg^{-1}), loaded with 250 μl of plasma diluted with ultrapure water, and eluting with 1 ml of methanol. In order to further decrease the matrix effect, the column, after sample loading, was washed with buffered solutions having different pH values: the former with an acid pH value and the latter with a basic one, to remove plasma components with different chemical characteristics.

Using this careful SPE procedure, the main plasma interference was eliminated as shown in Fig. 5(a) corresponding to the analysis of 'blank plasma', i.e. plasma from a healthy volunteer. The HPLC analysis of a blank plasma sample which was spiked with 100 ng ml^{-1} of FLU and N-FLU, and then subjected to the SPE procedure is provided in Fig. 5(b). The analytes were revealed by two neat chromatographic peaks at $t_{ret} = 9.7$ and 8.1 min., respectively. Extraction yield or 'absolute recovery' was very good for both substances, 96% for FLU and 94% for N-FLU. These values were the mean of the results obtained anlyzing plasma samples spiked with different amounts of fluoxetines (30, 50 and 100 ng ml^{-1}) in duplicate. These data are even more satisfactory if compared with previous data [5] which reported 55–60% for N-FLU and 79–81% for FLU.

The calibration curves of FLU and N-FLU in plasma were subsequently constructed: different amounts of analytes were added to human blank plasma samples. The mixtures were subjected to the SPE procedure described above and the resulting methanolic solutions were finally injected into the HPLC.

The calibration curve for the plasma assay was found to be linear over the concentration range 8–200 ng of FLU and N-FLU per ml of methanol for each 250 μl plasma sample. Considering 1 ml of plasma, this concentration range was multiplied by four and as a result, the linearity range was 32–800 ng of FLU and N-FLU per ml of plasma.

The linear regression equation, obtained by means of the least-square method, was found to be: $y = 2638\ x - 547$ ($Rc = 0.9997$) for N-FLU and $y = 2597\ x - 455$ ($Rc = 0.9995$) for FLU.

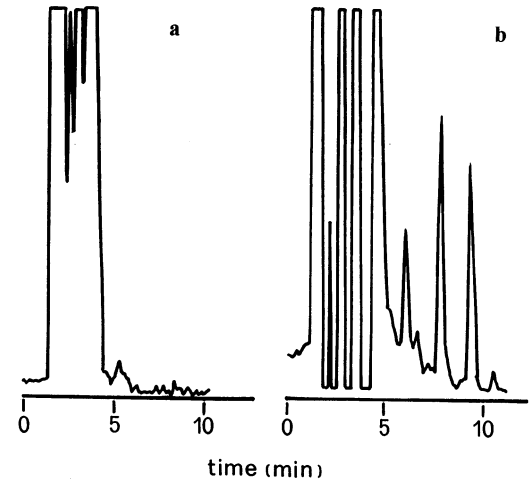

Fig. 5. Chromatograms of: (a) a 'blank' plasma sample; and (b) the same sample spiked with 100 ng ml^{-1} of both fluoxetine and norfluoxetine.

Table 1
Patient plasma concentrations

Patient	Daily dosage (mg)	Fluoxetine (ng ml^{-1})	Norfluoxetine (ng ml^{-1})
1	20	33	57
2	20	40	58
3	50	54	68
4	60	60	187
5	60	526	277

The values of fluoxetine plasma levels were obtained as the mean of three trials.

The intermediate precision of the proposed procedure was satisfactory, with RSD% values of 3 and 3.2 for FLU and N-FLU, respectively ($n = 6$). The detection limit (LOD) equalled 5 and 6 ng ml^{-1} for FLU and N-FLU, respectively, while the LOQ (quantitation limit), equalled 8 mg ml^{-1} for both analytes. Both LOD and LOQ were determined following the guidelines of USP 23 [16].

3.3. Application to plasma of treated patients

Once the analytical method was validated, it was used for the analysis of FLU and N-FLU plasma levels in patients treated with Fluoxetine. FLU was clearly detected at 9.7 min, while N-FLU was detected at 8.1 min. Thus, the method is suitable for the determination of FLU and its main metabolite.

Several drugs, such as clozapine, risperidone and diazepam, currently used in psychiatry, did not interfere with chromatographic analysis of FLU. In fact, no new peak was detected when standard solutions of these drugs were added to patient plasma samples (100 ng ml^{-1}, final concentration in plasma). This result was predictable, since clozapine is not detected under the reported chromatographic conditions. However, risperidone and diazepam are detected at $t_{rit.}$ 3.9 and 4.9 min, respectively, and are thus masked by the initial large peak corresponding to the plasma matrix.

The accuracy of this method was verified by recovery studies, by adding known amounts of FLU standard solutions to a known amount of a plasma sample from a patient whose FLU and N-FLU plasma levels had been previously determined, and by subjecting the mixture to the SPE and HPLC procedure above. A recovery of 94 and 93% for FLU and N-FLU, respectively was obtained for additions of FLU and N-FLU standard solutions (1 ug ml^{-1}), yielding an increase of 50 ng ml^{-1} in the analyte concentrations in plasma. A preliminary study was performed in order to verify if a correlation between the amount of FLU administered and FLU and N-FLU plasma levels exists. The data obtained for five patients receiving 20–60 mg of FLU, administered as Prozac® capsules, as an antidepressant drug, are listed in Table 1. The blood sample was withdrawn 12 hours after the final drug administration. As seen in Table 1, on increasing FLU dosage, the plasma levels of FLU and N-FLU also increased. The only abnormal increase of analyte plasma levels was observed for patient 5, perhaps due to the large number of coadministrated drugs. Experiments are in progress in order to extend this study to a larger number of cases, to improve verification if a correlation between FLU and N-FLU plasma levels and FLU amounts administered as Prozac® capsules exists.

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References

[1] P. Benfield, R.C. Heel, S.P. Lewis, Drugs 32 (1986) 481–550.

[2] Martindale, The Extra Pharmacopoeia, Royal Pharmaceutical Society, London, 31st edn., 1996.
[3] P. Thomare, K. Wang, V. Van Der Meersch-Mougeot, B. Diquet, J. Chromatogr. 583 (1992) 217–221.
[4] D.T. Wong, F.P. Bymaster, E.A. Engleman, Life Sci. 57 (1995) 411–441.
[5] R.N. Gupta, M. Steiner, J. Liq. Chromatogr. 13 (1990) 3785–3797.
[6] R.J. Lantz, K.Z. Farid, J. Koons, J.B. Tenbarge, R.J. Boop, J. Chromatogr. 614 (1993) 175–179.
[7] B.D. Potts, C.J. Parli, J. Liq. Chromatogr. 15 (1992) 665–681.
[8] S.H.Y. Wong, S.S. Dellafera, R. Fernandes, H. Kranzler, J.Chromatogr. 499 (1990) 601–608.
[9] J.H. Nichols, J.R. Charlson, G.M. Lawson, Clin. Chem. 40 (1994) 1312–1316.
[10] R.F. Suckow, M.F. Zhang, T.B. Cooper, Clin. Chem. 38 (1992) 1756–1761.
[11] I.M. McIntyre, C.V. King, S. Skafidis, O.H. Drummer, J. Chromatogr. 621 (1993) 215–223.
[12] S. Joron, H. Robert, Biomed. Chromatogr. 8 (1994) 158–164.
[13] R.N. Gupta, J. Liq. Chromatogr. 16 (1993) 2751–2765.
[14] E.M. Koves, J. Liq. Chromatogr. 692 (1995) 103–119.
[15] A.L. Peyton, R. Carpenter, K. Rutowski, Pharm. Res. 8 (1991) 1528–1532.
[16] United States Pharmacopeia, United States Pharmacopeial Convention, Rockville, 23rd edn., 1995.