ORIGINAL ARTICLE

Development and validation of an automated solid-phase extraction-LC-MS/MS method for the bioanalysis of fluoxetine in human plasma

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ABSTRACT

A wide-range, specific, and precise liquid chromatography tandem mass spectrometric (LC-MS/MS) technique for quantifying fluoxetine (FLX) in human plasma was developed using the RapidTrace® automated solid-phase extraction (SPE) method; the analyte and internal standard (IS) were extricated on Oasis MCX SPE cartridges. Acetonitrile and 5 mM ammonium formate buffer (90:10 v/v) were used as mobile phase to achieve chromatographic separation on the reverse phase (C_{18} column). The analyte and IS were ionized using +ve electrospray ionization approach which was further traced by multiple-reaction monitoring on a tandem mass spectrometer. To quantify the FLX and FLX-d5, the parent-to-daughter ion transition of m/z of 310.0/44.1 and 315.0/44.0 was used, respectively. The method demonstrated a linear active limit of 0.20–30 ng/ml with recoveries ranging from 63.04% to 79.39% for quality control samples and 61.25% for IS samples. The concentrations over the calibration range demonstrated acceptable precision and accuracy. Due to the high inconsistency of the FLX concentration data, the minimum threshold of the assay was kept at 0.20 ng/ml. The flow rate was maintained at 500 μL/min, and the time for sample analysis for each injection was 3.5 min. The method was found to be specific, sensitive, and faster with minimum utilization of organic solvents and was utilized further for metabolic and pharmacokinetic studies.

Key words: Automation, fluoxetine, human plasma, mass spectrometry, solid-phase extraction

INTRODUCTION

Fluoxetine (FLX) is mainly administered for the management of major depressive disorder (MDD). MDD is characterized by mood depression, losing interest in daily activities, altered cognitive functions, and deteriorating physical health, and these symptoms altogether result in a low quality of life.[1] The pharmacotherapy of MDD utilizes FLX and aims to elevate the mood without causing significant side effects and to prevent the relapse of the diseases.[2] Chemically, FLX "(R,S)-N-methyl-3-phenyl-3-[4-(tri luoromethyl) phenoxy] propan-1-amine" is containing both R and S racemates in equimolar amount [Figure 1] and categorized as an inhibitor of selective serotonin reuptake (SSRI).[3] SSRIs are considered safer than other antidepressant drugs due

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to mild adverse effects, hence approved for pregnancy, teenagers, and children populations.[4] FLX is metabolized by N-demethylation into active metabolite norfluoxetine in the liver. However, active metabolites' production takes a few days due to the longer half-life of the parent compound.[5] Thus, it becomes challenging to measure the active metabolite in the plasma. It requires conducting studies with a longer duration, which adds to limitations as these studies involving human volunteers. As a result, most of the analytical studies rely on the measurement of FLX levels in biological fluids for pharmacokinetic and pharmacodynamic extrapolations.[6]

Several methods are available for the quantification of FLX, including the high-pressure liquid chromatographic (HPLC) methods with fluorescence detection, electrochemical detection,[7,8] and gas chromatography, and HPLC along with MS detection.[9,10] However, all the methods involve manual extraction of the compound of interest from the biological samples and subsequent chromatographic separation and quantification. The sample preparation process in the chromatographic analysis is considered a very crucial step. When it is manual, it makes the process very tiresome and time-consuming and leads to compromised final results with reduced precision and accuracy.[11] In addition to compromised results, due to manual extraction, these methods have long run time, low sensitivity, narrow range, and require a large volume of biological samples. As per the available literature up to now, no methods are available for the quantification of FLX with this broad range and with automatic extraction process using the RapidTrace® (Biotage) solid-phase extraction (SPE) unit.

Here, we established a simple, fast, responsive, and robust tandem LC-MS/MS technique for the quantitative assessment of FLX in human K3EDTA plasma. An automated SPE system was used to increase the process accuracy and precision. Plasma concentrations of FLX were analyzed by using the +ve ion electrospray ionization with the multiple-reaction monitoring (MRM) technique. The approach was validated following the USFDA Bioanalytical Method Validation Guideline.

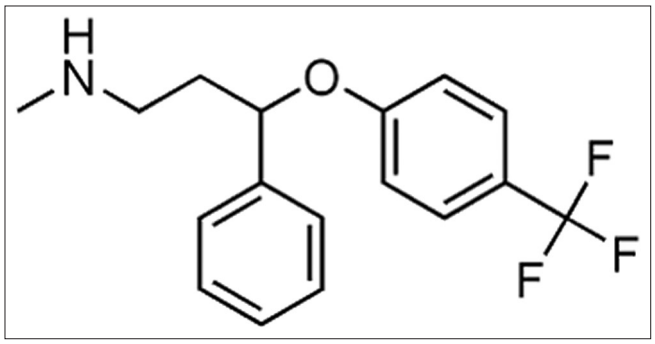

Figure 1: Chemical structure of fluoxetine

MATERIALS AND METHODS

Working standards and chemicals

The reference working standards of FLX (98.71%) and FLX-d5 (99.9%) were procured from Clearsynth Ltd., India. Acetonitrile and methanol, liquid ammonia (25% NH_3), orthophosphoric acid, and ammonium formate were procured from Sigma-Aldrich, Germany. In-house preparation used Milli-Q water (Direct-Q WPS) in the study.

Instrument and conditions

Chromatographic/mass spectrometric condition

Shimadzu HPLC was used to perform chromatographic separation on an Ascentis® Express C18 reverse-phase column, which includes a pump (LC-10ADVR), column oven (CTO-10AVP), and an autosampler (SIL-HTC). The column oven was integrated with an autosampler; 10 µL of extracted samples was directed to the column at 10°C. Mobile phase, acetonitrile (CAN) as well as 5.0 mM ammonium formate in a ratio of 90:10 (v/v) were included in isocratic mode, with a flow rate of 0.5 mL/min. Ions were identified in the +ve MRM mode by monitoring the m/z transition at 310.0/44.1 for FLX and 315.0/44.0 for the internal standard (IS) in unit-unit resolution. Analyst Software Version.1.4.2 (ABS Sciex, Framingham, MA0170,USA) was used to analyze and process the data.

Standard solution preparation

The stock solutions of FLX and FLX-d5 were prepared in ACN and Milli-Q water (70:30 v/v) at 1 mg/ml. All the stock dilutions were prepared in diluent, water, and methanol (50:50 v/v) under the low-light condition and stored in refrigerator. The calibration curve and quality controls (QCs) were obtained by diluting a sufficient stock solution to obtain plasma concentration between 0.201 and 30.00 ng/ml and for IS as 50 ng/mL in the diluent.

Sample preparation/extraction

The automated SPE technique was opted to process the plasma samples using RapidTrace® (Biotage) SPE system. The drug extraction from plasma samples was achieved

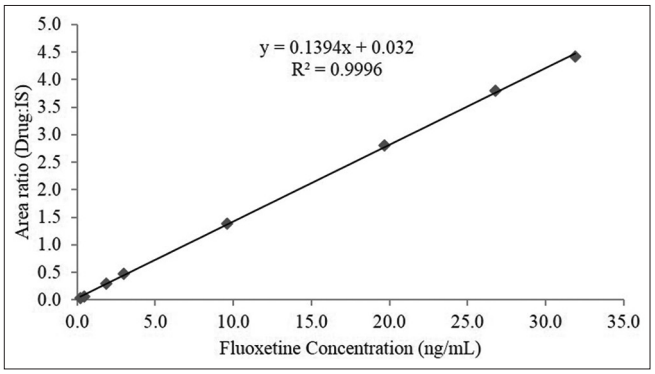

Figure 2: Calibration curve of fluoxetine concentration versus area ratio of drug/internal standard

through MCX cartridges (30 mg/cc). The extracted samples were evaporated to complete dryness at 45°C ± 2°C using a nitrogen evaporator (RapidVap Vertex evaporator). The dried extracts were reconstituted with mobile phase (500 µL) and transfer into vials for further examination [Table 1].

Method validation

USFDA guideline for analytical method validation was chosen to validate the method to test the selectiveness, linearity, precision, accuracy, matrix impact, and drug recovery.[12] In addition to this, other validation parameters tested were analyte and IS stability, benchtop stability, benchtop stability during extraction, freeze-thaw stabilities, long-term stabilities, and standard stock solutions and reference solution stabilities.[13] The reproducibility of the method was evaluated by determining reinjection reproducibility, dilution integrity, and ruggedness.

RESULTS AND DISCUSSION

For measuring the concentration of FLX in biological

Table 1: Extraction procedure (RapidTrace®)

Steps	Sources	Destination	Volumes (mL)	Flow rate (ml/min)
Conditioning	Methanol	Organic waste	1	15
Conditioning	Water	Aqueous waste	1	15
Loading	Sample	Biowaste	0.65	1
Purge cannula	Water	Cannula waste	2	30
Rinsing	Washing solution	Biowaste	1	2
Rinsing	Washing solution	Biowaste	1	2
Rinsing	Methanol	Biowaste	1	2
Rinsing	Methanol	Biowaste	1	2
Drying	Time (5 min)			
Collection	Elution solution	Fraction-1	1	30
Collection	Elution solution	Fraction-1	1	30
Purge cannula	Methanol	Cannula waste	2	30
Purge cannula	Water	Cannula waste	2	30

Table 2: Summary of chromatographic and mass spectrometric conditions

Parameter	Optimized Condition
Column name	Ascentis Express C18 column (75 mm×4.6 mm×2.7 µm)
Mobile phase	Acetonitrile: 5.0 mM ammonium formate (90:10 v/v)
Oven temperature	40
Injected volume (µl)	10
Flow rate (mL/min)	0.5
Detector	MS/MS
Gas-1 (nebulizer)	50
Gas-2 (heater gas)	50
Curtain gas	18
Collision gas	6
Ion source	Turbo-ion spray (positive ion mode)
Temperature	450
IS voltage	4500 V
DE clustering potential	18
Entrance potential	10
Collision energy	10
collision cells exit potential	9
Dwell time (ms)	200
M/z ion fluoxetine	310.0/44.1
M/z fluoxetine-d5	315.0/44.0
Sample cooler temperature	10±2
Rinsing solution	Methanol: Water (50:50 v/v)
Retention time (fluoxetine), min	1.5-1.8
Right (fluoxetine d5), min	1.5-1.8
Run time (min)	3.5

IS: Internal standard, MS: Mass Spectrometry

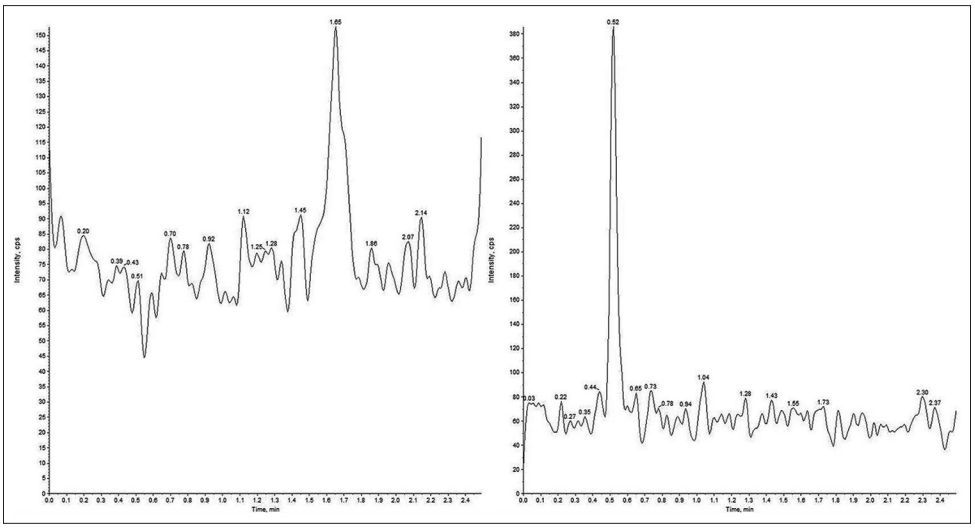

Figure 3: Representative chromatograms of blank (left) and blank internal standard (right) in plasma samples

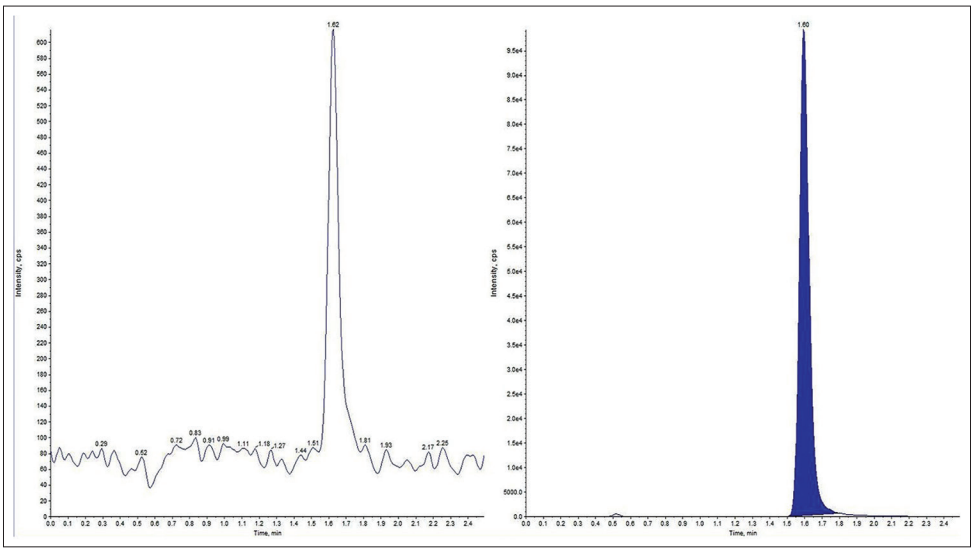

Figure 4: Representative chromatograms of blank (left) and internal standard (right) in spiked plasma samples

matrices, few HPLC, gas chromatography mass spectrometry (GCMS) and liquid chromatography mass spectrometry (LCMS) methods are available.[10,14,15] These methods, however, have some drawbacks, therefore, LC-MS/MS approach for measuring FLX was considered by utilizing the RapidTrace® automated SPE system,[16,17] and detailed processes are given in Table 1. To create a reliable, consistent, and reproducible LC-MS/MS method chromatographic, separation conditions were optimized after MS/MS optimization to offer adequate separation and peak symmetry with an appropriate response.[18,19] Chromatographic and mass spectrometric conditions are established in Table 2.

The method was developed and validated keeping in mind a bioequivalence pharmacokinetic approach, in which healthy subjects were involved. The evaluation of parameters such as selectiveness, linearity, precisions and accuracies, drug recovery, stabilities (freeze-thaw, benchtop, long-term, and stock solution stabilities), and ruggedness was completed for the validation of the method.[20,21] The selectivity was performed on different lots of hemolyzed and lipemic plasma samples. Insignificant interference was found in the various plasma sets tested at the RT of FLX and FLX-d5 [Table 3]. A graph of a representation of regression calibration curve is provided in Figure 2.

The coefficient of variation (CV) of the experiment was calculated by using QCs as a % mark scale of sample concentration. Table 4 shows the precision and accuracy statistics for the interday and intraday periods. The chromatograms of FLX and FLX-d5 blank samples, blank + IS samples, limit of quantitation (LOQ) and upper limit of quantitation (ULOQ) are presented in Figures 3-6, respectively.

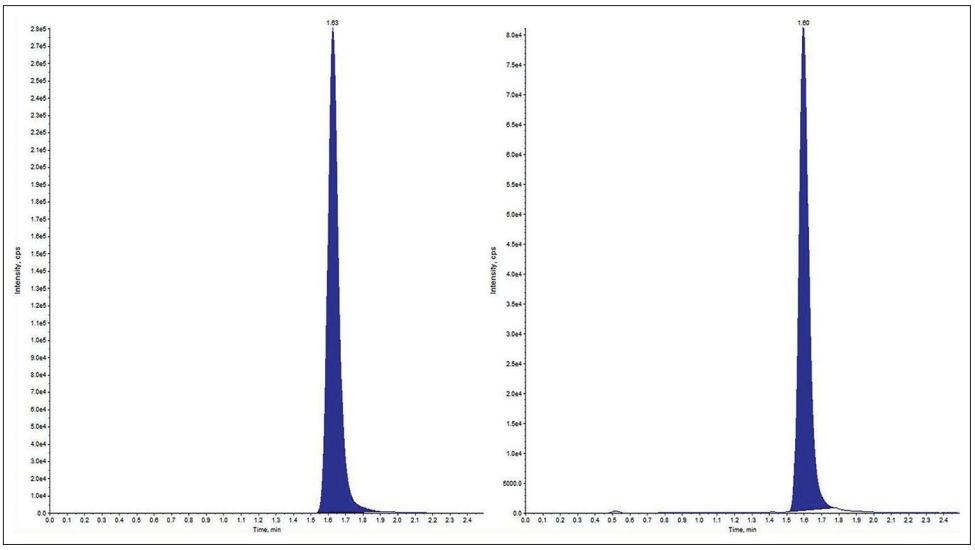

Figure 5: Representative multiple reaction monitoring chromatograms of lower limit of quantitation samples of fluoxetine (left) and fluoxetine-d5 (right) in spiked plasma

Table 3: Blank screening and selectivity

Blank ID	Interference fluoxetine	LOQ area-fluoxetine (%)	Interference fluoxetine-d5	IS area (%)
B-01	83	16,283 (0.50)	80	1,462,938 (0.005)
B-02	62	17,995 (0.30)	90	1,398,671 (0.006)
B-03	2	15,388 (0.00)	63	1,351,752 (0.005)
B-04	31	14,124 (0.20)	23	1,230,928 (0.002)
B-05	20	14,850 (0.10)	50	1,434,412 (0.003)
B-06	109	14,036 (0.80)	211	1,132,306 (0.019)
B-07	12	-	300	
B-08	6	-	230	
B-09	78	-	150	
B-10	45	-	186	
Mean		15,446		1,335,168
SD		1502.820		128,453
Percentage CV		9.730		9.6

IS: Internal standard, SD: Standard deviation, LOQ: Limit of quantitation, CV: Coefficient of variation

Table 4: Intraday and interday precision and accuracy of fluoxetine

Nominal concentration (ng/mL)	Intraday ($n=6$)			Inter day ($n=18$)		
	Mean concentration±SD	Precision (%)	Accuracy (%)	Mean concentration±SD	Precision (%)	Accuracy (%)
0.203 (LOQQC)	0.203±0.01	7.26	99.92	0.192±0.01	7.7	94.69
0.583 (LQC)	0.603±0.01	1.92	108.06	0.629±0.02	2.57	107.8
12.677 (MQC)	12.405±0.32	2.58	97.93	12.543±0.38	3.02	99.02
25.353 (HQC)	25.218±0.51	2.02	99.47	25.018±0.61	2.42	98.68

SD: Standard deviation, LOQQC: Lowest limit of quantification quality control, LQC: Low quality control, MQC: Medium quality control, HQC: High quality control

The presence matrix effect will alter the ionization pattern of analytes present in the matrix. Thus, the technique was designed to evade any effect on the matrix. It was found that the absolute matrix effect was 98.4%, 98.5%, and 99.5% at lower, middle, and higher levels, respectively. This indicated that matrix effect in the presence of plasma was found to be negligible. The mean overall recovery of FLX was found to be 68.63%, with a precision of 13.58%. Whereas, the mean overall recovery of FLX-d5 was found to be 61.2%, with a precision of 4.7%. Table 5 shows the comparative recoveries and complete matrix effects with procedure proficiencies for FLX and FLX-d5.

The stability was performed in both matrices, namely aqueous as well as plasma, and the results of the stability exercise are tabulated in Table 6. It is evident and apparent

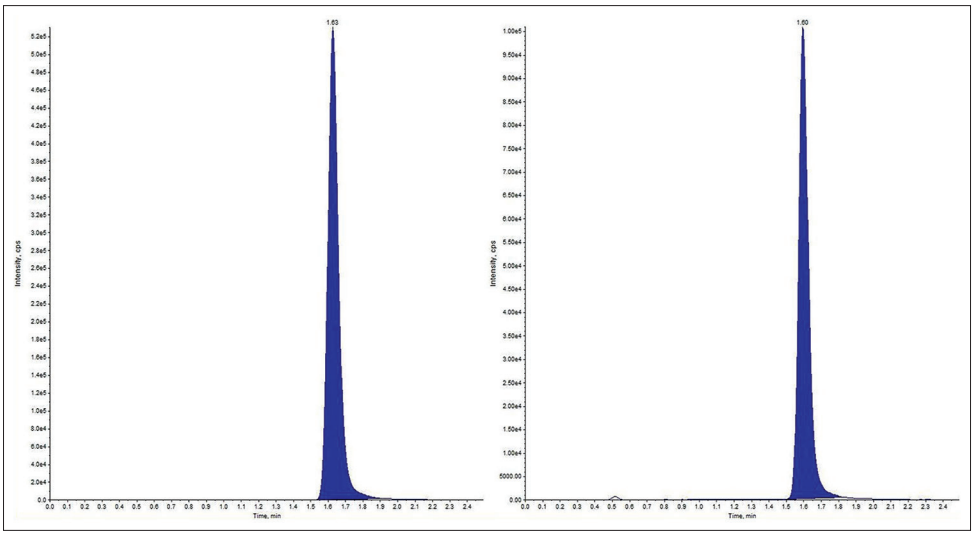

Figure 6: Representative multiple reaction monitoring chromatograms of upper level of quantification samples in plasma: fluoxetine (left) and fluoxetine-d5 (right)

Table 5: Matrix effect and recovery for fluoxetine and fluoxetine d5

QC	A (CV %)	B (CV %)	C (CV %)	Absolute ME (%)	RE (%)
LQC	45142.20 (4.6)	44423.70 (8.66)	35268.20 (6.51)	98.41	79.39
MQC	974522.10 (4.64)	960072.67 (2.48)	609186.83 (6.52)	98.52	63.45
HQC	1915432.40 (2.99)	1905052.00 (2.90)	1200998.20 (4.10)	99.46	63.04
ISD	1234584.60 (7.39)	1229850.70 (0.70)	753278.20 (4.25)	99.62	61.25

QC: Quality control, ME: Matrix effect, LQC: Low quality control, MQC: Medium quality control, HQC: High quality control, RE: Relative recovery, ISD: Internal standard dilution, CV: Coefficient of variation

Table 6: Stability data for fluoxetine and fluoxetine-d5

Storage condition	QC (ng/mL)	Precision (%)	Accuracy (%)	Stability (%)
Benchtop stability (14.50 h)	LQC	4.35	96.10	101.44
	HQC	2.52	100.71	98.14
Benchtop extraction stability (14.53 h)	LQC	2.38	98.54	98.37
	HQC	1.94	102.96	102.52
Long-term stability (15 days)	LQC	3.93	95.31	96.52
	HQC	1.34	102.50	98.97
Freeze-thaw stability (3 cycles)	LQC	2.81	102.8	95.45
	HQC	2.70	99.30	101.27
In-injector stability (52.50 h)	LQC	5.29	94.16	96.35
	HQC	2.74	95.21	93.05
Reinjection reproducibility (52.50 h)	LQC	1.21	91.00	98.61
	HQC	0.82	98.27	101.46
Short-term stability of reference standard (9.50 h)		0.87		100.24
Stock solution stability of fluoxetine (7 day)		2.32		94.80
Stock solution stability of fluoxetine-d5 (24 h)		0.44		100.15

QC: Quality control, LQC: Low quality control, HQC: High quality control

Table 7: Precision and accuracy batch for the ruggedness

Nominal concentration (ng/mL)	Mean±SD	Precision (%)	Accuracy (%)
0.203 (LOQQC)	0.192±0.01	2.82	94.33
0.583 (LQC)	0.615±0.02	3.95	105.55
12.677 (MQC)	12.847±0.12	0.93	101.34
25.353 (HQC)	25.480±0.04	0.16	100.51

SD: Standard deviation, LOQQC: Lowest limit of quantification quality control, LQC: Low quality control, MQC: Medium quality control, HQC: High quality control

that analytes as well as IS stability samples were found to be stable under different storage circumstances. The QC samples were prepared to determine dilution accuracy. The nominal percentage of dilution integrity was found to be 94.9% and 94.2% after 2–4 times dilution, whereas the percentage of CV was stated to be 2.9 and 6.8%, respectively. The method showed ruggedness for the selected validation range [Table 7].

CONCLUSION

In this method, a reverse-phase chromatographic method with tandem mass spectrometric detection using FLX-d5 as an IS was developed in the K3EDTA plasma. Chromatographic separation with a lower baseline was achieved using mobile phase with acetonitrile and ammonium formate buffer. The maximum response was observed using the reverse-phase Ascentis® Express C18 column at an optimized flow rate (0.5 ml/min). This technique was established and found to be rapid, specific, precise, and linear and can facilitate the biostudies of FLX. Chromatographic and extraction parameters have been designed to achieve a short-run time and a fast, automated plasma sample processing procedure due to the chromatographic run duration of 3.5 min and retention time of 1.6 min. Thus, the method makes a high sample throughput. This approach can also be implemented in a clinical laboratory for therapeutic drug control, enabling individual dosage optimization, drug interaction identification, and patient compliance evaluation.

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Conflicts of interest

There are no conflicts of interest.

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