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ORIGINAL RESEARCH

Determination of fluvoxamine in rat plasma by HPLC with pre-column derivatization and fluorescence detection using 4-fluoro-7-nitro-2,1,3-benzoxadiazole

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ABSTRACT: A sensitive, simple and reliable method using high-performance liquid chromatographic (HPLC) assay of fluvoxamine (FLU), a selective serotonin reuptake inhibitor (SSRI), in rat plasma after pre-column derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was developed in this study. Extracted plasma samples were mixed with NBD-F at 60°C for 5 min and injected into HPLC. Retention times of FLU and an internal standard (propafenone) derivative were 15.5 and 13.5 min, respectively. The calibration curve was linear over the range 0.015–1.5 μg/mL ($r^2 = 0.9985$) and the lower limits of detection and quantification of FLU were 0.008 and 0.015 μg/mL, respectively, in 100 μL of plasma. The derivative sample was stable at 4°C for 1 day. The coefficients of variation for intra-day and inter-day assay of FLU were less than 8.3 and 9.6%, respectively. Other SSRIs and centrally acting drugs did not interfere with the peak of the FLU derivative. The method was applied for analysis of the plasma samples from rats treated with FLU. These results indicate that the method presented is useful to determine the FLU levels in rat plasma of volumes as small as 100 μL and can be applied to pharmacokinetic studies. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: fluvoxamine; pre-column derivatization; 4-fluoro-7-nitro-2,1,3-benzoxadiazole; pharmacokinetic study

INTRODUCTION

In the past, tri- and tetracyclic antidepressant drugs such as imipramine, amitriptyline and mianserin have been widely used for the treatment of depression. After that, because of high cardiac toxicity, malin syndrome and other severe side effects, a new generation of compounds was developed with similar efficacy and fewer adverse effects (Kent, 2000). Fluvoxamine (FLU), 5-methoxy-1-[4-(trifluoromethyl)phenyl]-1-pentanone (*E*)-*O*-(2-aminoethyl)oxime, is one of several drugs developed as a selective serotonin reuptake inhibitor (SSRI; Benfield and Ward, 1986; Wakelin, 1986; Kent, 2000). It tends to be more frequently utilized for the treatment of depression than tri- and tetracyclic antidepressant drugs but it is structurally different from them. Under certain circumstances, it is important to determine FLU in biological fluids.

Several assays of FLU in plasma or serum were based on high-performance liquid chromatography (HPLC) with UV detection or gas chromatography with nitrogen phosphorus and mass spectrometric detections (Foglia *et al.*, 1989; Pommery and Lhermitte, 1989; Van der Meersch-Mougeot and Diquet, 1991; Hartter *et al.*, 1992; Gupta, 1993; Spigset *et al.*, 1995; Carrillo *et al.*, 1996; Eap *et al.*, 1996; Lacassie *et al.*, 2000; Duverneuil *et al.*, 2003; Lamas *et al.*, 2004; Suzuki *et al.*, 2004). However, the described methods required a large sample volume (0.7–2 mL; Foglia *et al.*, 1989; Pommery and Lhermitte, 1989; Van der Meersch-Mougeot and Diquet, 1991; Gupta, 1993; Spigset *et al.*, 1995; Carrillo *et al.*, 1996; Eap *et al.*, 1996; Duverneuil *et al.*, 2003). The pharmacokinetic study of FLU was shown by HPLC analysis after sampling 7 mL of blood in psychiatric patients (Suzuki *et al.*, 2004). The utilized detectors [fluorichrom detection (Pommery and Lhermitte, 1989), photodiode-array UV detection (Duverneuil *et al.*, 2003) or mass spectrometry (Lamas *et al.*, 2004)] and separation system [column-switching technique and gradient pump control system (Hartter *et al.*, 1992)] were too complicated, specialized and/or expensive for routine application. Although a micellar electrokinetic capillary chromatographic procedure was recently reported to measure FLU levels in blood and urine (Labat *et al.*, 2002), the sensitivity and blood sample (1 mL) were too poor and large, respectively, to study its disposition kinetics. There is much scope

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Abbreviations used: FLU, fluvoxamine; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; SSRI, selective serotonin-reuptake inhibitor.

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for improvement in the procedures described above, because to obtain a large sample volume usually causes pain in the patients and complicated equipment is not cost-effective.

When the pre-column derivatization of FLU was performed using dansyl chloride as a fluorescent reagent (Pullen and Fatmi, 1992; Suckow *et al.*, 1992), a large sample volume (1 mL) was required. 4-(*N*-chloroformylmethyl-*N*-methyl)-amino-7-nitro-2,1,3-benzoxadiazole as the reagent was adopted to measure levels of fluoxetine, another SSRI, and examine the disposition kinetics in rat plasma as small as 100 μL (Guo *et al.*, 2003). However, derivatization was time-consuming (2 h at 60°C). Thus, previous derivatization methods contain problems in terms of sample volume and speed.

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was synthesized as a fluorescent reagent by Imai *et al.*, and selectively reacts toward the primary or secondary amino group (Watanabe and Imai, 1981, 1983; Imai, 2003). Simultaneous determination of amino acids as well as amino glycoside was possible using NBD-F (Watanabe and Imai, 1981, 1983; Honda *et al.*, 2000; Aoyama *et al.*, 2004; Fukushima *et al.*, 2004). However, the procedure has not been applied for the determination of exogenous compounds in biological fluids.

In the present study, we investigated the quantitative determination of FLU in 100 μL of rat plasma by isocratic HPLC coupled with fluorescent detection using NBD-F as a labeling reagent according to the reaction shown in Fig. 1. Afterwards, we examined the disposition kinetics of FLU in rats using our HPLC method.

MATERIALS AND METHODS

Reagents. FLU maleate, NBD-F, fluoxetine hydrochloride, imipramine hydrochloride, norfluoxetine hydrochloride, paroxetine hydrochloride, sertraline hydrochloride, acetonitrile and general reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). Propafenone hydrochloride, amitriptyline hydrochloride, amoxapine, carbamazepine, clomipramine hydrochloride, desipramine hydrochloride, droperidol, ethosuximide, haloperidol, maprotiline hydrochloride, nortriptyline hydrochloride, phenytoin, primidone, protriptyline hydrochloride and spiperone were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Equipment. The HPLC system comprised a model L-6200 pump (Hitachi, Tokyo, Japan), a Rheodyne injection valve (Cotati. CA, USA) with a 20 μL loop and a model RF-10A fluorometer (Shimadzu, Kyoto, Japan) operating at an excitation wavelength of 470 nm and an emission wavelength of 540 nm. The HPLC column (Kanto Chemical, Tokyo, Japan) was 150 × 4.6 mm i.d. with 5 μm particles of C_{18} packing material. Quantification of the peaks was performed using a Chromatopac Model CR-8A integrator (Shimadzu, Kyoto, Japan). The mobile phase was prepared by the addition of acetonitrile (600 mL) to a solution of 400 mL containing trifluoroacetic acid (0.1% v/v) in water. The samples were eluted from the column at 25°C at a flow rate of 1.0 mL/min.

Extraction from plasma. Control plasma was collected from rats. A 100 μL aliquot of plasma sample was rendered alkaline by the addition of NaOH (4 M, 100 μL). Propafenone hydrochloride solution in water (25 μM, 100 μL) was added as an internal standard (IS) to produce the standard curve for FLU. Then the mixture was vortexed for 1 min and extracted with freshly distilled *n*-hexane (3 mL, twice). Each *n*-hexane

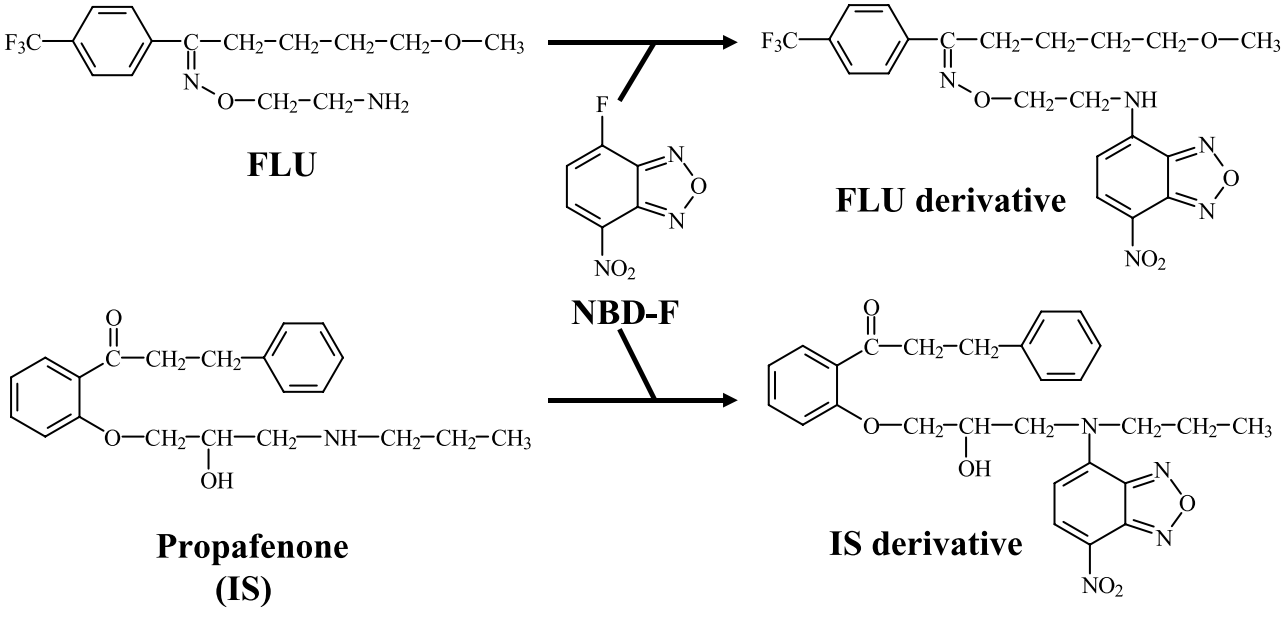

Figure 1. Derivatization scheme for FLU and IS with NBD-F.

phase was mixed and evaporated, and the derivatization was performed as follows.

Derivatization. Borate buffer (0.1 M) containing ethylenediaminetetraacetic acid, disodium salt (1 mM) was adjusted to pH 8.0 by the addition of NaOH. Borate buffer (300 μL) was added to the residue. NBD-F solution in acetonitrile (25 mM, 100 μL) was added and vortexed. The mixture was allowed to react for 5 min at 60°C. Then it was set on ice for 5 min to stop the derivatization reaction before HCl (0.05 M, 400 μL) was added. Derivative samples (20 μL) were injected into the HPLC system. NBD-F solution was prepared weekly in acetonitrile (100 mM), stored at −20°C as a stock solution and diluted with acetonitrile before use.

Calibration curve. The solution of FLU in water (1 mM) was added to drug-free plasma from rats. The range of concentrations of FLU was varied: 0, 0.015, 0.03, 0.075, 0.15, 0.3, 0.75 and 1.5 μg/mL. All samples were extracted and analyzed using the procedures described above. The calibration curve based on the peak area ratios of FLU to I.S. was analyzed in duplicate for each sample.

Animal study. Male Wistar rats (9–10 weeks, 272 ± 11 g; mean ± SD, Sankyo Laboratory Animals, Toyama, Japan) were used in the pharmacokinetic study. Rats were divided into three groups with each group receiving the dose of FLU maleate by a specific route (8 mg/kg i.v.; 8 mg/kg i.p.; 16 mg/kg p.o.). The drug was administered i.v. via the left jugular vein. After administration, the rats were unrestrained and freely able to take water but not food. Under light anesthetization by diethyl ether, blood samples (about 0.2 mL) were withdrawn with heparinized syringes from the right jugular vein at designated time intervals (0.03, 0.25, 0.5, 1, 2, 4 and 6 h) for i.v., i.p. or (1, 1.5, 2, 3, 4, 5 and 6 h) p.o. administration by a separate venous puncture and collected in tubes. Blood samples were centrifuged (1600 ***g***, 5 min) to obtain the plasma. In the same manner, drug-free pooled plasma samples were obtained from rats. The plasma samples were immediately frozen and stored at −20°C until assayed.

Pharmacokinetic and statistic analysis. Pharmacokinetic parameters of FLU after i.v., i.p. or p.o. administration were estimated by the moment analysis. The areas under the plasma concentration–time curves from zero to 6 h ($AUC_{i.v.}$, $AUC_{i.p.}$ or $AUC_{p.o.}$) were calculated using the linear trapezoidal rule. The absolute bioavailability after i.p. and p.o. administration was calculated from the mean values of the dose-adjusted $AUC_{i.v.}$ or $AUC_{i.p.}$ and $AUC_{i.v.}$ or $AUC_{p.o.}$, respectively. The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were determined from the actual data obtained after i.p. or p.o. administration. Data are expressed as the mean ± SD.

RESULTS AND DISCUSSION

Derivatization of FLU and IS with NBD-F

For the time course study, the reaction time was set at 1, 2, 3, 5, 7 and 10 min. FLU (0.3 μg/mL) and IS (10 μg/mL) in borate buffer were derivatized as described. The derivatization of FLU and IS reached each plateau level at 5 min (data not shown). Thus, a derivatization time of 5 min was chosen for a complete reaction in this study.

Chromatogram

Figure 2 shows the chromatograms obtained from (A) drug-free plasma, (B) plasma spiked with FLU and IS and (C) plasma at 2 h after the oral administration of FLU to rats (16 mg/kg). The retention times of FLU and IS derivatives were 15.5 and 13.5 min, respectively. The derivatized analytes were well separated from each other and from a large system peak, which was apparently produced by NBD-F hydrolysis. No interfering peaks from endogenous substances were observed in the chromatograms.

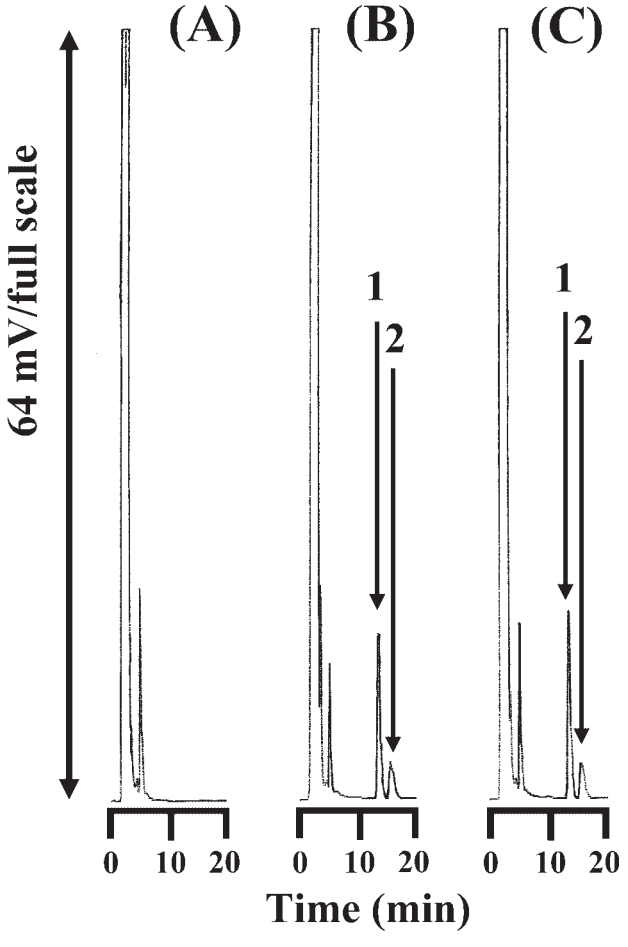

Figure 2. Chromatograms of fluorescence derivatives of FLU and IS from extracted rat plasma samples. (A) Drug-free plasma; (B) plasma spiked with FLU (0.075 μg/mL) and IS (10 μg/mL; peaks—**1**, IS derivative, **2**, FLU derivative); (C) plasma at 2 h after p.o. administration of FLU (16 mg/kg) to rats. The attenuation for all chromatograms is 64 mV/full scale.

Standard curve of FLU

The standard curve of FLU was constructed by plotting integrated peak area ratios of FLU to IS vs. FLU concentrations. Linearity was displayed for FLU ($y = 0.8691x + 0.0093$) concentrations ranging from 0.015 to 1.5 μg/mL. Square regression coefficients (r^2) of FLU were 0.9985. The lower limit of detection for FLU was established at 0.008 μg/mL (signal-to-noise ratio of 3:1). Previous methods using large sample volumes (0.7–2 mL) reported detection limits in the range 0.0005–0.025 μg/mL (Foglia *et al.*, 1989; Pommery and Lhermitte, 1989; Van der Meersch-Mougeot and Diquet, 1991; Pullen and Fatmi, 1992; Suckow *et al.*, 1992; Gupta, 1993; Spigset *et al.*, 1995; Carrillo *et al.*, 1996; Eap *et al.*, 1996; Lacassie *et al.*, 2000; Duverneuil *et al.*, 2003; Lamas *et al.*, 2004; Suzuki *et al.*, 2004). Our detection limit was in this range but used a much smaller sample volume. Automated determination of FLU in the same plasma volume (100 μL) by column-switching HPLC (Hartter *et al.*, 1992) showed the detection limit (0.01 μg/mL), suggesting that our assay system was slightly more sensitive. The HPLC assay described here proved sufficient for the study of FLU pharmacokinetics in rats. The method should also be suitable for a therapeutic drug monitoring of FLU in patients since the expected levels are greater than 50 ng/mL (Van der Meersch-Mougeot and Diquet, 1991).

Stability of FLU derivative

The storage stability of the FLU derivative was examined by analysis of derivative samples containing known amounts of analytes stored at 4°C for 1 day ($n = 3$). The stabilities of the FLU derivatives at 0.03 and 0.3 μg/mL were within the range 101–109% (average 105%) and 100–108% (average 104%), respectively. These data indicate that derivative samples are stable at 4°C for 1 day.

Precision and accuracy

Precision and accuracy for intra-day and inter-day assays of FLU derivative are shown in Table 1. In the intra-day assay, the range of standard deviation to the average of FLU was within 3.3–8.3%. These recoveries of FLU were within 99.3–104.0%. In the inter-day assay, the range of standard deviations for average FLU was within 3.4–9.6%. These recoveries of FLU were within 97.3–104.7%.

Table 1. Intra- and inter-day assay reproducibility for determination of FLU

Concentration (μg/mL)	Measured (μg/mL) (mean ± SD, $n = 4$)	CV (%)	Recovery (%)
Intra-day assay			
0.015	0.0156 ± 0.0013	8.3	104.0
0.15	0.149 ± 0.009	6.0	99.3
1.5	1.53 ± 0.05	3.3	102.0
Inter-day assay			
0.015	0.0157 ± 0.0015	9.6	104.7
0.15	0.152 ± 0.010	6.6	101.3
1.5	1.46 ± 0.05	3.4	97.3

Interference

As FLU is frequently given together with other SSRIs and centrally acting drugs, the potential interference from a number of such drugs was investigated. In this study, run time was prolonged up to 60 min. The retention times of these compounds are shown in Table 2. None of the compounds were found to pose a problem. When FLU and other compounds (not detected drugs) were co-administered, the determination of FLU levels was performed. However, co-administration of FLU with compounds exhibiting longer retention time than FLU derivative resulted in late eluting peaks overlapping with peaks in the next sample. In that situation, the time advantage for the resolution was lost. The low concentration of co-administered drug in plasma may not interfere with the detection of the FLU derivative. As for the application of our method in a clinical setting, further studies are needed on the concentration-dependent interference of tested or other drugs on the detection of FLU derivative.

Pharmacokinetic study of FLU in rats

The HPLC method was used to analyze plasma samples after a single i.v. (8 mg/kg, $n = 4$), i.p. (8 mg/kg, $n = 4$)

Table 2. Retention times of other SSRIs and centrally acting drugs tested for interference with FLU derivative

Compounds	Retention time (min)
FLU	15.5
Norfluoxetine	20.5
Paroxetine	20.6
Fluoxetine	22.3
Protriptyline	22.9
Nortriptyline	26.0
Maprotiline	27.1
Sertraline	59.8
Amitriptyline	ND
Amoxapine	ND
Carbamazepine	ND
Clomipramine	ND
Desipramine	ND
Droperidol	ND
Ethosuximide	ND
Haloperidol	ND
Imipramine	ND
Primidone	ND
Phenytoin	ND
Spiperone	ND

ND, not detected.

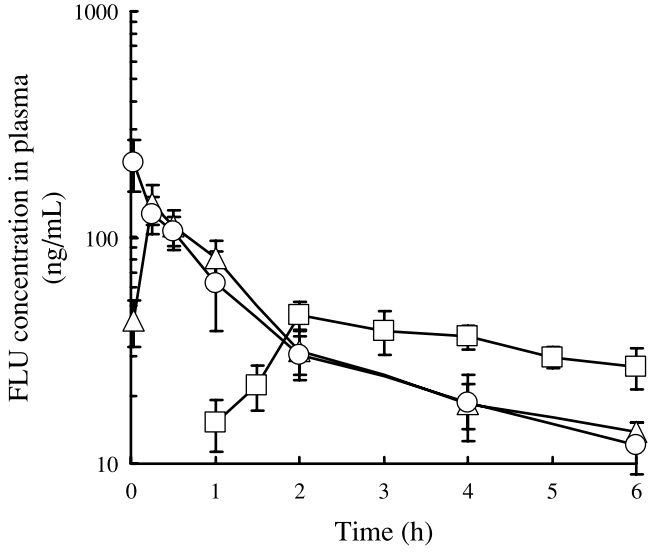

Figure 3. Plasma concentration-time courses of FLU after a single i.v., i.p. or p.o. administration of FLU maleate to rats. (○) i.v. administration (8 mg/kg, $n = 4$); (△) i.p. administration (8 mg/kg, $n = 4$); (□) p.o. administration (16 mg/kg, $n = 6$). Each point represents the mean ± SD of four to six rats.

Table 3. Pharmacokinetic parameters of FLU in rats

	Administration route		
	i.v. ($n = 4$)	i.p. ($n = 4$)	p.o. ($n = 6$)
Dose (mg/kg)	8	8	16
AUC (ng h/mL)	242 ± 60	239 ± 67	180 ± 38
Bioavailability (%)	(—)	98.8	37.6
T_{max} (h)	(—)	0.250	2.55 ± 0.55
C_{max} (ng/mL)	(—)	142 ± 28	49.0 ± 4.3

(—) Not calculated.

or p.o. (16 mg/kg, $n = 6$) administration of FLU maleate to rats. As shown in Fig. 3, concentration vs. time profiles were constructed for up to 6 h for the analytes. The pharmacokinetic parameters of FLU are listed in Table 3. The values of $AUC_{i.v.,}$ $AUC_{i.p.}$ and $AUC_{p.o.}$ were 242 ± 60, 239 ± 67 and 180 ± 38 ng h/mL, respectively. The absolute bioavailability value after i.p. and p.o. administration was 98.8 and 37.6%, respectively. After i.p. administration, the values of T_{max} and C_{max} were 0.250 h and 142 ± 28 ng/mL, respectively. On the other hand, after p.o. administration, the values were 2.55 ± 0.55 h and 49.0 ± 4.3 ng/mL. The $AUC_{i.p.}$ of FLU was consistent with the value reported in the literature (Van der Meersch-Mougeot and Diquet, 1991), which consists of only one report on the pharmacokinetic study of FLU in rats, to our knowledge.

CONCLUSION

The HPLC method by fluorescent derivatization of FLU with NBD-F is useful for the sensitive, simple, rapid and reproducible determination of FLU levels in rat plasma. The procedure, using a plasma volume as small as 100 μL, can be applied to the pharmacokinetic study of FLU in various animals. We expect the method to also be suitable for therapeutic drug monitoring of FLU in patients.

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