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Rapid and sensitive bioanalytical method for measurement of fluvoxamine in human serum using 4-chloro-7-nitrobenzofurazan as pre-column derivatization agent: Application to a human pharmacokinetic study

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Abstract

A sensitive and rapid high-performance liquid chromatographic method for the analysis of fluvoxamine, a selective serotonin reuptake inhibitor in human serum, is described using 4-chloro-7-nitrobenzofurazan as pre-column derivatization agent. The drug and an internal standard (fluoxetine) were extracted from 0.25 mL of serum using ethyl acetate as extracting solvent and subjected to pre-column derivatization by the reagent. A mobile phase consisting of methanol and sodium phosphate buffer (0.05 M; pH 2.8) containing 1 mL/L triethylamine (72:28 v/v) was used and chromatographic separation was performed on a Shimpack CLC-C18 (150 mm × 4.6 mm) column. The fluorescence derivatives of the drugs were monitored at excitation and emission wavelengths of 470 and 537 nm, respectively. The calibration curve was linear over the concentration range of 0.5–240 ng/mL with a limit of quantification (LOQ) of 0.5 ng/mL using 0.25 mL serum sample. The method validation was performed for its selectivity, specificity, sensitivity, precision and accuracy. In this method, which was applied in a randomized cross-over bioequivalence study of two different fluvoxamine preparations in 24 healthy volunteers, the sensitivity and run time of analysis were significantly improved.

Keywords: HPLC; Fluvoxamine; Serum; 4-Chloro-7-nitrobenzofurazan; NBD-Cl; Derivatization

1. Introduction

Fluvoxamine (FL) 5-methoxy-4(trifluoromethyl) valerophenone (E)-*O*-(2-aminoethyl) oxime maleate is a selective serotonin reuptake inhibitor which has been used for the treatment of depression with fewer adverse effects compared to the tricyclics [1]. The drug is well absorbed with time to peak plasma concentration of about 2–8 h, bioavailability of more than 90% and elimination half-life of about 18 h. FL undergoes extensive metabolism in the liver to produce a number of inactive metabolites [2]. In common with other antidepressants the role of therapeutic drug monitoring of FL in management of depressive patients is unknown [1], however, quantification methods of the drug in pharmacokinetic studies need to be sensitive and specific. There are several analytical techniques in the literature for quantification of the drug in pharmaceutical preparations and biological fluids. FL has been measured in the pharmaceutical dosage forms using capillary gas chromatography (GC) [3,4], capillary electrophoresis (CE) [5] and spectrophotometeric [6] methods. In the biological fluids, however, analysis of the drug has been reported using high-performance liquid chromatography (HPLC) coupled with UV [7–12], fluorescence [13–15] or visible [16] detectors. Determination of the drug in serum samples, using HPLC with UV detection with different sensitivities (10 ng/mL [7,8] and 25 ng/mL [9–11]) have been reported. Low-blood concentrations are achieved following single dose administration of the drug, thus, more sensitivity is needed in single dose human pharmacokinetic studies. A column switching HPLC-UV method (LOQ 0.8 ng/mL), using 1.5 mL serum sample and volume injection of 500 μL, has been reported [12]. In this method, however, toxic solvents (toluene–chloroform) has

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been used for drug extraction; sample preparation is tedious and time-consuming and long run time of analysis (25 min) has been reported. Different fluorogenic reagents including fluorescamine [13], dansyl chloride [14], 4-fluoro-7-nitrobenzofurazan (NBD-F) [15] and 1, 2-naphthoquinone-4-sulphonic acid [16] have been used to improve sensitivity of the assay. LOQ of 10 ng/mL has been reported using derivatization of 1 mL serum samples with either fluorescamine [13] or dansyl chloride [14], however, using of fluorescamine and dansyl derivatives are limited by their poor photo stability and band broadening of the resulted peaks. In pre-column derivatization of the drug with 2-naphthoquinone-4-sulphonic acid [16] sensitivity of 5 ng/mL and analytical run time of about 6 min have been reported. In this method, however, 30 min incubation of the reaction mixture and time-consuming multi steps extraction of the resulted derivative are needed. Pre-column derivatization of the drug using NBD-F as labeling agent has been reported by Higashi et al. [15]. Although derivatization of the drug has been achieved during 10 min yet in their method, which has not been tested in human studies, poor sensitivity (15 ng/mL using 100 μL serum sample) and long run time of analysis (18 min) have been reported. Present study describes a new, fast and sensitive method using NBD-Cl as fluorogenic agent. The procedure presented here, which has been approved in a bioequivalence study of two different FL preparations, is very sensitive with LOQ of 0.5 ng/mL using 250 μL serum sample and 20 μL injection, and fast with analytical run time of 6 min.

2. Experimental

2.1. Chemicals

FL (purity 99.7%) was from Neolux (England, UK) and was kindly provided by Bakhtar Bioshimi pharmaceutical company (Kermanshah, Iran). Fluoxetine (I.S.) was from Sigma (St. Louis, MO, USA). All reagents used were of analytical-grade except methanol which was HPLC grade and purchased from Merck (Darmstadt, Germany). Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Standard solutions

A stock solution of FL (1000 μg/mL) was prepared in methanol. Working standards of the drug (1.25–600 ng/mL) were prepared by serial dilution of the stock solution in methanol. Working standard solutions of the I.S. (1.2 μg/mL) and NBD-Cl (5 mg/mL) were prepared in methanol. A borate buffer (0.05 M) was prepared in water and adjusted to pH 8.0 with 0.05 M potassium hydroxide solution. All solutions were stored at 4 °C and were stable for at least 3 weeks.

2.3. Calibration curve, sample preparation and derivatization

Pooled blank human serum was used for construction of the calibration curve. After evaporation of 100 μL from each working solution of the drug, under a gentle stream of nitrogen at 50 °C, the residues were reconstituted in 250 μL of drug-free human serum. In an Eppendorf tube 250 μL serum samples (blank, calibration or unknown), 100 μL of the I.S. and 1 mL ethyl acetate were added. After briefly mixing for 20 s on a vortex mixer and centrifugation for 3 min at 12000 × *g*, the organic phase was removed, transferred into a 100 × 16 mm disposable glass tube and evaporated to dryness under a stream of nitrogen at 50 °C. To the residue 125 μL of the NBD-Cl solution, 100 μL dichloromethane and 25 μL of the borate buffer were added and after a brief mixing for 10 s on a vortex mixer, the samples were kept at 60 °C for 5 min. The NBD-Cl derivatives were then analyzed by injection of a 20 μL volume of the reaction mixture onto the chromatographic column.

2.4. Equipment

The HPLC system used consisted of two pumps of Shimadzu LC-10A solvent delivery system, a system controller (SCL 10AD), a spectroflurometric detector (RF-551) operated at excitation and emission wavelengths of 470 and 537 nm, respectively, a column oven (CTO-10A), a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. The analytical column was a Shimpack CLC-ODS (Shimadzu, Kyoto, Japan), 150 mm × 4.6 mm ID., 5 μm particle size, which was protected by a Shim-pack G-ODS guard column (1 cm × 4.0 mm I.D., 5 μm particle size). A mixture of methanol and sodium phosphate buffer (0.05 M; pH 2.8) containing 1 mL/L triethylamine (72:28 v/v) was used as the mobile phase. The column oven temperature was set at 58 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 2.0 mL/min.

2.5. Optimization of the derivatization conditions

Solutions of 1, 50 and 200 μg/mL of the drug were used to optimize derivatization of FL with NBD-Cl while the I.S. was reacted with the reagent at the concentration of 1.2 μg/mL. Concentrations of the NBD-Cl solutions ranging from 0.5 to 10 mg/mL, pH of the buffer solutions ranging from 6 to 12 and pH of the mobile phase ranging from 2.2 to 7 were tested to obtain optimal conditions for analysis. The polarity of the reaction solution was optimized using various organic solvent–water proportions, ranging from 1:1 to 10:1, and the reaction was allowed to proceed in a water bath at temperature ranging from 40 to 80 °C. Different organic solvents including acetone, ethyl acetate, dichloromethane, acetonitrile and chloroform were used to increase the yield of the reaction.

2.6. Method validation

Specificity of the method was tested by the analysis of 24 human blank serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure without the addition of the I.S. to ensure the absence of endogenous substances with the same retention times as the analytes of interest. Average recoveries of the extraction procedure for both FL and the I.S. were estimated by comparing the

peak areas obtained from derivatization of an extracted spiked blank sample with those obtained from derivatization of the similarly treated standard. The limit of detection (LOD) was defined as a peak height that produces three times of baseline noise, and LOQ was estimated as the lowest concentration that could be quantified with a coefficient of variation of less than 20%. Inter-day variation was measured by assessing the different controls in replicates of six. Intra-day variation was based on repeated analysis of the same concentration controls in 10 analytical runs performed on 10 consecutive days. The selectivity of the assay was verified by checking for interferences with a group of drugs that might be co-administered.

2.7. Application of the method

The present method was applied in a randomized cross-over bioequivalence study of two different FL preparations. Twenty-four male healthy volunteers aged 27.2 ± 3.1 years and weighing 67.7 ± 8.3 kg with normal biochemical parameters were enrolled in this study. All the subjects received a single oral dose of 100 mg FL from either Bakhtar Bioshimi (Kermanshah, Iran) or Solvay Healthcare Ltd. (Ireland) pharmaceutical companies on two working days separated by a wash-out period of 3 weeks. All the subjects were asked to refrain from food or water consumption for 3 h after drug administration. Blood sampling were carried out at suitable intervals up to 48 h and pharmacokinetic parameters including maximum concentration (C_{max}), area under the concentration time curve from zero to time of last sampling (AUC0 − t) and area under the concentration time curve from zero to infinity (AUC0–∞) were compared. Student's t-test was used for statistical analysis of the data and statistical significance was defined at the level of $P < 0.05$.

3. Results

3.1. Chromatographic separation

Typical chromatograms of (A) human blank serum (B) human blank serum spiked with FL (1.5 ng/mL) and the I.S. are presented in Fig. 1. The retention times for FL and the I.S. were 4.1 and 5.1 min, respectively. Endogenous components and excess of the reagent were chromatographed within 2.5 min and the

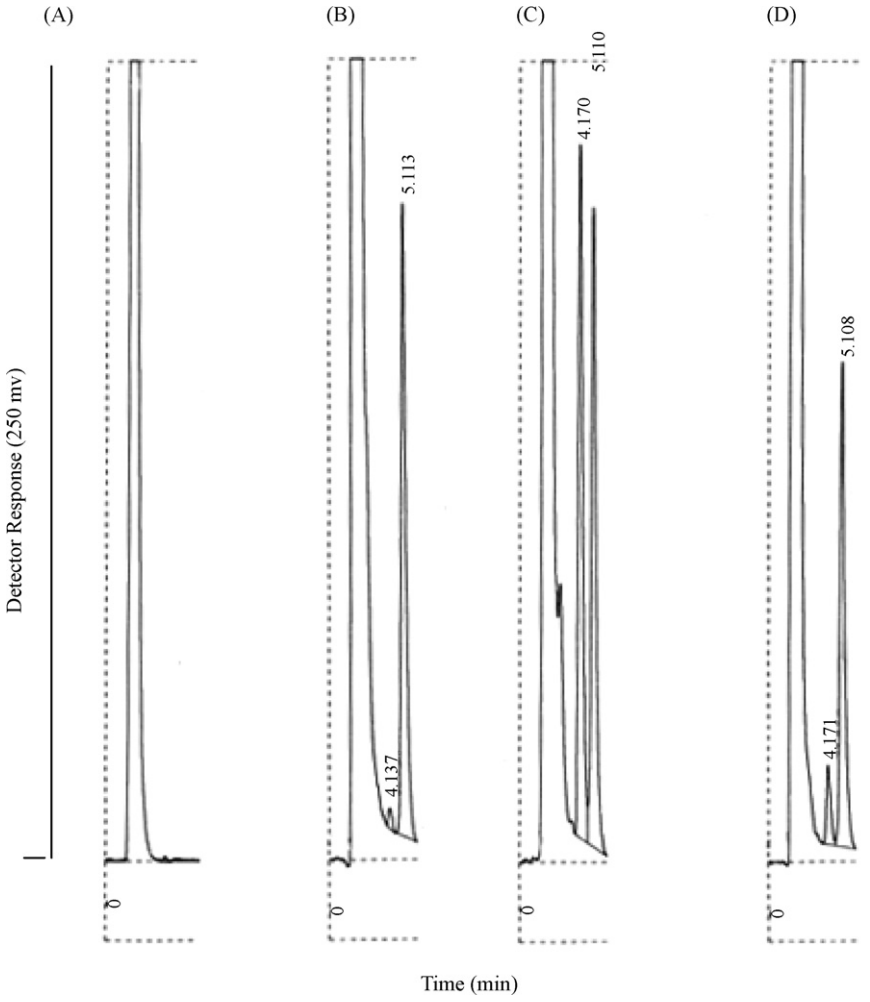

Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum; (B) human blank serum spiked with 1.5 ng/mL fluvoxamine and the I.S; and (C) and (D) serum samples obtained at 5 and 48 h after a single oral dose of 100 mg of the drug from a healthy volunteer containing 36.6 and 5.9 ng/mL of the drug, respectively. Peaks eluted at 4.1 and 5.1 min correspond to fluvoxamine and the I.S., respectively.

Table 1
Assay linearity for analysis of FL in human serum by the HPLC method

	Correlation coefficient of the linear regression analysis[a] ($r \pm$ SD)	Slope (b) (mean ± SD)	Intercept (a) (mean ± SD)
Inter-day reproducibility ($n = 4$)	0.996 ± 0.010 (C.V. = 0.1%)	3.05 ± 0.11 (C.V. = 4.8%)	0.652 ± 0.0.214 (C.V. = 14.2%)
Intra-day reproducibility ($n = 10$)	0.996 ± 0.011 (C.V. = 0.1%)	3.17 ± 0.18 (C.V. = 3.9%)	0.743 ± 0.2241 (C.V. = 13.5%)

r = correlation coefficient.
[a] Linear weighted regression, formula: $y = bx + a$.

peaks of analytes were well resolved from each other. Fig. 1C and D show the chromatograms of serum samples obtained at 5 and 48 h, respectively, after a single oral dose of 100 mg FL from a healthy volunteer.

3.2. Optimization of derivatization reaction

The reaction of NBD-Cl with the drug appeared to be highly dependent on pH of buffer solution, time, temperature, concentration of the labeling agent and polarity of the medium. NBD-Cl reacts with FL in alkaline medium and the reaction efficiently proceeds in the presence of dichloromethane within 5 min. In our method, maximal yield of the derivative was obtained with a NBD-Cl solution of 5 mg/mL, a buffer with pH of 8.0, a reaction temperature of 60 °C for 5 min and a reaction medium containing the buffer–acetonitrile and dichloromethane (1:5:4 v/v).

3.3. Validation of the method

The results of the selectivity study showed that there were no interfering peaks from any of the following drugs: citalopram, amitriptyline, sertraline, imipramine, desipramine, nortriptilyne, norfluoxetine, clomipramine, doxepine, trimipramine, amoxapine, maprotiline, haloperidol, acetaminophen, naproxen, diclofenac, codeine, caffeine, phenytoin, phenobarbital, carbamazepine, lamotrigine, zonisamide, topiramate, primidone, vigabatrin, ethosuximide and clonazepam. The calibration curve was linear over concentration range of 0.5–240 ng/mL using line-fit plot in regression analysis. LOD and LOQ were estimated to be 0.2 and 0.5 ng/mL, respectively, using a volume of 250 μL sample and 20 μL injection. Correlations coefficient greater than 0.996 were routinely obtained. Intra- and inter-day reproducibility for calibration curves were determined on the same day in replicate ($n = 4$) and on different days ($n = 10$), respectively, using same pooled serum sample. The results have been shown in Table 1.

The inter- and intra-day accuracy and precision values of the assay method are presented in Table 2. The coefficient of variation values of both inter- and intra-day analysis were less than 16%, whereas the percentage error was less than 5.8%. Stock solutions of FL and the I.S. were stable for at least 30 days when stored at 4 °C. Derivatized solutions were found to be stable (>95%) for at least 12 h if the samples were kept at room temperature. Stability of the drug was found to be 100% from the initial value, after 60 days' maintenance of the serum at −80 °C and following 3 thaw–freeze cycles. The recoveries of FL and I.S. were estimated by comparing peak areas in extracted spiked human drug-free serum samples with those in standard solutions and were found to be 100% for both FL and the I.S.

3.4. Application of the method

The present method has successfully been used for the determination of the drug in a randomized cross-over bioequivalence study following single oral administration of two different FL preparations in 24 healthy volunteers. Typical serum concentration–time profile and the resulted pharmacokinetic parameters of the drug have been shown in Fig. 2 and Table 3, respectively.

4. Discussion

Derivatization of FL in rat plasma using NBD-F as labeling agent has been previously reported by Higashi et al. [15]. However, poor sensitivity of their method (LOQ 15 ng/mL) do not allowed detection of low-drug serum levels which are usually found in single dose human pharmacokinetic studies. In their procedure derivatization of the extracted samples has

Table 2
Inter and intra-day precision and accuracy for determination of FL in human serum by the HPLC method

Known concentration (ng/mL)	Concentration found (mean ± SD)	Coefficient of variation (%)	Accuracy (%mean deviation)
Inter-day			
1	1.02 ± 0.16	15.3	−5.8
50	51.5 ± 2.6	5.0	3
200	198.2 ± 6.6	3.3	0.9
Intra-day			
1	1.02 ± 0.16	16.0	2.7
50	51.6 ± 1.8	3.6	3.3
200	202.5 ± 7.0	3.4	1.3

Accuracy has been calculated as a percentage of the nominal concentration

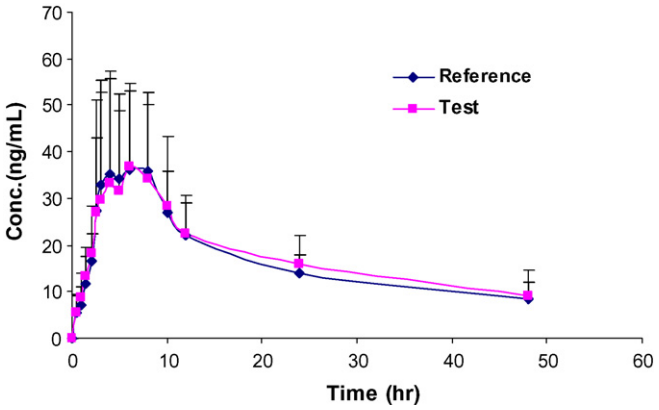

Fig. 2. Mean serum concentrations–time profiles of fluvoxamine for two preparations in 24 human volunteers after administration of a single 100 mg oral dose.

Table 3
Mean (SD) pharmacokinetic parameters of two FL preparations after single oral administration of 100 mg in 24 human volunteers

Parameter\Prep.	Test	Reference	*P*-value[a]
C_{max} (ng/mL)	46.2 (29)	48.5 (28)	NS
AUC0-48 (ngh/mL)	866.2 (480)	802.2 (360)	NS
AUC0-∞ (ngh/mL)	1308 (781)	1224.9 (430)	NS
$t_{1/2}$ (h)	27.8 (9.3)	29.6 (11.4)	NS
t_{max} (h)	5.3 (2.0)	5.6 (2.1)	NS

t_{max} = time to maximum concentration; C_{max} = maximum concentration; AUC = area under the curve; $t_{1/2}$ = elimination half life.
[a] NS = no significant difference ($P < 0.05$).

been achieved using a mixture of 300 μL borate buffer, 100 μL NBD-F (25 mM) and 400 μL HCl (0.05 M). The reactions of NBD-Cl with amines have been found to take place within an aqueous–organic phase system; hence, yield of the reaction is determined by polarity of the medium. Furthermore, sufficient amounts of the organic phase are needed to dissolve the resulted derivatives. Increasing of organic/aqueous phase ratio of the medium in our method significantly enhances the yield of reaction. Also to improve sensitivity of analysis higher temperature and time of reaction were examined; however, due to evaporation of the organic phase in the reaction mixture a significant reduction in the yield of reaction was observed when high temperature (more than 64 °C) or long incubation time was used. Effect of different organic solvents on the speed and yield of the reaction was tested and a significantly improved LOQ (0.5 ng/mL) was obtained when dichloromethane was added to the reaction mixture. It seems that the reaction is effectively proceeded in the presence of sufficient amounts of dichloromethane. In the method described by Higashi et al. [15] long run time of analysis (approximately 18 min) is reported. In our method, however, an improved analytical condition was validated in which the time for the chromatographic separation has been significantly reduced.

In conclusion, a sensitive and rapid HPLC method that allows determination of low blood FL levels, which are found following single dose human pharmacokinetic studies within 6 min, has been described.

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