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Determination of fluoxetine and its *N*-desmethyl metabolite in human plasma by high-performance liquid chromatography

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Abstract

A rapid and sensitive high-performance liquid chromatographic method has been developed for the simultaneous determination of the antidepressant fluoxetine and its active metabolite norfluoxetine in human plasma using paroxetine as internal standard. After liquid–liquid extraction, the compounds were separated on a C18 column using as mobile phase acetonitrile and 40 mM potassium dihydrogen phosphate buffer (pH 2.3) in the ratio 31:69 (v/v). The quantification of fluoxetine and norfluoxetine was made by fluorescence detection at Ex/Em 230/312 nm. The assay for each analyte was linear over the ranges 1–39 and 0.9–36 ng/ml, respectively. For both compounds intra- and inter-day accuracy and precision ranged between −7.9–12.4 and 0.7–14.7%, respectively. The method was applied to the analysis of plasma samples obtained from healthy subjects treated with one single oral dose of 40 mg fluoxetine.

Keywords: Fluoxetine; Norfluoxetine; Plasma; HPLC; Fluorescence

1. Introduction

Fluoxetine, (3RS)-*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine, (Fig. 1) is a selective serotonin reuptake inhibitor widely used in the treatment of depression. Fluoxetine (FLX) is extensively metabolized by *N*-demethylation in liver (CYP2D6), to its active metabolite norfluoxetine (NFL) [1,2]. Although in the past 20 years there are published works about determination of FLX and NFL by gas chromatography with electron capture detection (GC-ECD) [3], gas chromatography-mass spectrometry (GC–MS) [4] or high-performance liquid chromatography-mass spectrometry (HPLC–MS) [5,6], HPLC with ultraviolet or fluorescence detection remains the most accessible and widely used liquid-chromatographic method for FLX quantification in biological samples, with the overall limit of quantification about 3–10 ng/ml for both compounds [7–12]. The most widely applied extraction technique was liquid–liquid extraction but there are also works in which solid-phase extraction was used [13–15]. In a few studies special treatment of samples such derivatization [11,16] or column switching technique are involved [6,17].

In a single-dose pharmacokinetic/bioequivalence (PK/BE) study of a medicinal product, the extrapolated area of the plasma concentration of the drug versus time after administration curve must not be greater than 20% of total calculated area. To achieve this requirement a quantifiable concentration must be obtained after five half-life times. Taking into account this fact, the sensitivity of the involved analytical method plays an important role. In the case of FLX, after administration of a 40 mg single dose, the highest plasma levels for FLX and NFL are about 39 and 20 ng/ml, respectively [18]; therefore a quantification limit of 1 ng/ml is required for a single 40 mg dose design PK/BE study of FLX. As far as we aware, none of the most sensitive previously published HPLC methods using UV or fluorescence detection had such a lower limit of quantification [19–21], except the

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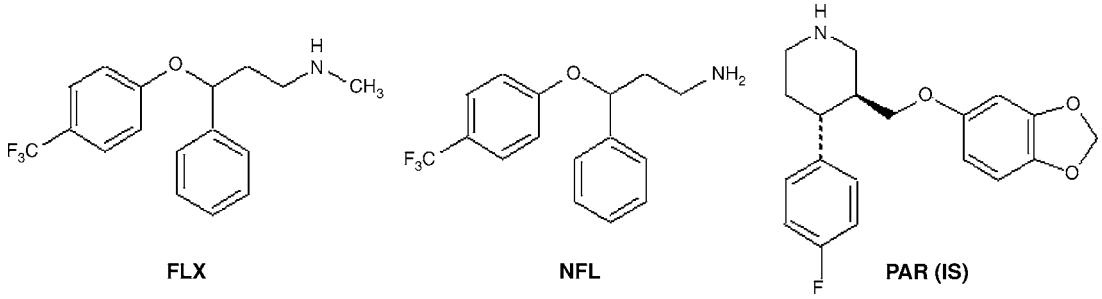

Fig. 1. Chemical structures of fluoxetine (FLX), norfluoxetine (NFL) and paroxetine (PAR)—(internal standard).

work of Peyton et al. [22] in which a chiral HPLC method is described.

The aim of this study was to develop and validate a simple, fast and sensitive HPLC method with fluorescence detection for quantification of fluoxetine and norfluoxetine in human plasma using paroxetine as internal standard (Fig. 1), a method which had to satisfy the requirements for bioequivalence studies.

2. Materials and methods

2.1. Chemicals

FLX and NFL metabolite, as hydrochlorides, were supplied by Pharmaceutical Research Institute (Poland). The internal standard paroxetine (PAR) was purchased from Dodler (Switzerland). All other chemicals were from Merck (Darmstadt, Germany).

2.2. Chromatographic conditions

The HPLC system was an Agilent 1100 Series HPLC system (Agilent Technology Co., Ltd.) consisting of a binary pump, degasser, autosampler, thermostat operating at 37 °C, fluorescence detector. Chromatographic separation was performed on a Zorbax SB-C18 column (150 mm × 3.0 mm i.d., 3.5 μm) (Agilent) preceded by a 0.5 μm online filter. The mobile phase consisted of acetonitrile and 40 mM potassium dihydrogen phosphate buffer, with the pH adjusted at 2.3 with 85% phosphoric acid, in the ratio 31:69 (V/V). The mobile phase was delivered at a flow rate of 1 ml/min. The column effluent was monitored at Ex/Em 298/340 nm up to 4.8 min and at 230/312 nm up to 8 min. The autosampler injection volume was settled to 100 μl.

2.3. Standard solutions

Stock solutions of FLX and NFL were prepared in acetonitrile and PAR solutions, in methanol. All the stock solutions were stored protected from light at 4 °C. The working solutions were prepared by diluting appropriate volumes of stock solutions with 0.01% phosphoric acid and used to obtain plasma concentrations between 1 and 39 ng/ml for FLX and between 0.9 and 36 ng/ml for NFL. Internal standard working solutions were prepared in a similar manner, providing finally a plasma concentration of 42 ng/ml paroxetine.

2.4. Sample preparation

In a glass centrifuge tube, to 1 ml plasma 100 μl solution of internal standard, 100 μl sodium hydroxide and 7 ml hexane containing 1.5% isoamilic alcohol were added. The tubes were capped and mechanically shaken for 10 min on a reciprocating mixer (Pulsing Vortex Shaker, Glas-Col, USA) and then centrifuged at a rotational speed of 4500 rpm for 6 min (Sigma 2–15 centrifuge, Sigma, Osterode am Harz, Germany). After centrifugation, the organic layer was transferred in a clean tube and 200 μl of phosphoric acid 0.3% solution were added. The tubes were vortex-mixed for 8 min and after centrifugation at 4500 rpm for 6 min, the organic layer was discarded. The remaining aqueous phase was transferred with the aid of an automatic pipette in an autosampler vial and a volume of 100 μl was injected onto HPLC system.

2.5. Analytical method validation

The method being developed for a bioequivalence study in which healthy subjects are involved, specificity was assumed only against anticoagulant (heparin). No other medicinal substances interference was verified. Quantification of fluoxetine and norfluoxetine was made using internal standard method (paroxetine as internal standard). Calibration curve was constructed by plotting peak area ratio of FLX or NFL against concentration ratio FLX or NFL/PAR. In order to calculate the main parameters of calibration curve (slope, intercept and correlation coefficient), linear regression method was used. The calibration model was accepted, if the residuals were within ±20% at the lower limit of quantification and within ±15% at all other calibration levels and at least 2/3 of the standards meat this criterion [23–25].

The intra- and inter-day accuracy and precision were determined for FLX and NFL by analysis five samples at each level of calibration in the same day and six samples in three different days (two samples per day at each level of calibration), respectively. Accuracy was calculated as

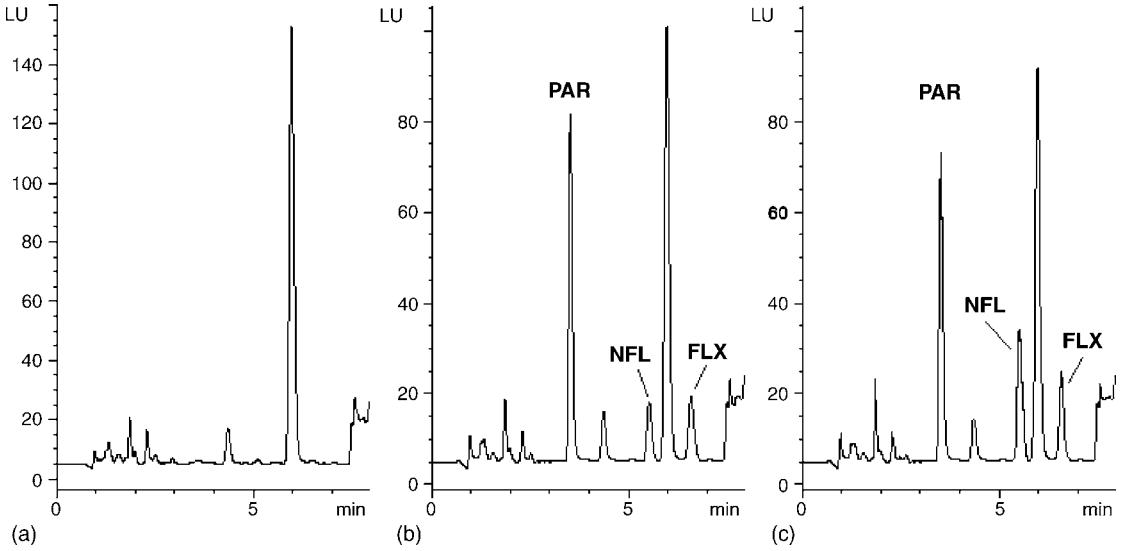

Fig. 2. Chromatograms of: (a) plasma blank; (b) plasma with NFL, FLX (3 ng/ml) and PAR—internal standard (42 ng/ml); (c) volunteer plasma 120 h after receiving 40 mg fluoxetine (5.2 ng/ml FLX, 8.5 ng/ml NFL, 42 ng/ml PAR).

percentage difference between the concentration of drug measured with calibration curve and the concentration of drug added to the blank plasma. Precision was expressed as the percentage variation (coefficient of variation CV%) of measured concentrations for each calibration level.

Recovery was determined by comparing the peak area of an extracted sample with the one obtained after the direct injection of a solution with the same drug concentration.

2.6. Clinical application

The validated method was successfully applied in a bioequivalence study of two dosage forms containing 20 mg fluoxetine. The collecting times ranged between 0 and 840 h after oral administration of a single 40 mg dose (two capsules of 20 mg) of fluoxetine.

3. Results and discussions

The chromatographic conditions and samples preparation for the proposed method were optimized in order to be suitable for PK/BE studies. Not only sensitive, accurate and precise methods are necessary but also fasts, because hundreds, sometimes thousands samples, are involved in such kind of studies. For these reasons developing a sensitive and fast analytical method was the primary goal of our research.

Table 1
Intra-day precision, accuracy and recovery for FLX ($n = 5$)

Concentration (ng/ml)	Mean measured concentration ng/ml (±S.D.)		Precision (C.V. %)	Accuracy (%)	Recovery % (± S.D.)	
0.97	1.05	0.06	5.71	8.25	109.0	5.4
1.94	1.96	0.12	6.12	1.03	100.2	5.6
3.49	3.29	0.15	4.56	−5.73	98.6	4.2
5.04	5.05	0.34	6.73	0.20	102.3	6.6
10.08	9.72	0.35	3.60	−3.57	93.3	3.3
19.38	19.84	0.14	0.71	2.37	101.6	0.7
38.76	38.87	0.61	1.57	0.28	92.6	1.4

Table 2
Inter-day precision, accuracy and recovery for FLX ($n = 6$)

Concentration (ng/ml)	Mean measured concentration ng/ml (±S.D.)		Precision (C.V. %)	Accuracy (%)	Recovery % (± S.D.)	
0.97	1.09	0.16	14.68	12.37	112.6	14.2
1.94	1.96	0.14	7.14	1.03	100.1	6.6
3.49	3.50	0.23	6.57	0.29	104.5	6.5
5.04	4.78	0.54	11.30	−5.16	97.1	10.5
10.08	9.96	0.43	4.32	−1.19	95.5	4.0
19.38	19.69	0.26	1.32	1.60	100.9	1.3
38.76	39.08	1.04	2.66	0.83	93.1	2.5

Table 3
Intra-day precision, accuracy and recovery for NFL ($n = 5$)

Concentration (ng/ml)	Mean measured concentration ng/ml (±S.D.)		Precision (C.V. %)	Accuracy (%)	Recovery % (± S.D.)	
0.90	0.95	0.05	5.26	5.56	94.7	4.4
1.80	1.88	0.04	2.13	4.44	85.0	1.9
3.24	3.28	0.13	3.96	1.23	83.5	3.3
4.68	4.56	0.12	2.63	−2.56	95.9	2.5
9.36	8.97	0.55	6.13	−4.17	88.3	5.4
18.00	18.40	0.67	3.64	2.22	96.6	3.5
36.00	35.97	2.57	7.14	−0.08	87.6	6.3

Table 4
Inter-day precision, accuracy and recovery for NFL ($n = 6$)

Concentration (ng/ml)	Mean measured concentration ng/ml (±S.D.)		Precision (C.V. %)	Accuracy (%)	Recovery % (± S.D.)	
0.90	0.97	0.08	8.25	7.78	97.3	8.0
1.80	1.86	0.10	5.38	3.33	84.2	4.6
3.24	3.45	0.28	8.12	6.48	87.8	7.0
4.68	4.31	0.27	6.26	−7.91	90.7	5.7
9.36	9.02	0.33	3.66	−3.63	88.7	3.2
18.00	18.42	0.58	3.15	2.33	96.7	3.0
36.00	36.57	2.12	5.80	1.58	89.0	5.2

The method proved to be specific, both analytes and internal standard being well resolved from endogenous compounds within 8 min of isocratic elution. A blank and a sample plasma chromatogram of FLX and NFL (3 ng/ml) are presented in Fig. 2a and b, respectively. The retention times were 3.8 min for PAR, 5.7 min for NFL and 6.8 min for FLX.

The calibration curves were linear over the ranges 1–39 ng/ml FLX and 0.9–36 ng/ml NFL, with a coefficient of correlation greater than 0.998.

The quantification limits (accuracy and precision less than 20%) for FLX and NFL were 1 and 0.9 ng/ml, respectively, and detection limit for both substances, 0.4 ng/ml (corresponding to a signal-to-noise ratio of 3). The lowest quantifiable concentrations of the proposed method are similar to those of Peyton et al. [22] for stereospecific determination of fluoxetine and norfluoxetine enantiomers in human plasma by applying liquid–liquid extraction and derivatization but the assay was linear using two concentration ranges.

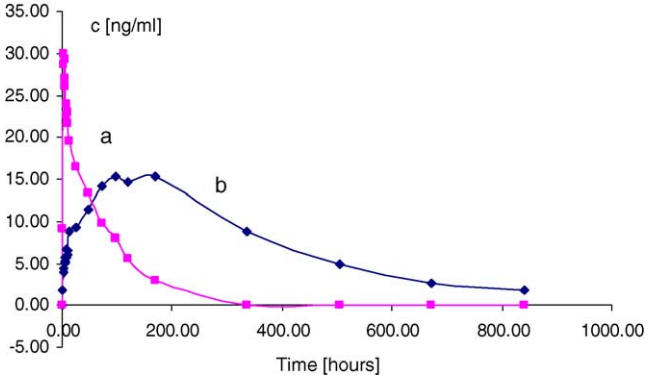

Fig. 3. Concentration vs. time profile of (a) FLX and (b) NFL in plasma of one volunteer after receiving 40 mg fluoxetine.

Intra-day precision, accuracy and recovery results for FLX are shown in Tables 1 and 2, and for NFL, in Tables 3 and 4.

Regarding clinical application, the method continued to perform satisfactorily during analytical assay (Fig. 2c) of the clinical samples obtained for the bioequivalence study. A representative concentration–time curve after oral administration of fluoxetine is shown in Fig. 3.

4. Conclusions

This paper describes a rapid and sensitive HPLC method with fluorescence detection for quantification of fluoxetine and norfluoxetine in human plasma, using paroxetine as internal standard, and its applicability to pharmacokinetic/bioequivalence studies.

Chromatographic and extraction parameters were optimized in order to obtain a short run-time and a rapid procedure of sample plasma processing. The method was successfully applied in a bioequivalence study of two pharmaceutical formulations containing 20 mg fluoxetine. The results and statistical comparison between tested drugs are not published yet.

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