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Sensitive, high-throughput gas chromatographic–mass spectrometric assay for fluoxetine and norfluoxetine in human plasma and its application to pharmacokinetic studies

R.S. Addison*, M.E. Franklin, W.D. Hooper

Centre for Studies in Drug Disposition, Department of Medicine, The University of Queensland, Royal Brisbane Hospital, Brisbane 4029, Australia

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Abstract

A sensitive, robust gas chromatographic–mass spectrometric assay suitable for use in pharmacokinetic or bioequivalence studies is presented for the selective serotonin reuptake inhibitor, fluoxetine, and its major metabolite, norfluoxetine (*N*-desmethylfluoxetine). This method employs solid-phase extraction followed by acetylation with trifluoroacetic anhydride and analysis of the derivatives using selected ion monitoring. The lower limit of quantification was 1.0 ng/ml, and the assay was linear for both analytes from 1 to 100 ng/ml. Mean recoveries following solid-phase extraction at concentrations of 5.0, 20 and 100 ng/ml were 91% (fluoxetine) and 87% (norfluoxetine). Assay precision (as mean RSD) and accuracy (as mean relative error) for both analytes were tested at the same three nominal concentrations and were found to be within 10% in all cases. Analysis of fluoxetine concentrations in plasma samples from 18 volunteers following administration of a single 40 mg dose of fluoxetine provided the following pharmacokinetic data (mean±SD): C_{max}, 32.73±9.21 ng/ml; $AUC_{0-\infty}$, 1627±1372 ng/ml h; T_{max}, 3.08 h (median); k_e, 0.022±0.007 h^{-1}; elimination half-life, 37.69±21.70 h.

Keywords: Fluoxetine; Norfluoxetine

1. Introduction

Fluoxetine [*N*-methyl-3-phenyl-3-(4-trifluoromethylphenoxy)propylamine, FLX, Fig. 1] is an antidepressant belonging to the class of selective serotonin reuptake inhibitors (SSRI), which is used in the treatment of major depression and obsessive compulsive disorder. Fluoxetine is extensively metabolized in the liver to the *N*-desmethyl derivative,

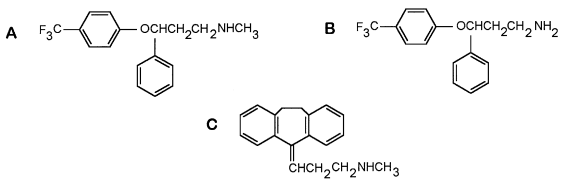

Fig. 1. Structures of (A) fluoxetine, (B) norfluoxetine and (C) nortriptyline.

*Corresponding author.

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norfluoxetine [3-phenyl-3-(4-trifluoromethylphenoxy) propylamine, NFX, Fig. 1], which has approximately equal SSRI activity to that of the parent drug [1]. There are, in addition, a number of hitherto unidentified metabolites.

A number of high-performance liquid chromatographic (HPLC) and gas chromatograpic (GC) methods are available for the assay of fluoxetine and norfluoxetine in biological samples, and these have been recently reviewed [2,3]. Most of the HPLC assays listed in these reviews quote a lower limit of quantification within the range of 5–20 ng/ml, as do the GC assays with electron-capture detection [2]. Meineke et al. [4] have recently described a non-enantioselective HPLC assay with UV detection for FLX and NFX, but were also unable to quantitate these two analytes at concentrations below 25 ng/ml. Two further reports have described GC methods using nitrogen–phosphorus detection. One involved co-assay of FLX, NFX and desipramine in liver and brain tissue with a lower quantification limit of 50 ng/g [5], while the other described assay of FLX and NFX in plasma with lower limits of quantification of 2 and 5 ng/ml, respectively [6]. Both fluoxetine and norfluoxetine are chiral compounds, and methods are available for analysis of their enantiomers, by GC with electron-capture detection following derivatization with (*S*)-trifluoroacetylpropyl chloride [7], and by HPLC after derivatization with *R*-1-(1-naphthyl)ethyl isocyanate [8]. Eap et al. [9] recently published a gas chromatographic–mass spectometric (GC–MS) assay for the enantiomers of fluoxetine and norfluoxetine, as well as for the related SSRI, fluvoxamine, with a lower limit of quantification for FLX and NFX of 1 ng/ml. However, to date, no results are available for the pharmacokinetics of fluoxetine and norfluoxetine enantiomers following administration of fluoxetine.

The conduct of bioequivalence studies provides a situation where accurate determinations of plasma drug and metabolite concentrations are required in multiple plasma samples following single doses of the drug in a panel of human subjects. Generally, determination of individual enantiomers of chiral drugs is not required in bioequivalence studies, unless the pharmacologically more active enantiomer undergoes greater first-pass metabolism (i.e. the drugs which Karim has classified as Category III [10]). Since fluoxetine does not undergo first-pass metabolism, there is no basis for suggesting that an enantioselective method would be required for fluoxetine.

We therefore wished to develop a convenient and specific non-enantioselective GC–MS method for the simultaneous measurement of fluoxetine and norfluoxetine in human plasma, with a lower limit of determination of 1 ng/ml or below, and which was rugged and suitable for high-throughput analysis of the large batches of samples obtained in the conduct of bioequivalence studies.

2. Experimental

2.1. Chemicals

Fluoxetine hydrochloride and norfluoxetine hydrochloride were supplied by Alphapharm (Brisbane, Australia). Nortriptyline hydrochloride (NTP, internal standard), trifluoro-acetic anhydride (TFAA) and ammonium hydroxide solution were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, isopropanol, ethyl acetate (chromatography grade) and hexane (nanograde) were from Mallinckrodt (Paris, KY, USA), and dichloromethane and chloroform were obtained from BDH (Auburn, Australia). All other chemicals were of analytical reagent or higher grade and were used as received. Solid-phase extraction cartridges (Bond Elut Certify, 130 mg packing/cartridge) were purchased from Varian (Brisbane, Australia).

2.2. GC–MS

A Hewlett-Packard Model 5890 Series II gas chromatograph was used in conjunction with a Model 7673 injector and a Model 5971A mass selective detector, operated in selected ion monitoring (SIM) mode with an electron impact source. The mass spectrometer utilized an ionizing electron energy of 70 eV and was autotuned using the maximum sensitivity option. The column used was a 25 m×0.2 mm I.D.×0.33 μm film thickness HP-5 capillary column preceded by a 0.5 m×0.53 mm I.D. deactivated methyl silica guard column (Hewlett-Packard). The operating conditions for the GC–MS were as follows: oven, initial temperature 80°C for 1 min, ramp at 40°C/min to 240°C, ramp at 5°C to

290°C, hold at 290°C for 0.5 min; injector, initial temperature 200°C, ramp at 60°C/min to 270°C, hold at 270°C; GC–MS transfer line 300°C. Helium was used as the carrier gas at 0.7 ml/min. Data were acquired from the detector using MS Chemstation software (Hewlett-Packard). The ions monitored for the trifluoroacetylated derivatives of each analyte were as follows: FLX, *m/z* 244; NFX, *m/z* 230; NTP *m/z* 232.

2.3. Standard solutions

Stock solutions (100 μg/ml) and appropriate dilutions of FLX, NFX and NTP as the hydrochlorides were prepared in water and stored in amber glass vials at 4°C. Under these conditions, the solutions were found to be stable for a minimum of 3 months.

2.4. Dosing and sample collection

A single 40 mg oral dose of fluoxetine (Prozac®, Eli-Lilly Australia, 2×20 mg capsules) was administered to 18 healthy young adult volunteers and blood samples were drawn at appropriate intervals until 624 h post-dose. Blood (10 ml) was collected by syringe and immediately transferred into lithium heparin tubes and gently mixed. Samples were centrifuged immediately at 2000*g* and the plasma transferred to 5 ml tubes and stored at −20°C until analysis. Drug-free plasma used during assay validation was collected from healthy volunteers and treated in an identical manner.

2.5. Sample assay

The solid-phase extraction procedure of Dixit et al. [11] was adapted and used for this procedure. Extractions were performed using either a Vac Elut SPS 24 Vacuum manifold (Varian, Sydney, Australia) or a purpose-built poly(vinyl chloride) extraction manifold 370 mm long×210 mm wide×135 mm high, fitted with a vacuum controller (Varian part number AI-122340-02). The manifold included a lid capable of accommodating up to 60 SPE cartridges and included a removable collector rack for holding up to 60 small glass tubes for eluant collection from the corresponding SPE cartridges. The extraction columns were conditioned by washing with methanol (2 ml) followed by phosphate buffer (pH 6.0; 0.1 *M*; 2 ml). To prevent drying of the columns, the vacuum source was disconnected before all of the buffer passed through the column bed. Plasma samples (1.0 ml) were dispensed into 5 ml polypropylene culture tubes. Internal standard solution (0.5 μg/ml, 100 μl) was added to the plasma samples, followed by phosphate buffer (pH 6.0; 0.1 *M*; 2 ml). The tubes were vortex mixed for 5 s and the samples decanted onto conditioned SPE cartridges. The samples were passed through the columns at low flow rates (<1 ml/min) by application of a moderate vacuum, and the columns were washed sequentially with the following solvents: methanol (2 ml), acetonitrile (2 ml), hexane–ethyl acetate (1:1, v/v, 2 ml). The columns were dried for 10 min by application of a moderate to strong vacuum (4–5 inches mercury) and the analytes were eluted from the column with 2 ml 2% ammonium hydroxide solution in dichloromethane–isopropanol (8:2, v/v). The eluant was passed through each column at a low flow rate (<1 ml/min), collected into clean 5 ml disposable glass culture tubes and decanted into clean 15 ml screw-cap Pyrex test tubes with PTFE-lined lids. Methanolic HCl (0.3 *M*, 20 μl) was added to each tube, which was then vortex mixed (5 s) and the solvent evaporated to dryness under a gentle stream of air. The residue was reconstituted in chloroform (100 μl).

For derivatization, trifluoroacetic anhydride (50 μl) was added and the tubes were capped and heated at 50°C for 30 min. The samples were allowed to cool and the reagents evaporated to dryness under a gentle stream of air. The residue was reconstituted in chloroform (100 μl) with vortex mixing (15 s) and this was transferred to a 250 μl glass insert contained in a 2 ml auto injector vial with a Teflon-faced rubber septum. An aliquot (1.0 μl) was injected directly onto the GC column and peak area ratios were determined electronically (HP Chemstation software, Hewlett-Packard).

2.6. Assay validation

Linearity of the assay was demonstrated for both FLX and NFX on three separate occasions by processing plasma standards in triplicate at seven separate concentrations over the range 1–100 ng/ml. Peak area ratios (FLX/NTP or NFX/NTP) were

plotted against FLX or NFX concentration and analyzed using weighted (1/concentration) least-squares linear regression. Intra-batch precision and accuracy were assessed by analysing six spiked plasma samples at each of three concentrations (nominally 5, 20 and 100 ng/ml) on one occasion, while inter-batch precision and accuracy were assessed from the results of quality control samples, at the same nominal concentrations, from 10 analytical batches performed on separate days. Measured concentrations were determined by application of the appropriate standard curve obtained on that occasion. Precision was assessed in terms of the relative standard deviation of the measured concentrations in a replicate set, while accuracy was determined from the mean relative error in a replicate set (i.e. difference between measured and nominal concentrations of the spiked samples).

Recovery of FLX and NFX from plasma was assessed by comparison of the slopes of calibration curves for FLX/NTP or NFX/NTP from extracted versus non-extracted samples. All samples were assayed in triplicate, and the internal standard was not extracted in both sample sets. The recovery of the internal standard was determined at the assay concentration in triplicate extracted versus non-extracted samples, with FLX serving as the unextracted reference standard. Specificity in relation to endogenous compounds was demonstrated by analysis of a series of randomly selected drug-free plasma samples ($n=10$). In addition, lignocaine, commonly used at the site of insertion of forearm venous cannulas, was also investigated for possible interference due to co-elution. The stability of FLX and NFX under conditions of storage and handling relevant to, and exceeding, those encountered in the conduct of clinical pharmacokinetic studies (6 h at room temperature and up to 617 days at −20°C, Tables 2 and 3) was also investigated.

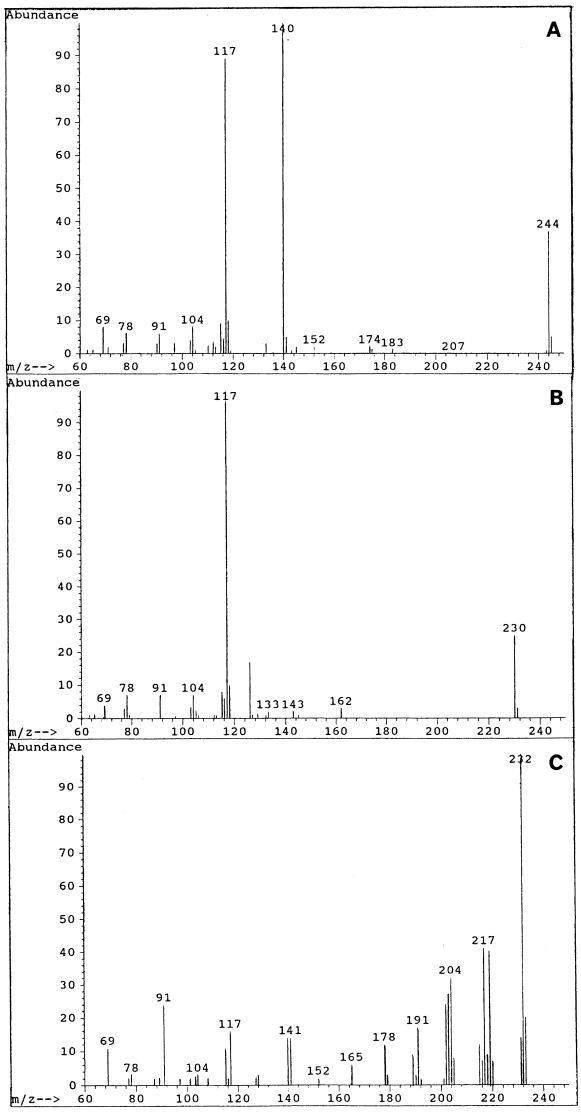

Fig. 2. Mass spectra of the trifluoroacetylated derivatives of (A) fluoxetine, (B) norfluoxetine and (C) nortriptyline.

3. Results and discussion

The mass spectra of the trifluoroacetylated derivatives of FLX, NFX and NTP are shown in Fig. 2. Reaction of fluoxetine with TFAA yielded the mono-*N*-trifluoroacetylated derivative, with a mass of 405.4. However, no parent ion was observed when full scan mass spectrometry was used to analyse this product (data not shown). The ion chosen for selected ion monitoring of trifluoroacetylated fluoxetine (m/z 244, M^+-161) was formed by the loss of the trifluoro-phenyl ether group. Cleavage of the corresponding bond of derivatised norfluoxetine resulted in the ion at m/z 230, also chosen for selected ion monitoring of this compound. Additional ions were observed at m/z 140 (FLX) and m/z 126 (NFX),

following cleavage of the CH_2–CH_2 bond. Both compounds gave ions at m/z 117, resulting from the alkyl-phenyl moiety of the molecules. This ion was also observed by Eap et al. [9] during mass spectral analysis of the enantiomers of both FLX and NFX following derivatization with (*S*)-(−)-*N*-trifluoroacetyl-propyl chloride.

The use of selected ion monitoring resulted in high signal-to-noise ratios for both compounds as evident in the total ion chromatogram of a plasma extract shown in Fig. 3B. Using the described chromatographic conditions, the retention times observed during the three separate occasions of linearity were: NFX, 6.9–7.2 min; FLX, 7.3–7.6 min; and NTP, 10.1–10.6 min. The intra-batch coefficients of variation for these retention times during the three occasions of linearity evaluation were <0.3% for each analyte. The assay was linear from 1 to 100 ng/ml with a typical calibration curve over this range producing a regression of $y = 0.012216x - 0.001446$; $r^2 = 0.9983$ (y = peak area ratio, x = concentration of analyte). The calibration range could easily be extended for assay of plasma samples collected at steady-state, but the upper limit of 100 ng/ml is substantially higher than the peak concentrations encountered following 40 mg single doses, which are usually below 50 ng/ml. Mean overall extraction recoveries for FLX, NFX and NTP were 91, 87 and 97%, respectively. These recoveries were considered acceptable given the requirement for multiple washes of the solid-phase extraction cartridges and subsequent elution and derivatization of the analytes. In contrast, Eap et al. [9] reported recoveries for the enantiomers of FLX and NFX using solvent extraction ranging from 50 to 66%. No interference from co-eluting endogenous compounds was observed from analysis of 10 drug-free plasma samples (Fig. 3A). Lignocaine showed no interference under the described conditions.

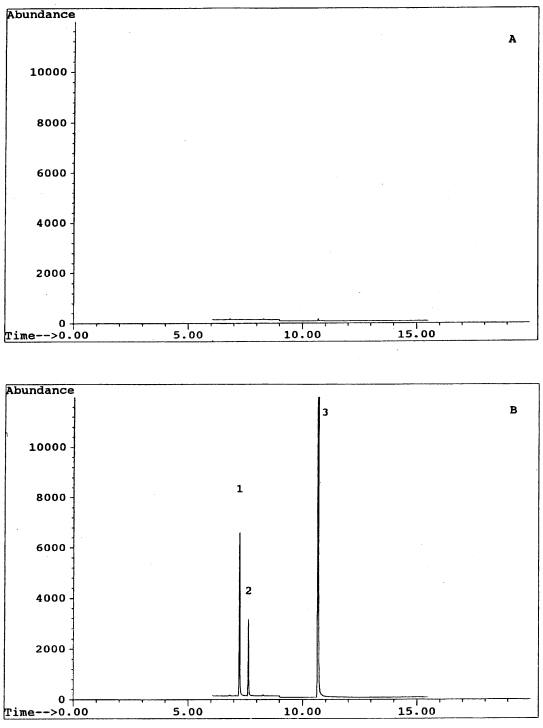

Fig. 3. Total ion chromatograms following analysis of (A) drug-free plasma and (B) plasma from a volunteer 48 h following ingestion of 40 mg fluoxetine. Peaks: 1 = norfluoxetine 31.5 ng/ml, 2 = fluoxetine 11.3 ng/ml and 3 = nortriptyline 50 ng/ml.

Intra-batch precision and accuracy of FLX and NFX were evaluated from assays of spiked samples ($n = 6$) at three concentrations, and are shown in Table 1. In all instances both precision and accuracy were well within 10%.

Inter-batch precision and accuracy of FLX and NFX were assessed from assays of quality control samples analyzed in conjunction with 10 analytical batches on separate days and are shown in Table 1. In all instances both precision and accuracy were within 7%. The limit of quantitation (LOQ), defined as the lowest concentration at which both accuracy and precision is within 20%, was deemed to be 1 ng/ml for each analyte during the use of this assay for pharmacokinetic studies. The data obtained for triplicate 1 ng/ml standards measured on three separate occasions (FLX: precision, 11.4%, accuracy, 10.0%; NFX: precision, 13.2%, accuracy 11.3%) provided results which easily satisfied these criteria, indicating that a somewhat lower LOQ may have been achievable if required. The mean signal-to-noise (peak–peak) ratio calculated over five separate analyses at 1 ng/ml was 25 for both FLX and NFX.

FLX, NFX and NTP (as their derivatives) were found to be stable in vials at three concentrations on the autoinjector carousel for at least 10 h (Table 2), with peak area ratios after this period differing by <3% from the initial observations. Additionally, FLX and NFX were shown to be stable in plasma at

Table 1
Intra- and inter-batch precision and accuracy for assay of FLX and NFX

	Nominal concentration (ng/ml)	Measured concentration (ng/ml)		Precision (%)		Accuracy (%)	
		FLX	NFX	FLX	NFX	FLX	NFX
Intra-assay[a]	5.0	4.73	4.70	2.7	5.5	5.3	6.4
	20	19.9	18.6	4.5	8.6	3.5	8.0
	100	104	101	5.6	5.7	5.8	4.1
Inter-assay[b]	5.0	4.94	4.95	2.3	3.2	2.1	2.5
	20	19.9	19.5	3.1	3.4	2.8	3.2
	100	99.3	95.5	7.0	3.8	5.1	4.5

[a]Results of six measurements of FLX and NFX obtained on one occasion.
[b]Results of single measurements of FLX and NFX obtained on 10 separate occasions.

Table 2
Stability of trifluoroacetylated derivatives for 10 h at room temperature on autoinjector carousel

Nominal concentration (ng/ml) FLX, NFX	Peak area ratio (FLX/NTP)				Difference (%)	
	Initial ($t=0$)		$t=10$ h			
	FLX	NFX	FLX	NFX	FLX	NFX
5.0	0.0602	0.0529	0.0617	0.0525	2.5	0.8
20	0.2326	0.2034	0.2300	0.1920	1.1	5.6
100	1.2101	1.0601	1.1830	1.0422	2.2	1.7

Mean±SD of duplicate measurements on three occasions ($n=6$).

room temperature for up to 6 h and also when subjected to three freeze–thaw cycles (data not shown). Analysis of spiked plasma samples which had been stored at −20°C for 617 days (Table 3) indicated differences of <11% over this period, with no evidence of matrix instability observed using this assay. This differs from the recent report of Zuccaro et al. [12], who, using a HPLC assay with UV detection, found significant levels of matrix degradation causing interfering peaks after only 21 days storage at −20°C.

Fig. 4 shows a plot of the mean FLX and NFX concentrations using data from 18 volunteers at each sample collection time. Analysis of subject plasma samples following a 40 mg dose provided the pharmacokinetic data as listed in Table 4. The relatively large variance for AUC_{0-t} for FLX was largely attributable to a single AUC result for one subject which was more than twice that of the greatest AUC result for the remaining 17 subjects. Omitting this abnormally high result from the data reduced the AUC result from 1627±1372.7 (mean±SD, $n=18$) to 1342.8±676.3 ($n=17$), effectively halving the standard deviation. This phenom-

Table 3
Stability of FLX and NFX following storage at −20°C for 617 days

Nominal concentration (ng/ml) FLX, NFX	Measured concentration (ng/ml)				Difference (%)	
	Initial ($t=0$)		$t=617$ days			
	FLX	NFX	FLX	NFX	FLX	NFX
5.0	5.34	4.96	5.11	5.25	4.3	5.8
20	21.5	20.6	20.8	22.7	3.3	10.2
100	109.3	98.8	100.9	103.2	7.7	4.4

Mean±SD of triplicate measurements.

Table 4
Pharmacokinetic parameters for FLX and NFX following administration of 40 mg fluoxetine

Drug	C_{max} (ng/ml)	t_{max} (h)	$AUC_{0-\infty}$ (ng/ml h)	K_{elim} (h^{-1})	$t_{1/2}$ (h)
FLX	32.73±3.45	3.08	1627.0±1372.7	0.021706±0.006625	37.69±21.70
NFX	23.19±6.97	60.15	6075.2±1866.4	0.006192±0.001382	117.27±25.78

Mean±SD, $n=18$. Median data for t_{max}.

enon has been observed previously in a bioequivalence study by Bergstrom et al. [13]. In that study, one individual, who was found to be a poor metabolizer of the sparteine type (i.e. CYP2D6), contributed largely to the abnormally broad range of AUC values observed in the study. The pharmacogenetics of the metabolism of fluoxetine requires further detailed study, preferably with the use of enantioselective analytical methods.

This assay has been used in our laboratory for the successful analysis of over 4000 plasma samples from bioequivalence studies. In this context, the use of nortriptyline as the internal standard posed no problems as the subjects were healthy volunteers taking no other drugs. If the method were to be used in populations of treated patients, care would have to be taken to ensure that no patients were co-medicated with nortriptyline. The use of a large capacity solid-phase extraction manifold allows a typical batch of approximately 55 samples to be prepared for GC–MS analysis in around 5 h.

In a study of the comparative pharmacokinetics of SSRIs, Baumann and Rochat [14] recently concluded that enantioselective analysis of FLX for therapeutic drug monitoring is of little relevance. Our preference for a non-enantioselective assay is also supported by the approach advocated by Karim [10], who has argued convincingly that enantioselective assays are required in bioequivalence studies only for chiral drugs in which the pharmacologically more active enantiomer undergoes the greater first-pass metabolism. There may be valid reasons for requiring an enantioselective method in other types of pharmacokinetic studies. The lack of necessity for enantiomeric analysis of FLX and NFX for bioequivalence studies allows the use of a simple derivatization procedure and expedites chromatographic manipulations and pharmacokinetic data analysis. This, combined with the above-mentioned reference of Karim [10] confirms our use of non-enantioselective analysis of FLX and NFX only.

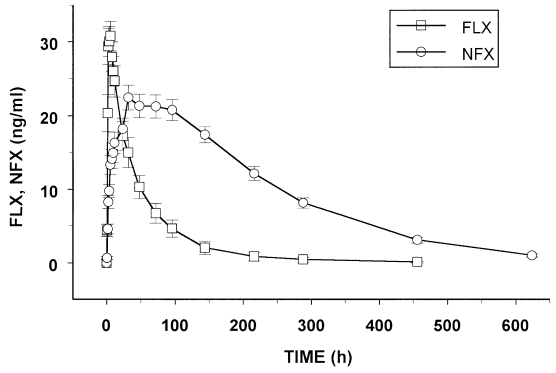

Fig. 4. Plasma concentration–time profile (mean±SEM) for fluoxetine and norfluoxetine following single oral 40 mg doses in 18 subjects.

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References

[1] R.W. Fuller, H.D. Snoddy, K.W. Perry, F.P. Bymaster, D.T. Wong, Biochem. Pharmacol. 27 (1978) 193.
[2] C.B. Eap, P. Baumann, J. Chromatogr. B 686 (1996) 51.
[3] P. Baumann, Clin. Pharmacokinet. 31 (1996) 444.
[4] I. Meineke, K. Schreeb, I. Kress, U. Gundert-Remy, Ther. Drug Monit. 20 (1998) 14.
[5] D.B. Goodnough, G.B. Baker, R.T. Coutts, J. Pharmacol. Toxicol. Methods 34 (1995) 143.
[6] P. Fontanille, N. Jourdil, C. Villier, G. Bessard, J. Chromatogr. B 692 (1997) 337.
[7] G.A. Torok-Both, G.B. Baker, R.T. Coutts, K.F. McKenna, L.J. Aspeslet, J. Chromatogr. 579 (1992) 99.
[8] B.D. Potts, C.J. Parli, J. Liq. Chromatogr. 15 (1992) 665.

[9] C.B. Eap, N. Gaillard, K. Powell, P. Baumann, J. Chromatogr. B 682 (1996) 265.
[10] A.J. Karim, Clin. Pharmacol. 36 (1996) 490–499.
[11] V. Dixit, H. Nguyen, V.M. Dixit, J. Chromatogr. 563 (1991) 379.
[12] P. Zuccaro, R. Pacifici, I. Altieri, A. Avenoso, M. Pellegrini, E. Spina, E. Perruca, S. Pichini, Ther. Drug Monit. 20 (1998) 20.
[13] R.F. Bergstrom, L. Lemberger, B.J. Cerimele, R.L. Wolen, J.C. Kisicki, Pharm. Res. 7 (1990) S251.
[14] P. Baumann, B. Rochat, Int. Clin. Psychopharmacol. 10 (1995) 15.