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Determination of fluvoxamine in human plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

Fluvoxamine and nortriptyline, the assay internal standard, were extracted from plasma with ethyl acetate, then reacted with dansyl chloride. The derivatives were quantitated by isocratic reversed-phase high-performance liquid chromatography with fluorescence detection. The assay calibration range for fluvoxamine was 10–1000 ng/ml using a 1-ml plasma sample. Pooled plasma quality control sample relative recoveries at 25 and 250 ng/ml were 103 and 105%, respectively. Estimates of quality control inter-day precision during validation were $\leq 3\%$ relative standard deviation. The assay was cross-validated with a gas chromatographic method and has been employed in therapeutic drug level monitoring.

INTRODUCTION

Fluvoxamine (Fig. 1), a "second-generation" antidepressant, is a selective serotonin reuptake inhibitor used in the treatment of a variety of depressed states [1]. The selective mode of action produces a different side-effect profile than demonstrated by the tricyclic antidepressants [2]. The

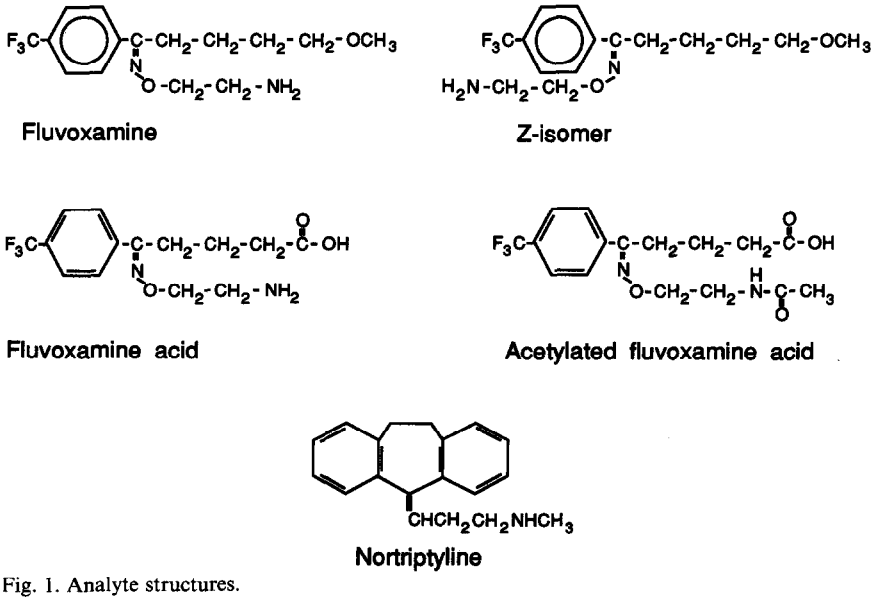

Fig. 1. Analyte structures.

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typical therapeutic dose range for fluvoxamine is 100–300 mg per day. Trough plasma levels for six subjects who received 50 mg of fluvoxamine twice a day for 28 days ranged from 15 to 380 ng/ml [3].

The goal of this work was to develop a rapid, inexpensive and sensitive fluvoxamine plasma assay for the routine determination of large numbers of samples from therapeutic drug monitoring studies. Several methods have been reported with limits of quantitation of 1–2 ng/ml to support single-dose pharmacokinetic studies with fluvoxamine [4–6]. However, these methods required extensive/costly extraction steps or lengthy chromatographic separation times to achieve sufficient selectivity. De Jong [7] reported a gradient high-performance liquid chromatographic (HPLC) technique for the direct determination of the fluorescamine derivative of fluvoxamine with a limit of detection of 3 ng/ml. However, it was necessary to replace the pre-column after five to ten samples, presumably due to adsorption of plasma components on the stationary phase and column frits. Foglia *et al.* [8] described a method which employed a two-step extraction followed by HPLC with UV detection at 215 nm. The reported limit of quantitation of 25 ng/ml does not provide sufficient sensitivity to encompass the anticipated range for trough levels at the 100 mg per day dose level.

Dansyl chloride was employed by Pommery and Lhermitte [6] to prepare a fluorescent derivative of fluvoxamine for subsequent determination by gradient normal-phase HPLC. The feasibility of coupling the dansyl chloride derivatization with an isocratic reversed-phase separation was investigated. Fluvoxamine and nortriptyline, the internal standard selected for this work, were extracted from plasma into ethyl acetate, then reacted with dansyl chloride. The fluorescent derivatives were separated on an isocratic reversed-phase system with a total run time of 12 min. This report describes the validation and application of this assay to representative clinical samples.

EXPERIMENTAL

Materials

Fluvoxamine maleate [4′-trifluoromethyl-5-methoxyvalerophenone(*E*)-O-(2-aminoethyl)oxime maleate] reference standard (100%), fluvoxamine acid hydrochloride, acetylated fluvoxamine acid, and the *Z*-isomer of fluvoxamine were supplied by Duphar Research Labs. (Weesp, Netherlands) (Fig. 1). Nortriptyline hydrochloride (100%), the assay internal standard (I.S.), was purchased from U.S. Pharmacopeial Convention (Rockville, MD, USA) (Fig. 1). Desipramine hydrochloride was obtained from Merrell Dow Research Institute (Cincinnati, OH, USA). "Chempure-grade" acetone was purchased from Curtin Matheson Scientific (Marietta, GA, USA). Deionized water was purified by reverse osmosis. "Spectrophotometric-grade" ethyl acetate was purchased from Mallinckrodt (Paris, KY, USA). "HPLC-grade" acetonitrile and potassium phosphate, dibasic (98%), were purchased from EM Science (Cherry Hill, NJ, USA). Phosphoric acid (85.4%) and sodium bicarbonate (100%) were "Baker-analyzed-grade" reagents (J. T. Baker, Phillipsburg, NJ, USA). Dansyl chloride (98%) was purchased from Aldrich (Milwaukee, WI, USA). Blank plasma was obtained from apparently healthy human volunteers by plasma phoresis using sodium citrate anticoagulant (Bio Lab., Birmingham, AL, USA). The plasma was stored at approximately −20°C.

Sample preparation

Standard preparation. Plasma calibration standards were prepared fresh daily by spiking 1 ml of blank human plasma with the appropriate amount (10–50 μl) of aqueous fluvoxamine standard solutions (20.0, 2.00, 0.200 μg/ml). Plasma calibration standards were prepared in duplicate at seven levels during three separate validation runs. The assay calibration range for fluvoxamine was 10–1000 ng/ml. Pooled plasma quality control (QC) samples were prepared at 250 and 25 ng/ml to assess assay accuracy and precision. QC samples were also prepared at 50 ng/ml to assess storage stability in the plasma matrix at approximately −20°C. In addition, stability to freezing and thawing, stability in the extraction solvent, and stability in plasma at room temperature were evaluated.

Extraction and derivatization. Plasma samples were spiked with 10 μl of an aqueous I.S. solution of nortriptyline (100 μg/ml). The samples were

buffered to pH 7.6 with 100 μl of 1 *M* K_2HPO_4 (pH 7.6) and mixed for 5 s on a vortex mixer. The analytes were extracted from plasma by adding 6 ml of ethyl acetate and vortex-mixing for 1 min. The samples were centrifuged for 5 min at 1900 *g* to separate the phases. The upper organic phase was transferred to polypropylene tubes for evaporation under nitrogen at 45°C. The dried extracts were reconstituted for derivatization by adding 250 μl of 40 m*M* $NaHCO_3$, 20 μl of a 10 mg/ml solution of dansyl chloride in acetone, and 750 μl of acetone. The tubes were capped and vortex-mixed for 30 s followed by incubation at room temperature (22 ± 2°C) for 15 min. After 15 min the samples were once again evaporated under nitrogen at 45°C. The samples were prepared for injection by adding 1 ml of mobile phase and vortex-mixing for 1 min. The samples were clarified by centrifugation at 1900 *g* for 10 min and the clear supernatant was poured directly into an autosampler vial. Fifty plasma samples were processed in 5.5 h.

High-performance liquid chromatography

The HPLC apparatus consisted of a Waters Model 510 pump, WISP Model 710B autosampler, and a Model 420 fluorescence detector (Milford, MA, USA), a NewGuard RP-8, 7 μm particle size, 15 mm × 3.2 mm I.D. cartridge guard column (Brownlee Labs., Santa Clara, CA, USA), and a 250 mm × 4.6 mm I.D. analytical column packed with Supelcosil LC-18-DB, 5 μm particle size (Supelco, Bellefonte, PA, USA). The mobile phase was acetonitrile–10 m*M* potassium phosphate (pH 7.2) (85:15, v/v). The mobile phase was filtered twice through a 0.45-μm Nylon 66 membrane filter. The sample injection volume was 100 μl. The chromatographic run time was 12 min.

Data acquisition and quantitation

Collection and analysis of the detector output (1 V full scale) was performed with an HP 1000 computer (Hewlett-Packard, Sunnyvale, CA, USA) using Beckman Computer Automated Laboratory System software (Waldwick, NJ, USA). The mean relative weight response factor (RWRF) [(peak height of fluvoxamine/peak height of I.S.) × (I.S. concentration/fluvoxamine concentration)] was calculated for the plasma standard calibration curve. Assay results were calculated by multiplying the peak-height ratio (fluvoxamine/I.S.) by the I.S. concentration and dividing by the mean RWRF.

Selectivity

Blood plasma was obtained from drug-free human volunteers using sodium citrate ($n = 2$), sodium heparin ($n = 11$) and EDTA ($n = 8$) as anticoagulants. The samples were stored at approximately −20°C until they were assayed to screen for endogenous fluvoxamine interferences. Analytical standards containing desipramine, fluvoxamine acid, acetylated fluvoxamine acid, and the *Z*-isomer of fluvoxamine were prepared at concentrations of 400–500 ng/ml.

Assay cross-validation study

Clinical samples from fifteen subjects were assayed by the HPLC method and a previously reported gas chromatographic (GC) technique using electron-capture detection (ECD) [4], modified to employ a capillary column separation [9]. Single determinations were performed by both methods. The clinical samples were obtained during a safety and efficacy study of fluvoxamine in the treatment of obsessive compulsive disorder [10]. The fluvoxamine dose was gradually increased over the first fourteen days of the study, then adjusted within the range 100–300 mg per day until the final assessment. Samples were taken at least 12 h after the last dose during double-blind assessment (study day 70 or earlier) as a measure of compliance.

RESULTS

High-performance liquid chromatography

Typical within-run retention times (and relative standard deviations, R.S.D.) for fluvoxamine and the I.S. were 5.3 min (0.9%) and 9.7 min (1.4%), respectively ($n = 36$). Injection of over 600 plasma extracts on the same guard and analytical columns did not produce a significant change in system back-pressure or retention times. Inter-day differences in mobile phase composition, as well as chromatography using two different analytical columns did not significantly alter chromatographic performance.

TABLE I

FLUVOXAMINE-SPIKED PLASMA CALIBRATION STANDARD POOLED RESULTS

Spiked plasma calibration standards were assayed in duplicate at each level during three separate validation runs. Assay values were calculated using the daily mean relative weight response calculated across the range of the calibration curve. $n = 6$.

Spiked concentration (ng/ml)	Mean assay concentration (ng/ml)	R.S.D. (%)	Accuracy (%)
9.98	9.8	7.8	99
20.0	19.2	4.4	96
49.9	48.4	3.3	97
99.8	96.8	3.3	97
200	208	2.9	104
499	512	3.6	103
998	1040	3.9	104

Calibration standard response, precision, and accuracy

Pooled validation results for fluvoxamine plasma calibration standards are presented in Table I. Intra-day RWRF precision ranged from 4 to 7% R.S.D. for all three validation runs. Accuracy for duplicate calibration standards from 10 to 1000 ng/ml ranged from 91 to 109%. The peak height for fluvoxamine at the quantitation limit of 10 ng/ml was consistently five-fold greater than co-eluting endogenous plasma components in blank calibration samples. Chromatograms of a plasma blank and a 10 ng/ml calibration standard are shown in Fig. 2. The absolute analyte procedural recoveries could not be determined due to the absence of dansylated reference standards for fluvoxamine and nortriptyline (the I.S.).

TABLE II

POOLED PLASMA QUALITY CONTROL SAMPLE PRECISION AND ACCURACY

Pooled plasma quality control samples were assayed in triplicate at both levels during three separate validation runs. $n = 9$.

Spiked concentration (ng/ml)	Mean assay concentration (ng/ml)	R.S.D. (%)	Accuracy (%)
24.7	25.4	3.4	103
247	258	2.1	105

Quality control sample precision and accuracy

Assay precision and accuracy were evaluated by assaying aliquots from pooled plasma QC samples in triplicate during each validation run (Table II). QC sample mean relative recoveries at 25 and 250 ng/ml were 103 and 105%, respectively. Pooled estimates of inter-day assay precision at these two concentrations were $\leq 3\%$ R.S.D. Inter-day QC precision was higher, but still acceptable, during three recent clinical studies (4–10% R.S.D., $n \geq 14$ in each study).

Stability

A stability study of 50 ng/ml plasma QC samples stored at $-20°C$ is currently underway. Mean assay results for QC samples stored for six months at $-20°C$, carried through three freeze–thaw cycles, stored for 2 h at room temperature, and left for 2 h in ethyl acetate extraction solvent were all within $\pm 10\%$ of the spiked concentration.

Selectivity

No quantifiable fluvoxamine interference peaks were present in plasma samples obtained from human volunteers using sodium citrate, sodium heparin and EDTA as anticoagulants. Analytical standards of desipramine and the fluvoxamine *Z*-isomer did not interfere with the analytes of interest (Fig. 3). No peaks were detected for acetylated fluvoxamine acid and fluvoxamine acid under the current experimental conditions.

Assay cross-validation study

The results of the assay cross-validation study are presented in Fig. 4. HPLC assay results were plotted as a function of GC–ECD assay results and a weighted perpendicular least-squares linear regression analysis was performed ($r = 0.91$) [11]. The *y*-intercept and slope were not significantly different from 0 and 1, respectively, at the 95% level of significance. A relatively low fluvoxamine plasma concentration (27.0 ng/ml) is shown in Fig. 2C. This sample was collected approximately 12 h post-dose from an outpatient on standard fluvoxamine therapy (100–300 mg per day). Over 600 clinical samples have been assayed to date using the HPLC procedure.

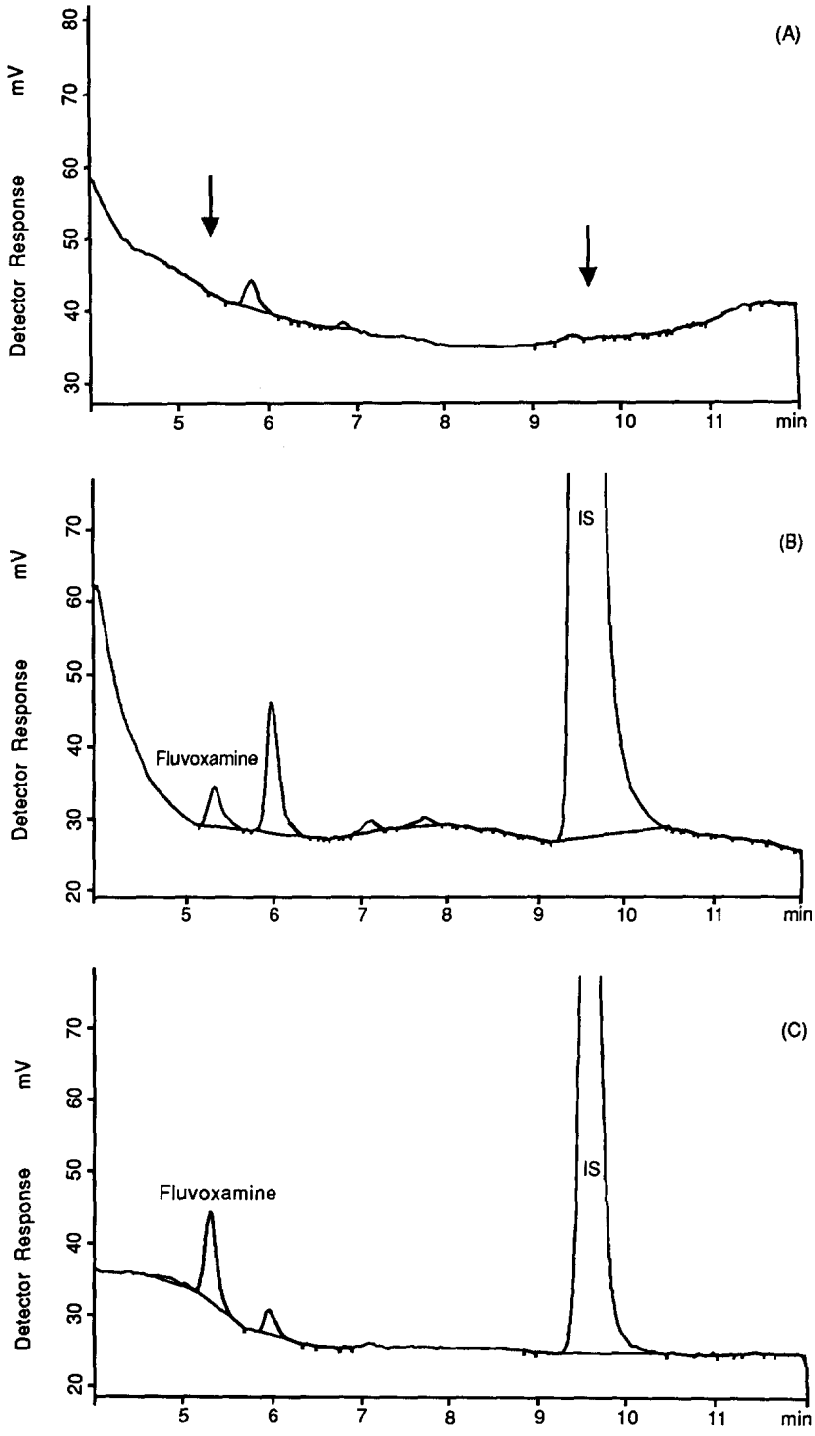

Fig. 2. Chromatograms of (A) a blank plasma sample, (B) a 10 ng/ml plasma calibration standard, and (C) a trough plasma sample (27.0 ng/ml) from a subject on standard fluvoxamine therapy (100–300 mg per day).

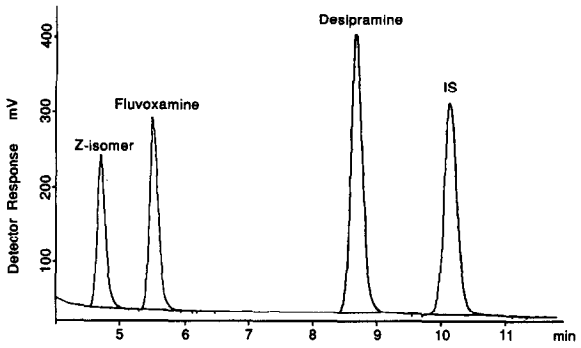

Fig. 3. Analytical standard chromatogram depicting analyte resolution from the fluvoxamine *Z*-isomer and desipramine.

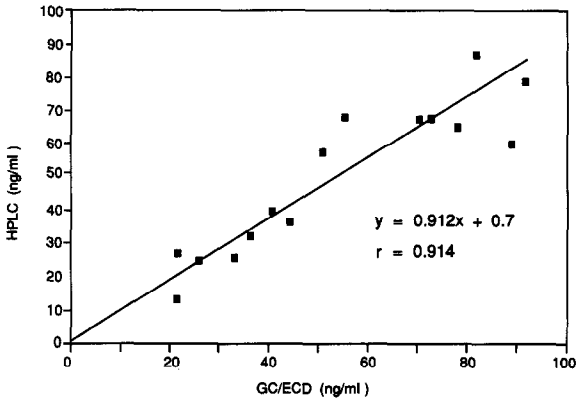

Fig. 4. Assay cross-validation results.

DISCUSSION

The high organic content of the mobile phase (85% acetonitrile) combined with the 1.5 ml/min flow-rate produced a relatively short chromatographic run time of 12 min. The aqueous portion of the mobile phase was buffered to ensure the reproducibility of the separation. A buffer pH of 7.2 and an endcapped analytical column were selected to minimize silanophilic retention, a possible source of column-to-column variability in reversed-phase separations [12].

Hurst *et al.* [13] previously reported a three-step ethyl acetate extraction for the separation of fluvoxamine and its 4′-chloro analogue, clovoxamine, from human plasma. The method proposed in this report employed a single-step ethyl acetate extraction which produced optimum recovery, a clean chromatographic baseline, and minimized the sample preparation time. Calibration standard RWRF precision and relative recoveries indicated that absolute procedural recoveries were constant over the range 10–1000 ng/ml. The sample preparation procedure was conducted at a neutral or basic pH to avoid any potential conversion of the oxime ether of fluvoxamine to the ketone under acidic conditions [4,7].

Fluvoxamine acid, acetylated fluvoxamine acid and the *Z*-isomer of fluvoxamine were resolved from the analytes of interest by HPLC. Fluvoxamine acid and acetylated fluvoxamine acid have been identified as two significant urinary metabolites of fluvoxamine in humans [14]. It was postulated *a priori* that these metabolites could potentially react with dansyl chloride to form fluorescent derivatives. Separation of the *Z*-isomer from fluvoxamine was important in order to verify the absence of *E* to Z conversion *in vivo* or *in vitro*.

CONCLUSIONS

The assay was evaluated for response, precision, accuracy, and specificity, and demonstrated adequate performance with respect to these parameters. An HPLC *versus* GC–ECD assay cross-validation study indicated that there was no significant difference in clinical assay results between the two methods. The quantitation limit of 10 ng/ml coupled with the rapid sample preparation procedure (50 plasma extracts in 5.5 h) enabled application of this method to routine therapeutic drug level monitoring. The assay has proven to be rugged and reproducible during the analysis of over 600 clinical samples to date.

Further efforts are being made to extend the assay limit of quantitation to 1–2 ng/ml to enable application of the method to single-dose pharmacokinetic studies. Solid-phase extraction is being explored as an alternative to multiple-step liquid–liquid extraction. A dual-monochromator fluorescence detector is being evaluated to achieve additional selectivity.

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