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# Simultaneous Estimation of Fluoxetine and Norfluoxetine in Plasma by RP-HPLC Employing Pre-Column Derivatization for UV-Sensitivity Enhancement

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## Abstract

A rapid high-performance liquid chromatographic method is described for the simultaneous estimation of the widely used antidepressant drug, fluoxetine and its principal metabolite norfluoxetine in human plasma. Pre-column derivatization of norfluoxetine was done using 4-dimethylaminobenzaldehyde (PDAB) to overcome the limitations of sensitivity of norfluoxetine. After liquid-liquid extraction the separation of analytes and internal standard from endogenous matrix interference was achieved using a reversed-phase HIQ sil ODS column (250 mm length x 4.6 mm internal diameter) KYA TECH (Japan) and assayed by ultraviolet absorption at 227 nm. The isocratic mobile phase (1 ml/min.) consisting of acetonitrile: water: triethylamine: 0.01 M Orthophosphoric acid (O.P.A.) (70:30:0.5:2) was used to separate fluoxetine, norfluoxetine and the internal standard, nebivolol. The relative retention times were 2.49, 4.24 and 7.29 min for norfluoxetine, fluoxetine and nebivolol, respectively. Chromatographic run time was 10 min and peak area ratios of analytes to IS were used for regression analysis of calibration curve. Linearity was obtained over the concentration range 10-60 µg/ml for both substances. The mean % recovery ± SD was found to be 101.23% ± 1.0 and 100.69 ± 0.67 for fluoxetine and norfluoxetine respectively. The method seems suitable, in terms of accuracy and precision, for the determination of fluoxetine plasma levels of patient; furthermore, it is rapid and sensitive.

**Keywords:** Fluoxetine; Norfluoxetine; Pre-column derivatization; Liquid chromatography; Plasma

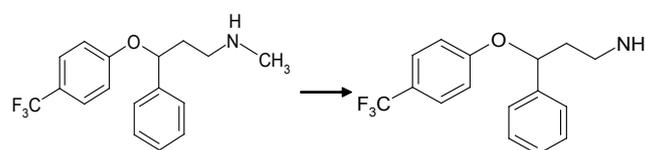
## Introduction

Fluoxetine (FLX), N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy], propan-1-amine exhibits selective inhibition of serotonin uptake in presynaptic neurons [1]. It is prescribed for a variety of psychopathological conditions including mood and eating disorders, obsessive-compulsive disorders, depression in the elderly and dysthymia [2]. It is official in United States Pharmacopoeia [3]. FLX is extensively metabolized by cytochrome P450 (CYP) isoenzymes in the liver to form an active N-demethylated metabolite Norfluoxetine (N-FLX) which has similar potency and selectivity with regard to the serotonin reuptake inhibiting effect of parent drug [2,4]. The degree of serotonin reuptake inhibition is correlated with the FLX plasma concentration. FLX is well absorbed after oral administration and disappears from plasma with half-time of 1-3 days; and its metabolite N-FLX has a plasma half-time of 7-15 days. After administration of FLX, approximately 65% of the administered dose of this drug is recovered in urine and about 15% in feces. The therapeutic dosage for fluoxetine is 20 mg/day which is metabolized in the liver to N-FLX and other unidentified metabolites. Overdoses of FLX have been reported to cause death. The plasma concentrations of the drug in these fatalities are 1.93-4.57 µg/ml [5,6].

The literature survey reveals that FLX and N-FLX have been simultaneously estimated by spectrophotometric [7,8], HPLC [9-22], GC [23-26], capillary electrophoresis [27], TLC with derivatization [28], LC-MS [29], HPLC-DAD [30].

For ready adaptation in clinical laboratory, a previously published HPLC procedure for quantitation of antidepressant was studied and compared to that of proposed RP-HPLC. The aim of present study was to develop an accurate, precise, specific and sensitive HPLC method

for quantitation of such metabolites in presence of the parent drug and other interfering components of the body fluids could be useful in a clinical laboratory for therapeutic drug monitoring, metabolic and bioequivalence studies (scheme 1).



Fluoxetine Norfluoxetine

 $C_{17}H_{18}F_3NO$   $C_{16}H_{16}F_3NO$ 

309.3 gm/mol 295.3 gm/mol

## Experimental

### Apparatus

a) The HPLC system was a PC based Jasco system comprising of a pump PU-2080 and a UV-2070 detector. Manual injections were carried out using a Rheodyne injector with a fixed 20 µl external loop.

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The mobile phase was a mixture of acetonitrile: water: triethylamine: 0.01M O.P.A. (70:30:0.5:2). Before analysis, the mobile phase was filtered through a 0.45  $\mu$  nylon filter, and then degassed ultrasonically for 15 min. The chromatographic separations were performed on a 5  $\mu$ m HIQ sil ODS column (250 mm length x 4.6 mm internal diameter) KYA TECH (Japan), operating at ambient temperature. The flow-rate was set at 1 ml/min and detection wavelength at 227 nm.

- b) Cata microwave synthesizer: RG – 31L was used for synthesis.
- c) Shimadzu AY 120 (max 120 g d = 0.1 mg) analytical balance was used for weighing.
- d) PCi Ultrasonicator
- e) Laboratory centrifuge Remi R- 8C was used for centrifugation.

## Materials

**Pure samples:** FLX, N-FLX and Nebivolol (NEB), the internal standard was kindly supplied by Cadila Pharmaceuticals Ltd., Ahmedabad. The purity given as % purity  $\pm$  SD was found to be 100.65  $\pm$  0.71 and 99.23  $\pm$  0.82 respectively for FLX and N-FLX.

**Chemical and reagents:** For HPLC work double distilled water was prepared in laboratory. Acetonitrile, triethylamine, orthophosphoric acid, chloroform, n-hexane and isopropanol were of HPLC grade from Lobachemie Pvt. Ltd. (Mumbai, India). Ammonium chloride and 4-dimethylaminobenzaldehyde were of analytical reagent grade from Lobachemie Pvt. Ltd. (Mumbai, India).

## Selection of pre-column derivatization reagent

As the metabolite had relatively poor sensitivity for UV-detection a pre-column derivatization of the metabolite was done for the HPLC method development. Pre-column derivatization of Norfluoxetine was done using 4-dimethylaminobenzaldehyde.

## Preparation of internal standard solution

Standard stock solution containing 100  $\mu$ g/ml of NEB was prepared by dissolving 10 mg of NEB in 50 ml of mobile phase and then sonicating for 10 minutes. The volume of solution was made up to 100 ml with mobile phase after filtration through a 0.45  $\mu$  nylon filter to get 100  $\mu$ g/ml of NEB in 100 ml volumetric flask.

## Studying the effect of human plasma constituents on assay of FLX and N-FLX

**Preparation of plasma solutions for calibration curve:** To 1-6 mg of FLX, 1-6 mg of N-FLX and 5 ml of plasma was added to 10 mg of 4-dimethylaminobenzaldehyde reagent and the reaction mixture was heated for 5 minutes in microwave synthesizer at power 5. In a 15 ml centrifuge tube, 5 ml of ammonium chloride buffer (pH 9.5) and 5 ml of extraction solvent chloroform: isopropanol: n-hexane (10:14:26) was added. Centrifuge at 2000 g for 5 min and aqueous layer was removed by freezing. The organic layer was transferred into clean brown glass test tube and evaporates under vacuum. The residue was redissolved in 10 ml of the mobile phase to give the stock plasma solution.

In to a series of 10 ml volumetric flasks, 1 ml of stock plasma solution pipetted and to each flask 2 ml of (100  $\mu$ g/ml) NEB was added and then final volume of the solutions was made up to 10 ml with mobile phase.

## Results and Discussion

The method describes a pre-column derivatization procedure along with a HPLC procedure employing a  $C_{18}$  column and mobile phase comprising of acetonitrile: water: triethylamine: 0.01M O.P.A. (70:30:0.5:2) for separation and quantitation of FLX, N-FLX and NEB. FLX and N-FLX are freely soluble in organic solvents like methanol and acetonitrile. Mobile phase containing acetonitrile- water at various ratios gave tailing effect and triethylamine was used as organic modifier to overcome this problem. Keeping in view the physicochemical properties of both the drugs few drugs were tested for use as an internal standard with respect to resolution suitability. Nebivolol was found to give good resolution, accuracy and precision of quantitative results.

Flow rate of 0.5 ml/min resulted in greater retention times and broader peaks while 1.2 ml/min resulted in very close retention times with poor resolution. A flow rate of 1 ml/min resulted in optimum retention times with good resolution, good peak shape and with all drug components and internal standard eluting within 10 minutes.

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is one that gives good response for the drugs to be detected. Optimization of wavelength was done at different wavelengths for detection by UV detector to get good response. In the present study, drug solutions of 50  $\mu$ g/ml of FLX and 50  $\mu$ g/ml of N-FLX pretreated with PDAB were separately prepared in mobile phase. After observing UV spectra of both the drugs and the internal standard, wavelength of 227 nm was selected for further study.

## Chromatography

A chromatogram of plasma spiked with 50  $\mu$ g/ml of FLX and N-FLX is shown in Figure 1. The relative retention times were 2.49, 4.24 and 7.29 min for N-FLX, FLX and NEB respectively. The three peaks were adequately resolved without any interference from endogenous compounds, as shown in Figure 1.

## Linearity, recovery and limit of detection

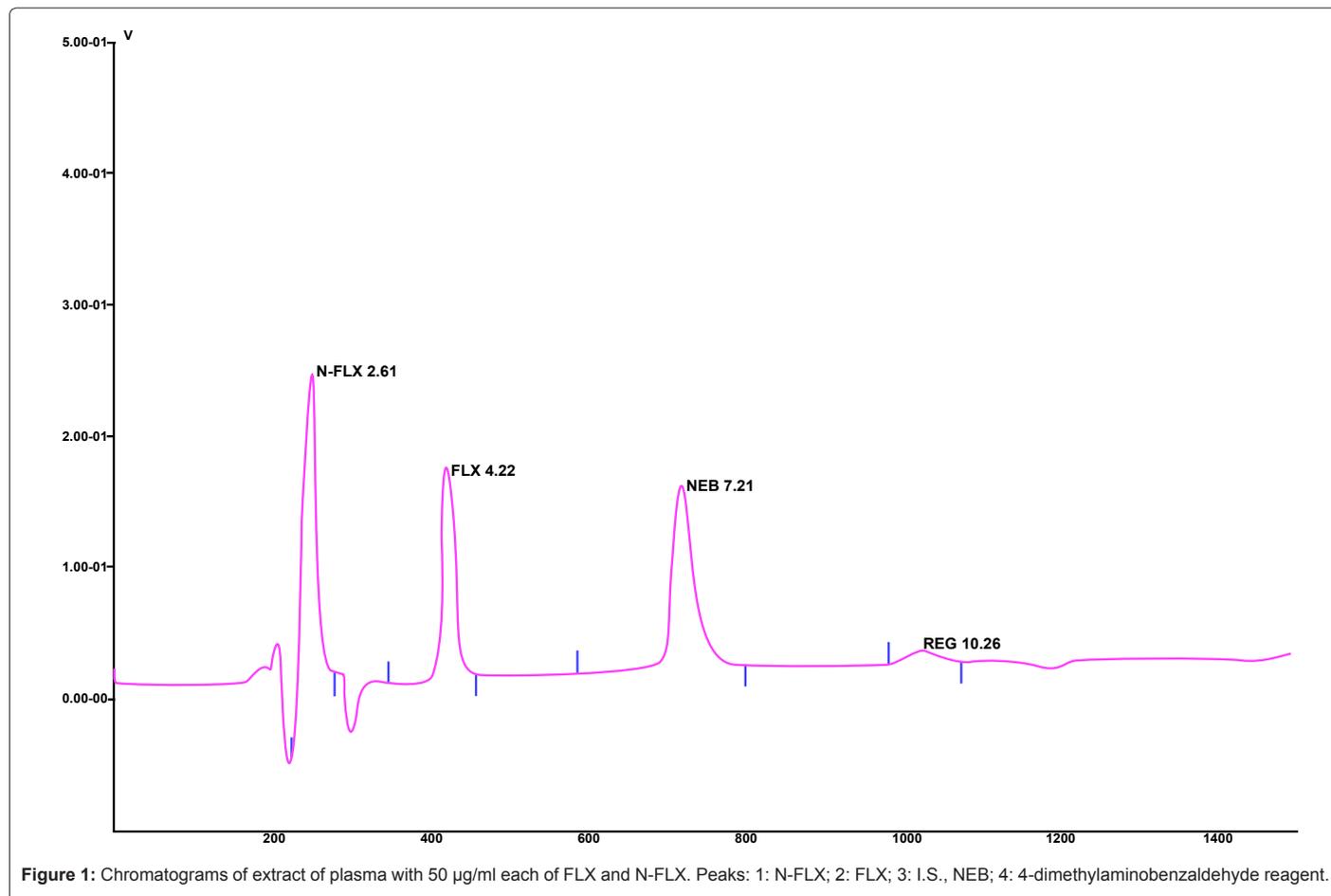
Calibration curves were linear over the range 10-60  $\mu$ g/ml ( $r^2 = 0.999$ ,  $n = 6$ ) for both FLX and N-FLX. The extraction recovery calculated as the mean ( $n = 6$ ) for FLX and N-FLX is shown in Table 1. The limits of detection (LOD) defined as three times the baseline noise were 0.83 and 1.54 for FLX and N-FLX respectively. The limits of quantitation (LOQ) were 2.75 and 5.87 for FLX and N-FLX respectively.

## Accuracy, precision and intra-assay reproducibility

Assays were performed over a 3-day period, using spiked human plasma samples ( $n = 6$ ). The results are listed in Table 2. The method was found to be reproducible, as indicated by the low values obtained for the coefficients of variation less than 2%. The proposed method can also be used to accurately determine FLX and N-FLX in plasma as there was no interference from the biological matrices.

## System suitability parameters

System suitability parameters were analyzed on freshly prepared standard stock solution FLX and N-FLX. Both the drugs were injected into the chromatographic system under the Optimization of Chromatographic conditions. Parameters that were studied to evaluate the suitability of the system are listed below and the results are given in Table 3.



- a) Height Equivalent to Theoretical Plate (H.E.T.P.)
- b) Theoretical plates
- c) Tailing/ asymmetric factor
- d) Resolution
- e) Retention time
- f) Selectivity
- g) Calibration Curve
- h) Limit of detection and limit of quantitation.

	Conc. added (µg ml <sup>-1</sup> )	Accuracy (%)	(%) C.V. intra-assay	(%) C.V. inter-day
Fluoxetine	30	98.7	0.92	1.25
	40	99.7	0.78	0.95
	50	99.9	1.21	0.51
Norfluoxetine	30	98.2	1.54	0.72
	40	98.9	0.68	1.82
	50	99.8	0.84	1.61

CV stands for Coefficient of variance

**Table 2:** Accuracy, intra-assay reproducibility and precision (n = 6).

Concentration (µg ml <sup>-1</sup> )	% Recovery (mean ± SD)	
	Fluoxetine	Norfluoxetine
10	101.8 ± 0.81	99.89 ± 1.35
20	98.88 ± 0.54	99.34 ± 0.48
30	99.92 ± 1.20	101.12 ± 0.74
40	99.94 ± 1.53	100.06 ± 0.65
50	100.86 ± 0.68	99.73 ± 1.59
60	100.30 ± 0.76	99.52 ± 1.69

SD stands for Standard Deviation

**Table 1:** Recovery of the analytical method (n = 6).

The proposed RP-HPLC procedure for quantitation of FLX & N-FLX was compared with a reported HPLC method [9] for the comparison data has been given in Table 4.

### Conclusion

The HPLC method described is selective, sensitive and reproducible for quantitation of FLX and N-FLX from plasma. The HPLC method involving pre-column derivatization with UV detection, owing to its sensitivity, seems to be very promising for the Norfluoxetine assays in biological fluids. Studies show that the HPLC method owing to its specificity could also allow for the detection and determination of some impurities, such as N-FLX and others which can be toxic, often present in the pharmaceutical formulations. The method proves

Sr. No.	Parameters	FLX	N-FLX
1.	Theoretical plates	4412.07	3670.35
2.	Tailing factor	1.275	1.275
3.	Resolution factor	6.625	8.161
4.	Retention time in minutes	4.24	2.49
5.	Selectivity	1.71	1.70
6.	Calibration range ( $\mu\text{g ml}^{-1}$ )	10-60	10-60
7.	LOD ( $\mu\text{g ml}^{-1}$ )	0.83	1.54
8.	LOQ ( $\mu\text{g ml}^{-1}$ )	2.75	5.87

Table 3: System suitability parameters from plasma.

	Previous Method		Proposed Method	
	FLX	N-FLX	FLX	N-FLX
Retention times	7.7	3.4	4.24	2.49
% Recovery (n = 6)	86-88	92-93	98-102	98-102
Tailing factor	-	-	1.275	1.275
Resolution	Poorer	Poorer	6.625	8.161
% Accuracy (n = 6)	97-100	98-100	98-100	98-100
(%) C.V. inter-day (n = 6)	7.56	5.7	0.90	1.41
(%)C.V. intra-assay (n = 6)	2.66	2.16	0.97	1.02

Table 4: The data of fluoxetine and norfluoxetine in presence of plasma by the previous method and proposed method.

that it is precise and accurate for the analysis of FLX and N-FLX in plasma. The proposed method could be applied for routine analysis in a clinical laboratory for therapeutic drug monitoring, metabolic and bioequivalence studies.

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