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Rapid quantitation of fluoxetine and norfluoxetine in serum by micro-disc solid-phase extraction with high-performance liquid chromatography–ultraviolet absorbance detection

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Abstract

A rapid, robust and sensitive method for the extraction and quantitative analysis of serum fluoxetine (FLX) and norfluoxetine (N-FLX) using a solid-phase extraction (SPE) column and high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was developed and validated. The sample clean-up step was performed by simple micro-disc mixed-mode (non-polar and strong cation exchange (SCX)) SPE cartridges. Separation of analytes and internal standard (IS) clomipramine (CLO) from endogenous matrix interference was achieved using a Waters Symmetry C_8 (150 mm × 2.1 mm i.d., 5 μm) reversed-phase narrow bore column. The relative retention times were 8.5, 9.6 and 10.5 min for FLX, N-FLX and CLO, respectively with a low isocratic flow rate of 0.3 ml/min. Chromatographic run time was completed in 15 min and peak area ratios of analytes to IS were used for regression analysis of the calibration curve. The latter was linear from 10 to 4000 nmol/l using 0.5 ml sample volume of serum. The average recovery was 95.5% for FLX and 96.9% for N-FLX. The lowest limit of quantitation (LLOQ) for serum FLX and N-FLX was 10 nmol/l (on-column amount of 200 fmol). The method described was used to analyse serum samples obtained from rats given chronic FLX treatment and to examine the relationship between steady state serum drug concentrations and neurochemical changes in several brain regions.

Keywords: Fluoxetine; Norfluoxetine

1. Introduction

Fluoxetine (FLX) is an antidepressant drug which has been shown to be a selective serotonin (5-hydroxytryptamine: 5-HT) reuptake inhibitor (SSRI) in presynaptic neurons [1]. The SSRIs have different chemical structures and pharmacological profiles as compared to the classical tricyclic antidepressants (TCAs), but are generally better tolerated with equivalent antidepressant efficacy [2]. FLX is extensively metabolised 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 the parent drug [3,4]. The chemical structures of fluoxetine and norfluoxetine are presented in Fig. 1.

FLX has a large volume of distribution (V_d) which indicates the drug is extensively accumulated in tissue. The long half-lives ($t_{1/2}$) of FLX (2–3 days) and N-FLX (7–15 days) are related to a significant accumulation of these active species in the body during chronic use and for a considerable duration after discontinuation of therapy. The monitoring of serum FLX and N-FLX levels may be beneficial in the clinical situation, particularly the concentrations at steady state, where variation in the pharmacodynamic effects of the drug might be due to non-compliance, interaction with other drugs or other possible causes. Although many studies have shown neurochemical and behavioural effects of chronic FLX treatment in rats, much less attention has been given to the steady state serum concentration of the drug required to produce such effects [5–10]. We report here the results of an analysis of FLX and N-FLX serum levels in rats and their relationship to neurochemical changes in serotonin (5-HT) and its principle metabolite

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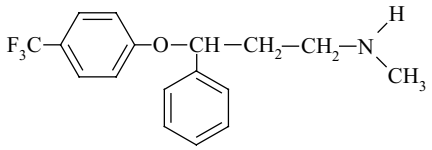
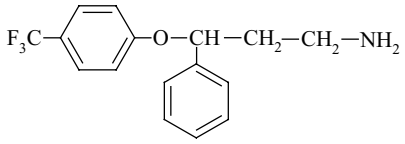


Fluoxetine



Norfluoxetine

Fig. 1. Chemical structures of fluoxetine and norfluoxetine.

5-hydroxyindoleacetic acid (5-HIAA) in various brain regions in rats.

Several different analytical methods have been used to measure serum FLX and N-FLX concentrations in rodents and humans. There are some reports using gas chromatography (GC) with electron capture detection (ECD) for measuring FLX and its metabolite but this technique is laborious and unsuitable for routine analysis and the processing of large numbers of blood samples [11,12]. Thus, high-performance liquid chromatography (HPLC) with either fluorescence or ultraviolet (UV) detection remains the most widely used analytical method for FLX measurement. Fluorescence detection has also been applied to the chiral separation and analysis of the enantiomers of FLX and N-FLX [13–16]. The complexity of additional sample derivatisation steps for fluorescence analysis renders it unsuitable as a desired routine analytical method for measuring FLX and its metabolites.

Most previously reported HPLC–UV methods involved multiple step liquid–liquid extraction (LLE) procedures for sample clean-up to remove interfering materials [16–21]. These conventional LLE techniques are labour intensive, time-consuming and difficult for batch processing and automation for routine analysis. They also require relatively large quantities of organic solvents that are expensive, toxic and environmentally hazardous. In this regard, sample clean-up step using solid-phase extraction (SPE) technique allows faster, more robust analytical procedure leading to lower detection limits while providing increased sample throughput and enhanced productivity. HPLC–UV methods using SPE with bonded phase of non-polar C_{18} [22] or C_8 [23] for sample clean-up procedures have been developed to measure serum FLX and N-FLX. However, the major problems encountered in these traditional non-polar SPE cartridges relate to the large volume of solvents used for sample work up steps and the relatively low extraction recoveries (60–80%) leading to lower sensitivity for subsequent HPLC–UV detection.

Recently, we have demonstrated that ion exchange SPE chemistry produces better analytical recovery and cleaner sample extract as compared to non-polar bonded phase chemistry in analysing ionic compounds [24]. In this report, we describe the use of highly selective micro-disc mixed-mode (non-polar and strong cation exchange (SCX)) SPE cartridges for rapid sample extraction and enrichment procedures for the determination of low level of FLX and its metabolites in rat serum samples. To explore the wider application of this novel method, spiked human serum samples were also assessed using the described extraction procedure. The developed method offers a simple and fast sample preparation procedure for quantitation of serum FLX and its metabolite in both rats and humans via HPLC–UV detection.

2. Experimental

2.1. Chemicals and reagents

All aqueous solutions were prepared in Milli-Q water (Millipore, Milford, MA). Fluoxetine was kindly supplied by Eli Lilly (Indianapolis, IN, USA). Norfluoxetine, clomipramine, glacial acetic acid, isopropanol and dichloromethane were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and methanol were obtained from Mallinckrodt Baker (Sellby-Biolab, Sydney, Australia). Ammonia solution was purchased from BDH (Poole, UK). APEC-DAU micro-disc (15 mg/disc) mixed-mode SPE cartridges were obtained from Varian (Sydney, Australia).

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a Shimadzu ADVP module (Kyoto, Japan) equipped with a SIL-10 autoinjector with sample cooler and LC-10 in-line vacuum degassing solvent delivery unit. Chromatographic control, data collection and processing were carried out using Shimadzu Class VP data software. Chromatographic separation of FLX, N-FLX and CLO was accomplished on a Waters Symmetry C_8 5 μm (2.1 mm × 150 mm) narrow bore reverse-phase column (Waters, Australia) coupled with a 3 mm Opti-Guard C_8 pre-column (Optimize Technologies, Alpha Resources, Thornleigh, Australia) maintained at 25 °C by a Shimadzu CTO-10AS column oven (Kyoto, Japan). The mobile phase consisted a mixture of 67 mmol/l potassium phosphate buffer (pH 3.0) and acetonitrile (67:33, v/v). The flow rate was maintained isocratically at 0.3 ml/min. The eluate from the HPLC column was directed via a GBC LC1200 UV-Vis detector (Melbourne, Australia) monitored at 226 nm and the total run time was 15 min.

2.3. Preparation of standards and control samples

Stock solutions of 5 mmol/l FLX and N-FLX were prepared in water. These solutions were further diluted with

water to give a series of spiking standard solutions with concentration of 0, 0.25, 2.5, 5.0, 12.5, 25 and 100 μmol/l. A standard stock solution of the internal standard (IS) clomipramine (5 mmol/l) was prepared in water and diluted to give a working standard solution of 50 μmol/l. Stock standard solutions were prepared monthly and stored at −20 °C. Working standard solutions were freshly prepared for each run. Blank serum was obtained from two different lots of untouched rats with similar body weight to the test rats, they were stored at −20 °C and subsequently used for standard calibration, preparation of quality control samples (100 and 1000 nmol/l of both FLX and N-FLX) and assay validation. The quality control samples were also stored at −20 °C.

2.4. Calibration and sample preparation

Calibration curves ranging from 10 to 4000 nmol/l of FLX and N-FLX in serum were constructed by spiking into blank serum (0.5 ml) with 20 μl of spiked standard solutions. For the unknown samples, at the time of decapitation, whole trunk blood from rat was collected in a pre-chilled plain blood collection tube, blood was allowed to clot and serum was separated from cells by centrifuging at 3300 × *g* at 4 °C for 15 min. Serum samples were stored at −20 °C and thawed just prior to analysis. The calibration curve of each standard was obtained by concentration versus the area ratio of the standard and IS.

2.5. Micro-disc solid-phase extraction

A 0.5 ml of the rat or human serum sample was spiked with 20 μl of IS in an Eppendorf tube to give a final serum concentration of 2 μmol/l. The sample was then diluted with 0.5 ml of 0.1 M potassium phosphate (KH_2PO_4) buffer (pH 6.0) and mixed gently. The SPEC-DAU micro-disc SPE cartridges were connected to a Vac Elut vacuum manifold (Analytichem, Harbor City, CA) and conditioned with 0.5 ml methanol followed by 0.5 ml 0.1 M KH_2PO_4 buffer (pH 6.0). Serum samples were then applied to each cartridge accordingly. The sample was allowed to run through the disc at a low flow rate of not more than 1 ml/min. The cartridge was then rinsed with 0.5 ml 1 M acetic acid (HAc) followed by 0.5 ml methanol (MeOH). The disc was dried under vacuum for about 2 min. The tips of the Vac Elut delivery needles were wiped and a rack with labelled collection micro tubes was placed in the Vac Elut. The analytes were eluted with 0.5 ml of dichloromethane–isopropanol–ammonia (80:20:2, v/v/v) at flow rate of not more than 1 ml/min. The eluant was then dried under vacuum in a SpeedVac vacuum evaporator (Savant Instruments, Farmingdale, NT, USA) and the dried residue was re-dissolved in 50 μl of mobile phase. The mixture was then vortexed and centrifuged at 16,000 × *g* for 5 min to remove particulates present, if any, in the tube. The supernatant was then transferred to micro insert vials and 20 μl of the reconstituted solution was automatically injected into the HPLC system. Quantitation of concentrations of FLX and N-FLX in unknown and control samples were obtained from the linear regression equation of calibration curves by plotting concentration versus the area ratio of the standard and IS.

2.6. Assay validation

2.6.1. Linearity, precision, accuracy, recovery and sensitivity

Peak area ratios of FLX and N-FLX to the IS measured at each nominal concentration were used to construct non-weighted least-square linear regression curves. The inter- and intra-run precision and accuracy of the method were evaluated on three separate days by triplicate analyses of rat serum containing FLX and N-FLX at concentrations of 10, 100, 1000 and 4000 nmol/l. An estimate of the inter-run precision for the analytes was obtained by one-way analysis of variance (ANOVA) for each test concentration using "run day" as the classification variable [25]. The day mean square (DayMS), error mean square (ErrMS) and the grand mean (GM) of the observed concentration across analytical run days were obtained. The inter-run precision was calculated for each calibrated standard as follows when n is the number of replicates within each analytical run:

$$\text{inter-run precision (\%)} = \frac{[[\text{DayMS} - \text{ErrMS}]/n]^{0.5}}{\text{GM}} \times 100$$

The intra-run precision was calculated for each calibration standard as follows:

$$\text{intra-run precision (\%)} = \frac{[\text{ErrMS}]^{0.5}}{\text{GM}} \times 100$$

The accuracy was assessed for each calibration standard and expressed as a percentage of bias ((mean value − nominal value)/nominal value × 100).

To study the effect of co-extracted plasma matrix, recovery was conducted by comparing peak areas of replicates from extracted serum samples (100 and 1000 nmol/l) with those of blank extraction eluants to which the same amounts of analytes had been added to post-extraction. The analytical limits of the assay were determined by analysis of five replicates of different serum FLX and N-FLX concentrations from a single pool of matrix. The lowest limit of quantification (LLOQ) was defined as the concentration which produced assay results within ±20% of the nominal concentration and a R.S.D. less than 20%.

2.6.2. Stability

For assessment of stability of serum samples upon storage, fresh pooled blank rat serum samples were spiked with FLX and N-FLX at 100 nmol/l (low concentration) and 1000 nmol/l (high concentration). They were then assayed on the day of preparation and the remainder of each control

was divided into multiple tubes and stored at −20 °C. These stability samples were removed and assayed periodically for up to 2 months. Triplicate control samples (low and high concentrations) were also subjected to three freeze/thaw cycles to assess stability over a 5-day period.

2.6.3. Pharmacodynamic studies

The method described was applied to a study of the steady state serum concentrations of FLX and N-FLX in male Wistar rats. The rats were given ad lib access to either FLX solution (175 mg/l in tap water) as their only fluid source (experimental condition) or tap water (control condition). The FLX concentration chosen equated a dose of 7 mg/kg assuming that a 500 g rat would drink approximately 20 ml of FLX solution per day. This dose is similar to effective doses for modifying behaviour with chronic administration in previous studies [26–31]. Serum samples and brain tissue were collected on 38th day of the treatment period. The developed HPLC–UV method was applied to quantify serum FLX and N-FLX concentrations. Neurotransmitter levels in various brain regions were measured by HPLC-electrochemical detection (ECD) method as previously described [32]. Blood collection procedures were as described in Section 2.4. The serum layer was removed and stored at −20 °C until analysis. All experimentation was approved by the University of Sydney Animal Ethics Committee in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*.

2.6.4. Statistical methods

The values shown are mean ± standard error (S.E.). Statistical analysis of data was achieved by ANOVA and unpaired student *t*-tests. The minimum significance level for all statistical tests was set at $P < 0.05$.

3. Results

3.1. Chromatographic separation

Typical chromatograms of extracted blank serum and serum spiked with 100 nmol/l of FLX and N-FLX with the IS CLO (2 μmol/l) are shown in Figs. 2 and 3. The analytes and the IS were well resolved and the relative retention times for N-FLX, FLX and CLO were 8.5, 9.6 and 10.5 min, respectively. No interfering endogenous peaks were observed in the blank serum samples in the HPLC–UV analysis. Under these conditions, no interference was observed with protriptyline, desipramine, imipramine and amitriptyline with the retention times of 4.5, 4.7, 5.1 and 5.7 min, respectively in both rat (Fig. 2) and human (Fig. 3) serum samples. The total chromatographic run time was 15 min. Fig. 4 shows the chromatograms obtained from rats after 38 days chronic ad lib access to FLX solution (175 mg/l tap water) or tap water.

3.2. Validation

3.2.1. Linearity, precision, accuracy, recovery and sensitivity

For the blank serum used in the study, there was no background difference between the two lots of pooled serum. The assay was validated for linearity and reproducibility of the calibration curve by running five separated serum standard of 0, 10, 100, 200, 500, 1000 and 4000 nmol/l of both analytes. The typical equations obtained by least squared regression were $y = 0.000381x - 0.013$ for FLX and $y = 0.000343x - 0.0074$ for N-FLX. Regression coefficients (r^2) were ≥0.999 for all calibration curves (Table 1). The intra- and inter-run accuracy (expressed as percentage bias) and

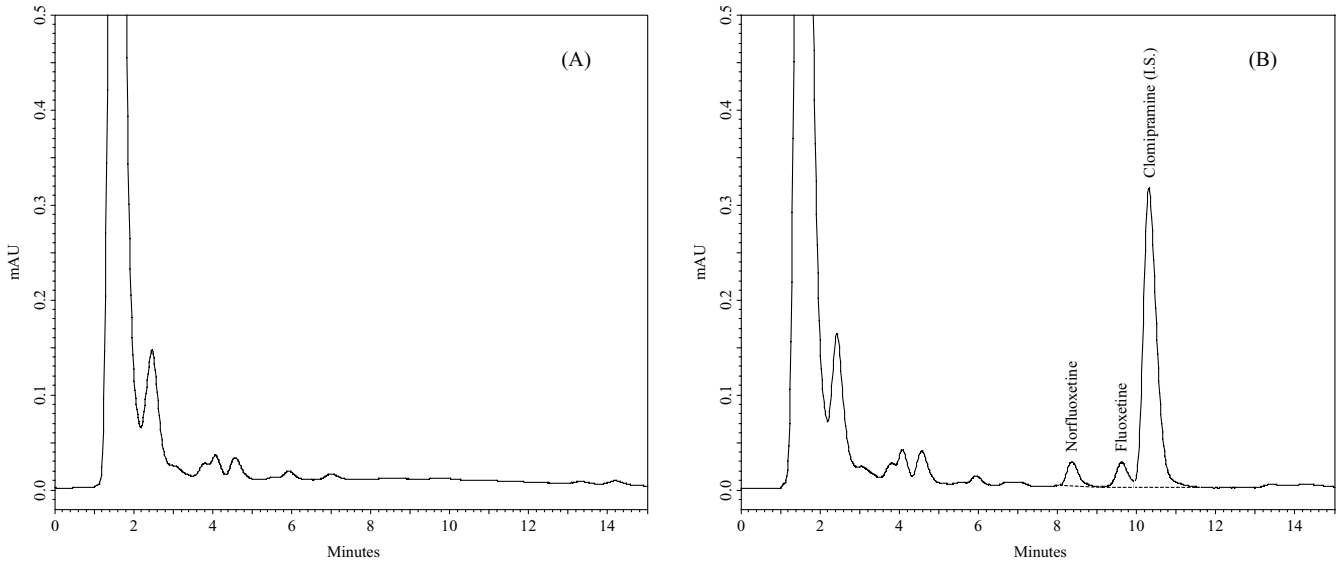


Fig. 2. Representative chromatograms of extracted rat serum sample of (A) blank sample, (B) blank sample spiked with 100 nmol/l of FLX and N-FLX with internal standard CLO (2 μmol/l).

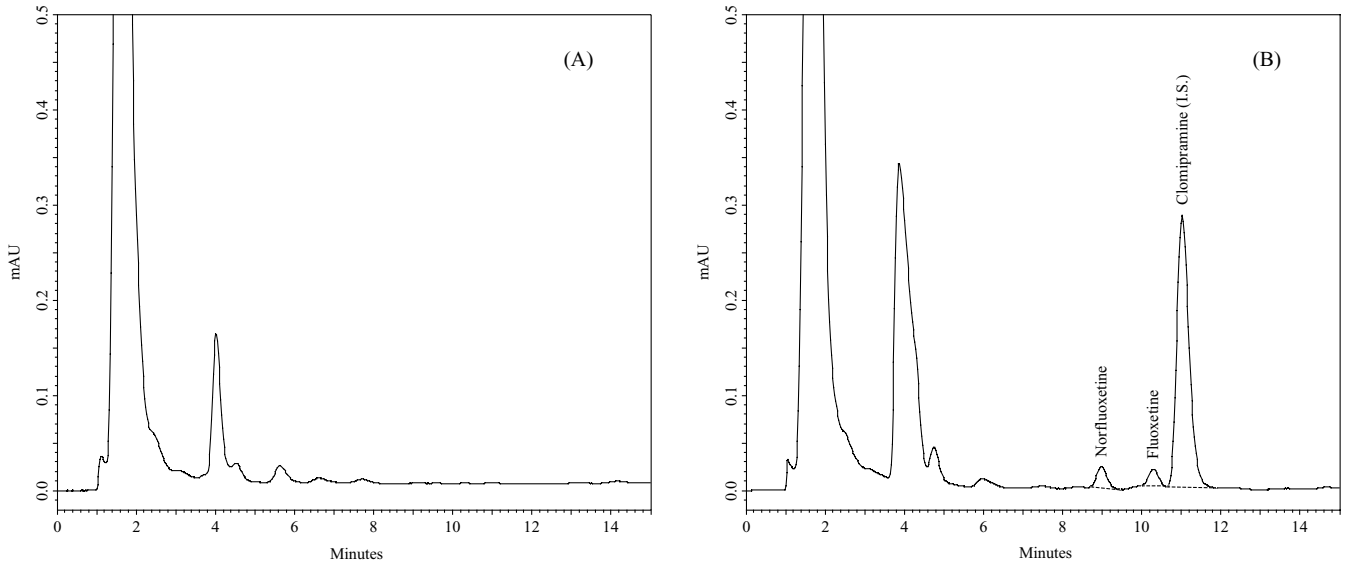


Fig. 3. Representative chromatograms of extracted human serum sample of (A) blank sample, (B) blank sample spiked with 100 nmol/l of FLX and N-FLX with internal standard CLO (2 μmol/l).

precision (expressed as R.S.D. (%)) for FLX and N-FLX based on peak area ratios are presented in Table 2. The assay bias ranged from 11% (10 nmol/l, assay LLOQ), to −3.9% (100 nmol/l) over the concentration range 10–4000 nmol/l. The inter-run precision for all concentrations was less than 10% and overall intra-run precision for FLX and N-FLX was less than 13%.

The analytical recoveries of serum FLX and N-FLX were calculated from pre-spiked and post-spiked extracted serum concentrations of 100 and 1000 nmol/l. The spiked serum was then analysed by the developed method. Average recovery from 10 separated batch assays over a 2-month period was 95.5 and 96.9% for FLX and N-FLX, respectively.

Table 1
Validation data of linear regression analysis ($n = 5$)

Parameter	FLX	N-FLX
Slope: mean (CV, %)	0.000381 (16.1)	0.000343 (11.8)
Intercept: mean ± S.D.	−0.013 ± 0.015	−0.0074 ± 0.0057
Correlation coefficient: mean (range)	0.999 (0.998–1.0)	0.999 (0.999–1.0)

The LLOQ was determined by analysis of five replicates of different concentrations of serum FLX and N-FLX. Using 0.5 ml of serum sample, the LLOQ for serum FLX and N-FLX was 10 nmol/l (on-column amount of 200 fmol) for which the R.S.D. ≤20% was found.

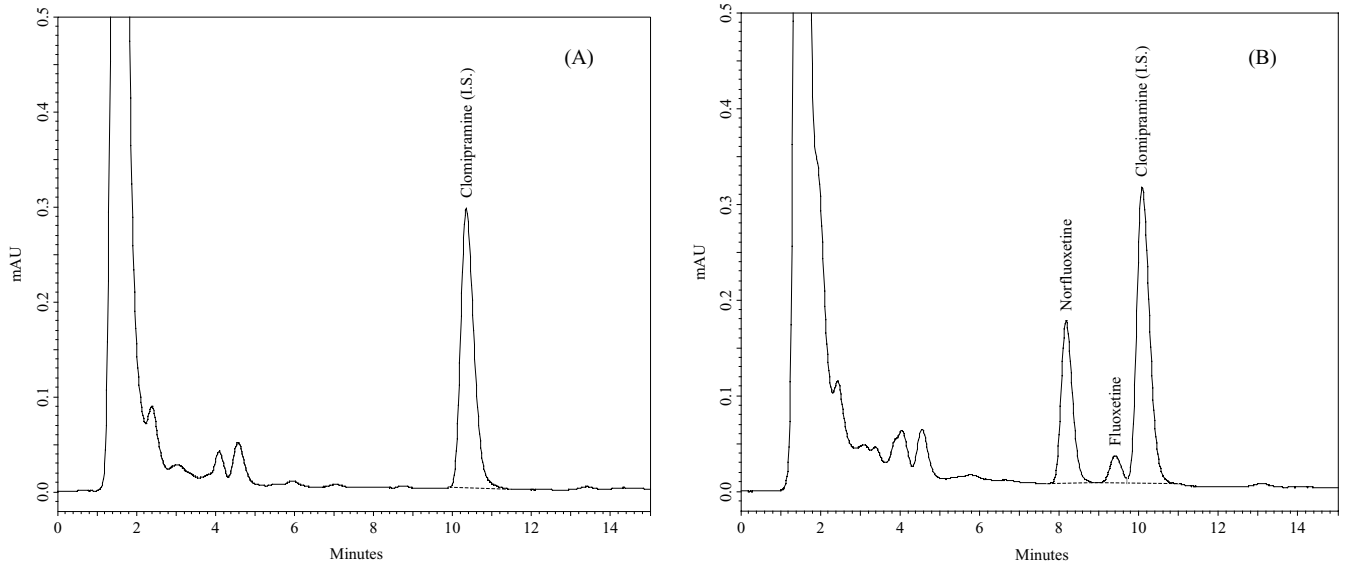


Fig. 4. Representative chromatograms of extracted rat serum samples of (A) tap water control treatment, (B) chronic fluoxetine treatment (N-FLX = 1068 nmol/l; FLX = 173.9 nmol/l).

Table 2
Validation data of precision and accuracy for serum FLX and N-FLX ($n = 3$)

Nominal concentration (nmol/l)	Measured concentration (nmol/l)		Intra-run precision (%)		Inter-run precision (%)		Accuracy (% bias)	
	FLX	N-FLX	FLX	N-FLX	FLX	N-FLX	FLX	N-FLX
10	11.1	10.9	12.9	11.5	8.20	9.56	11.0	9.0
100	96.1	103	5.54	5.62	4.26	4.38	−3.90	3.0
1000	985	978	3.44	3.27	1.33	1.84	−1.50	−2.20
4000	3884	3940	2.12	3.02	2.56	1.85	−2.90	−1.50

3.2.2. Stability

For the long-term stability of serum samples stored at −20 °C, repeated analysis of the control serum FLX and N-FLX samples (100 and 1000 nmol/l) showed no apparent change in concentration over 2 months. In addition, no significant change in concentration was also observed after three freeze/thaw cycles over a 5-day period (one-way ANOVA). FLX stability in aqueous solution has been studied extensively and shown no significant loss of FLX stored in water at both 4 and −20 °C for up to 3 months [33].

3.2.3. Pharmacodynamic studies

Serum FLX and N-FLX concentrations for rats in the chronic FLX treatment group are shown in Fig. 5. The serum concentrations of FLX, N-FLX and total in the treated group were 283±44, 1298±93 and 1581±120 nmol/l, respectively. The active N-demethylated metabolite N-FLX was five-fold higher than the parent drug ($P < 0.001$). No trace of the drug or its metabolite was observed in the control group given tap water only.

There were significant differences in 5-HIAA concentrations across the brain regions except between amygdala and hypothalamus in both control and FLX treated groups. Chronic FLX treatment significantly reduced 5-HIAA ($P < 0.001$) in all brain regions examined with reduction of 71.3, 73.4, 62.2, 74 and 65.3% of controls in the prefrontal cortex, striatum, hippocampus, amygdala and hypothalamus, respectively. Chronic FLX treatment also significantly reduced 5-HT to 84.6% of control in the hippocampus ($P < 0.01$) but did not significantly affect 5-HT in the other brain regions examined.

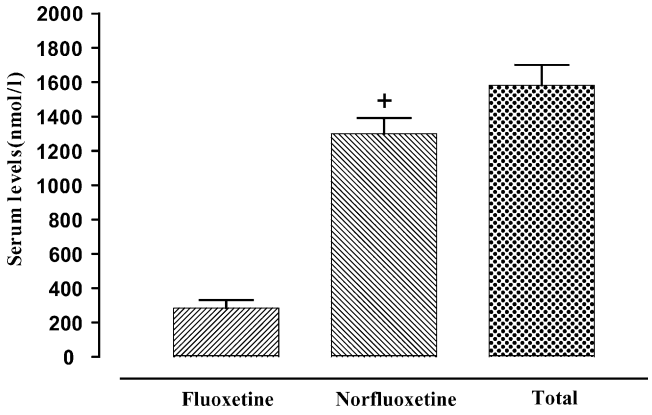


Fig. 5. Serum levels of FLX and N-FLX in chronic FLX treated rats ($n = 12$). Data are mean ± S.E.; $^{+}P < 0.001$ when compared with the parent drug FLX.

4. Discussion

The method described here provides major advantages in terms of simplicity of sample extraction and turnaround time as compared with other reported assays [17–21]. The removal of interference and the enrichment of analytes in sample preparation are often the most time consuming steps of an analytical process. Inadequate sample preparation procedures are often a contributing factor to error in subsequent HPLC analysis. There is a continuous need for the development of extraction methods for faster and more robust analytical techniques to achieve higher sensitivity of detection.

Conventional LLE extraction techniques are labour intensive, time-consuming and difficult for batch processing and automation in routine analysis. They also require relatively large quantities of organic solvents which are expensive, toxic and hazardous to the environment. The SPE technique solves many of the these problems and has been recognised as a major improvement in sample pre-treatment technique with a vast potential application area.

The sample clean-up procedure presented here involves a novel approach to the extraction of FLX and its metabolite using micro-disc mixed-mode of non-polar C_8 and SCX SPE cartridges which provides extremely clean serum extracts (Fig. 2). This cleanliness is due to the fact that most of the interferences are removed during the column rinsing steps using acetic acid and organic solvent (MeOH), while the analytes present in the serum are irreversibly retained on the column.

At pH 6.0, all amine groups of the analytes were virtually positively charged, so that retention of the drugs was from both ion-exchange and non-polar interaction mechanisms. The acetic acid in the washing step served to promote the ionic interaction between the amine groups and the SCX bonded phase whilst removing other polar and hydrophilic compounds in the sample by disrupting hydrogen bonding. Hydrophobic interactions and hydrogen bonding were disrupted by washing with the organic solvent MeOH which removed non-polar materials such as lipid and fatty acids and other hydrophilic compounds in the serum sample. The high pH of the elution mixture shut-off the ion exchange

retention as the amine groups of the drugs became unionised while the non-polar retention was disrupted with the isopropanol and dichloromethane. The bonded phase selectively retains and elutes the analytes by both non-polar and ionic interaction mechanism. Therefore, this mixed-mode extraction bed chemistry achieved better analytical recoveries for FLX (95.5%) and N-FLX (96.9%) as compared to other previously reported non-polar C_{18} (<70%) and C_8 (<87%) bonded phase SPE chemistries [22,23]. The described mixed-mode SPE procedures can also be applied to measuring FLX and N-FLX in human samples without modification (Fig. 3). In theory, the described extraction method can be applied for measuring other basic drugs in different biological matrices with slightly alternation of the pH and/or the organic solvents, which can be manipulated very predictably, in sample loading and washing steps. Up to 5% of triethyl amines in MeOH can also be used to increase the elution selectivity for tertiary and aromatic amines from primary and secondary amines.

This finding further supports our previous report on the supremacy of mixed-mode bed chemistry in analysing ionic compounds [24]. The good recovery obtained with the described method allowed us to use only 0.5 ml of rat serum sample for extraction, which is an advantage in sampling small animals for pharmacokinetic studies. The average binding capacity of the micro-discs used in the study was about 10% (1.3–1.7 mg) of the bed mass (15 mg). Ion exchange capacity of SCX was approximately 0.6 meq./g. Therefore, even a larger sample loading volume can be applied to the disc without overwhelming the capacity of the disc. One limitation for this particular micro-disc SPE is that excessive use of water for conditioning might reduce the disc capacity. The added advantage of mixed-mode SPE is in the diversity of its application as analysts can accomplish multiple extraction techniques in a single SPE cartridge. The use of small micro-disc sorbent mass requires only very small volumes of solvent (0.5 ml) during sample extraction procedures (conditioning, sample loading, rinsing and eluting) and offers shorter total cycle time, particularly in the solvent evaporation step that can sometimes limit sample throughput. The very small amount of dichloromethane used for elution step, therefore, should not pose any safety concerns to the operators.

The second feature of the assay method involves the use of a narrow bore column and low flow rate (0.3 ml/min) for the HPLC separation. Reduced column internal diameter results in smaller column volumes which not only allow an increase in detection sensitivity but also the reduction of mobile phase solvent consumption by almost 80% as compared to standard columns (4.6 mm i.d). The calibration range of 10–400 nmol/l of the assay is sufficient for both PK and steady state drug concentration study. The lowest limit of detection of 10 nmol/l for FLX and N-FLX achieved in the method was comparable to fluorometric detection [34] but better than previously reported UV methods [17–21,23].

The described HPLC–UV method was used to study steady state serum FLX and N-FLX levels in rats during chronic FLX administration and associated neurochemicals changes. N-FLX concentrations obtained in our study were comparable to those previously reported although residual FLX was undetectable in this previous assay method [35]. Our results indicate the steady state serum N-FLX was five-fold higher than the parent drug. These high N-FLX levels may significantly augment the antidepressant action of FLX. It is possible that FLX might be metabolised to a small number of other minor metabolites but there is no evidence that such unidentified metabolites produce a significant effect on the 5-HT uptake or other toxicity. Chronic FLX treatment significantly reduced 5-HIAA levels in all brain regions examined (prefrontal cortex, striatum, hippocampus, amygdala and hypothalamus) providing further evidence that FLX decreases re-uptake of 5-HT and prevents metabolism of 5-HT to 5-HIAA by intra-neuronal monoamine oxidase A [5,35]. These results confirm that the dose of FLX administered in the experiment was adequate in altering 5-HT uptake and metabolism.

In conclusion, the present assay is a simple and rapid method for the determination of serum FLX and N-FLX. The very small volume of serum required facilitates pharmacokinetic studies of the drug in small laboratory animals. The high efficacy of micro-disc extraction method also permits batch processing and automation for routine analysis of FLX and its metabolites in clinical samples.

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