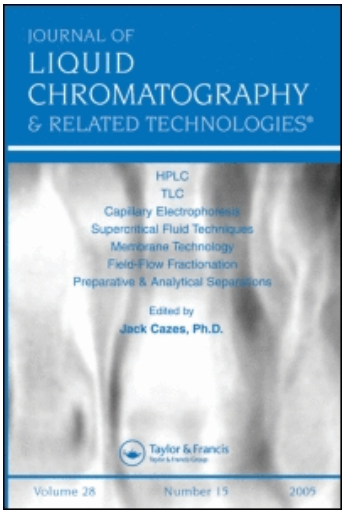

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DETERMINATION OF FLUOXETINE AND NORFLUOXETINE IN HUMAN PLASMA BY ION-INTERACTION RP-HPLC

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ABSTRACT

The paper reports a sensitive Ion-Interaction Reverse Phase HPLC-UV method for the separation and determination of fluoxetine ((±)N-methyl-(4-(trifluoromethyl)phenoxy) benzene-propanamine) in human plasma. An ODS silica-based column is used as the stationary phase and the mobile phase consists of 5.00 mM octylamine in water/acetonitrile (60/40 v/v), at pH = 6.4, with UV detection at 230 nm. The method is selective towards potentially interferent drugs, such as paroxetine, amylsulpride, fluvoxamine, mianserin, imipramine, amitryptiline, cromipramine, maprotiline, haloperidol, flunitrazepam and diazepam. The proposed method was successfully applied to the determination of fluoxetine and norfluoxetine in plasma of some patients being treated for obsessive-compulsive disorders.

INTRODUCTION

The use of selective serotonine-reuptake inhibitors (SSRI's) is widely prescribed in therapy for depression, obsessive-compulsive disorder, panic attack disorder, bulimia, social phobia, and post-traumatic stress disorder.[1-13] SSRI's are non-tricyclic antidepressants that enhance serotoninergic neurotrasmission process, through selective inhibition of neuronal reuptake of serotonine in presynaptic neurons. The chronic inhibition of serotonine reuptake leads to downregulation of serotonergic 5-HT1 presynaptic inhibitory autoreceptors and to increased serotonine release.[1] In particular, fluoxetine ((±)N-methyl-γ-(4-trifluoromethyl-phenoxy)benzene-propanamine), (prozac), has received widespread popularity in everyday clinical practice and is preferred with respect to classic tricyclic antidepressants, such as imipramine and amitryptiline.[2]

Fluoxetine, if administrated orally, exhibits few side effects in terms of frequence and severity. The major problem, as for other antidepressants, is its great inter-individual variability in clinical response which makes it difficult to value the correct posology. Fluoxetine posology ranges within 20 and 80 mg/day. The maximum concentration in plasma is generally attained after 4-8 hours, the elimination half-life varies between 1 and 9 days and steady-state plasma concentrations are achieved after 2-4 weeks.[1-4]

Metabolic processes in the liver convert some fluoxetine to norfluoxetine ((±)γ-(4-trifluoromethyl)phenoxybenzenepropanamine) which inhibits serotonine reuptake and significantly impacts to the overall clinical efficacy of fluoxetine. Norfluoxetine has a elimination half-life ranging between 3 and 10 days. Peak plasma concentrations are attained 3 days after the administration of the parent compound and steady-state plasma concentrations are reached after 4-5 weeks of fluoxetine administration.[1-2]

In order to determine whether the individual clinical response is correlated with the plasma concentrations, a sensitive and specific analytical method for determination of fluoxetine and norfluoxetine in plasma is required.

Some gas chromatographic[14-16] and HPLC methods (both normal-[7] and reversed phase modes[13,17-24] have been reported for determination of fluoxetine. Most methods employ derivatization reactions with dansyl chloride[21] and fluorescence detection[21,22] in order to have the desired sensitivity. A major problem[13,18,19,23] arises from interferences from other drugs prescribed as antidepressant (often in association with fluoxetine), which could be present in the plasma.

This paper reports an ion-interaction RP-HPLC method with UV detection (230 nm) which permits detection levels as low as 4.5 μg/L for fluoxetine and 2.3 μg/L for norfluoxetine in human plasma. The procedure does not require any derivatization reaction or pretreatment step. Moreover the method does not suffer any interference from the following drugs: paroxetine, amilsulpride, fluvoxamine, mianserin, imipramine, amitryptiline, cromipramine, maprotiline, haloperidol, flunitrazepam, and diazepam.

EXPERIMENTAL

Apparatus

The chromatographic analyses were carried out with a Merck-Hitachi (Tokyo, Japan) Lichrograph Chromatograph Model L-600, interfaced with a model L-4200 UV-Vis and a model L-3720 conductometric detectors.

A Metrohom 654 pH-meter equipped with a combined glass-calomel electrode was employed for pH measurements. A Hitachi (Tokyo, Japan) model 150-20 spectrophotometer was utilized for absorbance measurements.

A Minifuge T from Heraeus Sepatech (Hanau, Germany) centrifuge and a Minishaker (Duroni, Torino, Italy) rotator were used for sample preparation.

Chemicals and Reagents

Ultrapure water from a Millipore Milli-Q system (Millipore Corporation, Bedford, MA,USA) was used for the preparation of all the solutions. Octylamine and acetonitrile were Fluka (Buchs, Switzerland) analytical grade chemicals; NaOH and HCl were from Merck (Darmstadt, Germany); n-hexane and isoamylic alcohol from Farmitalia Carlo Erba (Milano, Italy).

(±)Fluoxetine hydrochloride and norfluoxetine maleate were kindly provided by Eli Lilly Co.(Indianapolis, In, USA). Fluoxetine hydrochloride is a white to off-white crystalline solid which is soluble at concentrations of 14 mg/mL in water, 250 mg/mL in methanol, and 125 mg/mL in chloroform. Less than 1% is soluble in benzene, ethyl acetate, and hexane. 10.0 mg/mL aqueous standard solutions of fluoxetine and norfluoxetine were prepared with the working aqueous solutions prepared by serial dilution.

Two sets of standard plasma respectively containing 0.0, 50.0, 100.0, 200.0, 400.0, 600.0, 800.0, and 1000.0 μg/L of fluoxetine and norfluoxetine were prepared by addition of the proper amount of standard solutions to 2.0 mL aliquots of drug-free plasma samples.[13]

Chromatographic Conditions

A 250 x 4.6 mm, 5 μm ODS-2 Phase Separations (Spherisorb) fully endcapped column with a carbon load of 12% (0.5 mmol/g), together with a 15.0 x 4.6 mm LiChrospher RP-18 5 μm guard pre-column were used. The mobile phase consisted of a mixture of 5.00 mM n-octylamine water solution / acetonitrile (60:40 v/v) brought to an "operational" pH value of 6.4±0.2 by the addition of o-phosphoric acid.[25,26] The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 h at a flow-rate of 1.0 mL/min was necessary. After use, the stationary phase was washed by flowing water (1.0 mL/min for 15 min), a 50/50 (v/v) water/acetonitrile mixture (1.0 mL/min for 15 min), and acetonitrile (1.0 mL/min for 5 min) through it.[25,26] Dead times were evaluated by injection of $NaNO_3$ solutions (10 mg/L) and conductometric detection of the unretained Na^+ ion.

Sample Collection and Extraction Procedure

Blood samples (10 mL) were collected from patients diagnosed with Obsessive-Compulsive Disorder (OCD) according to DSM-III-R criteria,[17] who were undergoing fluoxetine therapy at daily doses ranging from 20 to 60 mg. Blood was collected in evacuated tubes containing lithium-eparine; the tubes were centrifuged (3000 g, 10 min., 4°C) and plasma transferred to polypropylene tubes (Eppendorf, Hamburg, Germany) and kept frozen (-80°C) until assayed.

The extraction procedure, a modification of a previously published report,[20] consisted of three steps: alkalinization, organic extraction, and back-extraction. 2.0 mL aliquots of plasma were transferred to polycarbonate screw-capped tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) and 400 μL of 0.33 M NaOH solution were added. The samples were shaken for 5 sec and then extracted by rotating (MSA Clinomixer, Duroni, Torino, Italy) with 14 mL of n-hexane-isoamylic alcohol (985/15 V/V) for 20 min.

After centrifugation (2500 g, 5 min., 20°C) the organic phases were collected, adjusted to pH < 2.0 with 400 μL of HCl, shaken for 1 min, and centrifuged for 10 min (1500 g T=20°C). The organic phase was discarded and 100 mL of the aqueous phase was injected into the HPLC system. Each analysis was performed in triplicate. For routine analyses, only two injections were generally required with 1.0 mL of total blood samples necessary. When extracted according to the procedure described, the samples of plasma were stable for at least 24 h when stored at 4°C, and for at least 1 year when stored at -70°C.

RESULTS AND DISCUSSION

The different hydrophilicity of the aminic group present in the structures of fluoxetine and norfluoxetine (reported in Fig.1) suggests than ion-interaction HPLC method may prove useful for their separation.

This technique offers the advantage of good versatility since retention can be optimized as a function of many factors, as IIR (ion-interaction reagent) concentration, IIR alkyl chain lenght, acetonitrile percentage, pH. The effects of all these parameters have been systematically studied and reported in previous works (25,26).

Different compositions of the mobile phase were compared, ranging the percentage of acetonitrile between 30% and 50%, the octylamine concentration between 3.0 mM and 7.0 mM and the pH between 4 and 8. The optimized conditions resulted to be : 5.00 mM octylamine aqueous solution / acetonitrile (60/40 v/v) mixture, at pH = 6.4, that lead to a good resolution of the analytes within a reasonable total analysis time and minimize the interference of the matrix.

The proposed method was proved to be robust, being the resolution satisfactory with acetonitrile percentage ranging between 35% and 45%, octylamine concentration between 4.0 mM and 6.0 mM and pH between 6.0 and 7.0.

The separation obtained for a mixture of norfluoxetine and fluoxetine at concentration of 0.1 mg/L each is shown in Fig.1. The intra-day repeatibility was within 2% and the intra-day reproducibility for different mobile phase preparations was always within 5%. Detection limits (s/n = 3) were 4.5 μg/L and 2.3 mg/L, respectively for fluoxetine and norfluoxetine.

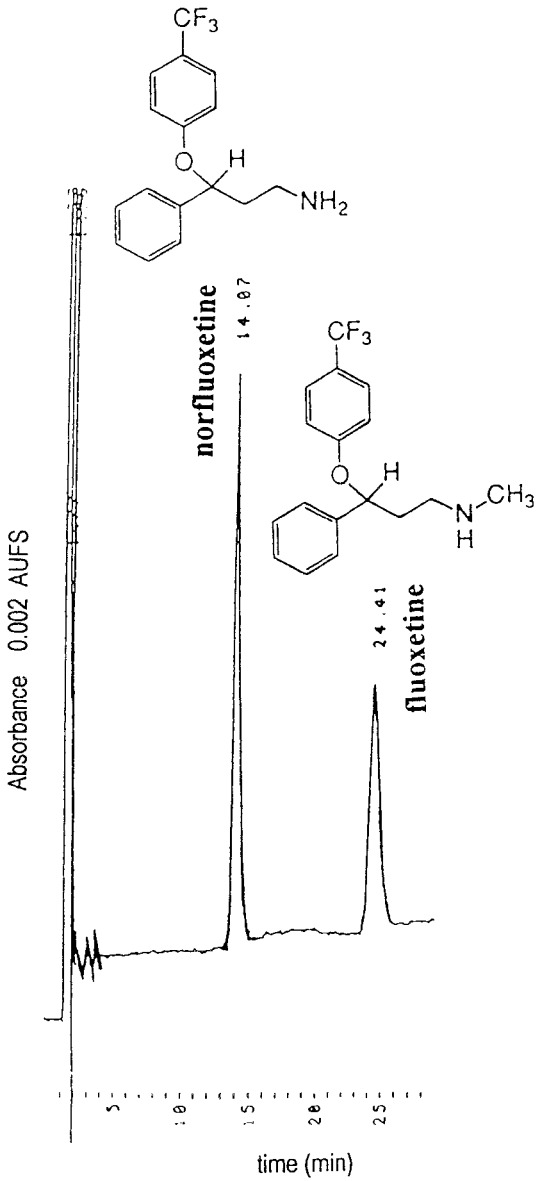

Figure 1. Separation of a mixture of norfluoxetine and fluoxetine (0.10 mg/L each). Stationary phase: Phase Separations ODS-2, (250 x 4.6 mm), endcapped, 5 µm. 100 µL injected. Mobile phase: 5.00 mM octylammonium o-phosphate in water-acetonitrile (60/40 v/v), pH 6.4. Flow-rate 1.50 mL/min. Spectrophotometric detection at 230.

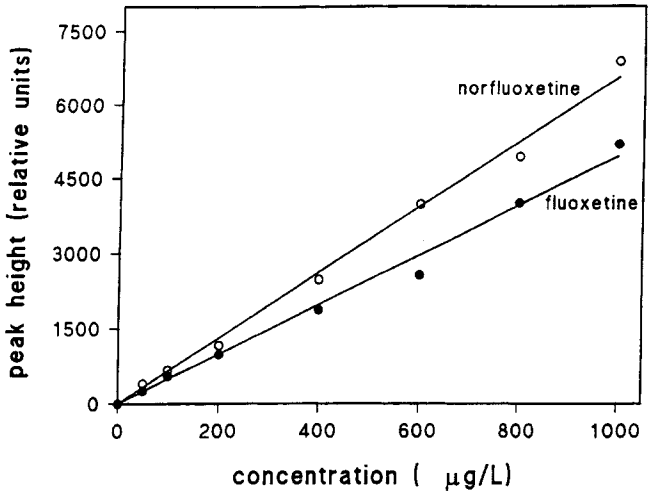

Figure 2. Peak height (relative units) vs. standard concentration in plasma for fluoxetine and norfluoxetine.

Calibration Plots and Recovery Data

Peak heights (relative units as given by the integrator) vs. standard concentration calibration plots for fluoxetine and norfluoxetine in drug-free plasma were constructed for concentrations ranging between 0.0 and 1000.0 µg/L. The plots (Fig.2), show very good linearity and were fitted by the following equations (*95%* confidence limits):

for fluoxetine: $y = 4.9518\ (\pm 0.1242)\ x$; $r^2 = 0.9906$

for norfluoxetine: $y = 6.5701\ (\pm 0.1288)\ x$; $r^2 = 0.9943$

where: y= peak height (relative units), x = the standard concentration (µg/L) and r^2 = correlation coefficient.

Some recovery tests were then performed on spiked plasma samples containing both analytes. Recoveries for both fluoxetine and norfluoxetine always greater than 92% were obtained.

Table 1

Capacity Factors k' (t_0 = 1.46) for Some Potentially Interfering Drugs

Drug	k'
Fluoxetine	14.89
Norfluoxetine	8.50
Paroxetine	13.80
Amylsulpride	1.60
Fluvoxamine	12.73
Mianserin	>70
Imipramine	58.67
Amitryptiline	>70
Clomipramine	>70
Maprotiline	20.86
Haloperidol	9.68
Flunitrazepam	3.95
Diazepam	6.94

Interference

In order to evaluate the matrix interference and recovery yield, experiments were performed for a fluoxetine-free plasma sample voluntarily offered by one of the authors. The sample was treated as described above and the chromatogram recorded under the optimized experimental conditions as in Fig.1, showed that the time-window corresponding to the retention of fluoxetine and norfluoxetine is interferent-free from plasma components.

In the therapy of obsessive-compulsive disorders, other drugs can be prescribed, both in co-diagnosis or in association with fluoxetine. To consider possible interferences in plasma analysis, under the optimized experimental conditions, the chromatographic retention of some common drugs, such as paroxetine, amysulpride, fluvoxamine, mianserin, imipramine, amitryptiline, clomipramine, maprotiline, haloperidol, flunitrazepam, and diazepam were determined (Table 1). The capacity factors indicate that the drugs can be separated well enough to guarantee very good resolution of the analytes of interest.

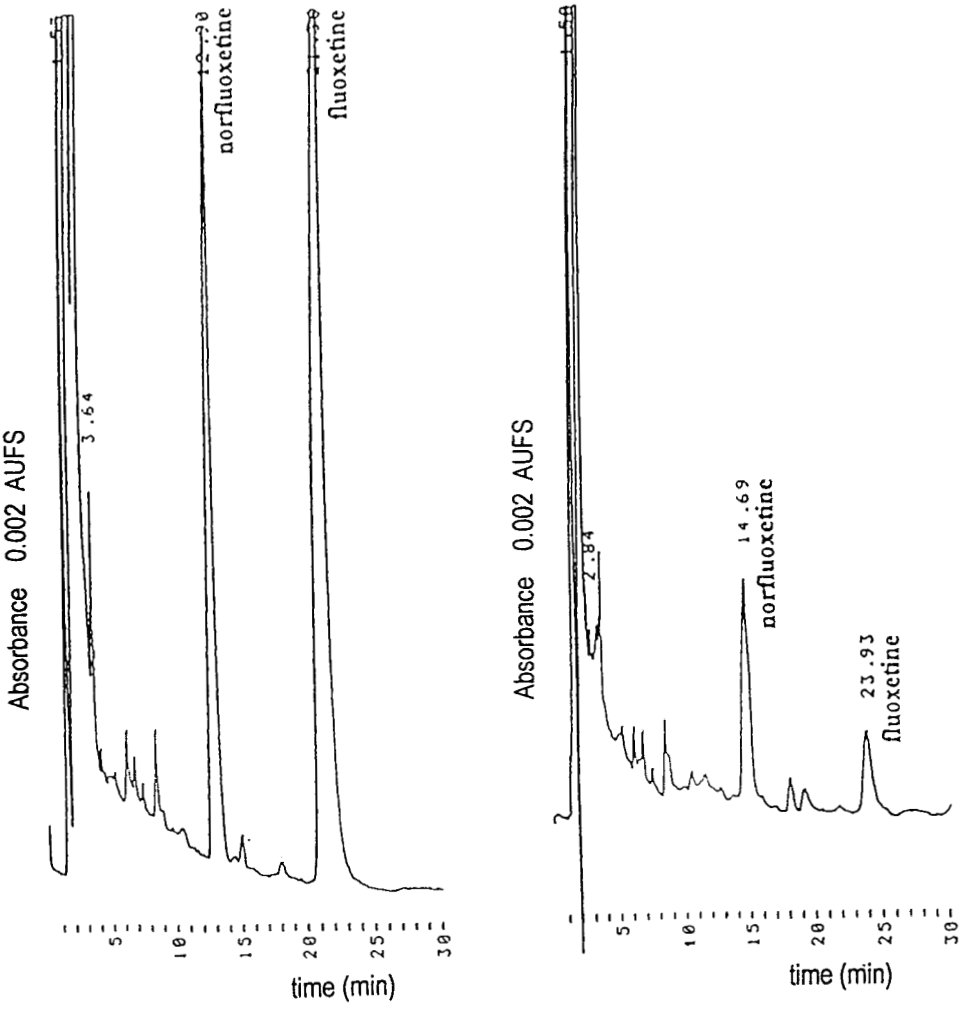

Figure 3. Examples of separation of norfluoxetine and fluoxetine in patient serum samples. Conditions as in Fig. 1.

Patient Plasma Analysis

In order to apply the method to real cases, samples of some patients plasma were analyzed. Typical chromatograms recorded for two patients

Table 2

Concentration of Fluoxetine and Norfluoxetine Found in Patient Plasma

Patient	Age (Years)	Sex	Daily Dosage (mg)	Duration of Therapy (Months}	Indication	FLX (μg/L)	NFLX (μg/L)	FLX/ NFLX Ratio
1	24	M	20	3	OCD	149±2	177±3	0.84
2	25	M	60	2	OCD	92±1	134±2	0.69
3	42	M	40	2	OCD	38±1	177±3	0.21
				3 later		30±1	153±2	0.19
4	24	F	40	4	OCD+GAD	620±3	318±4	1.95
5	25	F	40	3	OCD	87±1	155±3	0.56
6	45	F	20	1	OCD	321±3	98±1	3.27
				2 later		340±3	117±2	2.90

OCD = Obsessive Compulsive Disorder
GAD = Generalized Anxiety Disorder

(Fig.3) show that the plasma concentrations of fluoxetine and norfluoxetine are quite different despite the fact that the amount of time elapsed since drug administration were the same for both the patients. Furthermore, not only was the concentration of the two analytes different, but also the absolute values.

The fluoxetine/norfluoxetine ratio, appears to depend on the individual metabolism. The average results and standard deviations are reported in Table 2. As can be seen, the large variation of concentration was found in patient plasma, as well as different absolute concentration ratios.

Each analysis was repeated in triplicate and repeatibility was always within 2%. The quantitative determination was performed by means of the calibration curve. Some results were also obtained using the standard addition method. Such data confirmed the negligible matrix effects (correlation coefficients r^2 always > 0.99) and gave quantitative results which agreed within 5% with those obtained from the regular calibration plot method. As a conclusion, the HR-HPLC technique proved to be suitable for the separation of fluoxetine and norfluoxetine, leading to satisfactory separation and detection limits lower than 5 μg/L. The analytical method described here has been transferred to laboratories of the hospital where studies are in progress.

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