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Bioanalytical Method Development and Validation for Simultaneous Estimation of Brexpiprazole and Fluoxetine Hydrochloride in Human Plasma by RP-HPLC

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The present work aimed to develop a bioanalytical RP-HPLC technique for the simultaneous quantification of brexpiprazole (BREX) and fluoxetine hydrochloride (FLX) in human plasma. The chromatographic separation was performed on a Phenomenex C18 column (250 mm × 4.6 mm, 5 μm). The chromatogram was recorded at 224 nm. As an internal standard (IS), fimasartan was used. The mobile phase was composed of acetonitrile and 0.05% orthophosphoric acid in water (35:65), and with a flow rate of 1.0 mL/min. Separation was carried out in an isocratic mode. The protein precipitation method was used to isolate the analytes from the spiked plasma matrix. The retention times (RT) of BREX, IS, and FLX were 3.69, 4.91, and 6.82 min, respectively. With regression coefficients r^2 = 0.9996 and 0.9993 for BREX and FLX, respectively. The developed method demonstrated an acceptable linearity in the concentration range of 1.40–56 μg/mL. The extraction recovery of BREX was in the range of 82.57% to 85.26%, and for FLX, 91.74% to 94.05%. The stability studies showed no evidence of analyte degradation. Hence, the developed method can be used for the simultaneous quantification of BREX and FLX in biological samples.

Keywords: Brexpiprazole; Bioanalytical method validation; Fluoxetine hydrochloride; Mood disorders; Protein precipitation; Serotonin transporter.

Mood disorders and depression affect millions of people worldwide, endangering public health.[1] FLX, a selective serotonin reuptake inhibitor (SSRI), is primarily used to treat anxiety disorders, depression, and associated illnesses.[2] Chemically it is (3RS)-N-methyl-3-phenyl-3-[4-trifluoromethyl-phenoxy)-propan-1-amine hydrochloride.[3]. FLX inhibits the serotonin transporter (SERT), a protein that, once released into the synaptic cleft, reabsorbs serotonin (5-hydroxytryptamine) in the presynaptic neuron.[4] FLX increases serotonin in the synaptic cleft by blocking this transporter, which enhances serotonergic neurotransmission and improves mood and emotional stability. It has a long half-life and produces the active metabolite norfluoxetine, which extends its duration of action.[5] To improve treatment outcomes, augmentation techniques are necessary, as many patients do not respond well to SSRIs alone.[6,7] BREX, a second-generation atypical antipsychotic, has recently demonstrated promise as an adjuvant to SSRIs in the treatment of major depressive disorders.[8] Chemically it is 7-{4-[4-(1-benzo-thiophen-4-yl) piperazin-1-yl] butoxy} quinolin 2-(1H)-one.[9] Due

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to its partial agonist action at serotonin 5-HT1A and dopamine D2 receptors and antagonist action at serotonin 5-HT2A receptors, this serotonin-dopamine activity modulator (SDAM) has a positive side-effect profile as well as antidepressant and antipsychotic effects. [10,11] BREX and FLX co-administration is becoming more clinically significant, particularly for patients who only partially respond to monotherapy.

By utilizing the synergistic effects of serotonergic and dopaminergic regulation, the combination enhances treatment results for mood disorders.[12] This treatment approach also raises issues regarding safety monitoring and pharmacokinetic interactions, as FLX is a potent inhibitor of cytochrome P450 enzymes, mainly CYP2D6, which is involved with the metabolism of BREX. [13,14] Therefore, to prevent adverse effects and ensure therapeutic efficacy, it is necessary to monitor the plasma concentrations of both drugs simultaneously. Figure 1 illustrates the chemical structures of BREX and FLX.

A review of the literature revealed a few analytical techniques for estimating BREX and FLX, including the QbD-based UPLC method for estimating BREX[9], the bioanalytical method for estimation of BREX in dog plasma by UPLC–MS-MS,[15] estimation of BREX in bulk drug or formulation by RP-HPLC,[16-21] determination of BREX in bulk and formulation by HPTLC,[22] estimation of BREX in tablet formulation by spectrophotometry,[23] spectrofluorimetric methods,[24-26] simultaneous determination of FLX and BREX by HPLC to test the purity of both drugs in pharmaceutical formulations by monitoring fluoxetine-related impurities,[27] and estimation of FLX alone or with other drugs in bulk, formulation, and biological fluids by spectrophotometry.[28-30]

There is currently no validated bioanalytical RP-HPLC method reported for the simultaneous quantification of BREX and FLX in human plasma, even though several techniques have been reported for the individual estimation of FLX and BREX. The validated method was applied to real plasma samples.

MATERIALS AND METHODS

Reagents and chemicals

The chemicals and reagents were of analytical grade. FLX and fimasartan were purchased from Swapnroop Drugs, Sambhajinagar, India, while BREX was kindly supplied by Lupin Limited, Pune, India. The HPLC-grade acetonitrile, water, and methanol, as well as analytical-grade formic acid and orthophosphoric acids, were acquired from Shri Ganesh Services (Nashik, India). Human plasma was received from Arpan Blood Bank, Sangamner, India.

Instrumentation and chromatographic conditions

The separation was achieved by isocratic elution using a Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) on an Agilent HPLC provided with a quaternary gradient pump. The mobile phase consists of acetonitrile and 0.05% orthophosphoric acid (35:65). The separation was achieved within 10 min using a 1 mL/min flow rate, and the injection volume was 20 µL. Detection was performed at 224 nm using a UV detector.

Preparation of stock solution, calibration standards, and quality control samples

Accurately weighed quantities of BREX and FLX (purity 99.0%–101.0%) were diluted with methanol to prepare separate stock solutions (1600 ug/mL). The calibration stock solutions (1600 µg/mL) of BREX and FLX were diluted with methanol to prepare aqueous linearity solutions (28, 160, 400, 560, 840, and 1120 µg/mL) of BREX and FLX, respectively. These solutions were diluted with blank plasma to prepare calibration standards (1.40, 8, 20, 28, 42, and 56 µg/mL) of BREX and FLX, respectively. The LLOQ was set based on signal response. A concentration of 50 µg/mL of BREX and FLX gives 107 mAu and 116 mAu peak heights, respectively; therefore, 1.4 µg/mL yields approximately 3 mAu peak heights, which was selected as the LLOQ. This value represents d"5% of the Cmax (28 µg/mL) as per EMEA guidelines. The ULOQ is twice the Cmax, i.e., 56 µg/mL. Therefore, the linearity range was selected as 1.4–56 µg/mL. Aqueous quality control (QC) solutions of both drugs were prepared by diluting QC stock of each drug (1600 µg/mL) with methanol to get 28, 80, 560, and 896 µg/mL. Similarly, four QC samples at 1.40, 4, 28, and 44.80 µg/mL for BREX and FLX were prepared by diluting respective aqueous QC solutions in blank plasma and treated as Lower Limit of Quantification (LLOQ), Lower Quality Control (LQC), Middle Quality Control

(MQC), and Higher Quality Control (HQC), respectively. The calibration standards and QC samples were kept at -20°C and taken out during analysis. The proposed method is not suitable for low-level pharmacokinetic profiling but rather for higher concentration applications such as formulation studies and spiked plasma analysis.

Sample Preparation

BREX and FLX were extracted from plasma using protein precipitation. Plasma samples (calibration curve standards, QC samples) were vortexed for one minute and then placed in a deep freezer. All frozen samples were thawed and allowed to equilibrate to room temperature prior to analysis. 50 µL of IS solution was added to 500 µL of spiked plasma samples (calibration curve standards and QC samples), and the mixture was vortexed for one minute. After adding 20 µL of 2% formic acid, the mixture was vortexed for one minute. 1 mL of acetonitrile was added and vortexed for two minutes. The samples were centrifuged at 4000 rpm for three minutes. Supernatant, 0.5 mL, was withdrawn and injected. The absorption maxima of BREX and FLX were reported as 217 nm and 226 nm, respectively. The overlay spectrum of both drugs (Fig. 2) shows significant absorption at 224 nm as an isoabsorptive point. Fig. 3 illustrates the chromatogram of BREX, FLX, and IS that were extracted from plasma.

Method validation

The method was validated for sensitivity, selectivity, carryover, accuracy, precision, and recovery. Stability studies include bench-top, freeze-thaw, auto-sampler, stability of processed extracted samples, and stock solution stability. Validation of the developed method was performed according to the bioanalytical validation guidelines established by the US Food and Drug Administration (US-FDA) and European Medicines Agency (EMEA).[31,32]

RESULTS

Selectivity

Peak interference was determined using eight independent sources of blank plasma, including six human plasma samples along with lipemic and hemolyzed plasma. The selectivity was determined by comparing results with the lower limit of quantification (LLOQ). Chromatograms of blank and spiked plasma samples, containing drugs and internal standards (IS), were analyzed for interference (Figures 4 and 5).

Sensitivity

Six replicates of the LLOQ obtained from the same plasma sample were used in a sensitivity test. 475 µL plasma were transferred into six dry, clean 15 mL tarson tubes. The tubes were vortexed for one minute after adding 25 µL of aqueous LLOQ solution to each tube. 50 µL of IS solution was then added, followed by vortexing for one minute. The mixture was vortexed for another minute after adding 20 µL of 2% formic acid. In order to precipitate the proteins, 1.0 mL of acetonitrile was added, and the samples were vortexed for two minutes. The samples were centrifuged for three minutes at 4000 rpm. The supernatant 0.5 mL was withdrawn and injected. (Table 1)

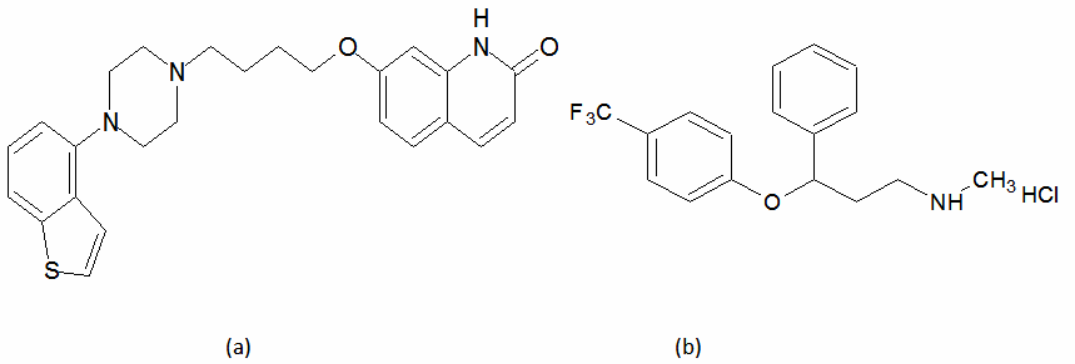

Fig. 1. Chemical structures of (a) BREX (b) FLX

Table 1. Observation summary of sensitivity

Area of BREX at LLOQ	Area of FLX at LLOQ
272531	512634
294635	490531
296352	496124
271531	505417
286524	480634
257451	526524
Mean 279837	Mean 501977
SD 15231.16716	SD 16424.08373
% CV 5.44	% CV 3.27

Carryover

In order to measure carryover, the same blank sample was injected three times. The upper limit of quantification (ULOQ) injection was preceded by one blank injection and followed by two blank injections (Table 2)

Accuracy and Precision

The accuracy and precision of the analytical method were determined using extracted blank samples, extracted blanks spiked with internal standard (IS), calibration curve standards, and QC samples. Four QC levels were examined in each analytical run: LLOQ, LQC, MQC, and

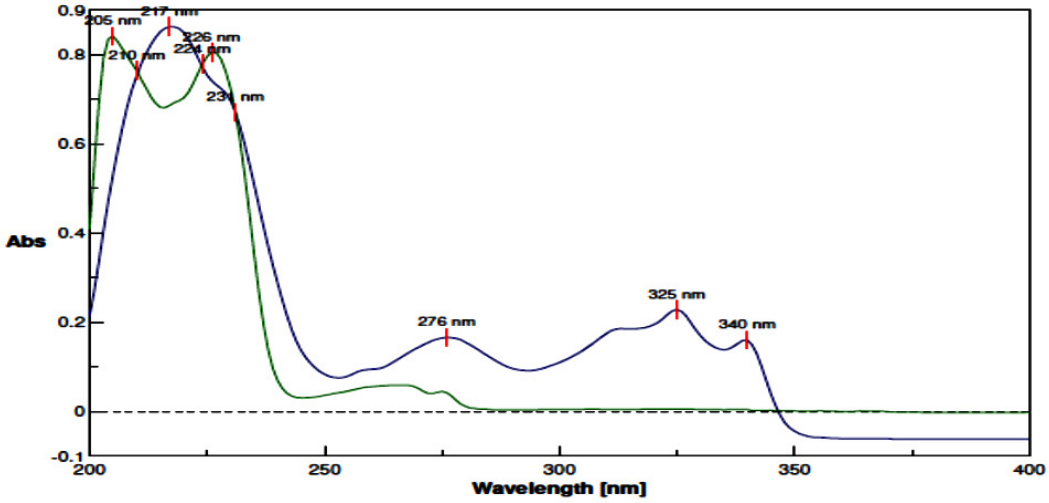

Fig. 2. Overlay UV spectrum of BREX and FLX

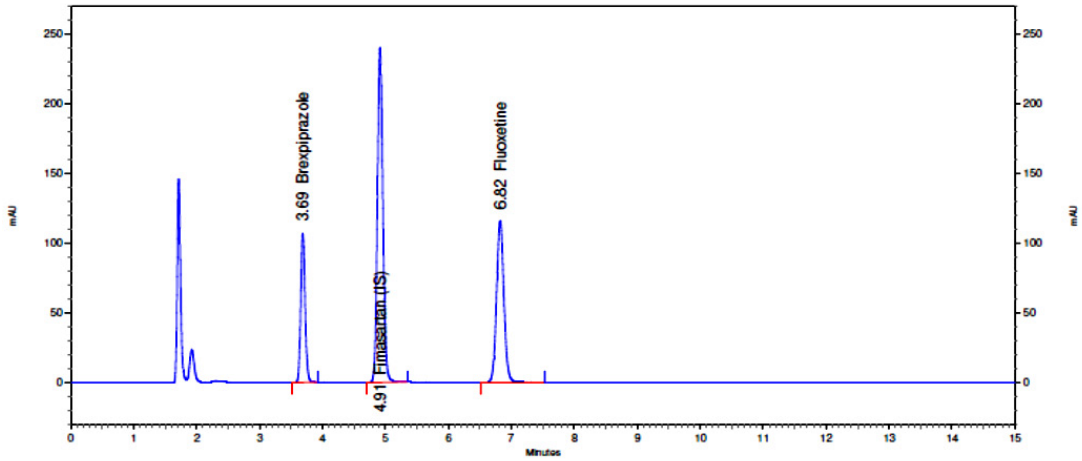

Fig. 3. Chromatogram of BREX (R.T. 3.69 min), IS, Fimasartan (R.T. 4.91 min), and FLX (R.T. 6.82 min) extracted from plasma.

HQC. Accuracy and precision were assessed by processing and analyzing six replicate samples at each QC level. Linearity was determined by plotting the analyte-to-IS peak area ratio against the actual concentrations. The accuracy and precision results are shown in Tables 3, 4, and 5.

Recovery

The extraction recovery of BREX, FLX, and IS was evaluated at low, medium, and high QC levels. A recovery vial was prepared by extracting blanks spiked with an analyte post-extraction. For recovery assessment, 952 µL of extracted plasma

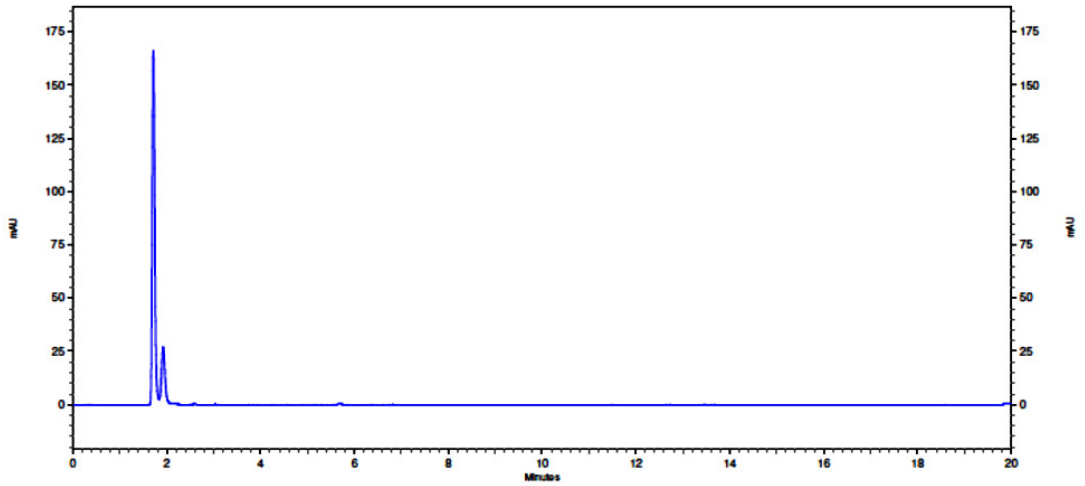

Fig. 4. Chromatogram of Blank Plasma

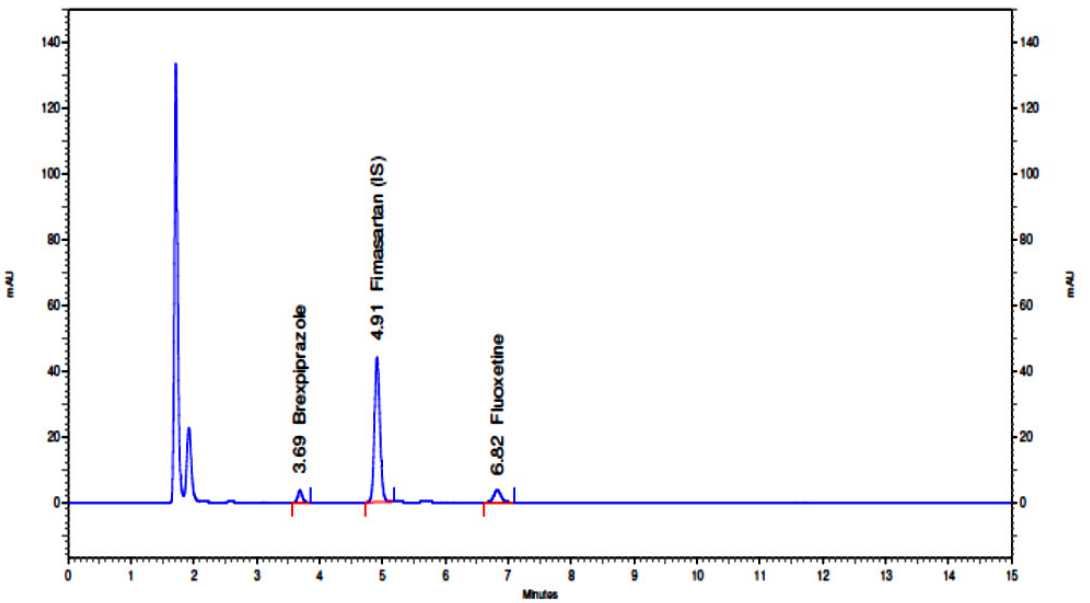

Fig. 5. Chromatogram of spiked plasma samples with the BREX (R.T. 3.69 min), IS, Fimasartan (R.T. 4.91 min), and FLX (R.T. 6.82 min) at LLOQ

was added with 16 µL of aqueous LQC/MQC/MQC solution and 32 µL of aqueous internal standard solution and injected (Table 6).

Stability

The stability of drugs in human plasma was determined at different conditions to confirm the suitability of the analytical technique. For stability studies, three replicates were analyzed at LQC and HQC levels. To evaluate benchtop stability, spiked plasma samples were kept at room temperature for six hours before being processed and analyzed. In freeze-thaw stability, the spiked plasma samples were processed for three freeze-thaw cycles to identify any possible degradation during repeated freezing and thawing. For autosampler stability, spiked plasma samples were reinjected 24 hours after the initial injection time. The stability of processed extracted samples was examined by keeping the processed LQC and HQC samples on the bench for 6 hours prior to injection into the analytical system. In addition, stock solution stability was investigated by storing aqueous LQC and HQC stock solutions for 24 hours, after which fresh LQC and HQC samples were prepared from these stored solutions and analyzed (Table 7).

DISCUSSION

Since there is no reported sensitive bioanalytical method for the simultaneous estimation of BREX and FLX, the validated RP-HPLC method was developed for routine analysis in human plasma. The available methods were developed to assess drugs individually. Therefore, there is a need to develop a bioanalytical method for the estimation of these drugs in combination. The current method aims to develop a simple, accurate, and reliable method for the simultaneous estimation of BREX and FLX in human plasma. The method used protein precipitation with acetonitrile for the extraction of drugs from human plasma. Protein precipitation is cost-effective (using inexpensive organic solvents such as acetonitrile), has less analyte loss during extraction, does not require drying, and allows for the direct injection of the supernatant. The chromatographic conditions have been optimized during method development to

Table 2. Observation summary of carryover

Particulars	Area of BREX	Area of FLX	% Interference
Blank 1	0	0	0
ULOQ	9648992	17608790	NA
Blank 2	0	0	0
Blank 3	0	0	0
LLOQ mean area from sensitivity	279837	501977	NA

Table 3. Accuracy of calibration curve

Standards	Actual Conc. (µg/mL)	Area of BREX	Area of FLX	Area of IS	% Accuracy (BREX)	% Accuracy (FLX)
Blank	0	0	0	0	-	-
Blank + IS	0	0	0	4534119	-	-
STD A	1.40	281369	507353	4551033	107.86	107.14
STD B	8.00	1466819	2608270	4703385	101.38	97.25
STD C	20.00	3615870	6518536	4685964	101.20	98.25
STD D	28.00	4781406	9130251	4594640	97.64	100.36
STD E	42.00	7285828	13964513	4603296	99.14	102.26
STD F	56.00	9648992	17608790	4507674	100.64	98.80

achieve excellent separation of BREX and FLX. Short retention times of both drugs result in a high-throughput method suitable for processing many samples.

For method development and optimization, different mobile phase compositions were used. In the first trial, methanol and water (70:30, v/v) were used as the mobile phase, but the shape of the peak was not proper. For the second trial (70:30, v/v), acetonitrile and water were used. Drugs were eluted, but proper peak shape was not observed. The chromatographic peak in the third trial, with methanol and 0.05% orthophosphoric acid in water (70:30, v/v), was broad. For the fourth trial, the mobile phase consisting of acetonitrile and 0.05% orthophosphoric acid in water (50:50, v/v), a Gaussian-shaped peak was not observed. In the fifth trial, acetonitrile and 0.05% orthophosphoric acid in water (35:65, v/v) were tried and showed a well-resolved peak with a satisfactory response, improved symmetry, and an acceptable RT. The optimized chromatographic conditions were used to evaluate Fimasartan as an IS. A mixed solution containing 100 µg/mL of BREX, FLX, and IS was injected. Fimasartan showed acceptable peak symmetry, without interfering with the analyte peaks.

Selectivity was confirmed if interfering substances were less than 5% of the IS signal and less than 20% of the analyte signal at LLOQ. The results met the acceptance criteria, showing no peaks in the blank plasma chromatograms corresponding to BREX and FLX retention times. For sensitivity, the acceptance criteria was achieved, and the % CV was observed as 5.44 and 3.27 for BREX and FLX, respectively. For carryover, the acceptance criterion was met when the peak areas at the retention times of BREX

Table 4. Summary of accuracy and precision of BREX from quality control samples

Level	Intra-day			Inter-day		
	Recovered Conc. (µg/mL)	% Accuracy	% CV	Recovered Conc. (µg/mL)	% Accuracy	% CV
LLOQ	1.28	91.43	5.65	1.28	91.43	4.83
	1.39	99.29		1.31	93.57	
	1.36	97.14		1.31	93.57	
	1.44	102.86		1.35	96.43	
	1.27	90.71		1.28	91.43	
	1.45	103.57		1.45	103.57	
LQC	3.85	96.25	5.6	3.77	94.25	2.81
	4.06	101.50		3.83	95.75	
	4.37	109.25		3.76	94.00	
	3.93	98.25		3.97	99.25	
	3.74	93.50		3.90	97.50	
	3.87	96.75		3.67	91.75	
MQC	27.84	99.43	1.38	26.72	95.43	1.92
	27.44	98.00		26.76	95.57	
	27.58	98.50		27.00	96.43	
	28.13	100.46		26.82	95.79	
	27.37	97.75		27.67	98.82	
	27.04	96.57		27.94	99.79	
HQC	44.61	99.58	3.01	43.04	96.07	2.75
	47.01	104.93		41.90	93.53	
	45.70	102.01		42.04	93.84	
	42.87	95.69		44.44	99.20	
	45.03	100.51		43.76	97.68	
	45.10	100.67		41.43	92.48	

Table 5. Summary of accuracy and precision of FLX from quality control samples

Level	Intra-day			Inter-day		
	Recovered Conc. (µg/mL)	% Accuracy	% CV	Recovered Conc. (µg/mL)	% Accuracy	% CV
LLOQ	1.59	113.57	4.74	1.37	97.86	5.45
	1.50	107.14		1.32	94.29	
	1.51	107.86		1.41	100.71	
	1.49	106.43		1.48	105.71	
	1.40	100.00		1.29	92.14	
	1.41	100.71		1.46	104.29	
LQC	4.06	101.50	3.06	4.14	103.50	4.22
	3.96	99.00		3.73	93.25	
	4.10	102.50		3.99	99.75	
	4.29	107.25		3.83	95.75	
	3.95	98.75		3.77	94.25	
	4.12	103.00		4.05	101.25	
MQC	29.49	105.32	2.07	28.88	103.14	3.05
	29.65	105.89		28.04	100.14	
	29.15	104.11		27.46	98.07	
	28.97	103.46		28.25	100.89	
	28.28	101.00		29.76	106.29	
	28.22	100.79		27.59	98.54	
HQC	43.81	97.79	2.61	42.64	95.18	2.94
	46.79	104.44		43.25	96.54	
	43.83	97.83		41.11	91.76	
	43.83	97.83		42.53	94.93	
	45.07	100.60		44.94	100.31	
	44.66	99.69		42.36	94.55	

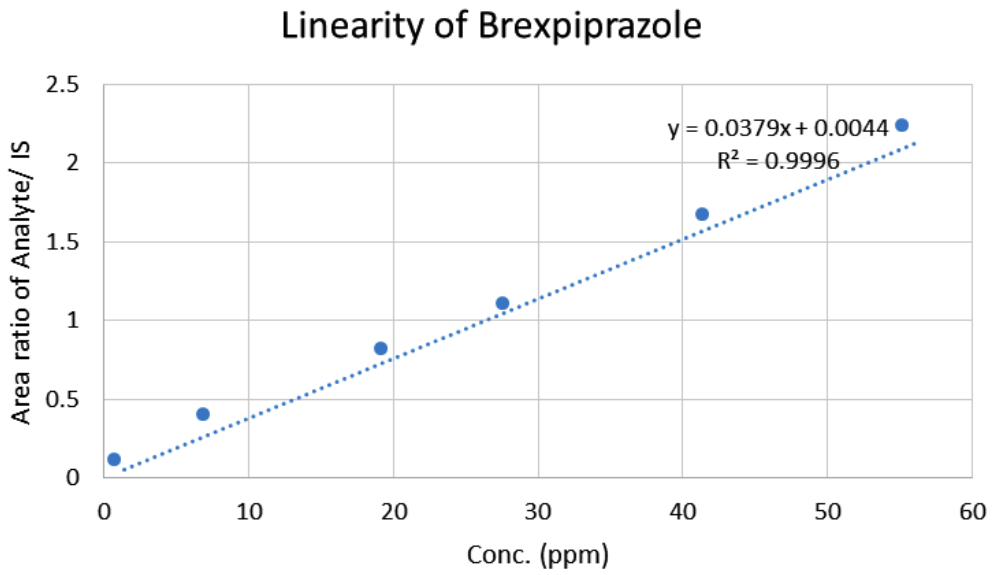

Fig. 6. Linearity of BREX

and FLX in the second and third blanks did not exceed 20% of the corresponding LLOQ peak areas. Acceptable carryover was confirmed by the observed responses, which were below this limit.

With a correlation coefficient (r^2) of 0.9996 and 0.9993 for BREX and FLX, respectively, the method showed good linearity over the concentration range of 1.40–56 µg/mL. The recovered concentration was used to determine the accuracy of the QC samples. Accuracy requirements for QC samples are ±20% for LLOQ and ±15% for other QC (LQC, MQC, HQC). The % CV for the recovered concentrations from six replicates at each QC level was used to determine precision. The % CV values were within permissible limits.

The extraction mean recovery was 84.34% for BREX, 93.22% for FLX, and 94.69% for IS. The % CV of BREX, FLX, and IS were found to be 1.82, 1.38, and 0.99, respectively. Across all evaluated stability conditions, the calculated accuracy at both LQC and HQC levels remained within 93.5% - 109.25% for BREX and 94.5% - 107.25% for FLX and the percent coefficient of variation (%CV) was observed from 1.04 to 6.39 for BREX and 0.69 to 4.13 for FLX. These results

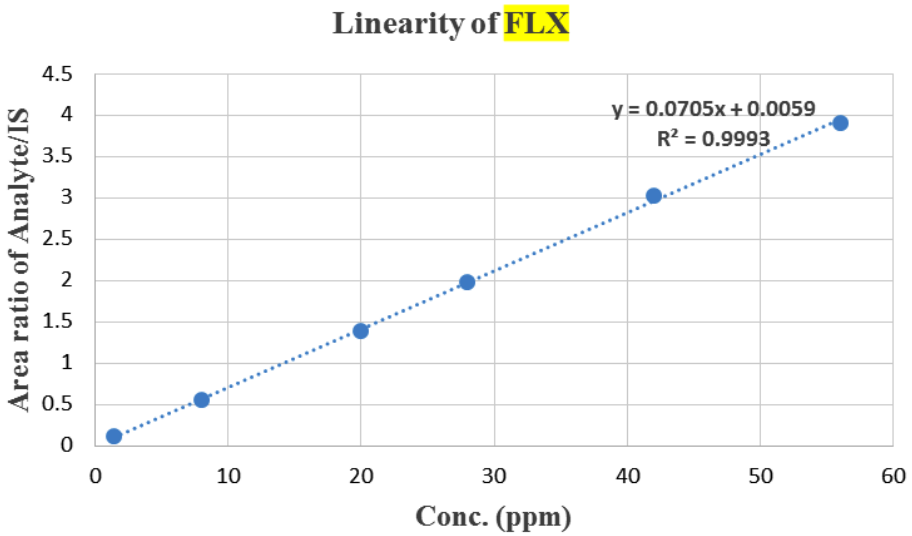

Fig. 7. Linearity of FLX

Table 6. Recovery of BREX, FLX, and IS in QC samples

Sample	BREX Area	FLX Area	IS Area	% Recovery (BREX)	% Recovery (FLX)	% CV (BREX)	% CV (FLX)
LQC 1	835214	1421177	4671829	85.26	91.74	1.82	1.38
LQC 2	840219	1432854	4752631				
LQC 3	867859	1459782	4805631				
Mean	847764	1437938	4743364				
MQC 1	5747275	9986527	4791791	82.57	94.05		
MQC 2	5685310	9925341	4785314				
MQC 3	5702894	9865718	4629746				
Mean	5711826	9925862	4735617				
HQC 1	9088968	15320092	4833144	85.19	93.86		
HQC 2	9153073	14024158	4798631				
HQC 3	9134667	15859642	4682157				
Mean	9125569	15067964	4771311				

Table 7. Stability tests for BREX and FLX

Sample solution	QC	Bench top stability		Freeze thaw stability		Autosampler Stability		Stability of processed extracted samples		Stock stability	
		Accuracy	% CV	Accuracy	% CV	Accuracy	% CV	Accuracy	% CV	Accuracy	% CV
BREX	LQC 1	96.25	6.39	99.75	2.90	104.50	2.27	98.25	2.52	102.25	2.52
	LQC 2	101.5		104.25		102.25		93.5		97.25	
	LQC 3	109.25		98.75		107.0		96.75		100.25	
	HQC 1	99.58	2.62	101.74	1.52	99.29	2.37	95.69	2.86	101.81	1.04
	HQC 2	104.93		101.88		103.71		100.51		102.01	
	HQC 3	102.01		99.15		99.89		100.67		100.09	
FLX	LQC 1	101.50	1.78	96	1.29	98	2.30	107.25	4.13	99	2.41
	LQC 2	99.00		98.5		100.5		98.75		94.5	
	LQC 3	102.50		97.5		96		103		95.75	
	HQC 1	97.79	3.83	98.55	0.69	96.14	1.54	97.83	1.42	98.53	1.79
	HQC 2	104.44		97.34		98.95		100.6		98.88	
	HQC 3	97.83		97.41		98.5		99.69		95.69	

Table 8. Comparison between the proposed method and the previously reported method

RT (min) Proposed method	RT (min) Reported method	LLOQ Proposed method	LLOQ Reported method	Detection method Proposed method	Detection method Reported method	Matrix Proposed method	Matrix Reported method	Reference
BREX – 3.69 FLX- 6.82	BREX- 4.538 FLX- 11.527	1.40 µg/mL	5 µg/mL	HPLC-UV	HPLC-UV	Blood Plasma	Pharmaceutical Formulation	27

confirm the adequate stability of BREX and FLX throughout the assay.

CONCLUSION

A sensitive and easy reverse phase-high performance liquid chromatographic method was developed for the simultaneous estimation of BREX and FLX in human plasma. For the first time, a novel bioanalytical method has been successfully developed by RP-HPLC for evaluating BREX and FLX simultaneously. The developed method comprises a simple sample pretreatment, cost-effectiveness, protein precipitation without drying or reconstitution, and shorter retention times of both analytes, compared to reported methods for individual estimation of these drugs, enabling rapid analysis. The LLOQ of the method was 1.40 µg/mL, confirming the sensitivity of the method, but it is not suitable for therapeutic drug monitoring or bioequivalence studies. Bioanalytical method validation was performed according to USFDA and EMEA guidelines, and all validation parameters were within acceptable limits. BREX and FLX remained stable at different conditions, including bench-top, freeze-thaw, autosampler, stability of processed extracted samples, and stock solution stability. Hence, the developed method was adequate for the simultaneous quantification of BREX and FLX in human plasma.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Permission to reproduce material from other sources

Not Applicable

Authors Contributions

Manoj Ramesh Kumbhare: Preparation of the manuscript draft, writing and editing; Pravin Rangnath Dighe: Data collection and analysis, writing and editing.

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