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# DETERMINATION OF FLUOXETINE AND NORFLUOXETINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

A high-performance liquid chromatographic assay was developed for a recently introduced atypical antidepressant, fluoxetine and its demethylated metabolite, norfluoxetine. Prior to analysis, aliquots of alkalinized plasma were extracted with *n*-hexane and isoamyl alcohol, followed by back-extraction with diluted phosphoric acid. These extracts were injected into a 10 $\mu$m, reversed-phase $C_{18}$ column with phosphate and acetonitrile as the mobile phase and detection at 214 nm. Peak height ratios were linearly correlated up to 800 $\mu$g/l. Acceptable coefficients of variation were demonstrated for both within-run and day-to-day studies. Selected drugs were checked for interference. The assay was used to monitor nine patients receiving 20 to 80 mg of fluoxetine per day. Plasma concentrations of fluoxetine and norfluoxetine ranged from 37 to 301 $\mu$g/l and 29 to 326 $\mu$g/l respectively.

## INTRODUCTION

Antidepressant measurement by high-performance liquid chromatography (HPLC) has been advocated for the monitoring of first generation, as well as the recently introduced atypical antidepressants such as trazodone and its metabolite, 1-metachlorophenyl piperazine[1–8]. Currently, the enzyme multiplied immunoassay technique (EMIT) with monoclonal antibodies may be used for quantitative measurement of selected first generation antidepressants such as imipramine, desipramine, amitriptyline and nortriptyline[9]. Rapid screening for these antidepressants may be achieved by using polyclonal antibody-based EMIT and fluorescence polarization immunoassay (FPIA)[4]. However, for newly introduced antidepressants, reversed-phase HPLC is still the method of choice for analysis due to its reproducibility. The current study demonstrated that a newly introduced antidepressant, fluoxetine (FLU) and its demethylated metabolite, norfluoxetine (N-FLU), may be quantitated in plasma by modifying a previously published, simple procedure[5].

Fluoxetine, N-methyl-8-[4-(trifluoromethyl)phenoxy] benzenepropanamine, is

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a non-tricyclic antidepressant[9–13]. It exhibits selective inhibition of serotonin uptake in presynaptic neurons, and is indicated for unipolar depression. After oral administration, peak plasma concentration is reached within 6 to 8 h and absorption is not affected by the presence of food. Mean elimination half-life of FLU is 2 days following a single dose, and 4 days following long-term administration, while that of N-FLU is 7 days, independent of dosage regimen[14]. Fluoxetine is metabolized to norfluoxetine, also an inhibitor of serotonin reuptake, via demethylation, as shown in Fig. 1 (ref. 10). Other unidentified metabolites and glucuronides are also detected in the urine.

FLU and N-FLU were quantified by gas chromatography with electron-capture detection according to Nash *et al.*[15]. Orsulak *et al.*[16] described a procedure using a multi-step extraction and a reversed-phase HPLC analysis with a phenyl column and detection at 226 nm. For 24 patients administered daily doses of 20–60 mg of fluoxetine during the preceding three weeks, the plasma concentrations of FLU and N-FLU were 47–469 and 52–446 μg/l, respectively. More recently, Kelly *et al.*[14] developed an HPLC assay in order to correlate plasma concentrations with clinical response. The assay involved an organic extraction, followed by reversed-phase analysis with a CN column and detection at 226 nm. From data on thirteen patients receiving daily doses of 20 to 60 mg of fluoxetine, the FLU and N-FLU serum concentrations were 73–453, and 54–362 μg/l respectively. Antidepressant response, however, was not correlated with serum concentrations.

For ready adaptation in the clinical laboratory, a previously published HPLC procedure for quantification of antidepressants[5] was modified by using a readily available internal standard, clomipramine, a $C_{18}$ column, a binary mobile phase and a readily available UV detection at 214 nm. This assay was used to quantitate plasma concentration of nine patients medicated with fluoxetine.

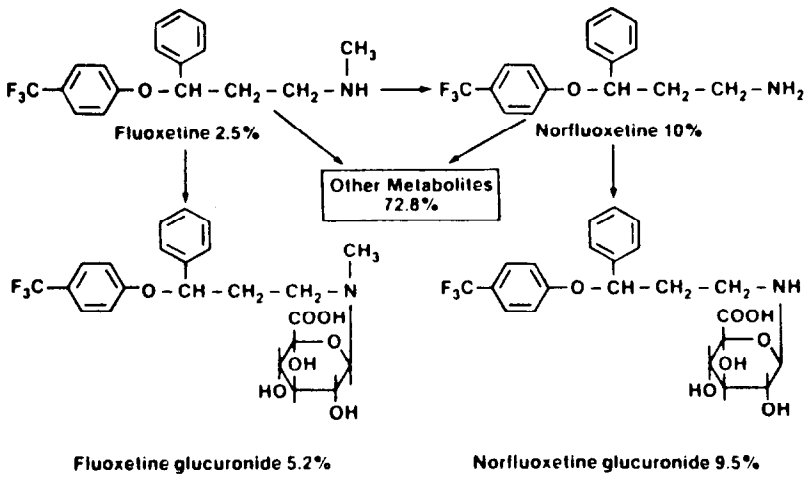


Fig. 1. Metabolites of fluoxetine identified in urine collected for 35 days from normal subjects given [$^{14}$C]fluoxetine. (From ref. 10.)

## EXPERIMENTAL

### *Chemicals*

Fluoxetine hydrochloride and norfluoxetine maleate were kindly provided by Eli Lilly (Indianapolis, IN, U.S.A.). Clomipramine, the internal standard, was obtained from Ciba-Geigy (Summit, NJ, U.S.A.). Primary stock solutions of fluoxetine and norfluoxetine, 10 mg/10 ml, were prepared by dissolving the appropriate amounts in 10 ml of distilled water inside volumetric flasks, while methanolic solution of clomipramine was similarly prepared. From these primary stock solutions, working aqueous stock solutions of FLU and N-FLU and working methanolic stock solution of clomipramine were prepared by diluting 100 $\mu$l of the above solutions in 10 ml of water or methanol. Acetonitrile, *n*-hexane and methanol were "UV" grade, distilled in glass (Burdick and Jackson, Muskegon, MI, U.S.A.). Isoamyl alcohol, potassium dihydrogen phosphate and orthophosphoric acid were "Baker Analyzed" reagent grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). The extraction solvent, *n*-hexane isoamyl alcohol (99:1), was prepared by mixing 990 ml of *n*-hexane with 10 ml of isoamyl alcohol, while 0.05% diluted phosphoric acid was prepared by diluting 59 $\mu$l of the orthophosphoric acid into 100 ml with distilled water. The mobile phase consisted of 0.05 *M*, pH 4.7, $KH_2PO_5$–acetonitrile (6:4). The phosphate solution was prepared by dissolving 13.61 g of monobasic phosphate in 2 l of distilled water, followed by adjusting the pH with diluted potassium hydroxide.

For checking the precision of the assay, quality control samples containing about 200 $\mu$g/l each of FLU and N-FLU were prepared by mixing 2 ml each of the working stock solutions of FLU and N-FLU with 96 ml of drug-free plasma in a silanized volumetric flask.

### *Chromatographic system*

The chromatograph was a Model 5000 liquid chromatography (Varian, Walnut Creek, CA, U.S.A.). The detector was a Spectro-Monitor III variable wavelength UV detector (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Chromatograms were recorded by an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). The analytical column was a $\mu$Bondapak $C_{18}$ (30 cm × 4.6 mm I.D.) column, connected to a guard column packed with Bondapack/Corasil $C_{18}$ (both from Waters/Millipore, Milford, MA, U.S.A.).

### *Sample collection*

A total of ten blood samples were collected from nine patients (one patient had repeat samples drawn), 2 days to 5 months after the initiation of fluoxetine therapy with daily doses of 20 to 80 mg. Sampling was performed at 8 h following a bedtime dose or just before the ingestion of a morning dose. Blood collection was performed with evacuated tubes containing EDTA. Afterward, the sample tubes were centrifuged, followed by transferring the plasma to a polypropylene tube, and kept frozen until subsequent analysis.

### *Procedures*

The extraction was a simple three step procedure: alkalinization, organic extraction and back-extraction. To a series of polypropylene tubes, 2-ml aliquots of

drug-free plasma were transferred, followed by addition of 0, 20, 40, 80 and 160 μl of each of the working stock solutions of FLU and N-FLU. The resultant plasma concentrations were 0, 100, 200, 400 and 800 μg/l. To these standards, quality control and patient samples, 160 μl of the working internal standard solution of clomipramine were added. These tubes were vortexed, followed by addition of 2 ml aliquots of 1 *M* sodium hydroxide and further vortexing. Then, these mixtures were extracted with 5 ml aliquots of n-hexane–isoamyl alcohol (99:1) by rotation for 5 min, and centrifugation for 10 min. The organic layers were transferred to another series of marked polypropylene test tubes, followed by addition of 200 μl of 0.05% dil. phosphoric acid for back-extraction. These tubes were rotated for 5 min and centrifuged for 10 min. The lower aqueous, acidic layer, containing FLU and N-FLU, was carefully pipetted into a small test tube for reversed-phase HPLC analysis.

*Chromatographic parameters*

Flow-rate was maintained at 2 ml/min. Column temperature was 50°C. Detection wavelength was 214 nm, 0.01 a.u.f.s. Injection volume ranged from 25 to 50 μl.

## RESULTS AND DISCUSSION

Fig. 2 shows the chromatograms for the extracts of drug-free plasma, a 200 μg/l standard, and a patient sample. Retention times of N-FLU, FLU and the internal standard were 5.8, 6.8 and 9.5 min respectively, with the corresponding capacity factors ($k'$) of 2.9, 3.5 and 5.3. Fig. 3 shows the linear calibration up to 800 μg/l for both FLU and N-FLU, with excellent correlation coefficients. Precision studies showed acceptable coefficients of variation for both within-run and day-to-day studies, as shown by Table I. Recoveries ranged from 55 to 60% for N-FLU, and 79 to 86% for FLU, comparable to those of other antidepressant assays[3–7]. Sensitivity, defined as signal-to-noise ratio = 3, is estimated to be 6 μg/l for both FLU and N-FLU. Selected drugs; checked for potential interference, their capacity factors are listed in Table II, showing the co-elution of imipramine with N-FLU and amitriptyline with FLU.

In modifying a previously published assay, our goal was to develop a simple and fast procedure that is adaptable by clinical laboratory personnel. Since clinical laboratory personnel would most likely be performing HPLC assays of the first generation tricyclics as well as the newer, atypical antidepressants, the simplicity of the described procedure would allow direct adaptation without lengthy daily change-over of extraction chemicals, solvents and columns. Since different personnel of a given laboratory would be using this procedure, the simplicity of the assay would ensure precision. This approach has been successfully applied in our clinical laboratory where the monitoring of first generation antidepressants, as well as new antidepressants and metabolites is routinely offered.

Simplicity of the procedure is evident in the use of a readily available $C_{18}$ column and detection wavelength of 214 nm. This was chosen instead of 226 nm, as in the assays of Orsulak *et al.*[16] and Kelly *et al.*[14], because 214 nm detectors are readily available in most clinical laboratories as a filter or variable-wavelength detector. In the procedure of Kelly *et al.*[14], protriptyline was used as an internal standard. In our procedure, clomipramine was used as the internal standard for this as well as other

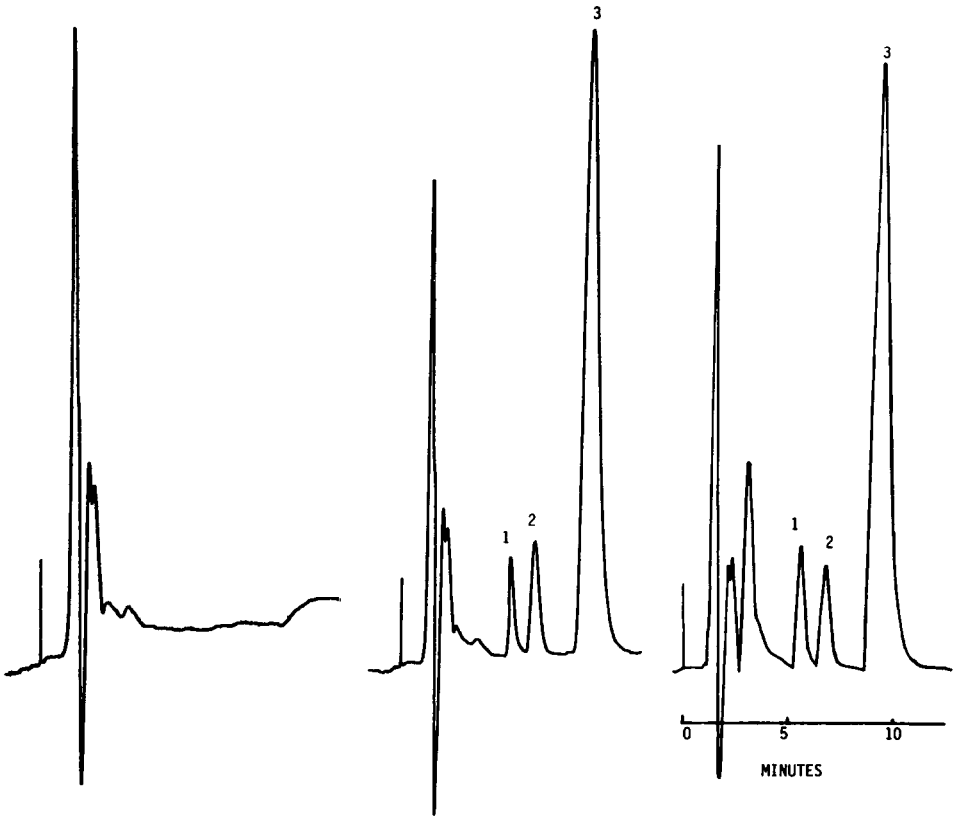


Fig. 2. Chromatograms of extracts of: (left) drug-free plasma (center) plasma with 200 μg/l each of FLU and N-FLU, and (right) a patient's plasma with 163 μg/l of FLU, and 208 μg/l of N-FLU. Peaks: 1 = N-FLU; 2 = FLU; 3 = internal standard, clomipramine.

clinical procedures for the first generation tricyclics. The choice of protriptyline and clomipramine as the internal standards enhances ready clinical adaptation of these procedures, even though the chemical structure of the tricyclic rings of both internal standards are different from those of FLU and N-FLU, as shown by Fig. 1.

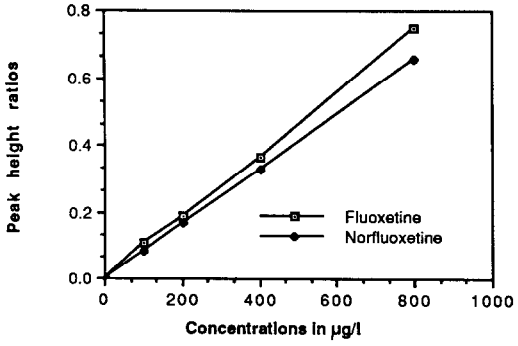


Fig. 3. Calibration curves for FLU and N-FLU. (FLU: $y = 9.24 \cdot 10^{-4}x - 0.0049$, $r = 0.9999$; N-FLU: $y = 8.19 \cdot 10^{-4}x - 0.0024$, $r = 0.9999$).

TABLE I

WITHIN-RUN AND DAY-TO-DAY COEFICIENTS OF VARIATION (C.V.) IN THE DETERMINATION OF FLU AND N-FLU

| | *Mean (μg/l)* | *C.V. (%)* | *n* |
|---|---|---|---|
| *Within-run* | | | |
| FLU | 197.8 | 1.6 | 6 |
| N-FLU | 201.8 | 0.7 | 6 |
| *Day-to-day* | | | |
| FLU | 191.4 | 4.1 | 18 |
| N-FLU | 193.1 | 5.5 | 18 |

Since the precision and recovery of the assay are comparable to those of other antidepressant assays, it was used to check the plasma FLU and N-FLU concentrations of a patient medicated with 80 mg of fluoxetine. Plasma concentration of FLU and N-FLU were 163 and 208 μg/l respectively. Table III lists the data for a group of nine patients medicated with 20–80 mg of fluoxetine and other medications, their FLU and N-FLU plasma concentrations, and relevant clinical information. Since FLU is a newly introduced antidepressant, its use in combination with other drugs may be enhanced by monitoring FLU and N-FLU. It is in this respect that our study differs from those of Orsulak *et al.*[16] and Kelly *et al.*[14] in terms of the observed concentrations. However, the range of FLU and N-FLU are comparable in all three studies.

In general, there was a direct relationship between dosage and the combined fluoxetine and norfluoxetine plasma concentrations measured by us. A similar relationship appears to exist between duration of treatment and plasma concentrations. Notable exceptions to these trends are evidenced by patients 3 and 4, who as outpatients were less likely to have been compliant with the precribed dosage, and by patients 7 and 9, who were on a variety of medications concomitant with fluoxetine.

TABLE II

CAPACITY FACTORS ($k'$) OF SOME COMMON DRUGS TESTED FOR INTERFERENCE

| *Drug* | *k'* | *Drug* | *k'* |
|---|---|---|---|
| Acetaminophen | 0.00 | Desipramine | 2.44 |
| Codeine | 0.00 | Nortriptyline | 2.74 |
| Meperidine | 0.12 | Norfluoxetine | 2.90 |
| Phenobarbital | 0.84 | Imipramine | 2.90 |
| Amoxapine | 1.67 | Propoxyphene | 3.48 |
| Phenytoin | 1.40 | Amitriptyline | 3.49 |
| Pentobarbital | 1.56 | Fluoxetine | 3.50 |
| Oxazepam | 1.79 | Diazepam | 3.99 |
| Lorazepam | 1.88 | Chlorpromazine | 4.15 |
| Secobarbital | 1.88 | Perphenazine | 4.92 |
| Flurazepam | 2.04 | Clomipramine | 5.30 |
| Chlordiazepoxide | 2.18 | Prochlorperaxine | 6.04 |
| Doxepin | 2.20 | Thioridazine | 8.44 |
| Cimetidine | 2.36 | Trifluoperazine | 8.44 |

TABLE III

PATIENT PLASMA CONCENTRATIONS

| *Patient* | *Age* | *Sex* | *Inpatient/ outpatient* | *Daily dosage (mg)* | *Duration of therapy* | *Indication* | *Other medications* | *FLU(μg/l)* | *N-FLU(μg/l)* |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 28 | M | I | 20 | 3 days | Alc. dep.[a] | Atenolol 50 mg | 37 | 29 |
| 2 | 27 | M | I | 20 | 13 days | MDD[b] | Perphenazine 4 mg | 63 | 33 |
| 3 | 52 | M | O | 60 | 5 months | Somatization disorder | Alprazolam 1.5 mg | 98 | 49 |
| 4 | 37 | M | O | 40 | 9 months | MDD[b] | Alprazolam 3 mg Chloral hydrate 2 g | 156 | 156 |
| 5 | 53 | F | I | 20 | 9 days | MDD[b] | Perphenazine 4 mg | 141 | 198 |
| 6 | 32 | F | O | 20 | 3 months | Adjustment disorder with depressed mood | None | 143 | 220 |
| 7 | 65 | M | I | 20 | 13 days | MDD[b] | Glyburide 2.5 mg Chlorpromazine 25 mg Triazolam 0.25 mg | 301 | 65 |
| | (patient 7, 2 months later) | | | 20 | 2 months | MDD[b] | Lorazepam 4 mg | 283 | 60 |
| 8 | 36 | M | I | 80 | 5 weeks | Bipolar disorder depressed | $LiCO_3$ 1500 mg | 163 | 208 |
| 9 | 34 | F | I | 40 | 2 days | Alc. dep.[a] Agoraphobia with panic disorder | Chlorzoxazone 600 mg Chlordiazepoxide 25 mg | 152 | 326 |

[a] Alcohol dependence.
[b] Major depressive disorder.

Repeated sampling from one patient (No. 7) resulted in very similar plasma values for both fluoxetine and norfluoxetine, following a 20 mg oral dose. While the limitations imposed by variability in compliance, concomitant medication, and individual pharmacokinetic factors cannot be disentangled in the present sample of patients, the present study demonstrated the suitability of the assay for typical clinical monitoring of FLU and N-FLU.

As shown by Kelly *et al.*[14], serum concentrations are not well correlated to clinical response. However, Orsulak[17] has suggested monitoring of fluoxetine may be helpful in the future for patients medicated with fluoxetine and other drugs. Recently, he observed that patients switching medications such as fluoxetine for tricyclics may benefit from monitoring due to possible drug-to-drug interactions. This may be explained by inhibition of the metabolism of first generation tricyclics by fluoxetine, resulting in possibly elevated levels of the first generation tricyclics. However, the described assay is not able to resolve the first generation tricyclics from fluoxetine and norfluoxetine due to co-elution with imipramine and amitriptyline as shown by Table II. Thus, there may be a need for future development of an alternative assay that can resolve combinations of tricyclics and serotonin-inhibiting antidepressants.

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