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Quantification of fluoxetine and norfluoxetine serum levels by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A rapid and sensitive high-performance liquid chromatography assay method was developed to determine serum fluoxetine and norfluoxetine levels by single extraction of 0.1 ml of serum with sodium hydroxide. The mobile phase (55% acetonitrile–45% distilled water containing 10 m*M* aqueous triethylamine) was used to separate fluoxetine and norfluoxetine (25–1000 ng/ml, using clomipramine as the internal standard) by ultraviolet detection at 226 nm. The inter- and intra-day variabilities of fluoxetine and norfluoxetine were 13–18%, and the recoveries of both drugs exceeded 89%. This assay method was applied to a pharmacokinetic disposition study of fluoxetine in mice. © 1997 Elsevier Science B.V.

Keywords: Fluoxetine; Norfluoxetine

1. Introduction

Fluoxetine hydrochloride is a novel selective serotonin reuptake inhibitor widely utilized in the treatment of depression. Marketed as a racemate, the ratio of the activities of the (*R*) and (*S*) enantiomers of fluoxetine are reported to be near unity [1]. Fluoxetine is extensively metabolized by the cytochrome P450 enzyme system to form its active metabolite, norfluoxetine [2,3]. The issue of chirality is also important for norfluoxetine, in that the (*S*) enantiomer of norfluoxetine is virtually equipotent to fluoxetine while the (*R*) enantiomer is relatively inert [4]. The percentage of (*S*) norfluoxetine varies among individuals. However, the percentage of the (*S*) enantiomer has been reported to constitute 60–75% of total serum norfluoxetine levels [5]. Norfluoxetine is known to undergo elimination-rate limited elimination in rats and humans [3,6] and subsequently, its biological half life is much longer than the parent compound (7–15 vs. 1–3 days, respectively) [6]. Therefore, the monitoring of serum norfluoxetine and fluoxetine levels would be beneficial in the clinical setting, especially at steady state, which occurs in humans about 1–2 weeks after initiation of fluoxetine therapy [7].

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Fluoxetine and norfluoxetine have been quantified by gas chromatography with electron-capture detection [3,8] or nitrogen–phosphorus detection [9] and liquid chromatography with fluorescence detection [10,11] or UV detection [12–15]. Furthermore, other methods have offered the advantage of simultaneously monitoring fluoxetine, norfluoxetine and tricyclic antidepressant serum levels [9,12]. Several of these previously published procedures involve lengthy, multiple step extraction procedures with long run times and relatively large sample volume requirements (1–2 ml). Therefore, a need exists for an high-performance liquid chromatography (HPLC) assay method which is suitable for the determination of total fluoxetine and norfluoxetine serum concentrations, that is simple, rapid and applicable to small sample volumes. We have developed an isocratic, reversed-phase HPLC method for the simultaneous quantitation of fluoxetine and norfluoxetine in serum utilizing clomipramine as the internal standard.

2. Experimental

2.1. Chemicals

Fluoxetine and norfluoxetine were kindly supplied by Eli Lilly (Indianapolis, IN, USA). Clomipramine hydrochloride, triethylamine and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol and hexane (all HPLC grade) were obtained from Baxter Healthcare (Muskegon, MI, USA) and phosphoric acid 85% from Mallinckrodt (Paris, KY, USA). Standard stock solutions of fluoxetine (1 μg/ml), norfluoxetine (1 μg/ml) and clomipramine (5 μg/ml) (all as free base) were prepared in methanol and stored at −20°C. All stock solutions were used within one month of preparation.

2.2. Chromatography

Chromatographic analysis was performed on a Shimadzu component system consisting of a LC-10AS pump, CR501 integrator, SPD-10AV UV–Vis detector (Shimadzu, Columbia, MD, USA) and an RH 7215 Rheodyne semi-auto manual injector. A Microsorb MV (Rainin, Woburn, MA, USA) octadecyl column (15×0.46 cm I.D., 5 μm) connected to a RP-C_{18} guard column (Brownlee, Santa Clara, CA, USA) was used in the assay. The mobile phase consisted of 55% acetonitrile and 45% distilled water containing 10 m*M* aqueous triethylamine, with the pH adjusted to 4.8 by dropwise addition of 85% phosphoric acid. The mobile phase was filtered via a Millipore (Milford, MA, USA) system and degassed. The flow-rate was set at 1.0 ml/min and the effluent was monitored for UV absorption at 226 nm.

2.3. Standard curve and extraction procedures

Standard curves were constructed using serum harvested from adult (C57BL/6×DBA/2)F_1 mice via cardiac puncture. Aliquots of the stock solutions of fluoxetine and norfluoxetine (each as 1 μg/ml in methanol) were added to 75×12 mm, disposable, snap-capped borosilicate glass tubes containing 0.1 ml of serum to achieve drug concentrations equivalent to 25, 50, 100, 250, 500 and 1000 ng/ml serum. Following addition of 20 μl of the internal standard (clomipramine, 5.0 μg/ml in methanol) and 100 μl of 5 *M* sodium hydroxide, the tubes were vortex-mixed for 30 s. Hexane (2 ml) was then added to each tube, vortexed for 30 s and centrifuged for 3 min at 3000 *g* (Heraeus Sepatech, Am Kalkberg, Germany). The organic layer was transferred into a fresh tube and dried under a gentle stream of nitrogen gas at 20°C using an N-Evap Evaporator (Organomation, Berlin, MA, USA). The resulting residue was reconstituted with 50 μl of mobile phase prior to injection. The samples were vortex-mixed for 30 s and an aliquot (20 μl) was injected into the chromatographic system. Standard curves were obtained daily by plotting the peak area ratios of the drug to internal standard against drug concentrations. For sample analysis, 0.1 ml of serum was subjected to extraction and drug concentrations were determined from the (unweighted) linear regression line of the standard data.

2.4. Recovery and variability studies

For the determination of extraction efficiency, aliquots (2.5, 5 and 10 μl, respectively) of the prepared methanolic stock solutions of fluoxetine and norfluoxetine were added separately to 0.1 ml of

serum to yield drug concentrations of 25, 100 and 1000 ng/ml for fluoxetine and norfluoxetine. Similarly, 25 and 100 μl of the prepared clomipramine stock solution was added to 0.1 ml serum to yield serum concentrations of 250 and 1000 ng/ml. The drugs were then extracted as described above. The peak areas of the extracted and non-extracted samples were compared. Recovery was determined as the mean (±s.d.) of four samples. Intra- and inter-day variabilities were determined at 25, 100 and 1000 ng/ml drug concentrations ($n=4$).

2.5. Animal study

To study the pharmacokinetic disposition of fluoxetine, adult (C57BL/6×DBA/2)F_1 mice (male and female, 2–4 months old, 20–30 g) were administered fluoxetine (10 mg/kg in saline) by tail vein injection ($n=4$ for each sampling time). Whole blood samples (~0.7 ml) were harvested by cardiac puncture at 0, 5, 15, 30 min and 1, 3, 6, 12, 24, 36 and 48 h post injection. Serum was collected and stored at −20°C until analysis. Serum drug concentrations were determined as the mean of duplicate samples.

3. Results and discussion

Representative chromatograms of extracted blank serum, serum spiked with fluoxetine and norfluoxetine, and a chromatogram obtained 12 h after intravenous administration of 10 mg/kg fluoxetine are shown in Fig. 1. Norfluoxetine, fluoxetine and clomipramine eluted with retention times of 4.0, 4.7 and 7.4 min, respectively. With a total run time of 10 min, there were no interfering peaks from serum constituents observed in this assay. Spiked drug concentrations were linearly related to the peak area ratios of drug vs. internal standard throughout the concentration range studied, with a correlation coefficient greater than 0.999 for fluoxetine and 0.998 for norfluoxetine. Typical equations obtained by least squared regression were $y=0.00058x-0.0079$ for norfluoxetine and $y=0.00075x+0.0086$ for fluoxetine. The retention factors were calculated to be 1.66 for norfluoxetine and 2.13 for fluoxetine.

Other previously published assay procedures have utilized serum sample volumes of 0.25 ml [13], 1 ml [10,11,14,15] and 2 ml [16,17]. Our assay was suitable for measuring serum drug concentrations of fluoxetine and norfluoxetine in situations where sample volume was limited (0.1 ml). Recoveries (Table 1) of norfluoxetine (>89%) and fluoxetine (>90%) were found to be greater than several previous methods which have utilized various extraction methods [11,14,15]. The inter-assay and intra-assay accuracy and precision values are given in Table 2, in which the coefficients of variation were less than 10.6% for all but the lowest concentration studied for fluoxetine and norfluoxetine.

A pH of 4.8 was used in this assay, which is similar to that found in other assays [14–16]. However, a lower pH (3.0–3.5) resulted in interfering peaks after drug extraction from serum. Several

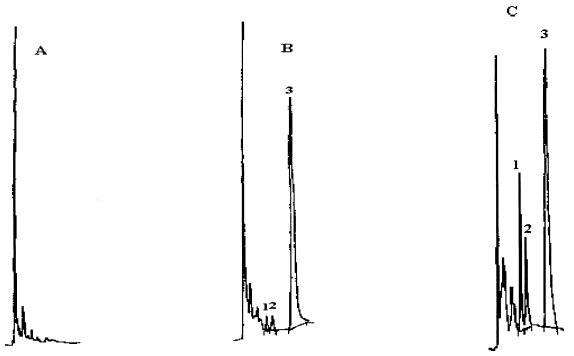

Fig. 1. Representative chromatograms of (A) extracted blank serum, (B) blank serum containing 25 ng/ml of fluoxetine and norfluoxetine, and (C) serum sample (fluoxetine=203.1 ng/ml; norfluoxetine=413.6 ng/ml) obtained 12 h after an i.v. injection of fluoxetine 10 mg/kg in (C57BL/6×DBA/2)F_1 mice. Peaks: 1=norfluoxetine (t_R=4.0 min); 2=fluoxetine (t_R=4.7 min); 3=clomipramine (t_R=7.4 min).

Table 1
Recovery of fluoxetine, norfluoxetine and clomipramine ($n=4$)

Drug	Concentration (ng/ml)	Recovery (mean±s.d.) (%)
Fluoxetine	25	90.7±3.2
	100	90.1±3.9
	1000	97.5±1.0
Norfluoxetine	25	92.5±11.2
	100	95.1±1.0
	1000	89.4±4.3
Clomipramine	250	97.1±1.4
	1000	96.8±2.6

Table 2
Intra-day and inter-day assay variability for fluoxetine and norfluoxetine

Theoretical concentration (ng/ml)	Actual concentration[a] (mean±s.d., n=4) (ng/ml)	
	Fluoxetine	Norfluoxetine
Intra-day variability		
25	22.8±3.1 (13.6)	25.8±3.9 (15.1)
100	101.1±3.56 (3.52)	95.8±9.5 (6.1)
1000	990.1±104.2 (10.6)	996.76±79.5 (7.9)
Inter-day variability		
25	27.6±4.3 (15.6)	26.4±4.9 (18.5)
100	96.7±8.4 (8.7)	91.5±8.5 (9.3)
1000	989.6±46.5 (4.7)	1006.2±54.3 (5.4)

[a] Values in parentheses are the percent coefficients of variation.

previous methods have employed a buffered mobile phase [13,14,17]. A buffered mobile phase was not used in this assay, eliminating the possibility of buffer salt precipitation, which may clog system tubing and irreversibly damage the packing of the column.

Pharmacokinetic data describing the disposition of fluoxetine and norfluoxetine in mice is limited despite the widespread use of this animal model in routine laboratory experiments [18]. The applicability of the assay method was demonstrated in a pharmacokinetic disposition study in which adult (C57BL/6×DBA/2)F_1 mice were intravenously injected with fluoxetine (10 mg/kg). However, this assay did not separate the enantiomers of fluoxetine or norfluoxetine. Fig. 2 shows the concentration time profile of fluoxetine and norfluoxetine after a single i.v. injection of fluoxetine (10 mg/kg). Fluoxetine disposition was best described by a bi-exponential equation as determined by the nonlinear least squares regression program WinNonlin (Scientific Consultants, Cary, NC, USA) and fluoxetine concentration at any time was described by the equation: $C=1178$ (ng/ml)·$e^{-0.032(h)\cdot t}+315$ (ng/ml)·$e^{-0.0008(h)\cdot t}$. Table 3 displays the pharmacokinetic parameters of fluoxetine (10 mg/kg) obtained after i.v. administration. The mean ratio of the metabolite to parent drug area under the concentration curve (AUC) was determined to be 2.4, which is comparable to the ratio found in rats [3]. As in rats [3] and humans [7], norfluoxetine elimination is limited by its formation from fluoxetine in mice (Fig. 2). In humans, monitoring serum norfluoxetine levels may become important at steady state since metabolite levels exceed

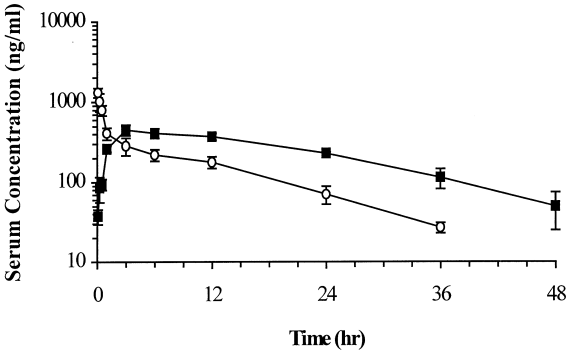

Fig. 2. Mean serum fluoxetine (open cirlces) and norfluoxetine (closed squares) concentration vs. time profiles following i.v. injection of fluoxetine (10 mg/kg) in (C57BL/6×DBA/2)F_1 mice.

Table 3
Pharmacokinetic parameters (mean±s.d.) of fluoxetine and norfluoxetine in (C57BL/6×DBA/2)F_1 after an i.v. injection of fluoxetine (10 mg/kg) (n=4)

Parameter	Fluoxetine	Norfluoxetine
V_β (l/kg)	28.8±4.3	N/A
Cl_s (min/ml/kg)	25.5±3.7	N/A
$T_{1/2,\lambda 1}$ (min)	21.1±2.7	N/A
$T_{1/2,\lambda 2}$ (h)	13.0±4.1	12.4±3.2
AUC (μg×min/ml)	391.1±81.2	928.1±157.7
C_{max} (ng/ml)	1493.3	451.1
T_{max} (h)	N/A	3.1

N/A denotes value not determined. V_β, volume of distribution; Cl_s, systemic clearance; $T_{1/2,\lambda 1}$, distributional half life; $T_{1/2,\lambda 2}$, dispositional half life; AUC, area under the drug concentration vs. time profile; C_{max}, maximal drug concentration, T_{max}, time to maximal drug concentration.

parent drug levels. Consequently, the high norfluoxetine levels may significantly augment the antidepressant action of fluoxetine.

In summary, a rapid and simple HPLC assay was developed to determine total serum fluoxetine and norfluoxetine concentrations when sample volume is limited, as is the case with mice.

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