ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
23 (2000) 989–996

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Determination of the antidepressant fluoxetine in human plasma by LC with UV detection

Manuela T. Maya [a,*], Constantino R. Domingos [b], M. Teresa Guerreiro [b], José A. Morais [b]

[a] *Centro de Patogenese Molecular, Faculdade de Farmácia da Universidade de Lisboa, Av. Forças Armadas, 1600 Lisbon, Portugal*
[b] *Faculdade de Farmácia da Universidade de Lisboa, Lisbon, Portugal*

Received 16 September 1999; received in revised form 4 April 2000; accepted 27 April 2000

Abstract

A selective and sensitive isocratic high-performance liquid chromatographic (HPLC) method was developed for the quantitative analysis of low concentrations of fluoxetine (FLX) in human plasma, with ultra-violet detection at 226 nm. A reversed-phase column, LiChrospher® 60 RP-Select B (125 × 3 mm i.d., 5 μm) (Merck), was used to resolve FLX and diazepam (DZP) (internal standard) from endogenous matrix interferences. FLX was isolated from plasma by liquid-liquid extraction. Two identical HPLC systems were used, both validated under the same study conditions. Each chromatographic separation was completed in 30 min and the results showed a mean relative recovery of 101 and 99.3% and an overall precision (RSD%) of 4.78 and 6.09 for each HPLC system. The standard curve was linear for FLX concentrations over the range of 5.00–50.0 ng ml^{-1} ($R = 0.997$ and 0.998).The limit of quantitation of FLX was 5 ng ml^{-1} for both HPLC systems. The method described was applied to the analysis of plasma samples obtained from healthy subjects treated with one single oral dose of 40 mg of fluoxetine.

Keywords: High-performance liquid chromatography (HPLC); Quantitative analysis; Fluoxetine (FLX)

1. Introduction

Fluoxetine, *N*-methyl-3-phenyl-3-(2,2,2-trifluoro-*p*-tolyloxy) propylamine hydrochloride, $C_{17}H_{18}F_3NO$, HCl, MW = 345.8, is an antidepressant which differs structurally and pharmacologically from the tricyclic agents. It has been shown to selectively inhibit the reuptake of serotonin in presynaptic neurons [1].

Fluoxetine (FLX) is also used in a variety of disorders in addition to depression [1,2]. Beneficial responses have been reported in obsessive compulsive disorders, pain syndromes including diabetic neuropathy and fibrositis, panic disorders and nervous bulimia (American Hospital Formulary Service, Drug Information 93) [3–5].

FLX is extensively metabolised, by demethylation in liver, to its primary active metabolite norfluoxetine (NFLX). The half-lives of FLX and NFLX are ~2–3 and 7–9 days, respectively.

* Corresponding author. Tel./fax: +351-217933064.
E-mail address: mmaya@correio.ff.ul.pt (M.T. Maya).

0731-7085/00/$ - see front matter
PII: S0731-7085(00)00367-8

Up to the present, analytical methods involving gas chromatography (GC) with electron capture [6,7] have been developed for FLX quantitation in human plasma. Nevertheless, this technique its not available in many laboratories and is too time consuming (extraction, purification and derivatization) to be used when a large number of samples have to be processed. Therefore, HPLC methods with ultra-violet or fluorescence detection have been published in the last few years [8–12] as they are less complicated and faster than GC but still adequate in terms of sensitivity and reliability, for FLX quantitation in biological samples.

This report describes a sensitive and selective method using an isocratic HPLC procedure for separating and quantifying FLX in plasma, to be mainly used in absorption pharmacokinetic studies. As a matter of fact, the method has been successfully used on a bioequivalence study of two different fluoxetine formulations. The bioavailability of the drug was calculated on the basis of fluoxetine plasma concentrations and not on the basis of its metabolite plasma concentrations.

For validation of the assay, recommendations from the conference on 'Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies', were followed [13].

2. Materials and methods

2.1. Chromatographic conditions

Analysis was performed on Hewlett Packard (HP) and Shimadzu (SH) liquid chromatographs both equipped with reversed-phase columns, LiChrospher® 60 RP-Select B (125 × 3 mm i.d., 5 μm) (Merck, Darmstadt, Germany) protected by a guard column, LiChrospher® 60 RP-Select B (5 μm) (Merck, Darmstadt, Germany).

The solvent was delivered by means of a Hewlett-Packard pump, Model series 1050 (Avondale, PA, USA) as well as a Shimadzu pump, Model LC-6A (Kyoto, Japan) which were coupled respectively to Hewlett-Packard, (Model series 1050) and Merck-Hitachi, (Model AS-2000 A, Merck, Darmstadt, Germany) automatic injectors. Two variable wavelength UV-VIS detectors, a Hewlett-Packard, Model series 1050 and a Shimadzu, Model SPD-6A, operating at 226 nm, were used.

Peak-heights were measured by two Hewlett-Packard integrators, Model 3395 with a chart speed at 0.1 cm min^{-1}.

The mobile phase consisted of potassium dihydrogen phosphate buffer (pH 6.0; 0.1 M) acetonitrile (70:30, v/v). The final apparent pH was 4.0. The eluent was delivered at a flow rate of 0.8 ml min^{-1}. The mobile phase was filtered by passing through a 0.45-μm membrane filter (S-Pak filter, Millipore) under vacuum, and was degassed in an ultrasonic bath for 15 min.

2.2. Reagents and standards

Purified water (Barnstead E-pure purification system, Barnstead Thermolyne, IA, USA), HPLC-grade hexane (Fisons, Loughborough, England), acetonitrile and extrapure isoamyl alcohol (Merck, Darmstadt, Germany) were used throughout.

Diethylamine (Sigma, St. Louis, USA), hydrochloric acid (37%), *ortho*-phosphoric acid (85%) potassium dihydrogen phosphate and sodium hydroxide pellets (Merck, Darmstadt, Germany), were of analytical grade.

Diazepam (Bial, Porto, Portugal) was used as internal standard (I.S.) at 1 mg ml^{-1} in methanol (stock solution). Fluoxetine hydrochloride (Dodler, Basle, Switzerland) was used as reference substance at 1 mg ml^{-1} in methanol (stock solution). The standard solutions were stored protected from light at 4°C.

Control plasmas were prepared from working solutions diluted with blank plasma at concentrations of 5, 10, 20 and 50 ng ml^{-1}.

Blank plasma was obtained from healthy subjects undergoing no drug therapy.

2.3. Sample preparation

To plasma (1 ml) in a 16 × 125-mm screw-cap tube with Teflon lining, 25 μl of methanol containing 25 μg of internal standard (diazepam), 250

μl of sodium hydroxide 0.2 N and 4 ml of hexane:isoamyl alcohol (97:3) were added.

The tubes were capped and vigorously shaken for 30 s (Vortex), followed by mechanical shake on a reciprocating mixer at 300 strokes per minute for 20 min and then centrifuged at 4000 rpm for 10 min at 10°C. After centrifugation the organic layer was transferred into another clean tube for back extraction with 500 μl of hydrochloric acid 0.1 N.

The tubes were vortex-mixed for 1 min and centrifuged at 4000 rpm for 10 min at 10°C. The upper organic layer was discarded by aspiration. The remaining aqueous phase was injected onto the HPLC system (60 μl).

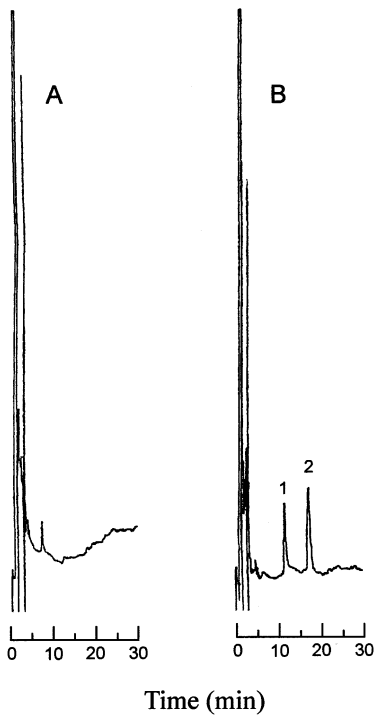

Fig. 1. HPLC of FLX: peak 1, FLX; peak 2, diazepam (internal standard). UV detection: 226 nm. Column: LiChrospher® 60 RP-Select B (125 × 3 mm i.d., 5 μm); mobile phase: potassium dihydrogen phosphate buffer (pH 6.0, 0.1 M)-acetonitrile (70:30, v/v). Final apparent pH was 4.0. (A) Blank extracted plasma; (B) extracted spiked plasma with 20 ng ml^{-1} of FLX and 25 μg ml^{-1} of internal standard.

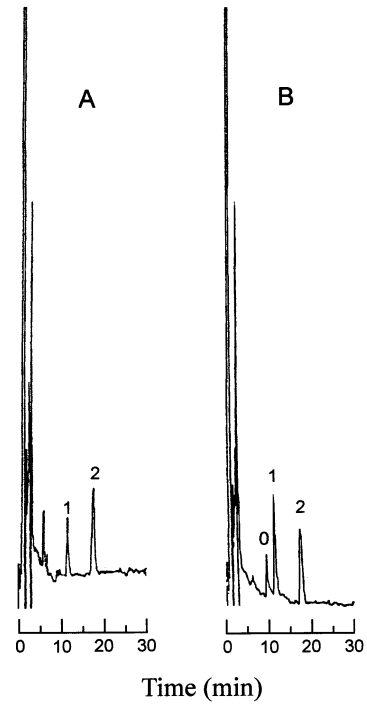

Fig. 2. Chromatograms obtained from one healthy volunteer after oral administration of fluoxetine at two different collection times: (A) 2.0 h after the oral administration of 40 mg of fluoxetine; (B) 12 h after the oral administration of 40 mg of fluoxetine. Peak 0, NFLX (?); peak 1, FLX; peak 2, internal standard.

2.4. Quantitation

Plasma concentrations of FLX in unknown and control samples were determined by using the linear regression equation from daily calibration curves, constructed by plotting the peak height ratio (FLX/I.S.) over the concentration range of 5.00–50.0 ng ml^{-1}.

3. Results

3.1. Chromatography

Fig. 1 shows the chromatograms obtained from drug-free human plasma (A) together with plasma supplemented with FLX (20 ng ml^{-1}) and the internal standard (25 μg ml^{-1}) (B) obtained by means of the described methodology.

Table 1
Results of linear regression analysis of calibration data

Parameter	HP[a] value	SH[b] value
Slope (b)	0.04069	0.04082
Intercept (a)	0.05132	0.06521
Standard error of slope (s_b)	0.00083	0.00079
Standard error of intercept (s_a)	0.02240	0.01949
Range (ng ml^{-1})	5.00–50.0	5.00–50.0
Correlation coefficient (r)	0.99709	0.99757

[a] Hewlett Packard HPLC.
[b] Shimadzu HPLC.

Fig. 2 shows the chromatograms obtained from one healthy volunteer after oral administration of fluoxetine at two different collection times.

The retention times were: fluoxetine, 11.4 min; diazepam, 17.3 min.

3.2. Linearity

The linearity of the method was checked for FLX in plasma (5.00–50.0 ng ml^{-1}). Peak height ratios (reference to internal standard) and analyte concentrations were found to be linearly related over this range (Table 1). Linear regression was used to determine the slope and intercept. The correlation coefficients were 0.997 and 0.998 for HP and SH, respectively.

3.3. Between-day precision

The between-day precision of the assay was determined from the analysis of pooled plasma spiked with FLX (5.00, 20.0 and 50.0 ng ml^{-1}) along the period of analysis. Results are summarised in Table 2.

3.4. Within-day precision/accuracy

The analysis of FLX was evaluated for within-day precision and accuracy by analysing replicate determinations of plasma pools ($n = 12$) at concentrations of 5.00, 20.0 and 50.0 ng ml^{-1}. The samples were extracted and injected once on the same day. Accuracy was measured as the percent difference from theoretical according to the following equation: % difference from theoretical = $((X/C_T) - 1)*100$, where X is mean determined concentration of a quality control pool and C_T is theoretical concentration. The results are summarised in Table 3.

3.5. Relative recovery

The relative analytical recovery from plasma for fluoxetine was measured by spiking drug-free plasma with known concentrations of the drug ($n = 8$). The spiked plasma was then analysed by the developed method. The relative recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with the actual added amounts. The mean values for

Table 2
Between-day precision of the analytical method for determination of FLX in plasma for both HPLC systems

Nominal concentration (ng ml^{-1})	HP[a]				SH[b]			
	n[c]	Mean[d] (ng ml^{-1})	S.D.	RSD (%)	n[c]	Mean[d] (ng ml^{-1})	S.D.	RSD (%)
5.00	43	4.78	1.13	23.6	59	4.13	1.17	28.3
10.0	43	9.60	1.58	16.4	59	8.94	1.31	14.6
20.0	43	20.2	2.95	14.6	58	20.6	1.74	8.44
50.0	44	51.0	7.14	13.9	60	52.5	6.94	13.2

[a] Hewlett Packard HPLC.
[b] Shimadzu HPLC.
[c] Number of plasma samples analysed for each value. Experimental conditions as described in the text.
[d] Mean values of different spiked plasmas analysed on different days.

Table 3
Summary of within-day precision and accuracy data for fluoxetine

Nominal concentration (ng ml^{-1})	HP[a]			SH[b]		
	Mean found (ng ml^{-1})	Within-day precision (%)[c]	Within-day accuracy % difference from theoretical	Mean found (ng ml^{-1})	Within-day precision (%)	Within-day accuracy % difference from theoretical
5.00	5.45	14.9	9.00	4.44	17.5	8.88
20.0	19.2	5.64	4.00	18.4	16.7	9.22
50.0	50.3	4.54	1.01	44.6	10.1	8.92

[a] Hewlett Packard HPLC system.
[b] Shimadzu HPLC system.
[c] Relative standard deviation (%).

Table 4
Percentage of nominal fluoxetine concentration from calibration data

	Spiked value (ng ml^{-1})	HP[a]		SH[b]	
		Estimated concentration (E.C.) (ng ml^{-1})	Mean relative recovery[c] (%)	Estimated concentration (E.C.) (ng ml^{-1})	Mean relative recovery[c] (%)
Fluoxetine	5.00	5.12	102.5	4.47	89.5
	8.00	8.17	102.1	8.78	109.8
	10.0	10.5	105.3	9.73	97.3
	15.0	15.7	104.5	15.3	102.3
	20.0	18.0	90.2	19.1	95.5
	30.0	30.5	101.8	30.2	100.8
	40.0	39.1	97.7	41.2	102.9
	50.0	50.9	101.7	48.3	96.6
Mean			100.7		99.3
RSD (%)			4.78		6.09

[a] Hewlett Packard HPLC system.
[b] Shimadzu HPLC system.
[c] [(E.C.)/(spiked value)] × 100. A total of eight replicate determinations for each concentration. Experimental conditions as described in the text.

HP and SH systems were 101 and 99.3%, with relative standard deviations of 4.78 and 6.09%, respectively (Table 4).

3.6. Limit of quantitation

The limit of quantitation (LOQ) was estimated on the basis of the analysis of at least five replicates of different concentrations of FLX, considering as LOQ the lowest concentration value for which an RSD of less than 20% was found. When 1 ml of the sample was used, the LOQ was 5 ng ml^{-1}. The relative standard deviation was 14.9 and 17.5% for HP and SH systems, respectively.

3.7. Specificity

The specificity of the analytical method was checked by analysis of six different independent sources of the same biologic matrix, obtained from healthy blood donors. No endogenous interfering peaks were visible on the retention times of FLX and internal standard (Fig. 1B).

3.8. Between systems and between operators variability

Two HPLC systems (HP and SH) were used and two operators performed the analysis. Variability was assessed on the results of five replicates obtained in paralleled for each instrument with the same operator and for each operator using the same instrument. Results show no significant difference using a t-test in the operators case, therefore it was assumed that the results could be pooled. For systems the values were significantly different so we reported them separately. The results are summarised in Tables 5 and 6.

4. Discussion and conclusions

Different mobile phase compositions phosphate buffer (pH 3.0, 0.067 M)-acetonitrile (60:40, v/v; 70:30, v/v; 68:32, v/v) at different pH values and phosphate buffer (pH 6.0, 0.1 M)-acetonitrile (70:30, v/v) at final pH 5.0 and different columns (Nova Pak® Phenyl 60 Å (3.9 × 150 mm i.d.; 4 μm), Waters, Milford, MA, USA) were tested in the present study for their ability to separate FLX from the I.S. and from plasma interfering substances. No systematic method of optimization was followed except for a trial and error procedure, following the general rules of solvent dependent order of elution. Initially, fluorescence

detection was used (excitation 260 nm, emission 310 nm), but a very noisy baseline was achieved and a high plotter attenuation was required to obtain a more stable baseline resulting in a poor response. Better results were obtained when an absorbance detector was used at 226 mn.

The HPLC method described here is selective, sensitive and reproducible for quantitation of fluoxetine in human plasma samples. Extraction procedure is time consuming as successive back extractions into water and the organic phase have been followed, but clean chromatograms are obtained.

With this method no endogenous interfering peaks were visible in blank plasma (Fig. 1A). The two peaks of fluoxetine and diazepam (internal standard) are well separated with an isocratic mobile phase with retention times of 11.6 and 17.2 min, respectively (Fig. 1B).

The principal aim of the developed method was apply to it a pharmacokinetic study with two different fluoxetine formulations. A single oral dose of 40 mg of fluoxetine was administered to 24 healthy volunteers enrolled on the bioavailability study. Each subject gave informed consent and agreed to refrain from drinking alcohol-containing beverages and taking any other drug for the duration of the study. Thus, there was no intention to apply the method in routine therapeutic drug monitoring, where an association of fluoxetine with diazepam can be observed in some depressive patients. Consequently, no special concern was observed concerning the choice of the internal standard unless to select a substance that showed good resolution from FLX and plasma interferents and that simultaneously showed good extraction efficiency and an acceptable retention time.

After oral administration of FLX a first peak, immediately before FLX peak, can be seen (Fig. 2B, peak 0). The fact that it only appears in volunteers' samples after oral administration of FLX and never in blank or supplemented plasma suggests that it represents its demethylated

Table 5
Summary of the *t*-test results applied to instrument data

	HP[a]	SH[b]	HP	SH	HP	SH
Theoretical (ng ml^{-1})	5	5	20	20	50	50
Mean	5.45	4.44	19.3	18.4	50.5	45.0
Obs.[c]	12	12	12	12	11	11
S.L.[d]	0.0052	0.0052	0.405	0.405	0.0024	0.0024

[a] Hewlett Packard HPLC system.
[b] Shimadzu BPLC system.
[c] Number of plasma samples analysed for each value.
[d] Significance level (critical value $P \leq 0.05$).

Table 6
Summary of the *t*-test results applied to operator data

	HP[a]	SH[b]	HP	SH	HP	SH
Theoretical (ng ml^{-1})	5	5	20	20	50	50
Mean	5.70	6.54	21.0	21.3	50.4	49.2
Obs.[c]	11	11	11	11	11	11
S.L.[d]	0.0933	0.0933	0.802	0.802	0.477	0.477

[a] Hewlett Packard HPLC system.
[b] Shimadzu HPLC system.
[c] Number of plasma samples analysed for each value.
[d] Significance level (critical value $P \leq 0.05$).

metabolite, norfluoxetine (NFLX). Accordingly, we were able only to identify and quantify FLX since NFLX reference standard was unavailable. To prolong the lifetime of the column a guard-column was used, in order to protect it from some interfering substances.

The calibration plot of peak height ratio (FLX/internal standard) is linear over the range 5.00–50.0 ng ml^{-1} and the precision of the method, calculated from the calibration curve, shows a relative standard deviation (RSD) of 4.78–6.09% (HP and SH, respectively). The limit of quantitation (LOQ) of the assay is 5.00 ng ml^{-1} which is lower than that reported by Orsulak et al. [9], Thomare et al [10] and Tokmakjian et al. [11]. Our limit of detection was 3.0 ng ml^{-1} which is higher than that of Thomare et al. [10].

The utilization of an automatic injector allows the processing of over 40 samples in 1 day, which is an advantage in a bioavailability study as a large number of samples have to be analysed.

Acknowledgements

This work was supported by Laborterapia-Produtos Farmacêuticos, S.A., Portugal.

References

[1] P. Benfield, R.C. Heel, S.P. Lewis, Drugs 32 (1986) 481–508.
[2] S.P. Roose, A.H. Glassman, Treatment Strategies for Refractory Depression. Progress in Psychiatry, vol. 25, American Psychiatry Press, Washington, DC, 1990, pp. 157–161.
[3] F.R. Schneider, et al., J. Clin. Psychopharmacol. 10 (1990) 119–121.
[4] L.C. Ramirez, et al., Am. J. Med. 88 (1990) 540–541.
[5] G.L. Cooper, Br. J. Psychiatry 153 (1988) 77–86.
[6] J.F. Nash, R.J. Bopp, R.H. Carmichael, K.Z. Farid, L. Lemberger, Clin. Chem. 28 (1982) 2100–2102.
[7] V. Dixit, H. Nguyen, V.M. Dixit, J. Chromatogr. 563 (1991) 379–384.
[8] S. Joron, H. Robert, Biomed. Chromatogr. 8 (1994) 158–164.
[9] P.I. Orsulak, et al., Clin. Chem. 34 (9) (1988) 1875–1878.
[10] P. Thomare, K. Wang, Van der Meersch-Mougeot, B. Diquet, J. Chromatogr. 583 (1991) 217–221.
[11] S. Tokmakjian, M. Haines, M. Edmonds, Clin. Chem. 39 (8) (1993) 1749–1750.
[12] R.F. Suckow, M.F. Zang, T.B. Cooper, Clin. Chem. 38 (9) (1992) 1756–1761.
[13] P. Shah, K. Midha, et al., J. Pharm. Sci. 3 (1992) 309–312.