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Sensitive and high-throughput bioanalysis of fluoxetine and nor-fluoxetine in rabbit and human plasma using SPE-LC-MS/MS

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Abstract

Fluoxetine is a commonly prescribed antidepressant agent in psychotherapy. A rapid, selective and sensitive LC-MS/MS method was developed and validated for simultaneous estimation of fluoxetine and its metabolite nor-fluoxetine in rabbit and human plasma. The assay was linear over the concentration range of 0.048–100 ng/mL with a lower limit of detection of 32 pg/mL (0.032 ng/mL) for both fluoxetine and nor-fluoxetine. Separation and detection of analytes were achieved on a reversed phase Waters Symmetry Shield™ C<sub>18</sub> column, with an isocratic mobile phase consisting of methanol and 0.5% formic acid (80:20, v/v) at a flow rate of 0.75 mL/min. A turnover rate of 2.5 min per sample enables the high-throughput bioanalysis of fluoxetine. An automated solid phase extraction method was employed for efficient extraction of analytes from matrix. Thereafter, analytes were monitored by using MS/MS with electrospray ionization source in positive multiple reaction monitoring mode. The method was successfully applied to in-vivo pharmacokinetic studies in rabbit and in-vitro protein binding studies in human plasma. Due to high sensitivity and low requirement of sample volume, the method could be applicable for preclinical and clinical applications such as therapeutic drug monitoring in special population (children and geriatric patients) using only 0.03 mL of plasma.

Keywords: Fluoxetine, Norfluoxetine, Solid phase extraction, LC-MS/MS, Pharmacokinetics
1. Introduction

Fluoxetine (FXT, Fig.1a), known as Prozac®, is one of the highly prescribed selective serotonin reuptake inhibitors (SSRIs) intended to curb the effects of major depression. It was the first SSRI approved by the US-FDA in 1987 for the treatment of depression in adults and for children in 2003. It is also used to treat obsessive compulsive disorder, panic disorder, premenstrual dysphoric disorder, bulimia nervosa. Among 43 million antidepressant prescriptions in UK in 2011, six million prescriptions (14%) were of FXT\(^1\). Besides the antidepressant activity, it is also reported to have antifungal\(^2,3\) antimicrobial\(^4\), antioxidant\(^5\), antiviral\(^6\), spermicidal and antitrichomonas activities\(^7\). FXT is metabolized by the liver to its major active metabolite nor-fluoxetine (NFXT, Fig.1b) by N-demethylation. Both FXT and NFXT were reported to have long half-lives (averaging 5 days for FXT and 10 days for NFXT)\(^8\). Although FXT is a revolutionary antidepressant with relative lack of side effects, an important concern associated with the overdose of FXT is the suicidality. In recent past, this tendency is increasing in number of people, including children, who are being given this drug by their general practitioners for mild depression and who are not seriously clinically ill. Moreover, children are reported to have twofold higher levels of FXT and NFXT relative to adolescents\(^9\). It has been reported that NFXT is slightly more potent inhibitor of serotonin neuronal reuptake than FXT\(^10\).

Due to the high prescriptions and frequent involvement in clinical investigations, reliable bioanalytical techniques should be available in clinical and toxicological laboratories to monitor these drugs in biological samples. Several bioanalytical methods such as HPLC with UV detection\(^11,12\), diode array detection\(^13,14\), fluorescence detection\(^15,16\); GC-MS\(^17\) and LC-MS/MS\(^18-25\) have been reported for the determination of FXT and NFXT in biological matrices. However, all of these methods require more than 100 µL of plasma\(^19,24,26\) which is difficult to collect in case of children particularly when multiple samples are needed for
therapeutic monitoring. Decreasing the plasma volume often results in low sensitivity for effective quantitation of drug. Because of relatively high polarity and very low levels of analytes in plasma some methods require derivatization process. Li Y et al has reported a LC-MS/MS method for simultaneous determination of fluoxetine and norfluoxetine with a dynamic range of 0.05–20 ng/mL using 100 µL of human plasma. Since therapeutic monitoring of fluoxetine is often limited by low sample size, it would be beneficial to have a bioanalytical method with low volume of plasma so as to have application in special population like children and geriatric patients. So, there is a clinical need to develop a bioanalytical method with high extraction efficiency and sensitive to be useful for the bioanalysis of FXT and NFXT in minimum volume of blood sample.

We report a highly sensitive LC-MS/MS method that simultaneously estimates FXT and NFXT in picogram level using automated solid phase extraction that requires low plasma volume (0.03 mL) than existing methods. The primary advantage of this method is short run time (~2.5 min for each sample), made it possible to analyze more than 200 samples per day. Since LLOQ is 48 pg/mL (0.048 ng/mL) for both parent and metabolite, the method enables the detection of analytes over a longer period of time in blood.

2. Experimental

2.1 Chemicals and reagents

Fluoxetine hydrochloride, norfluoxetine hydrochloride, phenacetin and formic acid (FA) for mass spectrometry (purity ~98%) were purchased from Sigma-Aldrich (St Louis, MO, USA). HPLC grade methanol was purchased from Spectrochem Pvt. Ltd (Mumbai, India). Human plasma was purchased from Bioreclamation, USA. Ultrapure water (18.2 MΩ*cm) was obtained from a Milli-Q PLUS PF water purification system. Oasis® hydrophilic-lipophilic balance (HLB) cartridges were purchased from Waters Corporation Milford, Massachusetts
USA (Lot No. 117A33036A). All other reagents were of analytical grade and purchased from standard chemical suppliers.

2.2 Preparation of stock, standard and quality control samples

Primary stock solutions (1 mg/mL) of FXT, NFXT and phenacetin (Internal standard, IS) were prepared in methanol. A mixed stock solution of FXT and NFXT was prepared at 100 µg/mL concentration. From the mixed stock, working standards were freshly prepared and spiked (3 µL) into blank plasma (27 µL) containing IS (100 ng/mL) to obtain a final calibration standards (CS) of 0.048–100 ng/mL. Four different quality control (QCs) samples viz., lower limit of quantification (LLOQ-0.048 ng/mL), low quality control (LQC-0.140 ng/mL), middle quality control (MQC-45 ng/mL) and high quality control (HQC-75 ng/mL), were prepared daily in five replicates. All the primary stocks and mixed stock were kept in refrigerator at 2–8 °C and brought to room temperature prior to use.

2.3 Extraction procedure

The extraction of analytes from plasma samples was carried out using Oasis® HLB cartridges (1cc, 30 mg) in a Rapid Trace® automated solid phase extraction (SPE) assembly (Caliper Life Sciences, USA). Prior to the extraction, the cartridges were preconditioned with 2 mL of methanol through the column twice followed by 2 mL of 0.5% v/v FA. All the CS, QCs along with pharmacokinetic and protein binding samples were diluted with 0.3 mL of 0.5% v/v FA solution. All the samples were vortexed for 5 min on a cyclomixer (Spinix Tarsons, Kolkata, India) and loaded onto preconditioned cartridges. The cartridges were then washed with 1 mL of 0.5% v/v FA containing 5% v/v methanol. Analytes were eluted in fresh tubes with 2 mL of methanol and subsequently dried at 50 °C for 30 min using nitrogen dryer (Turbovap). Dried samples were reconstituted with 50 µL of methanol, vortex mixed and loaded into LC-MS/MS for analysis.
2.4 LC-MS/MS conditions

A Shimadzu (Japan) SIL series LC system (comprising an autosampler (SIL-HTc), a binary pump and column oven) coupled to an API 4000 Q-trap mass spectrometer (Applied Biosystems, Canada) equipped with an electrospray ion (ESI) source was used for method development. The analytes and IS were separated on a Waters Symmetry Shield™ C₁₈ column (150 x 4.6 mm, 5µm) with an isocratic mobile phase consisting of methanol and 0.5% FA (80:20, v/v) delivered at a flow rate of 0.75 mL/min. Mobile phase was filtered through 0.22 µm membrane filter (Millipore, USA) and degassed ultrasonically for 15 minutes prior to use. The autosampler temperature was kept at 6 ± 2 °C and the column oven temperature was maintained at 30 °C. The injection volume was 20 µL and the total LC run time was 2.5 min. Other chromatographic parameters, viz., rinsing volume, rinsing speed, needle stroke, sampling speed, purge time and rinse dip time were set to 300 µL, 25 µL/s, 52 mm, 3.0 µL/s, 1.0 min and 10 s respectively. Rinsing solution was methanol and water (80:20, v/v). Rinsing mode was set before and after aspiration to ensure negligible carryover effect.

The mass spectrometric detection of analytes and IS was performed in multiple reaction monitoring (MRM) mode using an ESI source in positive ionization mode with gas 1, gas 2, and curtain gas set at 60, 50 and 15 psi, respectively. The ion source temperature was maintained at 500 °C and ion spray voltage was set at 5500 V. The dwell time was 250 ms. Zero air was used as source gas while nitrogen was used as both curtain and collision gas.

Compound dependent parameters, viz., declustering potential (DP) 46 and 44 V; entrance potential (EP) 10 and 10 V; collision energy (CE) 13 and 07 eV and collision cell exit potential (CXP) 12 and 10 V for FXT and NFXT, respectively. The MS/MS system was operated at unit resolution, monitoring precursor ion → product ion combinations of m/z 310.3 → 148.1 for FXT, m/z 296.2 → 133.9 for NFXT and m/z 180.1 → 138.1 for IS. The
equipment control, peak integrations and data analysis were accomplished by using PE SCIEX Analyst Software (Version 1.4.2) from Applied Biosystems.

2.5 Assay validation

The assay validation was performed in rabbit and human plasma according to US-FDA guidelines with respect to selectivity, accuracy, precision, linearity, extraction efficiency, lower limit of quantification (LLOQ), reproducibility, matrix effect and battery of different stability studies.29

The selectivity was investigated by analyzing processed blank plasma collected from six individual rabbits and human. Specificity was established by the lack of interfering peaks at the retention time of the analytes and IS.

The extraction recoveries were evaluated at three QC levels (LQC, MQC and HQC) by comparing the peak areas of extracted QC samples with those of analytical standards at corresponding concentration. Samples were prepared in minimum of five for each level where as extraction efficiency of IS was determined at single concentration of 100 ng/mL.

Matrix effect was evaluated at three different QC levels (LQC, MQC and HQC) each in six replicates. Blank plasma was processed using SPE method and after extraction, analytical standard solution equivalent to QCs was spiked into dried extract. Mean peak area of each post extracted sample was compared with corresponding analytical standard solution. If the ratio of peak area of post extracted spiked samples to analytical standard is less than 85% or more than 115%, the matrix effect is implied.26, 28, 30

Five replicates of QC samples at four levels (LLOQ, LQC, MQC and HQC) were included in each run for five consecutive days to determine the intra-and inter-day accuracy and precision.
Dilution effect was investigated to ensure that samples could be diluted with blank plasma without affecting the concentration. Analytes spiked plasma samples prepared at 500 ng/mL concentrations were diluted with pooled plasma at dilution factors of 5 and 10 in five replicates. As a part of the validation, five replicates had to comply with both precision of ≤ 15% and accuracy of 100 ± 15% similar to other QC samples.

Stability experiments were carried out at LQC and HQC levels (n=5) to examine the stability of analytes in stock solutions and in plasma samples under different conditions. Stability studies included bench-top stability for 6 h, freeze-thaw stability (up to three freeze-thaw cycles), auto-sampler stability for 24 h using wet extracted samples, dry extract stability (at –70 ± 10 °C for 30 days) and long term stability (at –70 ± 10 °C for 30 days).

Each validation run consist of system suitability test samples, calibration curve samples including blank sample (matrix sample processed without IS), zero sample (matrix sample processed with IS), eight non-zero points (0.048–100 ng/mL) and QC samples were prepared. The calibration curves were established by plotting the peak area ratio (analyte/IS) versus nominal concentration by using least-square linear regression analysis with a weighting factor of $1/x^2$. The acceptance criteria for each back calculated standard concentration was set at ±15% deviation, except for the LLOQ where it was set at ± 20% deviation of the nominal concentration.

2.6 Plasma protein binding

The purpose of plasma protein binding (PPB) study was to evaluate the applicability of the method in human plasma. PPB was performed in human plasma as per previously reported equilibrium dialysis method. Prior to study, equilibrium dialysis cells were coated with sigmacote (chlorinated organopolysiloxane in heptane) to avoid any non-specific binding of drug with apparatus. Dialysis membrane ($M_w$ cut-off:12–14 kDa) was purchased from Himedia and activated as per manufacturer’s protocol. The study was conducted in triplicate
(human plasma pH was adjusted to 7.4 with 0.1 N HCl and 0.1 N NaOH). Both the analytes were spiked at 50 ng/mL concentration and kept in oscillating thermostatic water bath for 30 min at 37 ± 2 °C. After incubation, spiked plasma samples were kept in donor cell and immediately covered by activated dialysis membrane. Tris buffer (100 mM, pH 7.4) was kept in receiver cell. Both the cells were closed tightly and kept in water bath for overnight incubation. At the end of the experiment, samples from donor and receiver side were taken out carefully. Protein binding was calculated using following equation. Organic content for spiking solution was kept at 1% for both the analytes. PPB was calculated by using Eq. (1). Moreover, non specific binding of FXT and NFXT in the dialysis blocks, membrane and equilibrium establishment was evaluated in dialyzed buffer using previously reported method\textsuperscript{32}.

\[
\text{%Bound} = \left( \frac{\text{Conc in donor cell} - \text{Conc in receiver cell}}{\text{Conc in donor cell}} \right) \times 100
\] (1)

2.7 In-vivo pharmacokinetic studies

Per oral (PO) and intravenous (I.V.) pharmacokinetic studies were performed in New Zealand (NZ) rabbits (n=3, weight range 2.7 ± 0.2 Kg). The rabbits were housed in restraining cages and kept in hygienic conditions, temperature 23–25 °C, relative humidity 50–70% and 12/12 h light/dark cycle. Prior to dosing, the rabbits were fasted overnight (12 hr) with free access to water and standard rabbit food was given 4 h post dose. The FXT was administered through oral and I.V. bolus at a single dose of 10 mg/kg and 1 mg/kg, respectively. The oral dose is given by oral gavage as 0.5% w/v carboxymethyl cellulose (CMC) suspension. The I.V. bolus formulation was prepared on the day of dosing by dissolving required amount of FXT in a mixture of N, N-dimethylacetamide and saline (10:80, v/v) and administered (1.0 mL) through ear vein. Blood samples (~100 µL) were collected from the marginal ear vein in heparinized tubes at 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0, 48.0, 73.0, 96.0, 120.0, 144.0 and 168.0 h post dose. Plasma was harvested by centrifugation of the blood samples at
4000 rpm for 10 min and stored at \(-70 \pm 10 \, ^\circ\mathrm{C}\) until the analysis. The pharmacokinetic parameters were calculated by non-compartmental analysis using Phoenix WinNonlin Version 6.3 (Pharsight Corporation, Mountain view, USA). All the animal experiments were conducted in accordance with current legislation on animal experiments as per Institutional Animal Ethics Committee at CSIR-Central Drug Research Institute (IAEC approval no IAEC/2012/91Ns).

3. Results and discussion

3.1 LC-MS/MS method development

Several HPLC parameters were optimized during the method development in order to get symmetrical peak shapes with best sensitivity. The optimized mobile phase consisted of methanol and 0.5% FA (80:20, v/v). The suitability and robustness of method was evaluated by using different reversed phase HPLC columns ranging from 50 to 150 mm in length. A Waters Symmetry Shield™ C18 column (150×4.6 mm, 5 µm) gave good peak shapes and sensitivity.

The ionization was performed in both negative and positive ion modes and compared in terms of intensity of the ions produced. The response observed in positive mode was much higher for both analytes compared with negative mode. The full scan parent (Q1) and product ion (Q3) spectra of FXT and NFXT were shown in Fig.2. The most abundant fragment ions of FXT (m/z 310.3/148.1), NFXT (m/z 296.2/133.9) and IS (m/z 180.1/138.1) were chosen in the MRM acquisition in terms of better selectivity and sensitivity.

Stable isotope labelled analyte or structural analogue is desirable as IS in mass spectrometry. Due to the unavailability of isotope labelled analyte and structural analogue, phenacetin was selected as IS based on the similar chromatographic behaviour, ionization response and extraction characteristics.

3.2 Assay validation
3.2.1 Selectivity

Selectivity of the intended method was established by screening the blank plasma from six independent sources. Chromatograms of six batches blank plasma contain no co-eluting peaks at >20% of analytes area at LLOQ level and no co-eluting peaks at >5% area of IS. The retention time for analytes and IS was 1.26 and 2.18 min, respectively. The representative HPLC chromatogram of blank plasma was shown in Fig.3 indicating no endogenous interfering peaks were observed at the retention times of analytes and IS. The peaks of analytes and IS showed less variability with a relative standard deviation (R.S.D.) within the acceptable limit of ± 15%.

3.2.2 Calibration curve and linearity

Calibration curve in rabbit and human plasma was constructed using eight calibrators ranging from 0.048–100 ng/mL. The standard curve was reproducible over the tested concentration range. It was evaluated by best fit for peak area ratio (analyte/IS) versus standard nominal concentration using \( y = mx + c \) with \( 1/x^2 \) weighting factor. The average intra-day and inter-day regression coefficient was found to be >0.99. With this method, the LLOQ with a signal to noise (S/N) ratio of >10 and %RSD <20% was found at 0.048 ng/mL and limit of detection with S/N ratio of >3 was found to be 0.032 ng/mL. An SPE processed zero sample was injected after HQC samples showed peak area <5% of LLOQ indicating there was no carry over effect.

3.2.3 Extraction efficiency and matrix effect

Extraction efficiency of FXT at three QC levels was found 94.5 ± 3.8% and 89.2 ± 2.9 % in rabbit and human plasma, respectively. The recovery of NFXT was 91.3 ± 2.5% and 84.9 ± 4.1% in rabbit and human plasma, respectively. Extraction efficiency of IS was found to be 92.4 ± 4.1% in rabbit plasma and 90.1± 2.9% in human plasma. Matrix effect was evaluated by calculating matrix factor. Six different screened blank plasma lots were processed as per
extraction procedure. Matrix factor was calculated by dividing mean peak area in the presence of matrix ions with mean peak area in the absence of matrix ions. The coefficient of variation (CV) for three QC levels was found within ± 15% (94.34–102.46 %) in both the plasma. Thus no significant matrix effect was observed. Significant amount of efforts were put on different extraction procedures such as protein precipitation and liquid-liquid extraction. The major issue was not only recovery but also matrix effect. Only SPE given better results in terms of recovery and minimal matrix effect. Thus finally SPE was adopted as an extraction method.

3.2.4 Accuracy and precision

Accuracy and precision (intra- and inter-day) were calculated at four different QC levels LLOQ, LOQ, MQC and HQC (n=5) for five days (Table 1). The intra- and inter-day accuracy and precision values were less than 15% at any of the concentrations, indicating that the proposed bioanalytical method was accurate and precise over the concentration range of 0.048–100 ng/mL.

3.2.5 Dilution integrity

The dilution integrity of 1:5 and 1:10 dilutions were within the acceptance limits of ± 15% for precision (CV) and 85.0–115.0% for accuracy. The results suggest that plasma samples whose concentrations above upper limit of quantitation can be determined by appropriate dilution.

3.2.6 Stability

The results of stability studies were shown in Table 2 and Table 3 for FXT and NFXT, respectively. The concentrations of FXT and NFXT were found within acceptable limits at different storage conditions. The analytes were found stable in rabbit and human plasma up to 30 days after three freeze thaw cycles, bench top for 6 h and auto-sampler for 24 h after sample preparation.
4. Plasma protein binding

FXT and NFXT were well equilibrated and negligible non-specific binding was observed with membrane and equilibrium device. The total recovery comprising of both the compartments (donor and receiver) was more than 95%. The FXT binding with human plasma was $96.34 \pm 2.85\%$ while NFXT binding was $92.45 \pm 6.35\%$. The results were in accordance with previously published literature$^{33-36}$. Thus, present method was successfully applied to study PPB in human plasma.

5. In-vivo pharmacokinetic studies

The pharmacokinetic data was analyzed by non-compartmental model using Phoenix WinNonlin software Ver 6.3 (Pharsight Corporation, Mountain view, USA). The linear trapezoidal method with linear interpolation was used to calculate pharmacokinetic parameters. The mean peak concentration ($C_{\text{max}}$) 342.50 ± 34.65 ng/mL was achieved at 1 h after oral administration, indicating rapid absorption. Absolute oral bioavailability (% F) of FXT was 18.12 ± 3.92%. AUC$_{0-\infty}$ was 1529.88 ± 201.85 and 9282.00 ± 5245.97 respectively for FXT and NFXT after oral administration. The mean plasma concentration-time profiles of FXT after oral and I.V. bolus administration along with the converted metabolite NFXT were shown in Fig. 4 and main pharmacokinetic parameters were summarized in Table 4. Because of its high sensitivity, short run time and low sample volume the outlined method enables the quantification of FXT and NFXT over a longer period of time in blood.

Conclusion

A rapid, sensitive and precise bioanalytical method was developed and validated for simultaneous estimation of fluoxetine and its active metabolite norfluoxetine in rabbit and human plasma using LC-MS/MS. The sample processing technique involves an automated SPE and the method is sensitive enough to quantify the drug and metabolite in picogram level by using only 0.03 mL of plasma. A turnover rate of 2.5 min per sample enables the high-
throughput bioanalysis of fluoxetine. The applicability of this method in human was checked by conducting plasma protein binding study. Also, the method was successfully applied to the pharmacokinetic study of fluoxetine in rabbits following intravenous and oral administration at a dose of 1 and 10 mg/kg, respectively. The method could be useful for future clinical studies and therapeutic drug monitoring of fluoxetine.

**Funding**

This work was supported by the Department of Biotechnology’s Twinning programme [BT/363/NE/TBP/2012].

**Acknowledgements**

Authors H.C. acknowledge the Council of Scientific & Industrial Research (CSIR) and Y.S.C., V.B. acknowledge the Indian Council of Medical Research (ICMR) for providing research fellowship. CSIR-CDRI communication number of this article is ----/----/RSB.
References


# Tables

**Table 1** Accuracy (% Bias) and precision (% R.S.D.) of FXT and NFXT in rabbit and human plasma

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Rabbit plasma</th>
<th>Human plasma</th>
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<tbody>
<tr>
<td></td>
<td>LLOQ</td>
<td>LQC</td>
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<tr>
<td>Theoretical</td>
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</table>

**FXT**

| % Bias intra-assay | –2.22 | 5.23 | 1.22 | –1.33 | –0.87 | 6.12 | 5.56 | 1.22 |
| % Bias inter-assay | –3.11 | 6.34 | 2.34 | 3.33 | –2.99 | 8.45 | 4.24 | 5.22 |
| % RSD intra-assay  | 5.44 | 4.45 | 4.34 | 4.56 | 10.33 | 9.34 | 10.39 | 7.89 |
| % RSD inter-assay  | 7.44 | 9.56 | 5.34 | 6.56 | 11.45 | 5.34 | 1.82 | 6.20 |

**NFXT**

| % Bias intra-assay | 4.24 | 4.90 | 3.12 | 3.14 | 2.59 | –0.89 | 2.11 | 2.11 |
| % Bias inter-assay | 2.91 | 7.11 | 6.45 | 8.11 | 5.24 | 2.11 | 5.65 | 3.87 |
| % RSD intra-assay  | 6.11 | 0.89 | 2.11 | 3.11 | 4.12 | 3.98 | 9.11 | 1.24 |
| % RSD inter-assay  | 3.19 | 0.96 | 3.14 | 2.14 | 3.23 | 6.56 | 3.35 | 2.67 |
### Table 2 Stability data of FXT in rabbit and human plasma

<table>
<thead>
<tr>
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<th>Nominal conc (ng/mL)</th>
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<th>Human plasma</th>
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<tr>
<td></td>
<td></td>
<td>%CV</td>
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Table 3 Stability data of NFXT in rabbit and human plasma

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</table>
**Table 4** Pharmacokinetic estimates after intravenous (I. V.) and oral administration of FXT

in NZ-rabbits

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimates</th>
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<tbody>
<tr>
<td></td>
<td>FXT</td>
</tr>
<tr>
<td></td>
<td>I. V. (1 mg/kg)</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>–</td>
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<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>–</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (hr*ng/mL)</td>
<td>966.55 ± 321.75</td>
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<tr>
<td>V&lt;sub&gt;d&lt;/sub&gt; (L/kg)</td>
<td>73.43 ± 19.48</td>
</tr>
<tr>
<td>Cl (L/hr/kg)</td>
<td>0.97 ± 0.38</td>
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<tr>
<td>MRT (hr)</td>
<td>69.19 ± 2.62</td>
</tr>
<tr>
<td>F (%)</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: C<sub>max</sub>: maximum concentration achieved in plasma, AUC: area under the curve from 0 to ∞ hr, V<sub>d</sub>: volume of distribution, Cl: clearance, MRT: mean residence time. Each value represents mean ± SD.
Graphical abstract

Fluoxetine

HPLC Chromatogram

Plasma Concentration profile

Graphical abstract
111x125mm (300 x 300 DPI)
Figures

Fig. 1 Chemical structures of (a) FXT and (b) NFXT.

Fig. 2 Parent ion spectra of (a) FXT at \(m/z\) 310.2, (b) Product ion spectra of FXT at \(m/z\) 148.0, (c) Parent ion spectra of NFXT at \(m/z\) 296.2 and (d) Product ion spectra of NFXT at \(m/z\) 134.1.
Fig. 3 Representative MRM chromatograms including a) blank plasma (FXT), b) blank plasma (NFXT), c) blank plasma (IS), d) FXT at LLOQ (0.048 ng/mL), e) NFXT at LLOQ (0.048 ng/mL), f) IS chromatogram, g) plasma sample obtained at 0.5 h post PO administration for FXT, h) plasma sample obtained at 0.5 h for NFXT and i) IS chromatogram of pharmacokinetic samples.

Fig. 4 Plasma concentration time profile of FXT and NFXT (a) after 10 mg/kg PO and (b) 1 mg/kg I.V. administration (n =3).