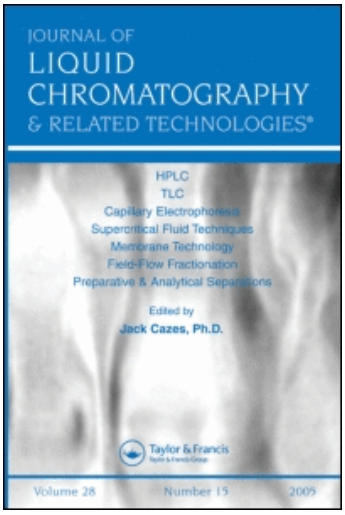

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Stereoselective Determination of Fluoxetine and Norfluoxetine Enantiomers in Plasma Samples by High-Performance Liquid Chromatography

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To cite this Article Pichini, Simona , Pacifici, Roberta , Altieri, Ilaria , Pellegrini, Manuela and Zuccaro, Piergiorgio(1996) 'Stereoselective Determination of Fluoxetine and Norfluoxetine Enantiomers in Plasma Samples by High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 19: 12, 1927 — 1935
To link to this Article: DOI: 10.1080/10826079608014016
URL: http://dx.doi.org/10.1080/10826079608014016

J. LIQ. CHROM. & REL. TECHNOL., 19(12), 1927–1935 (1996)

STEREOSELECTIVE DETERMINATION OF FLUOXETINE AND NORFLUOXETINE ENANTIOMERS IN PLASMA SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The simultaneous liquid chromatographic determination of the enantiomers of both fluoxetine and its metabolite norfluoxetine in plasma samples of treated patients is described. The compounds are subjected to solid phase extraction before chromatography. The separation of the analytes is achieved using two chiralcel ODR columns on-line coupled and a mobile phase consisting of acetonitrile-$NaClO_4$ 0.3M (66/34 v/v, pH 2.5) at a flow rate of 0.6 mL/min. The compounds were detected by ultraviolet absorbance at 220 nm. The limit of quantification for each compound was 10 ng/mL.

INTRODUCTION

Fluoxetine (FLU) (± N-methyl-3-phenyl-3-[α,α,α-trifluoro-p-tolyl)oxy]-propylamine) (Fig. 1) and its N-demethylated metabolite norfluoxetine (N-FLU) are antidepressant drugs. Their activity is based on the selective inhibition of 5-

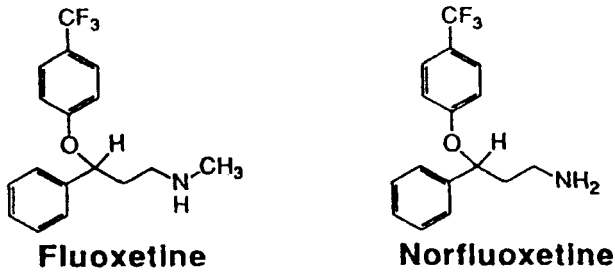

Figure 1. Structures of fluoxetine and norfluoxetine.

hydroxytryptamine (5-HT) recapture in the presynaptic neurons of the central nervous system.[1,2] Fluoxetine is sold, at present, as a racemate, despite different time courses in 5-HT uptake inhibition and rates of metabolism of fluoxetine R and S forms have been shown.[3-5] Similarly, (S)-N-FLU appears to be more potent than (R)-N-FLU in the inhibition of 5-HT uptake in vitro.[6]

Several chromatographic methods have been developed for the determination of fluoxetine and norfluoxetine in plasma or serum after administration of the parent drug,[7-13] but only two recent assays provide the measurement of the enantiomers of both fluoxetine and its metabolite, using chiral derivatization.[14-15]

In this paper we describe an enantioselective high performance liquid chromatographic (HPLC) method to determine the stereoisomers of fluoxetine simultaneously with the stereoisomers of norfluoxetine using solid phase extraction and spectrophotometric detection. The method was applied to plasma samples of treated patients using clozapine as an internal standard.

MATERIAL AND METHODS

Chemicals

Pure standards of (R, S)-FLU · HCl, (R, S)-N-FLU · HCl, (R)-N-FLU-HCl were provided from Eli Lilly Pharmaceuticals (Indianapolis, USA). The (R)-enantiomer of fluoxetine was obtained in our laboratory as described afterwards. Clozapine was provided from Sandoz S.p.A (Milano, Italy).

Extrelut-3 extraction columns were from Merck (Bracco, Milan, Italy). All solvents were of analytical reagent grade.

Chromatographic Instrumentation and Conditions

The HPLC system consisted of a Merck-Hitachi L6200 intelligent pump, a Merck-Hitachi L4200 UV-VIS detector set to 220 nm and a Merck-Hitachi D2000 chromato-integrator (Bracco, Milan, Italy). The columns used, from Daicel, Inc. (Schilling, Milan, Italy) were two Chiralcel ODR, both 25 cm x 4.6 mm i.d. and 10 μm particle size, on-line coupled with the second column heated at 40°C.

Resolution of the substances was achieved with acetonitrile-$NaClO_4$ 0.3M (66/34 v/v) containing 0.5 % triehylamine (pH 2.5 with perchloric acid). The mobile phase was left to equilibrate at least 2 hours before injections.

Isolation of the (R)-Enantiomer of Fluoxetine

In order to collect a purified enantiomer of fluoxetine, three Chiralcel ODR columns were on-line coupled, using the same mobile phase of the analytical separation.

This coupling permitted a 5 min interval between the peak baselines of the two fluoxetine stereoisomers, allowing the collection of the purified last-retained stereoisomer.

Subsequently, the stereoisomer dissolved in the mobile phase, alkalinized with NaOH 1N, was extracted with the same volume of n-hexane-isoamyl alcohol (97:3); the organic phase was evaporated under nitrogen and redissolved in 2 mL physiologic solution.

The absolute configuration of the extracted stereoisomer was determined comparing the optical rotation of its hydrochloride salt, measured with a Perkin Elmer 241 polarimeter (Perkin Elmer, Monza, Italy), to data reported in the literature.[4] It resulted to be the (R)- enantiomer of fluoxetine.

Sample Collection

Plasma samples were obtained from ten subjects treated for major depression with (R,S)-fluoxetine (Prozac). These participants received 20 mg of (R,S)-FLU once daily, usually taken between 7:00 and 8:00 a.m. Blood samples were taken after at least three weeks of treatment. Sampling was performed between 9:00 and 10 a.m., using evacuated tubes containing EDTA. Blood was then centrifuged, plasma was transferred to polypropylene tubes and kept frozen at - 20° C until analysis.

Solutions and Sample Preparation

Solutions of stock reference standards of racemic FLU, racemic N-FLU, (R)-N-FLU and clozapine (1 mg/mL, 10 μg/mL and 1 ug/mL) were prepared in methanol and stored below 0°C. Dilutions were made fresh daily for each analysis. Plasma standards were prepared daily by adding known amounts of the stock standards to blank human plasma.

A 1.5 mL aliquot of plasma, with 150 μl of clozapine as internal standard (200 ng/mL methanolic solution) and 500 μl 1N NaOH added, was vortex-shaken for 30 sec and transferred to an Extrelut-3 glass column. After 10 min, the analytes were eluted under gravity with 5 mL n-hexane-isoamyl alcohol (97:3). The organic phase was evaporated to dryness under a stream of nitrogen and redissolved in 150 μl of HPLC mobile phase. A 100 μl volume was injected into the HPLC column.

Calibration, Analytical Recovery and Precision

Spiked plasma carried through the entire procedure was used to create calibration curves and to determine analytical recoveries, intra-day and inter-day variabilites. The linearity of the calibration curves was studied in the range of 10-2000 ng/mL for each analyte.

Analytical recoveries were perfomed at three different concentrations (10, 100, and 500 ng/mL for each substance) with 5 samples for each concentration. The same concentrations were used to test the analytical imprecision, performing analyses of serum samples for up to six days.

Drugs Interferences

Several drugs commonly administered to individuals with depressive disorders were examined for their possible interference with the determination of fluoxetine and its metabolite stereoisomers. The substances tested were: imipramine, amitriptyline and their active metabolites desipramine and nortriptyline.

One microgram of each drug was added to blank plasma and to plasma spiked with FLU and N-FLU enantiomers carried through extraction procedure and analyzed by HPLC.

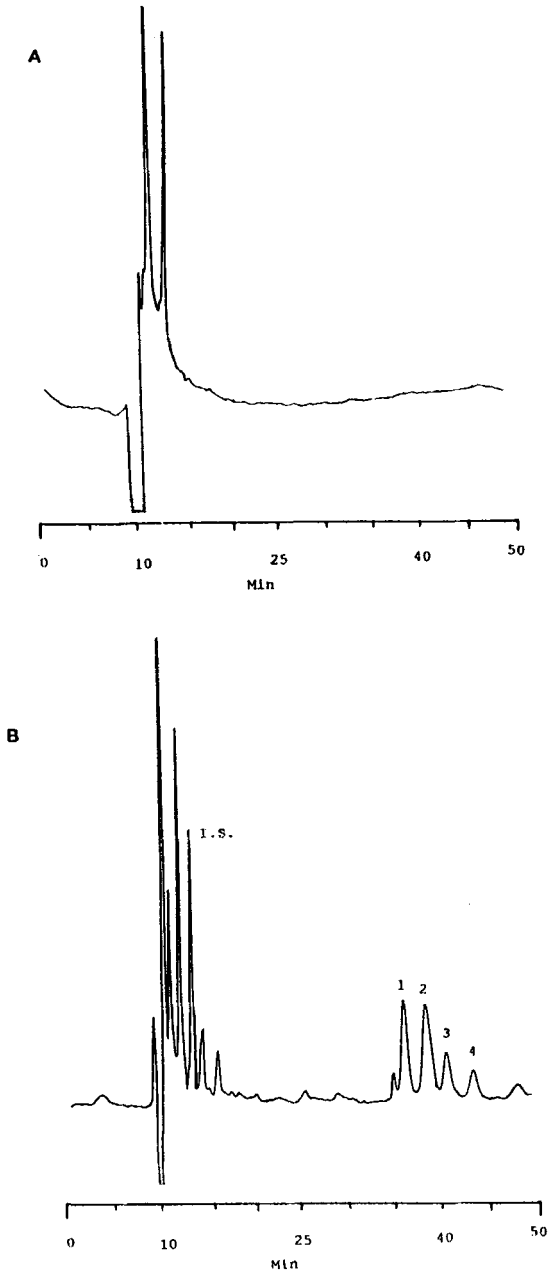

Figure 2. Chromatogram of (A) blank plasma; (B) an extract of a plasma sample containing 148 ng/mL (S)-N-FLU (1), 147 ng/mL (R)-N-FLU (2), 52 ng/mL (S)-FLU (3), 39 ng/mL (R)-FLU (4) and 200 ng/mL clozapine (I.S.).

Table 1

Recovery and Variability (n=5)

Concentration (ng/mL)	Recovery (mean + S.D.) (%)	Variability (%)	
		Intraday	Interday
(R)-FLU			
10	90.9 ± 3.4	3.7	3.9
100	91.2 ± 3.4	3.7	3.9
500	91.5 ± 3.6	3.9	4.1
(S)-FLU			
10	94.0 ± 3.4	3.6	3.8
100	94.1 ± 3.4	3.6	3.8
500	94.4 ± 3.5	3.7	4.0
(R)-N-FLU			
10	84.0 ± 2.3	2.7	2.9
100	84.3 ± 2.3	2.7	3.0
500	84.7 ± 2.4	2.8	3.1
(S)-N-FLU			
10	81.9 ± 2.1	2.5	2.9
100	82.3 ± 2.3	2.8	3.0
500	82.9 ± 2.4	2.9	3.1

RESULTS AND DISCUSSION

Fig. 2 depicts representative chromatograms of extracts of blank plasma sample and plasma sample containing both the enantiomers of FLU and N-FLU. Under the conditions outlined here, the retention times of (S)-N-FLU and (R)-N-FLU were 37 and 40 minutes while those of (S)-FLU and (R)-FLU were 41.6 and 44.6 minutes.

Extract of blank plasma showed that no chromatographic peak interfered with the analysis of enantiomers of FLU and N-FLU and with the internal standard clozapine, whose ritention time was 13 minutes.

The chiral separation was achieved only using an on line-coupled two column system, with the second column heated at 40°C for the improvement of peak width and shape.

Table 2

Detection Limit and Liability

Compound	Retention time (min)	Detection limit (ng/mL)	Linearity
(S)-Fluoxetine	41.6	10	$y = 3.7x + 0.7$
(R)-Fluoxetine	44.6	10	$y = 3.4x + 0.7$
(S)-Norfluoxetine	37.0	10	$y = 5.6x + 1.3$
(R)-Norfluoxetine	40.0	10	$y = 5.2x + 1.2$

y = peak height (cm)
x =m amount of the analytes (μg/mL)

Table 3

Patient Plasma Concentration of (S)-FLU, (R)-FLU (S)-N-FLU, (R)-N-FLU

Patient	Plasma Concentration (ng/mL)				Ratio	
	(S)-FLU	(R)-FLU	(S)-N-FLU	(R)-N-FLU	(S)/(R)FLU	(S)/(R)N-FLU
1	100	100	156	123	1.01	1.26
2	38	33	68	37	1.15	1.83
3	40	33	42	38	1.21	1.10
4	67	50	78	46	1.34	1.69
5	62	37	74	161	1.68	0.35
6	121	85	101	73	1.42	1.37
7	112	66	125	113	1.71	1.10
8	60	37	50	64	1.63	0.78
9	128	114	91	80	1.12	1.13
10	96	83	84	147	1.16	0.56
Mean	82.4	63.8	86.9	88.2	1.34	1.12
S.D.	33.1	30.1	34.0	45.2	0.26	0.46

S.D. = Standard Deviation

None of the other antidepressant drugs tested interfered with the assay.

The analytical recoveries of all the analytes, and the intra-day and inter-day variabilities are shown in Table 1.

The detection limit (signal-to-noise ratio of 3), the linearity of the method and the ritention times of the peaks are shown in Table 2. The calibration curves were linear over the range 10-2000 ng/mL for both the enantiomers of FLU and N-FLU with correlation coefficients always higher than 0.99.

This method has been applied in our laboratory to monitor the concentrations of enantiomers of fluoxetine and its metabolites in ten patients treated with 20 mg/die of the drug. Table 3 lists the data obtained, which are comparable with those reported in the literature.[15,16] Since plasma levels of (S)- and (R)- enantiomers of FLU and N-FLU were enough different, a stereospecific metabolism is confirmed.

In summary, the HPLC method described here permits quick and simple extraction and simultaneous determination of both the enantiomers of fluoxetine and norfluoxetine, without any derivatization and interference from two first-generation tricyclics. The development of this enantiospecific assay could be of great help in future studies on stereospecific pharmacokinetics and pharmacodynamics of fluoxetine, which can improve its clinical use.[17]

ACKNOWLEDGEMENTS

We thank Mrs. Simonetta Di Carlo and Mrs. Antonella Bacosi for expert technical assistance.

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Received November 24, 1995
Accepted December 15, 1995
Manuscript 4034