

Retraction Notice

Title of retracted article: Validated UPLC-MS/MS Method for the Simultaneous Quantification of Vortioxetine and Fluoxetine in Plasma: Application to Their Pharmacokinetic Interaction Study in Wistar Rats

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- All authors
 Some of the authors:
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Retraction type (multiple responses allowed):

- Unreliable findings
 Lab error Inconsistent data Analytical error Biased interpretation
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- honest error
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* Also called duplicate or repetitive publication. Definition: "Publishing or attempting to publish substantially the same work more than once."

History

Expression of Concern:

yes, date: 2020-6-4

no

Correction:

yes, date: yyyy-mm-dd

no

Comment:

The article has been retracted due to the conflicts of interests between all authors to straighten the academic record. Aim is to promote the circulation of scientific research by offering an ideal research publication platform with due consideration of internationally accepted standards on publication ethics. The Editorial Board would like to extend its sincere apologies for any inconvenience this retraction may have caused.

Validated UPLC-MS/MS Method for the Simultaneous Quantification of Vortioxetine and Fluoxetine in Plasma: Application to Their Pharmacokinetic Interaction Study in Wistar Rats

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Abstract

A sensitive and rapid, ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the determination of vortioxetine (VTX) and fluoxetine (FLX) in rat plasma has been developed and validated. The analytes and the internal standard (letrozole) were separated on Agilent eclipses, plus C18 analytical column (50 mm × 2.1 mm, 1.8 μm particle size) in isocratic mode. The mobile phase consisted of 60% of 10 mM ammonium formate and 40% acetonitrile at pH 4.0 with a flow rate of 0.25 mL/min. The total run time was 7 min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode to monitor the precursor-to-product ion transitions of m/z 299.2 → 150.1 for VTX, m/z 310.1 → 44.2 for FLX, and m/z 286 → 217 for letrozole (LTZ). Method validation was assessed as per the FDA guidelines for bioanalytical methods for VTX and FLX determination within the concentration range of 2.5 - 500 ng/mL ($r \geq 0.999$) with a low lower limit of detection (LLOD) of 1.0 ng/mL for both VTX and FLX, respectively. The analytical method exhibited excellent performance in terms of specificity, linearity, accuracy, precision, % recovery, dilution integrity, and stability. The developed method was successfully applied to a pharmacokinetic interaction study of VTX and FLX when the doses of VTX and FLX in rats were administered orally. A significant drug interaction between VTX and FLX in rats was reported. Thus, reduce VTX dose by two thirds when the FLX is co-administered could be considered with a necessity to perform more detailed clinical investigations.

Keywords

UPLC-MS/MS, Vortioxetine, Fluoxetine, Rat Plasma, Pharmacokinetic

1. Introduction

Vortioxetine is 1-[2-(2,4-Dimethylphenylsulfanyl)-phenyl]-piperazine (**Figure 1**), which is a novel antidepressant approved in the USA and EU for the treatment of major depressive disorder [1]. It administered in a dose of 5, 10, 15 and 20 mg [2]. It is a type of drug with a multimodal action specific to the serotonin neurotransmitter system. Its metabolism is mainly by cytochrome P450 (CYP450) enzymes that are responsible for oxidative metabolism of most drugs in the liver [3]. VTX has dual pharmacological modes of action, it makes inhibition of the serotonin transporter and makes immediate alteration of receptor efficiency [3]. Clinical investigations suggested that VTX has a good safety and tolerability profile [4]. The most common adverse events associated with it were nausea, headache, and dizziness [4]. VTX has been shown to be a substrate for several of the CYP450 isoforms in clinical investigations, in spite of no influence on CYP2C19 was spotted [5]. VTX had no influence on the steady-state pharmacokinetic parameters of aspirin or its metabolite salicylic acid, and had no effect on the platelet aggregation and co-administration of VTX did not alter the pharmacokinetics of warfarin and no pharmacodynamics interactions with oral contraceptives were shown [6] [7]. The clinical investigations of drug—drug interaction have shown that co-administration of bupropion (CYP2D6 inhibitor) can elevate the exposition of VTX about 2-folds [8]. Fluoxetine is considered as selective serotonin reuptake inhibitor (SSRI). It is chemically designated as N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine hydrochloride (**Figure 1**). It acts by increasing the extracellular level of the neurotransmitter serotonin by inhibiting its reuptake into the cell [9]. FLX is subjected to significant hepatic metabolism by cytochrome P450 enzymes (CYP2D6), thus, considered as strong CYP2D6 inhibitor [10]. The oxidative metabolism pathway of the enzyme CYP450 is implicated in drug-drug interaction mechanisms, since it is substantial for metabolism of many drugs, therefore, that interactions bring the major adverse effects with pharmacotherapy [11] [12]. Therefore, it is important to identify and quantify those interactions *in vivo* in order to avoid and reduce the side effects promoted from such interactions related to certain drug combination treatments. The FLX as SSRI antidepressant drug can be used in treatment-resistant depression when used in combination with other antidepressant like VTX. Hisaka *et al.*, [13] reported that FLX is a potent CYP2D6 inhibitor and can cause some inhibition of VTX metabolism resulted in an increased VTX blood level, causing the worse VTX side effects including a significant status called the serotonin syndrome. The symptoms of this syndrome included of seizure, confusion, hallucination, elevated heart rate, quite changes in blood pressure, too much sweating, fever, blurred vision, muscle spasm,

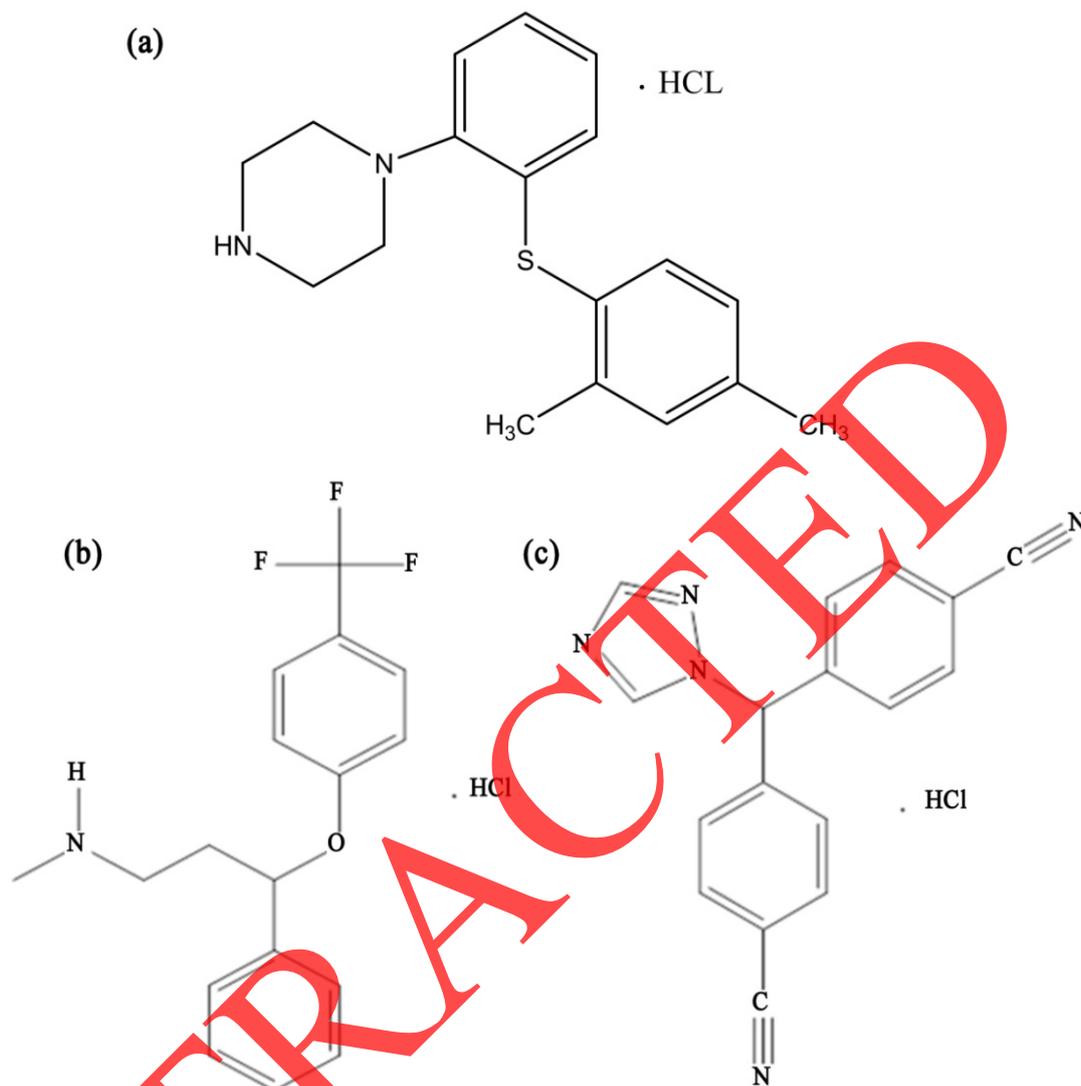


Figure 1. The chemical structures of study drugs: (a) vortioxetine hydrochloride (b) fluoxetine hydrochloride and (c) letrozole hydrochloride (IS).

shivering and shaking, tremor, stomach cramp, nausea, vomiting, and diarrhea and in critical situation may cause the coma and even death [14]. For these reasons, it is necessary to perform a pharmacokinetic interaction study of VTX and FLX in rats when being administered orally alone or being co-administered. An extensive literature review revealed that, VTX has been determined in biological samples by HPLC [15] [16] [17] and an LC-MS/MS technique [18] [19] [20] [21]. Also, several analytical methods have been cited for the quantification of FLX alone or in combination with other drugs, using HPLC [22] [23] [24], GC-MS [25] [26] and LC-MS/MS [27]-[32]. However, reports describing an UPLC-S/MS-based method for simultaneous determination of VTX and FLX in plasma are not available.

In this study, a sensitive and validated UPLC-S/MS method was developed to determine the concentrations of VTX and FLX in rat plasma and pharmacoki-

netic interaction between them was studied to supply some proposals for clinical practice.

2. Experimental

2.1. Materials and Reagents

Reference standards of vortioxetine (purity > 99%), and fluoxetine (purity > 99%), were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Letrozole reference standard (purity > 99%), (IS), was obtained from the Sigma Aldrich Co. (St. Louis, MO, USA). HPLC grade solvents including methanol and acetonitrile (Panreac, Barcelona, Spain) were used in the study. Also, formic acid, and ammonium formate (analytical grade) have been bought from Sigma-Aldrich (West Chester, PA, USA). Ultrapure water involved in the study was obtained from Ultrapure water Milli-Q Advantage water purification system, 0.22 μm filter (Millipore, Molsheim, France). The rats were provided from the Experimental Animal Care Centre, College of Pharmacy, King Saud University. Riyadh, Saudi Arabia.

2.2. Instrumentation and Analytical Conditions

The chromatographic separation of FLX, VTX and IS had been done on the Agilent HPLC-MS/MS (6410 QqQ) which consisting of binary pump (G1311A), degasser (G1322A), Autosampler (G1367B), thermostatted column compartment (G1316), and an Agilent 6410 QqQ LC/MS (Agilent Technologies, Palo Alto, CA, USA) with an electrospray ionization (ESI) interface. The detector was triple-quadrupole mass spectrometric detector (STEP WAVE™, Ultra-performance LC) with multiple reaction monitoring (MRM)-mode equipped with positive and negative ionization modes (Zspray™ ESI-APCI-ESCI, Ultra-performance LC). The C18 Agilent eclipses, plus analytical column (50 mm \times 2.1 mm, 1.8 μm particle size) was used for chromatographic analysis was purchased from (Agilent Technologies, Palo Alto, CA, USA) and its temperature was preserved at 22°C \pm 1°C. Data acquisition has been processed by Masslynx™ Version 4.1 (Micromass) software. The analytical separation of the analytes was carried out isocratically with a flow rate of 0.25 mL/min. The mobile phase used consisted of 60% of 10 mM ammonium formate and 40% acetonitrile at pH 4. The injection volume was 5.0 μL and the total run time was 7 min. The auto-sampler temperature was maintained at 5°C - 8°C. Mass spectrometric detection was carried out using positive ion electrospray ionization (ESI) source. The employed MS parameters were; drying gas nitrogen flow of 11 L/min, collision nitrogen gas turned on a pressure of 50 psi, source temperature and capillary voltage adjusted at 350°C and 4000 V respectively. Data acquisition was performed on Mass Hunter software (Agilent Technologies, Palo Alto, CA, USA). Quantification was carried out utilizing the mode of multiple reaction monitoring (MRM) for the transitions of m/z 299.2 \rightarrow 150.1 for VTX, m/z 310.1 \rightarrow 44.2 for FLX, and m/z 286 \rightarrow

217 for (LTZ). Fragmentor voltage was suited at 145 V with collision energy of 15 for LTZ, FLX and 140 V with collision energy 15 for VTX.

2.3. Stock Solutions Preparations, Calibration Standards, and Quality Control (QC) Samples Preparations

Stock solutions of VTX and FLX were prepared by dissolving 10.0 mg of each analyte in 10.0 mL methanol. Further dilutions were carried out with methanol to yield working solutions at several concentration levels. One mL of stock solution of both VTX and FLX were diluted to a volume of 10.0 mL to produce 100 µg/mL concentration (working standard 1), then dilute 1 mL of this solution to 10.0 mL to give the 10 µg/mL concentration (working standard 2). The IS stock solution was prepared by dissolving the reference of LTZ in methanol to produce a concentration of 1 mg/mL, then a standard solution of concentration 100 µg/mL was diluted with methanol to give a concentration of 10 µg/mL. All solutions were found to be stable for 1 month if kept in refrigerator at 2°C - 8°C, and no evidence of degradation of VTX, FLX, and LTZ was observed in the chromatograms obtained during this period. Drug free rat plasma samples spiked with pre-determined amounts of each of VTX and FLX, along with IS were used to construct matrix-based calibration curves. Ten calibration standards in plasma at concentrations of 2.5, 5, 20, 40, 60, 100, 150, 200, 300 and 500 ng/mL for VTX and FLX were prepared by spiking appropriate aliquots of drugs standard solution in the plasma. The samples of QC were prepared at four different concentration levels; 2.5 ng/mL (LLOQ), 7.5 ng/mL (LQC), 250 ng/mL (MQC), and 450 ng/mL (HQC) for both FLX and VTX along with IS.

2.4. Sample Preparation

Before analysis, rat plasma samples were defrosted at room temperature. An aliquot quantity of 50 µL of rat plasma was taken in a 1.5 mL Eppendorf tube, spiked with 50 µL of working IS solution at 500 ng/mL, and spiked with appropriate aliquots of drug standard solutions to give required concentrations. Each tube was diluted to 500 µL with deionized water and gently mixed for at least 30 s. The mixture was treated with 500 µL of acetonitrile for deproteinization [33]. The tubes were subsequently vortexed at high speed for 1 min and centrifuged at 6000 rpm for 30 min. The supernatant (upper layer) from each tube was loaded into autosampler tray and 5 µL of it were injected (in triplicate) into the UPLC-MS/MS system. The peak area ratios of each compound to IS were processed to obtain the calibration graph of each compound. Alternatively, the corresponding regression equation was derived.

3. Assay validation

3.1. Specificity

The drug-free plasma samples were examined for the existence of any interfering peaks at the times of elution of the tested drugs. Method specificity was esti-

mated by testing six various batches of plasma samples. This was done by comparing the chromatograms of blank plasma samples of drug-free with the plasma samples that were spiked with concentrations of the LLOQ of VTX and FLX along with the IS. Then a comparison was done between the results obtained from blank plasma samples and those analytical results gained at the retention times of the tested analytes at LLOQ and of IS.

3.2. Linearity

The rat plasma samples (50 μ L) were spiked with ten various concentrations of the VTX and FLX in the range 2.5 - 500 ng/mL, along with 50 μ L of 500 ng/mL LTZ (IS) in order to construct the calibration graphs of both drugs. Following the analysis of each sample, the peak area ratios of VTX and FLX to that of IS were related to the spiked analytes concentrations to get the matrix-based calibration graph and the corresponding regression equations.

3.3. Lower Limit of Detection (LLOD) and of Quantification (LLOQ)

The LLOD and LLOQ of both VTX and FLX were established on the concentrations that make analytical responses of at least three and ten times that of the blank signals, for LLOD and LLOQ, respectively. Moreover, the analytical responses at the LLOQ should yield acceptable accuracy and precision within $\pm 20\%$.

3.4. Precision and Accuracy

Intra-day accuracy and precision were computed through the analysis of QC samples at the four different concentration levels, very low LLOQ (2.5, ng/mL), low (7.5 ng/mL), medium (250 ng/mL) and high (450 ng/mL) during the same day ($n = 6$). While the inter-day evaluations were done on three successive days ($n = 18$). For each solution, VTX and FLX peak area ratios to that of LTZ (IS) was used to calculate the actual VTX and FLX concentrations by substitution into the regression equations and then compared with the nominal values. The percentage relative error (Er %) values were used to calculate the accuracy, while the relative standard deviation (% RSD) values were evaluated the precision. The (% RSD) = (SD/Mean) $\times 100$ and percentages relative error (Er %) = [(average measured concentration - nominal concentration)/nominal concentration] $\times 100$].

3.5. Extraction Recovery

The rat plasma samples were spiked with previously calculated volumes of VTX and FLX along with IS to prepare the four different QC levels as in precision and accuracy section. The developed UPLC-MS/MS conditions were used to analyze the prepared samples and the peak area ratios obtained from spiked plasma samples pre-extraction were compared to those gained from plasma samples after extraction with the same nominal concentration levels. Then calculate the mean percentage recoveries ($n = 6$) for both analytes. Also, the extraction recovery of

the IS at the same concentration level of the assay was also calculated.

3.6. Matrix Effect

The matrix effect computed by comparing the ratio of the mean peak area of each of VTX and FLX spiked after extraction to those of standard solutions prepared at the four different QC concentration levels (2.5, 7.5, 250 and 450 ng/mL). Similarly, the matrix effect of LTZ (IS) at the same concentration level used in the analysis was evaluated.

3.7. Dilution Integrity

Dilution of highly concentrated plasma samples, with concentrations beyond the linear range of the proposed method, was evaluated for its effect on VTX and FLX recoveries. Plasma samples spiked with high concentrations of (800 ng/mL) for VTX and FLX were used following dilution with blank plasma samples, dilution folds (1:2 and 1:5). Diluted samples were then treated as under "Sample preparation". Three replicates of each dilution were examined and the resulted concentrations were then compared with the expected concentrations. The integrity of both drugs was maintained if recovery % (\pm RSD) results of the diluted samples were within the acceptable limits (\pm 15%).

3.8. Stability Studies

QC samples spiked at four concentration levels of VTX and FLX were analyzed ($n = 6$) in order to assess the drug stability in plasma. Stability testing was performed by exposing the QC plasma samples to different conditions; stability in the injection medium (extracted samples left in the autosampler at 10°C for 48 h before injection), short-term stability (samples left at room temperature (25°C) for 6 h), long-term stability (samples left at -30°C for 30 days). Moreover, freeze-thaw stability was assessed as follows, plasma samples were frozen at around -30°C and then thawed at room temperature for three cycles. For each sample, VTX and FLX concentrations were related to the nominal concentrations to calculate the % recovery.

3.9. Application to Pharmacokinetic Studies

All animal procedures employed complied with the standards set forth in the guidelines for care and use of experimental animals by the Committee for Purpose of Supervision of Experiments on Animals (CPCSEA) [34], and the National Institutes of Health (NIH) protocol [35]. The study protocol was approved by the Animal Ethics Committee of Pharmacology Department, College of Pharmacy, King Saud University, Kingdom of Saudi Arabia (No. KSU-SE-18-19). Wistar healthy male rats weighing 250 ± 30 g were obtained from the Experimental Animal Care Center, College of Pharmacy, King Saud University. The animals were placed in cages kept in a well-ventilated room and subjected to a regular 12 h day-night cycle at a relative humidity of 40% - 60% and average temperature of

24°C - 27°C. All the rats could access the water freely while diet was prohibited for 12 h before drug administration. The rats were acclimatized for 7 days to laboratory conditions before conducting the experiment. Four groups of four rats each were involved in this study. Rats in Group 1 were orally administered saline by oral gavage to provide the blank rat plasma; rats in Group 2 were orally administered vortioxetine (4.0 mg/kg); rats in Group 3 were given fluoxetine (16.0 mg/kg) and rats in Group 4 were given vortioxetine (4.0 mg/kg) plus fluoxetine (16.0 mg/kg). For each group, volumes of 0.2 mL blood samples were withdrawn from the retro-orbital sinus of each rat into heparinized 1.5 mL polythene tubes. Blood samples were collected at different time intervals; 0 (prior to dosing), 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, and 24 h after oral administration, respectively. All collected blood samples were centrifuged immediately at 3000 rpm (10 min, 4°C). The plasma obtained (100 µL) were kept frozen at -20°C till the day of analysis. For the fate of animals, we leave the animals for a washout period (*i.e.* two weeks) then we use them in other animal studies in our laboratory. PK Solver 2.0 Add-in, Excel 2010 was used to process the VTX and FLX plasma concentrations as a function of the analysis time. Data were expressed as mean ± SD. Statistically significant differences of data from two sets were compared using one-way analysis of variance. In all statistical analyses, $P < 0.05$ was considered to indicate a statistically significant result.

4. Results and Discussion

4.1. Optimization of Chromatographic Conditions

Method development was begun with the optimization of chromatographic conditions, including mobile phase composition, and flow rate. Various mobile phases consisted of mixtures of different ratios of acetonitrile—water (30% - 90%), and formic acid (0.05% - 0.2%). Methanol—water mixtures (30 - 90%), and formic acid (0.05% - 0.2%) were investigated. The ammonium formate buffer at a concentration ranged from 5 - 20 mM and different pH ranges from 3 - 6 was examined with different mixtures of both organic modifiers to get better separation, lower retention times and good peak shapes. Mobile phase consisted of acetonitrile-water (40%:60%) containing 10 mM ammonium formate and pH was 4.0 was shown to improve signal-to-noise ratio and thus found to be suitable for the chromatographic separation of the studied analytes. Farther investigated of selected mobile phase showed that the acetonitrile percentage of less than 40% resulted in distortion of the tested analytes peaks, more concentration of acetonitrile from 45% - 80%, resulted in overlapping of the tested drugs and decreased the separation. The analysis was thus performed with an isocratic elution using a mobile phase consisted of 60% of 10 mM ammonium formate and 40% acetonitrile at pH 4.0 with a flow rate of 0.25 mL/min, for the whole run time of 7 min. Under the above optimized chromatographic conditions, sharp and symmetric peaks of all drugs were obtained (LTZ eluted at 3.22 ± 0.04 min, FLX at 4.67 ± 0.08 min, and VTX at 6.35 ± 0.07 min). No carryover was observed in the blank

matrix sample. Representative chromatograms of LLOQ, middle QC (MQC) and high QC (HQC) of FLX and VTX were shown in **Figure 2**.

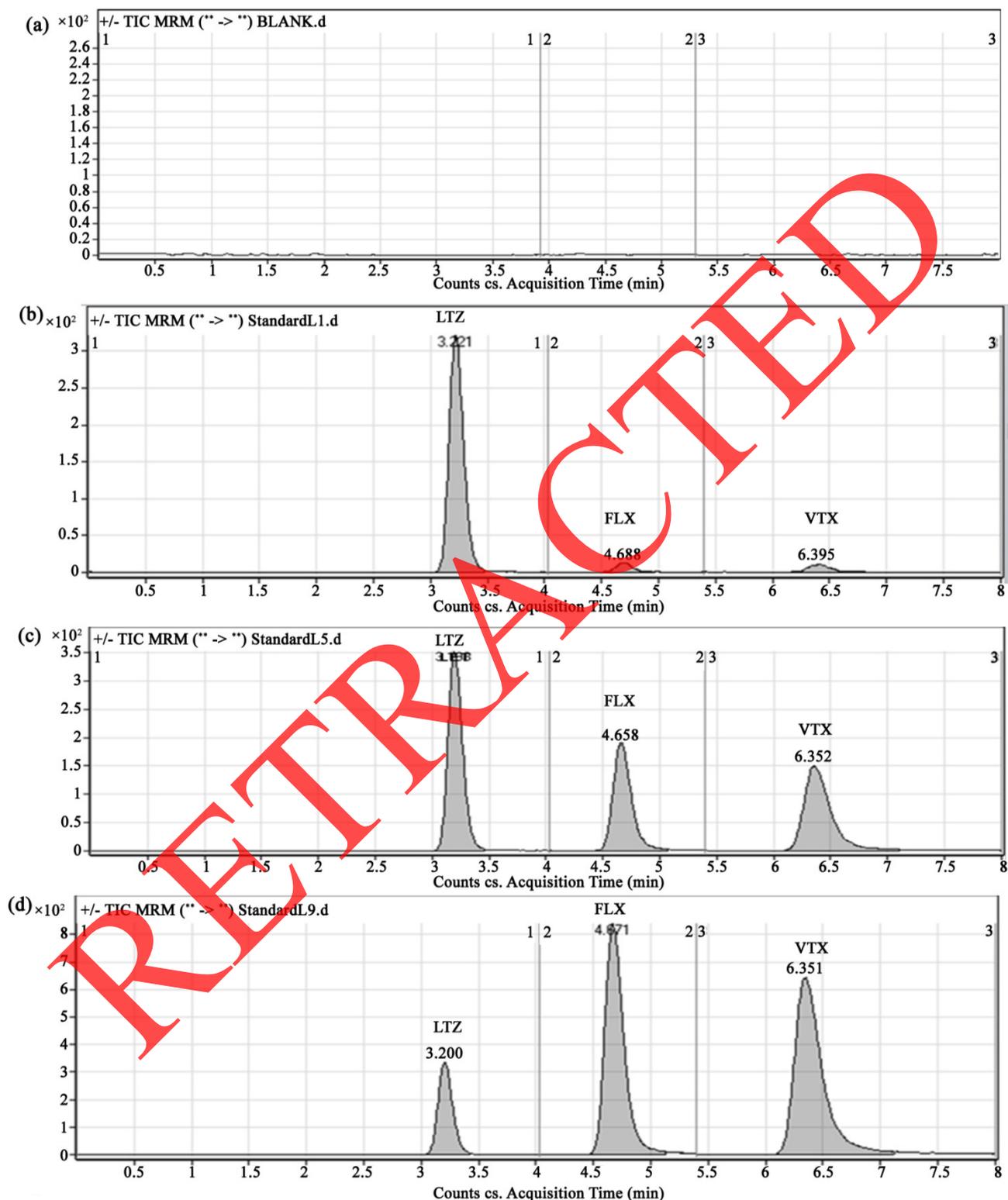


Figure 2. Representative chromatograms of blank plasma: (a) plank plasma spiked with 2.5 ng/mL (LLOQ); (b) 250 ng/mL (MQC); and (c) 450 ng/mL (HQC); (d) of FLX and VTX.

4.2. Optimization of Mass Spectrometric Conditions

The \pm ESI ionization mode was operated to assess the best MS/MS conditions of the injected standard solutions of VTX, FLX and the IS. The positive ionization mode provided better response for FLX, VTX, and IS relative to the negative ionization mode, under different MS parameters. Therefore, the optimization was carried out in the positive ionization mode in order to monitor the precursor as well as the product ions. MRM mode was defined in this research to clear any potential interference signals and improve the sensitivity of the procedure. For the highest intensity of the protonated molecular ions, different MS/MS parameters were adjusted as follows: an ESI source temperature of 350°C and desolvation gas flow rate of 11 L/min was found optimum in the analysis. On the other hand, the collision energy is an important parameter to get reasonable responses of the daughter fragment ions. However, increasing the collision energy resulted in an increased in the intensity of the particular fragment ion till optimum values after which a decrease in the intensity would be observed. The selected collision energies that produced maximum intensities of the selected daughter ions of all studied drugs were summarized in Table 1. Moreover, the intensity of the particular fragment ion increased gradually with increased the cone voltage till certain optimum values after which a dramatic decrease was recorded. The selected optimum values for cone voltages for all studied drugs were shown in Table 1. Full scans mass spectra were recorded in order to select the most abundant m/z value. The most abundant precursor is m/z 286.1 \rightarrow 216.8 for LTZ, m/z 310.1 \rightarrow 44.2 for FLX and m/z 299.1 \rightarrow 150.2 for VTX. Full scan product ion spectra of protonated precursor ions $[M + H]^+$ for LTZ, FLX and VTX were shown in Figure 3. The separation of VTX, FLX and IS was attained in 7 min with peaks were well separated. The overlaid MRM chromatograms of VTX, FLX and LTZ were shown in Figure 4.

4.3. Method Validation

Validation of this study was performed according to the “Guidance for Industry-Bioanalytical Method Validation” recommended by the US Food and Drug Administration [36] to evaluate the specificity, linearity, accuracy and precision, extraction recovery, matrix effects, dilution integrity and stability studies.

4.3.1. Specificity

The specificity of the method was assessed by comparing the chromatograms obtained from six batches of blank and plasma samples with those spiked with low

Table 1. UPLC-MS/MS optimized parameters for the determination of the studied drugs.

Molecules	Ion mode	MRM Transitions (Da)	Collision energy (eV)	Cone voltage (V)
Letrozole (IS)	ES+	286.2 > 190.0, 217.0	15	4000
Fluoxetine	ES+	310.1 > 44.2	18	4000
Vortioxetine	ES+	299.1 > 256.1, 150.2	15	4000

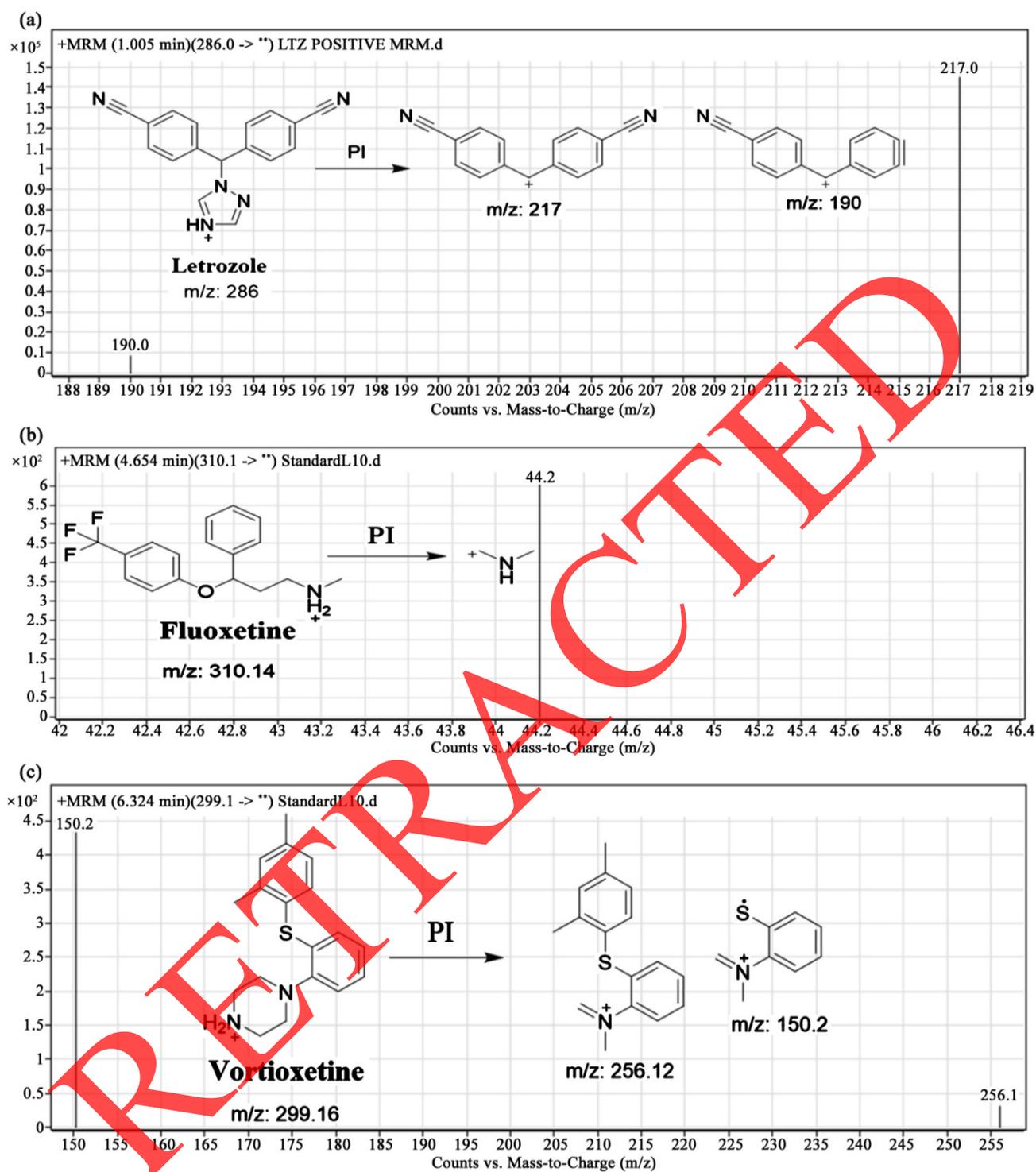


Figure 3. Product ion spectra of letrozole (a), fluoxetine (b) and vortioxetine (c).

concentrations equivalent to LLOQ of all studied drugs. No interference peak was detected at the retention time of VTX, FLX and IS indicates a high degree of method specificity. Representative MS chromatograms of blank rat plasma samples and plasma samples spiked with VTX and FLX at its LLOQ levels were shown in **Figure 2**.

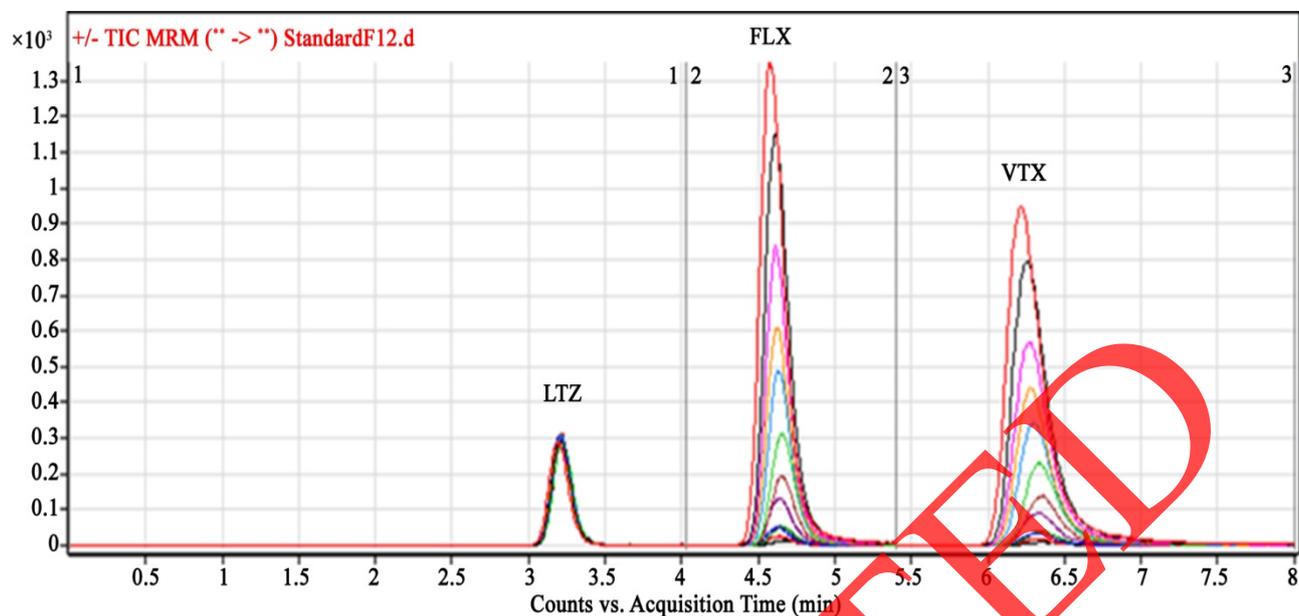


Figure 4. TIC chromatogram overlay of MRM of fluoxetine (2.5 - 500 ng/ml), vortioxetine (2.5 - 500 ng/ml) and letrozole (500 ng/ml).

4.3.2. Linearity

The spiking blank plasma samples with standard solutions of FLX and VTX along with LTZ (IS) were analyzed to assess the linearity. Peak area ratios of each drug to the IS were established linear in the concentration range of 2.5 - 500 ng/mL for both FLX and VTX. The least-square method was utilized for analyzing the linear regression results. High degree of linearity for both drugs was indicated by high values of the correlation coefficients ($r \geq 0.999$) for all analytes along with small intercepts. The regression equations obtained by least squared regression where $Y = 1.11 \times 10^{-2} \times X + 6.50 \times 10^{-3}$ for FLX, while the regression equation for VTX was $Y = 1.25 \times 10^{-2} \times X + 2.20 \times 10^{-3}$, where Y represents the peak-area ratio of an analyte to IS and X represents the plasma concentration of the analyte. The valid linearity indicated with an elevated value of the (r), and the lower values of standard deviations of the intercept and the slope. Other statistical parameters included standard deviations of residuals ($S_{y/x}$), standard deviations of the intercept (S_a), and standard deviations of the slope (S_b) were listed in **Table 2**. The RSD % values of each concentration point ($n = 6$) were not more than 1.86% for FLX and 1.88% for VTX in rat plasma. Calibration of both FLX and VTX (ten points) were back-calculated to ensure the best performance of the developed method. The precision values were 0.17% - 1.86% for FLX and 0.50% - 1.88% for VTX, while the accuracy values were ranged between -0.19% to -2.12% for FLX and -0.40% to -2.13% for VTX (**Table 3**).

4.3.3. Lower Limit of Detection (LLOD) and Lower Limit of Quantification (LLOQ)

The lower limit of quantification (LLOQ) was established as 2.5 ng/mL for both FLX and VTX, while the LLOD for FLX was 1.00 ng/mL and for VTX and FLX.

Table 2. Statistical data of the regression equations for the determination of VTX and FLX obtained from rat plasma by the proposed UPLC-MS/MS method.

Parameters	FLX	VTX
Concentration linear range (ng/ml)	2.5 - 500	2.5 - 500
Intercept (a)	1.11×10^{-2}	1.25×10^{-2}
Slope (b)	6.50×10^{-3}	2.20×10^{-3}
Correlation coefficient (r)	0.9995	0.9993
$S_{Y/N}$ ^a	2.01×10^{-2}	1.38×10^{-1}
S_a ^b	7.63×10^{-3}	4.43×10^{-2}
S_b ^c	3.12×10^{-2}	1.57×10^{-2}
LLOQ (ng/mL) ^d	2.50	2.50
LLOD (ng/mL) ^e	1.00	1.00

^aStandard deviation of the residual. ^bStandard deviation of the intercept. ^cStandard deviation of the slope. ^dThe lower limit of quantification. ^eThe lower limit of detection (the smallest amount of an analyte that can reliably be detected).

Table 3. Data of back-calculated fluoxetine and vortioxetine concentrations of the calibration standards from rat plasma.

Nominal Concentration (ng·mL ⁻¹)		Mean ^a ± SD		Precision (RSD %)		Accuracy E _r (%) ^b	
FLX	VTX	FLX	VTX	FLX	VTX	FLX	VTX
2.5	2.5	2.45 ± 0.04	2.46 ± 0.03	1.65	1.22	-2.00	-1.60
5	5	4.92 ± 0.06	4.93 ± 0.07	1.22	1.42	-1.60	-1.407
20	20	19.92 ± 0.11	19.91 ± 0.10	0.55	0.50	-0.40	-0.45
40	40	39.74 ± 0.21	39.53 ± 0.44	0.52	1.10	-0.65	-1.18
60	60	59.88 ± 0.10	59.68 ± 0.41	0.17	0.69	-0.19	-0.53
100	100	97.88 ± 1.83	99.22 ± 0.86	1.86	0.86	-2.12	-0.78
150	150	148.13 ± 2.34	146.80 ± 2.75	1.58	1.87	-1.25	-2.13
200	200	199.43 ± 0.81	197.10 ± 3.7	0.40	1.88	-0.29	-1.45
300	300	295.43 ± 4.80	294.60 ± 4.99	1.62	1.68	-1.52	-1.80
500	500	493.07 ± 1.93	494.37 ± 4.58	0.39	0.93	-1.39	-1.13

^aAverage of six determinations; ^bPercentage relative error calculated as (mean determined concentration - nominal concentration)/nominal concentration × 100.

The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were calculated according to the FDA guidelines [36]. The MRM chromatograms of plasma samples spiked with FLX and VTX at their LLOQ were approached in **Figure 3(b)**. The low values of LLOQ performed the succeeded implementation of the developed method in the trace analysis of the two drugs in clinical investigations.

4.3.4. Precision and Accuracy

The developed method was approved to be reproducible according to the resulted values of precision and accuracy of the intra- and inter-day assessment process of FLX and VTX QC samples. The data for intra-day and inter-day precision and accuracy were expressed in **Table 4**. The calculated intra-day relative errors were in the range -1.21% to -2.40% for FLX and -0.04% to -1.60% for VTX, while the calculated inter-day relative errors were in the range of -0.56% to -2.00% for FLX and -0.92% to -1.20% for VTX. The intra-day and inter-day RSD values were in the range 2.98% - 4.881% and 3.96% - 4.78% for FLX and VTX, respectively. According to the accepted values of the relative errors and the RSD values (not more than $\pm 15.0\%$), this specified that this study was with a high degree of accuracy and precision and was dependable and reproducible for the simultaneous quantitative analysis of vortioxetine and fluoxetine in rat plasma samples.

4.3.5. Extraction Recovery

Analyzing of the rat plasma samples with the tested analytes at four different concentration levels; very low (2.5 ng/mL), low (7.5 ng/mL), medium (250 ng/mL), and high ($450 \text{ ng}\cdot\text{mL}^{-1}$), along with LTZ (IS) followed by calculating the mean recovery percentage ($n = 6$) values of the FLX and VTX. The presented samples were processed according to the optimized UPLC-MS/MS conditions and the peak area ratios of each analyte to the IS were contrasted with those of standard solutions of the drugs having the same concentrations. Also, the extraction recovery of LTZ (IS) at the concentration level used in actual analysis was estimated. The mean recoveries of all the analytes ranged from $98.00\% \pm 3.87\%$ and $99.54\% \pm 4.55\%$. In addition the, the mean recovery of the IS was $98.56\% \pm 3.46\%$. Recovery results were summarized in **Table 5**.

Table 4. Intra-day and inter-day precision and accuracy results of fluoxetine (FLX) and vortioxetine (VTX) in rat plasma (Mean \pm SD, $n = 6$).

Analyte	Actual Conc. (ng/mL)		E _r (%) ^a		Mean recovery (%) \pm RSD	
	FLX	VTX	FLX	VTX	FLX	VTX
Intra-day ^b	2.5	15	-2.40	-1.60	97.60 \pm 4.35	98.40 \pm 3.96
	7.5	7.5	-1.86	-1.20	98.13 \pm 4.88	98.80 \pm 4.75
	250	250	-1.29	-0.04	98.71 \pm 3.99	99.96 \pm 4.78
	450	450	-1.21	-1.10	98.78 \pm 2.98	98.89 \pm 4.20
Inter-day ^c	2.5	2.5	-2.00	-1.20	98.00 \pm 3.55	98.80 \pm 3.87
	7.5	7.5	-0.80	-0.93	99.20 \pm 4.01	99.00 \pm 4.76
	250	250	-0.59	-0.92	99.43 \pm 4.84	99.08 \pm 4.77
	450	450	-0.56	-1.02	99.44 \pm 3.01	98.97 \pm 4.13

^aPercentage relative error calculated as (mean determined concentration-nominal concentration)/nominal concentration $\times 100$. ^bMean concentrations \pm SD based on $n = 6$. ^cMean concentrations \pm SD based on $n = 6$.

Table 5. Recovery of QC samples for determining the concentration of fluoxetine (FLX) and vortioxetine (VTX) in plasma matrix.

Nominal concentration (ng·mL ⁻¹)	FLX				VTX			
	2.5 ng/mL	7.5 ng/mL	250 ng/mL	450 ng/mL	2.5 ng/mL	7.5 ng/mL	250 ng/mL	450 ng/mL
Mean recovery (%) ± RSD ^a	98.40 ± 4.09	99.20 ± 4.32	98.58 ± 3.54	98.87 ± 4.21	98.80 ± 3.97	98.00 ± 3.87	99.40 ± 4.67	99.54 ± 4.55
E _r (%) ^b	-1.60	-0.80	-1.42	-1.12	-1.20	-2.00	-0.60	-0.46

^aMean recovery (%) ± RSD of six determinations; ^bpercentage relative error.

4.3.6. Matrix Effect

The matrix effect assessment is very valuable in the analytical method, this due to the significant effect of the biological samples on the ionization of the tested drugs either by inhibition or elevating of the ionization. This issue was carried out by the same process as per recovery assessment, but the processed samples (without plasma) were used as a reference for comparison. Four different concentration levels of both drugs along with actual IS concentration were utilized to evaluate the % matrix. Six different batches of plasma were extracted and spiked separately with the LLOQ concentration of 2.5 ng/mL of VTX and IS. Other six various batches of plasma were extracted and spiked with 2.5 ng/mL of FLX and IS, these considered as set 1. Also, set 2 was intended and implicated with six replicates of same concentrations of FLX and IS but were dissolved in the mobile phase and another six replicates of the same concentration of VTX and IS, were dissolved in the mobile phase. The same process was followed for the other three QC concentrations (7.5 ng/mL, 250 ng/mL and 450 ng/mL) for both VTX and FLX, respectively. For estimation of matrix effect, the mean peak area ratio of set 1/set 2 × 100 was calculated separately for each tested analyte. Mean recovery (%) ± RSD of six determinations of FLX exhibited between 97.22 ± 3.10 and 99.31 ± 3.54, while for VTX exhibited between 99.20 ± 4.76 and 99.54 ± 4.44. Consequently, the % matrix factors (% relative error) at the four selected concentration levels were found not more than -2.78% for FLX and -0.91% for VTX, the results were presented in Table 6. The % matrix factor for the IS at the actual concentration applied in the assay was found -2.51%. These results replied matrix effect had negligible influence on the ionization of the tested compounds.

4.3.7. Dilution Integrity

Dilution integrity was assessed to evaluate the dilution effects of plasma samples containing very high concentrations of FLX and VTX beyond the linear range of the presented method. Fold dilutions (1:2 and 1:5) of concentrated samples yielded acceptable recoveries with error values (RSD) not more than 3.21%. For both drugs, the recovery% (± RSD) following the dilution process were calculated and presented in Table 7. The recovery % values of FLX were ranged between 98.31% - 99.10% and for VTX were 98.02% - 98.66%. The RSD % values of the tested analytes were within the accepted rang and the error values ≤ 15%. The integrity of both FLX and VTX up to five fold dilution of concentrated plasma samples was revealed by the accepted values of the obtained results.

4.4. Stability Studies

Stability studies were assessed using plasma samples spiked at two different FLX and VTX concentrations, namely 7.5 and 450 ng/mL. The results were presented in **Table 8**, **Table 9** explained that all the resulted values of recovery did not exceeded the permitted limits (± 15), where the recoveries values of FLX were ranged between 97.19% - 99.92% and for VTX were 97.56% - 99.67%. The RSD % values of the results did not exceed the accepted limits, 4.32% for FLX and 4.75% for VTX. Negligible loss of the tested compounds during sample storage under different conditions and during sample handling of the QC samples at the analysis conditions indicating a high degree of sample stability.

Table 6. Evaluation of the matrix effect for determination of fluoxetine (FLX) and vortioxetine (VTX) in rat plasma by the proposed UPLC-MS/MS method.

Nominal concentration (ng·mL ⁻¹)	FLX				VTX			
	2.5 ng/mL	7.5 ng/mL	250 ng/mL	450 ng/mL	2.5 ng/mL	7.5 ng/mL	250 ng/mL	450 ng/mL
Mean recovery (%) \pm RSD ^a	98.11 \pm 3.78	98.53 \pm 4.65	97.22 \pm 3.10	99.31 \pm 3.54	99.25 \pm 4.34	99.20 \pm 4.76	99.28 \pm 3.95	99.54 \pm 4.44
E _r (%) ^b	-1.89	-1.47	-2.78	-0.68	-0.75	-0.80	-0.72	-0.91

^aMean recovery (%) \pm RSD of six determinations; ^bpercentage relative error.

Table 7. Evaluation of the dilution integrity of fluoxetine (FLX) and vortioxetine (VTX) in rat plasma.

Analyte	Spiked concentration (ng/mL)	Dilution fold	Mean recovery (%) \pm RSD ^a	E _r \pm (%) ^b
FLX	800	1:2	99.10 \pm 1.25	-0.86
		1:5	98.31 \pm 1.94	-1.68
VTX	800	1:2	98.66 \pm 1.37	-1.38
		1:5	98.02 \pm 3.21	-1.97

^aMean recovery (%) \pm RSD of six determinations; ^bEr%, Percentage relative error.

Table 8. Stability fluoxetine in rat plasma under different storage conditions.

Stability	Concentration added (ng/mL)	Mean recovery (%) \pm RSD ^a
Auto-sampler stability (10°C, 56 h)	7.5	99.92 \pm 3.22
	450	98.65 \pm 0.89
Short-term stability (25°C, 6 h)	7.5	98.22 \pm 3.33
	450	99.43 \pm 4.19
Long-term stability (-20°C, 30 days)	7.5	97.19 \pm 1.88
	450	98.82 \pm 3.64
Freeze-thaw stability (-20°C, 3 cycles)	7.5	97.81 \pm 2.75
	450	98.91 \pm 3.11
Refrigerator (4°C, 3 months)	7.5	98.75 \pm 4.01
	450	99.35 \pm 4.32

^aMean recovery (%) \pm RSD of six determinations.

Table 9. Stability vortioxetine in rat plasma under different storage conditions.

Stability	Concentration added (ng/mL)	Mean recovery (%) \pm RSD ^a
Auto-sampler stability (10°C, 56 h)	7.5	98.98 \pm 2.62
	450	98.43 \pm 0.76
Short-term stability (25°C, 6 h)	7.5	97.56 \pm 2.33
	450	99.31 \pm 4.09
Long-term stability (-20°C, 30 days)	7.5	99.29 \pm 1.78
	450	98.45 \pm 3.54
Freeze-thaw stability (-20°C, 3 cycles)	7.5	98.21 \pm 4.75
	450	99.41 \pm 1.11
Refrigerator (4°C, 3 months)	7.5	98.45 \pm 3.44
	450	99.67 \pm 4.02

^aMean recovery (%) \pm RSD of six determinations.

4.5. Comparison between the Developed Method over the Earlier Published Bioanalytical Methods

To our knowledge, this experiment is first time to use UPLC-MS/MS method to determine the concentration of VTX and FLX in rat plasma and its application to a pharmacokinetic interaction study. This validated technique has been utilized, evaluated and study the probable PK interactions between VTX and FLX that can occur subsequent to their co-administration as a proposed treatment agenda in the management of severe depression. Furthermore, no earlier LC-MS/MS methods were published for the bioanalysis assessments of VTX and FLX in plasma. This developed method has several characteristics over the earlier reported LC-MS/MS methods reported for the determination of either VTX or FLX in biological samples. First of all, this study had a remarkable sensitivity with a capability to quantify and detect a very low concentration of the tested drug. The current study provided the lowest LLOQ (2.5 ng/mL) designed for the analysis of VTX and FLX, compared with earlier reported liquid chromatography methods for the determination of VTX in biological fluids [14] [15] [16] or FLX [22] [23] [24] [25] [26]. The developed method was marked with a sensitivity and built on the analysis of extremely small plasma sample volume (50 μ L) which is very important in conditions where only small volumes of samples are obtainable, and this volume of plasma samples was lower than those used in the previous VTX or FLX analytical LC-MS/MS literatures [17] [18] [19] [20] [21] [27]-[32]. This is strongly recommended when cooperation with children, biological samples or population-based bio banks, where restricted volumes of samples are obtainable. Furthermore, shorten analysis run time (7 min) which approved the convenience of this method in high throughput bioanalysis.

4.6. Pharmacokinetic Interaction between Vortioxetine and Fluoxetine Study

This study was the firstly reported method utilized the UPLC-MS/MS technique for simultaneous determination of VTX and FLX in rat plasma and its application to a pharmacokinetic interaction study. According to the studies of the VTX metabolism bath way, it's extensively metabolized primarily through oxidation via multiple cytochrome P450 (CYP) isozymes (predominantly CYP2D6) and subsequent glucuronic acid conjugation [37]. In pharmacokinetic interactions, the majority of clinically relevant pharmacokinetic interactions with antidepressants arise as a consequence of drug induced changes in hepatic metabolism, through inhibition or induction of CYP isoenzymes [38]. In line with the previous observations, only the co-administration of a CYP2D6 inhibitor was able to increase the area under the plasma concentration curve (AUC), and the maximum plasma vortioxetine concentration in healthy adults, also increased the incidence of adverse effects when co-administered with vortioxetine [39]. Since the fluoxetine is an antidepressant drug with strong CYP2D6 inhibition effect due to its metabolism at the CYP2D6 isoenzyme [40] [41], and since VTX drug metabolized by the same mechanism, therefore, FLX co-administration with VTX expected to cause synergistic increase in the VTX concentration resulting in worse SSRI side effects [42] [43]. For this reason the presented UPLC-MS/MS method developed in this work was utilized to explore the probability of PK interaction between VTX and FLX. The present method was successfully applied to pharmacokinetic study of vortioxetine with/without fluoxetine in rats and this assay was designed for the purpose of comparison between the rats groups. The groups II and III were given oral doses of only VTX (4 mg/kg) and FLX (16 mg/kg), respectively, while the group IV were administered with a combination of VTX and FLX in a dose of 4 mg/kg for VTX and 16 mg/kg for FLX. The mean plasma concentration-time profiles of vortioxetine with/without fluoxetine were shown in Figure 5. The main relevant pharmacokinetic parameters from non-compartment model analysis were listed in Table 10. The typical MRM chromatograms gained from rat plasma 1 h after VTX oral administration alone/with FLX were shown in Figure 6, while MRM chromatograms gained from rat plasma 4 h after administration of FLX alone/with VTX were presented in Figure 7. After oral administration of vortioxetine with/without fluoxetine, the standard pharmacokinetic variables for VTX and FLX were derived. The mean value of T_{max} and C_{max} for VTX and FLX when administered alone were 1 h, 107.19 ± 5.14 ng/mL for VTX and 4 h, 81.92 ± 4.12 ng/mL for FLX, respectively. While the T_{max} and C_{max} values when both drugs were co-administered orally together in group IV were 1 h, 365.97 ± 17.23 ng/mL for VTX and 4 h, 75.83 ± 4.02 ng/mL for FLX, respectively. The AUC_{0-24} for VTX when administered alone was found to be 522.36 ± 26.12 ng.h/mL; while when administered simultaneously with FLX found to be 1665.85 ± 67.23 ng.h/mL; Moreover, The AUC_{0-24} for FLX when administered alone was found to be 794.62 ± 35.91 ng.h/mL; while when administered simul-

taneously with VTX was found to be 925.77 ± 42.71 ng.h/mL. The $AUC_{0-\infty}$ for VTX and FLX when each administered alone in group II and III were found to be 527.37 ± 25.43 and 1097.22 ± 55.11 ng.h/mL, respectively. While $AUC_{0-\infty}$ when administered simultaneously of both FLX and VTX were 1251.38 ± 32.12 ng.h/mL for FLX and 1756.88 ± 45.78 ng.h/mL for VTX. The elimination half-life ($T_{1/2}$) for VTX when administered alone without FLX was found to be

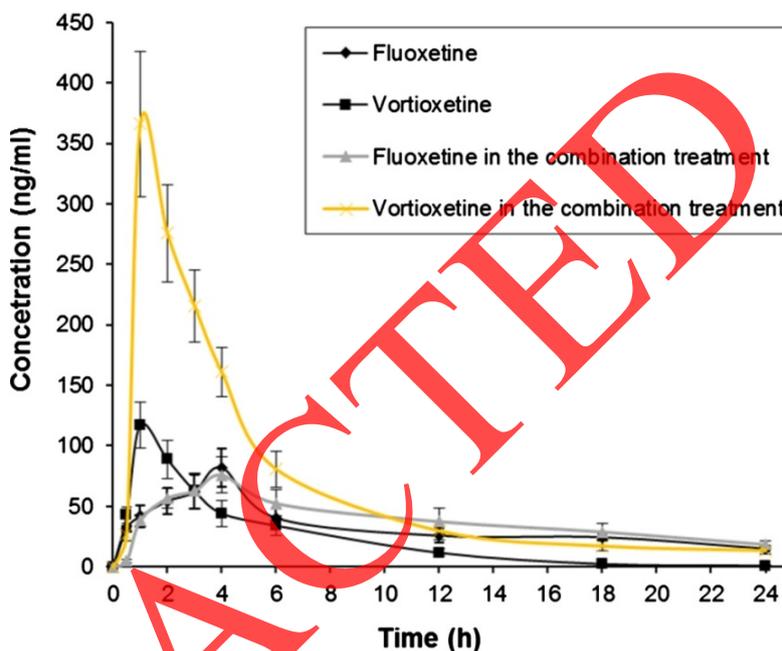


Figure 5. Mean plasma concentration-time profiles of vortioxetine in rats after oral administration of 4.0 mg/kg vortioxetine alone or in combination with 16.0 mg/kg fluoxetine. Data are expressed as mean \pm SD.

Table 10. The pharmacokinetic parameters of vortioxetine in rat plasma after oral administration of 4.0 mg/kg vortioxetine alone or in combination with 16.0 mg/kg fluoxetine (n = 6, Mean \pm SD).

Parameter	Unit	Fluoxetine	Fluoxetine combination	Vortioxetine	Vortioxetine combination
AUC_{0-24}^a	ng.h/mL	794.62 ± 35.91	925.77 ± 42.71	522.36 ± 26.12	1665.85 ± 67.23
$AUC_{0-\infty}^b$	ng.h/mL	1097.22 ± 55.11	1251.38 ± 32.12	527.37 ± 25.43	1756.88 ± 45.78
C_{max}^c	ng.h/mL	81.92 ± 4.12	75.83 ± 4.02	107.19 ± 5.14	365.97 ± 17.23
T_{max}^d	h	4.00 ± 0.09	4.00 ± 0.08	1.00 ± 0.04	1.00 ± 0.03
Cl/F^e	L/h/kg	0.0150	0.0132	0.0078	0.0023
$t_{1/2}^f$	h	13.88 ± 4.78	12.26 ± 3.99	12.51 ± 0.70	13.67 ± 1.01
$MRT_{0-\infty}^g$	h	18.87 ± 0.81	18.17 ± 0.76	5.32 ± 0.23	6.91 ± 0.29

*Data are presented as the mean \pm SD; ^aArea under the curve up to the last sampling time; ^bArea under the curve extrapolated to infinity; ^cThe maximum plasma concentration; ^dThe time taken to reach the maximum plasma concentration; ^eTotal clearance of drug from plasma after oral administration; ^fHalf-life; ^gMean residence time.

12.51 ± 0.70 h and was 13.67 ± 1.01 h when VTX co-administered with FLX in experimental rats group IV. While the elimination half-life ($T_{1/2}$) for FLX when administered alone without VTX was found to be 13.88 ± 4.78 h and was 12.26 ± 3.99 h when FLX co-administered with VTX in the rats group IV.

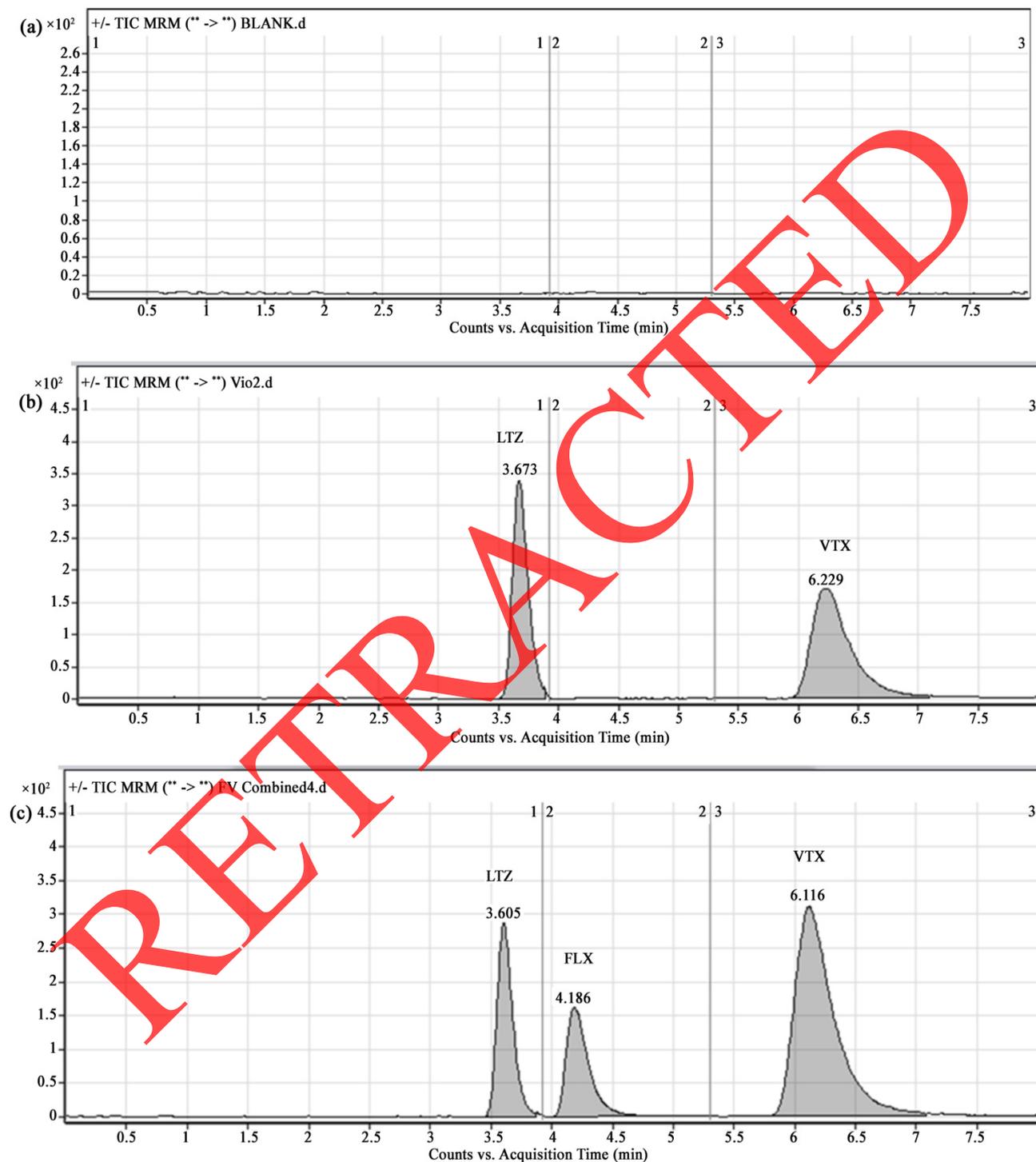


Figure 6. Representative MRM chromatograms of rat blank plasma (a), plasma sample obtained from oral administration of 4.0 mg/kg vortioxetine (b) and plasma sample obtained from oral administration of 4 mg/kg vortioxetine and 16.0 mg/kg of fluoxetine in rats (c).

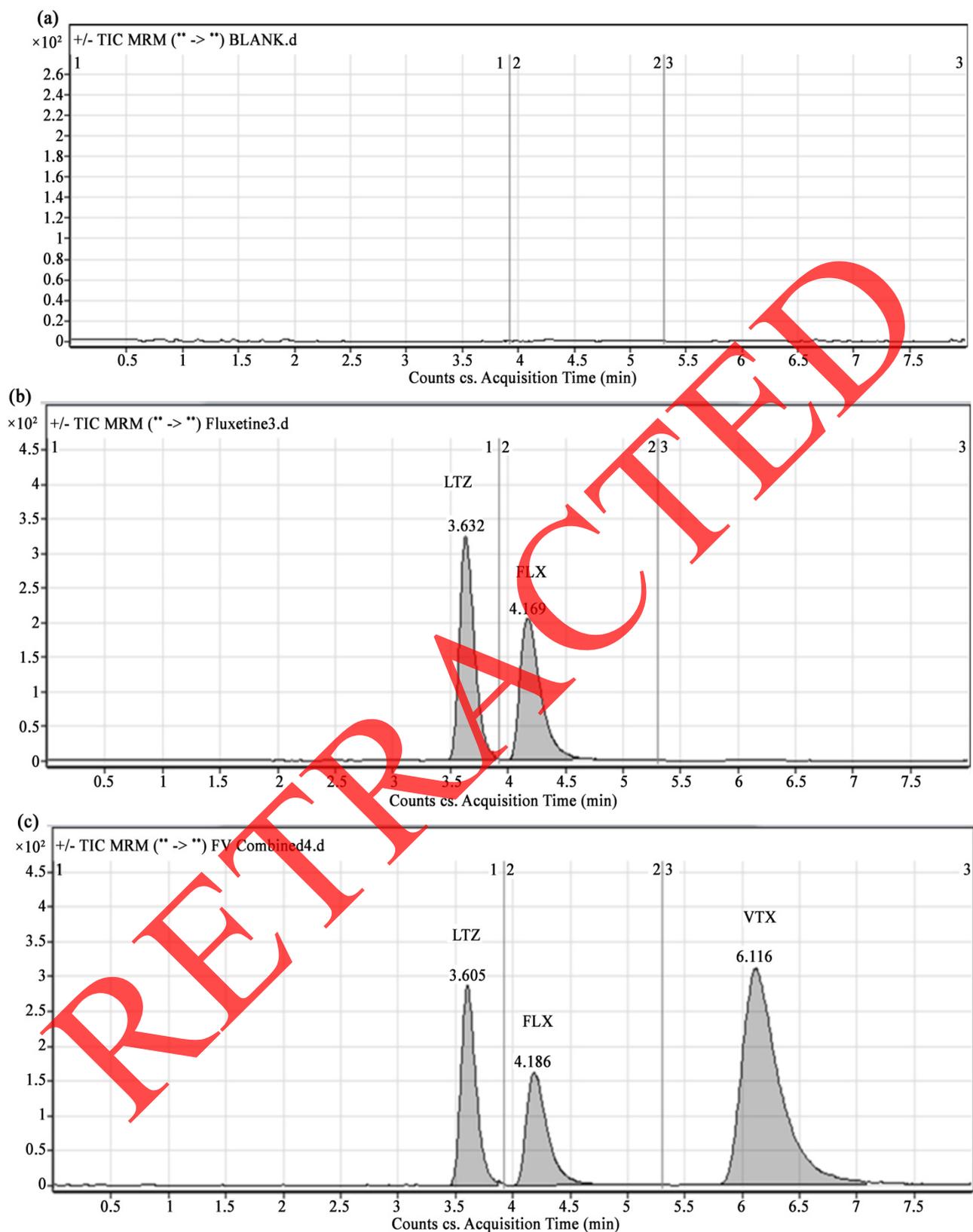


Figure 7. Representative MRM chromatograms of rat blank plasma (a), plasma sample obtained from oral administration of 16.0 mg/kg fluoxetine (b) and plasma sample obtained from oral administration of 4 mg/kg vortioxetine and 16.0 mg/kg of fluoxetine in rats (c).

Upon comparing the presented values of pharmacokinetic parameters of VTX and FLX with that values of the earlier reported studies, the VTX pharmacokinetic results reported from different studies with respect to C_{max} , $AUC_{0-\infty}$ and $T_{1/2}$, were ranging from 9.26 to 789.11 ng/mL, 157.13 to 2524.13 ng.h/mL, and 11.78 to 66.23 h; respectively, while the T_{max} values were ranged from 1.5 to 12 h [44] [45] [46]. For FLX, the C_{max} and $AUC_{0-\infty}$, $T_{1/2}$ and T_{max} results from different reported studies showed values were ranging from 194.82 to 1465 ng/mL, 23.33 to 1472 ng.h/mL, 11.98 to 87 h and 1.3 to 4.8 h; respectively [47] [48] [49] [50]. It was found that the results were in close agreement to that represented in our study. The PK parameters calculated for VTX and FLX given in combinations for rats in group IV were compared with those obtained following single administration of either of the two drugs in group II and III. Table 10 revealed that about 240% and 226% increase in C_{max} and AUC ($AUC_{0-\infty}$ and AUC_{0-t}) of VTX respectively were recorded with the co-administration of FLX together with VTX in studied rats group IV. Half-life of VTX was slightly longer, for T_{max} , it has been observed that there was no disparity between the obtained values of the rat groups treated with VTX and FLX combination in group IV, and those obtained following single administration of either of the two drugs in group II and III. This study concluded that when co-administration of FLX and VTX, the FLX could promote higher concentration of VTX in blood due to the inhibition of FLX to the liver enzyme responsible for VTX metabolism leading to increase in the plasma concentration the VTX. Therefore, reduce VTX dose by two thirds when the FLX is co-administered could be considered. The aim of antidepressant therapy is to induce remission and prevent relapses of major depressive disorder with minimum adverse effects during the treatment [51].

5. Conclusion

This work was the first analytically scanned the influence of FLX on VTX in rat plasma. A sensitive and simple UPLC-MS/MS method for simultaneous quantification of VTX and FLX in rat plasma has been developed and validated as per FDA guidelines. This method showed a linear range between 2.5 - 500 ng/mL for both studied drugs FLX and VTX with LLOD of 1.00 ng/mL. The developed simple and sensitive ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for quantification of the tested drugs. It is noticeable remind that the high sensitivity of the method permitted the accurate assessments of the PK parameters and the high capacity for the precise measurements and estimations for the very low doses of the tested medications that could be applied in any further clinical investigations. The short run time (7.0 min), and simple preparation process of the developed method was instituted to be accurate, precise and specific, and was succeeded to be used in the pharmacokinetic interaction study of vortioxetine and fluoxetine in rats. Results indicate that co-administration of vortioxetine and fluoxetine might bring a considerable change in vortioxetine plasma level. According to the product labeling, adminis-

tration of vortioxetine with the potent CYP450 2D6 inhibitor such as FLX resulted in greater than 2 fold increases in vortioxetine peak plasma concentration (C_{max}) and systemic exposure (AUC) compared to administration of vortioxetine alone. Accordingly, we recommended that the dosage of vortioxetine should be reduced by two thirds when used in combination with potent CYP450 2D6 inhibitors such as fluoxetine. Further investigations required to study that pharmacokinetic interaction on human in order to adjust the dose regimen of VTX when combined treatment with FLX in some cases of depression.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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