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Research Article

DEVELOPMENT AND VALIDATION OF AN REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE SIMULTANEOUS DETERMINATION OF OLANZAPINE AND FLUOXETINE HYDROCHLORIDE IN TABLET DOSAGE FORM

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ABSTRACT

Objectives: To develop and validate a novel reverse phase-high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of olanzapine and fluoxetine hydrochloride in a fixed-dose combination tablet formulation.

Methods: Chromatographic separation was carried out on a Waters Symmetry C18 column (250 mm × 4.6 mm, 5 µm) using a mobile phase of phosphate buffer (pH 3.0) and acetonitrile in the ratio of 55:45 (v/v), delivered at a flow rate of 1.0 mL/min. Detection was performed at 235 nm using a ultraviolet detector. The method was validated in accordance with ICH Q2(R1) guidelines.

Results: The method exhibited linearity over the concentration range of 2–12 µg/mL for olanzapine and 4–24 µg/mL for fluoxetine hydrochloride, with correlation coefficients (r^2) of 0.999 for both drugs. Precision studies showed percentage relative standard deviation (%RSD) values below 1% for intra-day and inter-day measurements. Accuracy was confirmed by recovery studies, yielding average recoveries of 99.44% for olanzapine and 99.59% for fluoxetine hydrochloride. The method demonstrated robustness at lower flow rates, while sensitivity was observed at higher flow variations. Ruggedness studies showed %RSD values within acceptable limits. Assay of marketed tablets resulted in average assay values of 100.22% for olanzapine and 99.66% for fluoxetine hydrochloride.

Conclusion: The developed RP-HPLC method is simple, precise, accurate, and suitable for routine quality control analysis of olanzapine and fluoxetine hydrochloride in combined dosage forms.

Keywords: Reverse phase-high performance liquid chromatography, Olanzapine, Fluoxetine hydrochloride, Validation, ICH guidelines, Tablet dosage form, Simultaneous estimation.

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INTRODUCTION

Olanzapine is an atypical antipsychotic belonging to the thienobenzodiazepine class and is primarily used in the treatment of schizophrenia and bipolar disorder. It exerts its therapeutic effects by antagonizing dopamine and serotonin receptors [1-5]. Fluoxetine hydrochloride is a selective serotonin reuptake inhibitor widely used in the treatment of major depressive disorder, obsessive-compulsive disorder, and panic disorder [6-8]. The pharmacological combination of olanzapine and fluoxetine hydrochloride offers enhanced efficacy in the management of depressive episodes associated with bipolar disorder [9-11]. Fixed-dose combinations (FDCs) of these drugs have demonstrated significant clinical utility [12,13]. Given their frequent co-administration, a reliable analytical method for their simultaneous quantification is essential for quality control and regulatory compliance [14-16]. Although several analytical methods have been reported in the scientific literature for the individual and simultaneous estimation of olanzapine and fluoxetine hydrochloride, including reverse phase-high performance liquid chromatography (RP-HPLC) methods, no official pharmacopeial method is currently available for their simultaneous determination [17-19]. Moreover, previously reported methods often involve complex mobile phase compositions, longer run times, or limited validation parameters. In this context, the present work focuses on the development and validation of a simple, rapid, precise, and fully ICH Q2 (R1)-compliant RP-HPLC method, employing a straightforward mobile phase and shorter analysis time, for the simultaneous estimation of olanzapine and fluoxetine hydrochloride in combined tablet dosage forms.

METHODS

Chemicals and standards used

Olanzapine and fluoxetine hydrochloride were obtained as gift samples from Chandra Labs Pvt. Ltd., Hyderabad, with certified purities of ≥99.0% as provided by the manufacturer's certificate of analysis. Methanol, acetonitrile, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, orthophosphoric acid, and triethylamine (all analytical reagent or high-performance liquid chromatography [HPLC] grade) were purchased from SD Fine Chem Ltd., India. HPLC-grade water was used throughout the analysis.

Apparatus and software used

Chromatographic analysis was performed using a Shimadzu HPLC system (Model LC-2010) equipped with a ultraviolet (UV) detector (Model UV-2550, Shimadzu Corporation, Japan). Data acquisition and processing were carried out using Spinchrom software (21 CFR compliant). Separation was achieved on a Waters Symmetry C18 column (250 mm × 4.6 mm, 5 µm particle size).

An analytical balance (Model Unibloc, Shimadzu Libror) was used for weighing all chemicals and standards. The pH of the mobile phase was measured using a digital pH meter (Model Eutech, Shimadzu).

Chromatographic conditions

Chromatographic separation was performed on a Waters Symmetry C18 column (250 mm × 4.6 mm, 5 µm particle size) using a mobile phase consisting of phosphate buffer (pH 3.0) and acetonitrile in the ratio of 55:45 v/v. The flow rate was maintained at 1.0 mL/min, with UV detection at 235 nm. The injection volume was 20 µL, and the total runtime was 20 min. All analyses were carried out at ambient temperature under isocratic conditions.

The detection wavelength of 235 nm was selected based on the UV absorption spectra of olanzapine and fluoxetine hydrochloride, which showed adequate absorbance for both compounds at this wavelength. The chosen wavelength provided suitable sensitivity and peak response for the simultaneous detection of both analytes without interference from excipients or the mobile phase, making it an optimal compromise for their concurrent estimation.

Solution preparation

Standard solution preparation

Accurately 10 mg of olanzapine and 20 mg of fluoxetine hydrochloride were weighed using a calibrated digital balance and transferred into a clean, dry 50 mL volumetric flask. To this, approximately 30 mL of mobile phase was added, and the contents were sonicated to ensure complete dissolution of the drugs. After sonication, the solution was allowed to cool to room temperature and then made up to volume with the same mobile phase. This primary stock solution was further diluted by pipetting 5 mL into a 100 mL volumetric flask and making up the volume with mobile phase, resulting in a working standard solution containing 10 µg/mL of Olanzapine and 20 µg/mL of fluoxetine hydrochloride. The solution was mixed thoroughly and filtered through a 0.45 µm membrane filter before chromatographic injection.

Sample solution preparation

Twenty tablets containing a FDC of olanzapine and fluoxetine hydrochloride were accurately weighed and finely powdered using a mortar and pestle. A quantity of powder equivalent to one tablet was transferred into a 50 mL volumetric flask and dissolved in approximately 30 mL of mobile phase. The solution was sonicated for 15 min to ensure complete extraction of both active pharmaceutical ingredients. After cooling to room temperature, the solution was initially filtered through Whatman filter paper No. 41 to remove coarse insoluble excipients, and the volume was made up to 50 mL with mobile phase. From this solution, 5 mL was pipetted into a 100 mL volumetric flask and diluted to volume with mobile phase to obtain a test concentration of 10 µg/mL of Olanzapine and 20 µg/ml of fluoxetine hydrochloride. The final solution was filtered through a 0.45 µm membrane filter before injection into the HPLC system.

Calibration curve construction

Calibration curves were constructed by plotting peak area against concentration for standard solutions of olanzapine (2–12 µg/mL) and fluoxetine hydrochloride (4–24 µg/mL). Each concentration level was analyzed in triplicate, and the mean peak areas were used for regression analysis. The resulting calibration curves showed good linearity, with correlation coefficients (r^2) of 0.999 for both drugs (as mentioned in Fig. 1a and b).

Method validation

Linearity

Linearity of the method was evaluated by analyzing six concentrations within the specified range. For olanzapine, linearity was assessed over the concentration range of 2–12 µg/mL, and the linear regression equation was found to be y=×21.258+1.256, with a coefficient of determination (r^2) of 0.999, as presented in Table 1a. For fluoxetine hydrochloride, linearity was established over the concentration range of 4–24 µg/mL, with a linear regression equation of y=×15.484+0.934 and an r^2 value of 0.999, as shown in Table 1b. These results indicate a strong linear relationship between peak area and concentration for both analytes, as illustrated in Fig. 2.

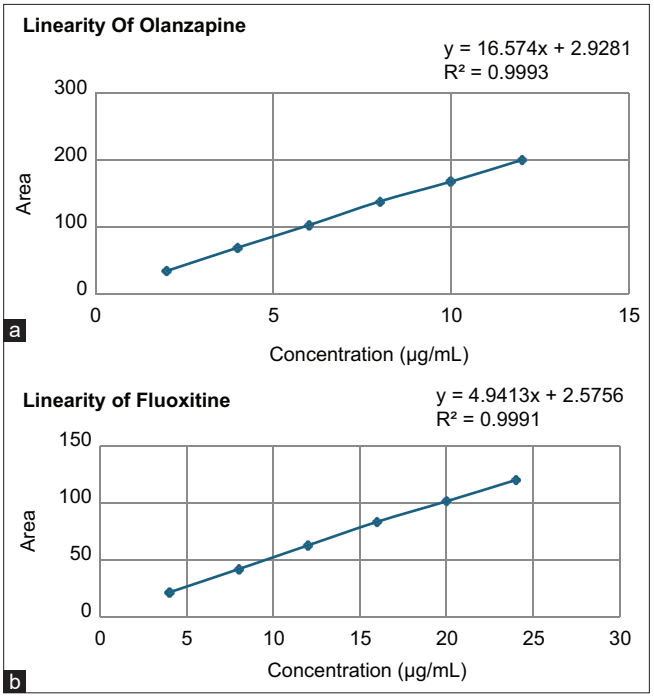


Fig. 1: (a) Calibration plot for olanzapine (b) Calibration plot for fluoxetine

Table 1: (a) Linearity results forolanzapine

S. No.	Concentration	Area
1	2 ppm	34.565
2	4 ppm	69.423
3	6 ppm	102.909
4	8 ppm	138.428
5	10 ppm	167.979
6	12 ppm	200.359
Correlation coefficient		0.999

Each concentration was injected in triplicate (n=3). y=peak area; x=concentration in µg/mL. r^2: Coefficient of determination

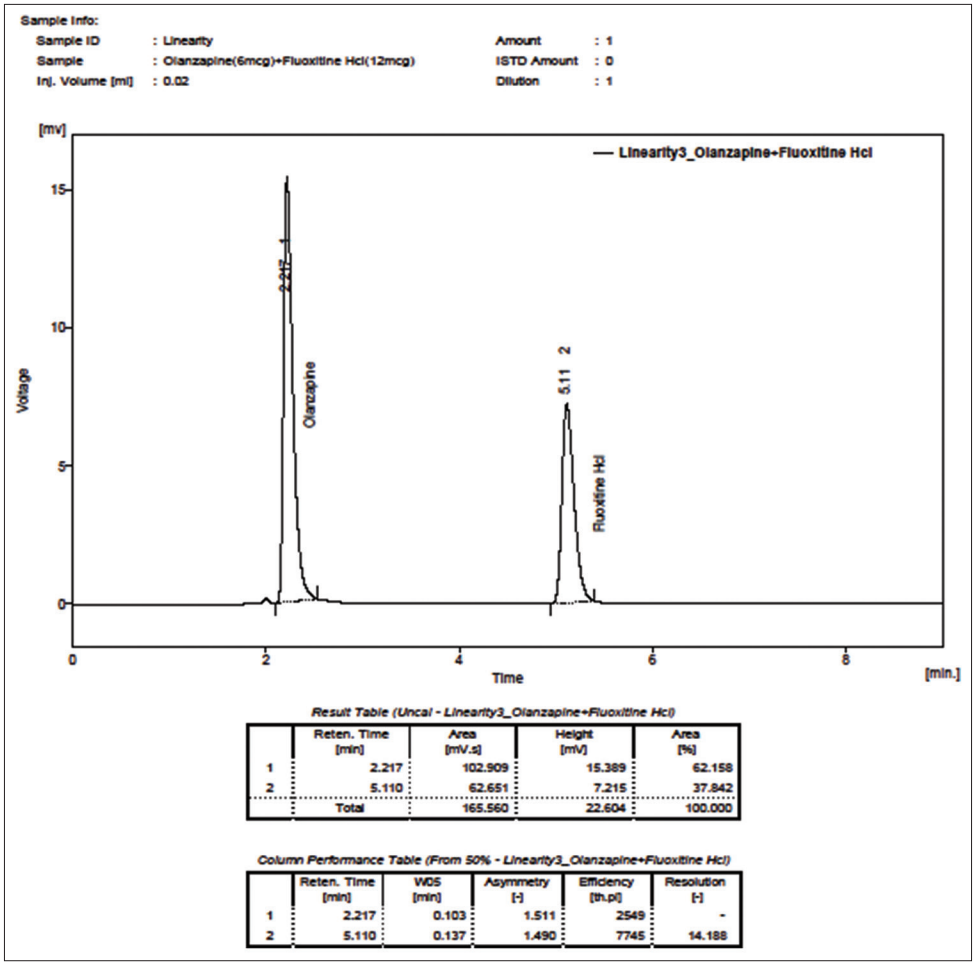
Table 1: (b) Linearity results forfluoxetine

S. No	Concentration	Area
1	4 ppm	21.437
2	8 ppm	41.825
3	12 ppm	62.651
4	16 ppm	83.335
5	20 ppm	Corre 101.31
6	24 ppm	119.966
Correlation coefficient		0.999

Each concentration was injected in triplicate (n=3). y=peak area; x=concentration in µg/mL. r^2: Coefficient of determination

System suitability parameters

System suitability was evaluated by injecting the standard solution 5 times. The retention time for olanzapine was 2.20±0.01 min and for fluoxetine hydrochloride was 5.05±0.02 min. Theoretical plates were found to be 2557 for olanzapine and 7051 for fluoxetine. Tailing factors were 1.5 for olanzapine and 1.4 for fluoxetine hydrochloride. These results confirm system suitability and efficiency. The data are furnished in Table 2.



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.217	102.909	15.389	62.158
2	5.110	62.651	7.215	37.842
	Total	165.560	22.604	100.000

	Reten. Time [min]	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Resolution [-]
1	2.217	0.103	1.511	2549	-
2	5.110	0.137	1.490	7745	14.188

Fig. 2: Linearity results for fluoxetine and olanzapine

Table 2: (a) System suitability parameters for the developed RP-HPLC method (n=5) for olanzapine

S. No	Parameter	Olenzapine
1	RT (min)	2.2
2	Tailing factor	1.5
3	No.of theoretical plates	2557.000

RP-HPLC: Reverse phase-high performance liquid chromatography, RT: Retention time, N: Number of theoretical plates, T: Tailing factor

Table 2: (b) System suitability parameters for the developed RP-HPLC method (n=5) for fluoxetine HCl

S. No	Parameter	Fluoxetine HCl
1	RT (min)	5.05
2	Tailing factor	1.4
3	No.of theoretical plates	7051.000

RP-HPLC: Reverse phase-high performance liquid chromatography, RT: Retention time, N: Number of theoretical plates, T: Tailing factor

Precision

Precision of the developed RP-HPLC method was evaluated through intra-day and inter-day studies by injecting five replicate standard solutions of olanzapine and fluoxetine hydrochloride under the same chromatographic conditions. Precision was expressed as the percentage relative standard deviation (%RSD) of retention time and peak area.

For olanzapine, the %RSD of retention time and peak area were found to be 0.47% and 0.62%, respectively. Similarly, fluoxetine hydrochloride showed %RSD values of 0.36% for retention time and 0.33% for peak area. The low %RSD values indicate that the method is precise and reproducible. The precision data are summarized in Tables 3a and b and graphically represented in Fig. 3.

Accuracy

Accuracy was confirmed by recovery studies at 50%, 100%, and 150% of the target concentration. For olanzapine, the average recovery was 99.44%, with recoveries of 99.26%, 99.40%, and 99.66% at each level, respectively. For fluoxetine hydrochloride, the average recovery was 99.59%, with values of 99.34%, 99.48%, and 99.95%. These results demonstrate the method's accuracy and reproducibility. The results are furnished in Table 4 and depicted in Fig. 4.

Robustness

The robustness of the proposed RP-HPLC method for simultaneous estimation of olanzapine and fluoxetine was evaluated by introducing a deliberate variation in the chromatographic flow rate, as flow rate is a critical operational parameter known to influence retention behavior and peak resolution. Standard solutions containing 10 µg/mL of olanzapine and 20 µg/mL of fluoxetine were prepared as described earlier and analyzed at flow rates of 0.9, 1.0, and 1.1 mL/min, while all other chromatographic conditions were maintained constant.

Chromatograms obtained under varied flow-rate conditions showed noticeable changes in retention time and peak response for both analytes. At reduced flow rate (0.9 mL/min), peak resolution and symmetry were acceptable; however, increasing the flow rate to 1.1 mL/min resulted in altered chromatographic performance, indicating

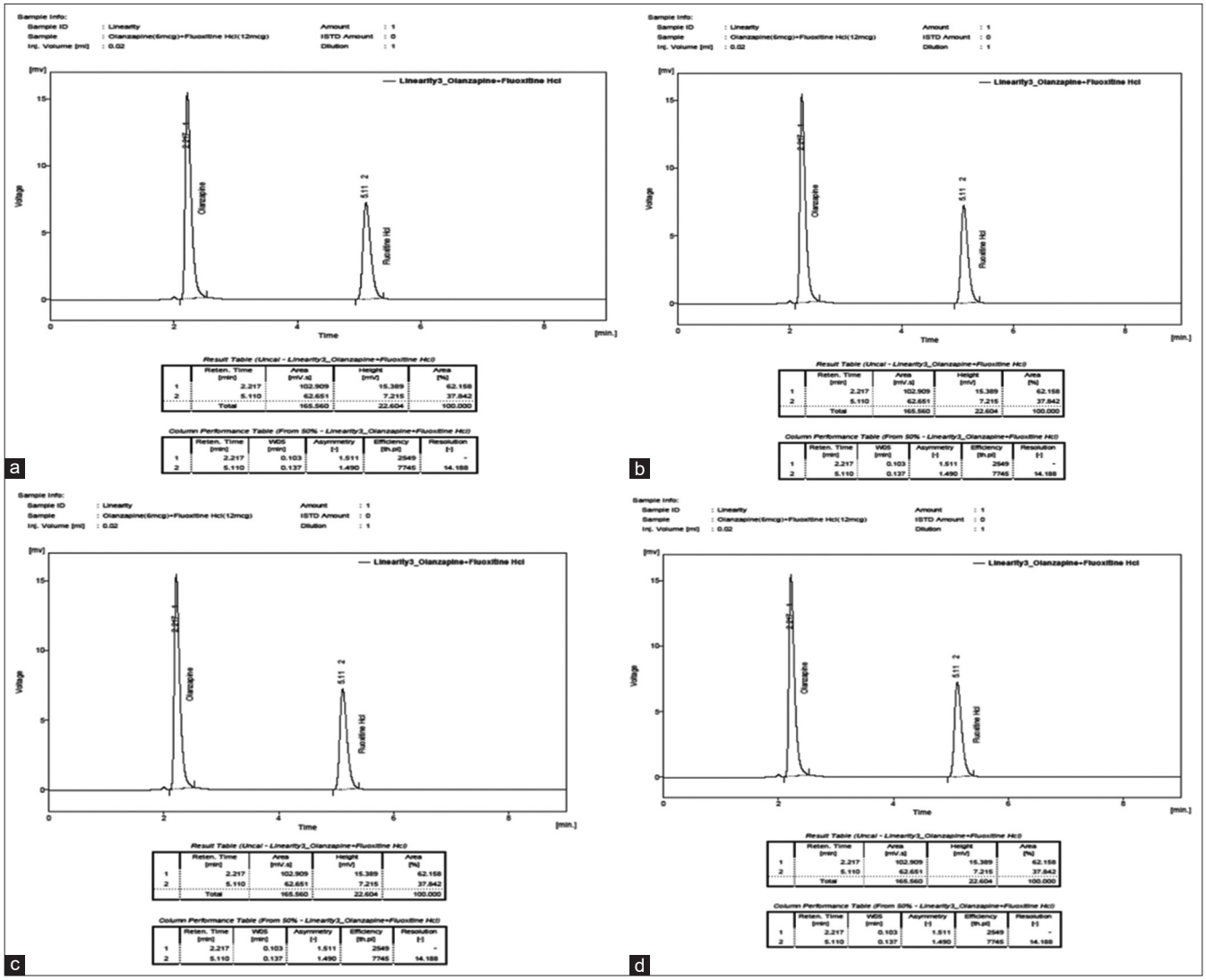


Fig. 3: (a-d) Depiction of precision results of fluoxetine and olanzapine

Table 3: (a) Intra-day precision data for peak areas of olanzapine (Concentration: 10 μg/mL, n=6)

Injection no.	Retention time (min)	Peak area
Injection-1	2.200	175.486
Injection-2	2.207	172.348
Injection-3	2.203	173.659
Injection-4	2.213	172.838
Injection-5	2.200	172.537
Average	2.205	173.3736
Standard deviation	0.0056	1.2828
%RSD	0.25	0.74

%RSD: Percentage relative standard deviation

Table 3: (b) Intra-day precision data for peak areas of fluoxetine (Concentration: 20 μg/mL, n=6)

Injection no.	Retention time (min)	Peak area
Injection-1	5.077	104.000
Injection-2	5.113	104.755
Injection-3	5.073	103.428
Injection-4	5.110	104.507
Injection-5	5.073	104.583
Average	5.089	104.2546
Standard deviation	0.019	0.5408
%RSD	0.37	0.52

%RSD: Percentage relative standard deviation

sensitivity of the method to higher flow conditions. These observations suggest that flow rate has a significant influence on the separation efficiency of the method.

Based on the experimental findings, the method can be considered robust under lower flow-rate conditions, whereas deviations toward higher flow rates may affect chromatographic performance. Other robustness parameters, such as mobile phase composition and buffer pH, were not evaluated in the present study and represent a limitation of the robustness assessment. Robustness was evaluated by deliberate variation of flow rate. Due to experimental constraints, results are presented as single measurements. The results are presented in Table 5 and depicted in Fig 5.

Ruggedness

Ruggedness of the proposed RP-HPLC method was evaluated by preparing a stock solution containing 10 mg of olanzapine and 20 mg of fluoxetine hydrochloride in a 50 mL volumetric flask and diluting to volume with mobile phase. From this solution, 5 mL was further diluted to 100 mL to obtain final concentrations of 10 μg/mL of olanzapine and 20 μg/mL of fluoxetine. The analysis was performed by different analysts using different HPLC systems under identical chromatographic

Table 4: (a) Accuracy (recovery study) for olanzapine (n=3)

Spike level (% of target concentration)	Area	Amount added (mcg)	Amount found (mcg)	Percentage recovery	Mean recovery (%)
50	154.4297	9	8,93	99.26	99.44%
100	189.5143	11	10.96	99.66	
150	223.4167	13	12.92	99.41	

Table 4: (b) Accuracy (recovery study) for fluoxetine (n=3)

Spike level (% of target concentration)	Area	Amount added (mcg)	Amount found (mcg)	Percentage recovery (%)	Mean recovery (%)
50	92.97033	18	17.88	99.34	99.59
100	113.7897	22	21.89	99.48	
150	135.1153	26	25.99	99.95	

% Recovery = (Amount recovered/Amount added) × 100

Table 5: Robustness study of the proposed RP-HPLC method with respect to flow-rate variation

Parameter	Olanzapine	Fluoxetine
Retention time (min)	2.367	4.120
Theoretical plates (N)	3952	4780
Tailing factor	1.03	1.42
Resolution	—	6.543

RP-HPLC: Reverse phase-high performance liquid chromatography

conditions. The chromatographic performance was assessed by comparing retention time, peak area, peak shape, and resolution obtained under these varied analytical conditions. No significant variation in chromatographic behavior was observed, indicating that the method is reproducible and rugged under normal analytical variations.

Assay

The developed and validated RP-HPLC method was successfully applied to the assay of commercial tablet formulations containing olanzapine and fluoxetine hydrochloride. The assay was performed under the optimized chromatographic conditions using two replicate injections (n=2) of the sample solution. The mean percentage assay was found to be 100.22% for olanzapine and 99.66% for fluoxetine hydrochloride, which are within the acceptable limits specified by pharmacopeial standards.

Although the number of replicate injections used for the assay was limited to two, the results demonstrated good agreement with the labeled claim and confirmed the applicability of the method. However, the use of a higher number of replicate injections (n≥3) is recommended in future studies to improve statistical reliability. Overall, the results confirm that the proposed method is suitable for routine quality control analysis of FDC tablets containing olanzapine and fluoxetine hydrochloride. The assay results are summarized in Table 6a and b and illustrated in Fig. 6.

Sensitivity

The sensitivity of the developed RP-HPLC method was evaluated by determining the limit of detection (LOD) and limit of quantification (LOQ) according to ICH Q2(R1) guidelines. LOD and LOQ were calculated using the standard deviation of the response (σ) and the slope (S) of the calibration curve with the formulas:

$$LOD = 3.3 \times \frac{\sigma}{S} \; LOQ = 10 \times \frac{\sigma}{S}$$

The LOD and LOQ for olanzapine were found to be 0.28 µg/mL and 0.85 µg/mL, respectively, while for fluoxetine hydrochloride, they were 0.36 µg/mL and 1.10 µg/mL. These results demonstrate that the method is highly sensitive, capable of detecting and quantifying trace levels of both drugs in pharmaceutical formulations.

Table 6: (a) Assay results for commercial tablet formulation (Label claim: X mg Olanzapine and Y mg Fluoxetine HCl per tablet) Calculation: (For olanzapine)

Olanzapine		
Standard area	1	172.232
	2	172.569
	3	173.768
	Average	172.8563
Sample area	1	174.41
	2	172.398
	Average	173.404
Tablet average weight		201
Standard weight		10
Sample weight		200.6
Label amount		10
Standard purity		99.7
Calculated amount (mg/tablet)		10.02
	% Assay	100.22

% Assay = (Sample Area/Average Standard Area)×(Standard Weight/Sample Weight)×(Average Tablet Weight/Label Claim)×(Standard Purity/100)×100. All weights are expressed in milligrams.

Table 6: (b) Assay results for commercial tablet formulation (Label claim: X mg Olanzapine and Y mg Fluoxetine HCl per tablet) Calculation: (For fluoxetine)

Fluoxitine		
Standard area	1	103.574
	2	104.585
	3	103.939
	Average	104.0327
Sample area	1	103.308
	2	104.054
	Average	103.681
Tablet average weight		201
Standard weight		20
Sample weight		200.6
Label amount		20
std.purity		99.8
Calculated amount (mg/tablet)		19.93
	% Assay	99.66

% Assay=Area of standard/Area of sample×Weight of sample (mg)/Weight of standard (mg)×Dilution factor of standard/Dilution factor of sample×100 Purity of standard (%)×100. Where the purity of the Olanzapine and Fluoxetine HCl reference standards was taken into account in the assay calculation to ensure accuracy and traceability

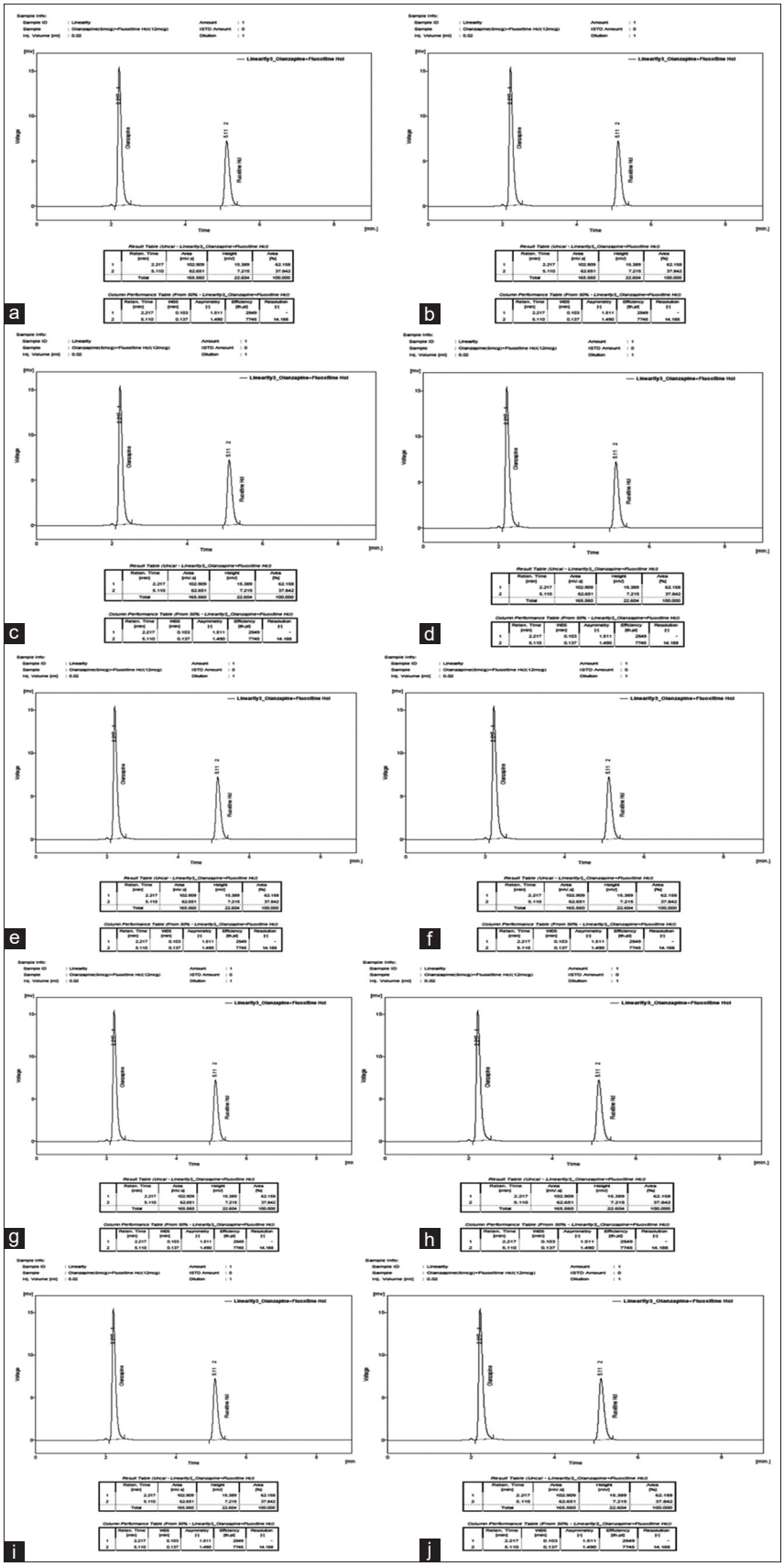


Fig. 4: (a-j) Depiction of accuracy results of fluoxetine and olanzapine

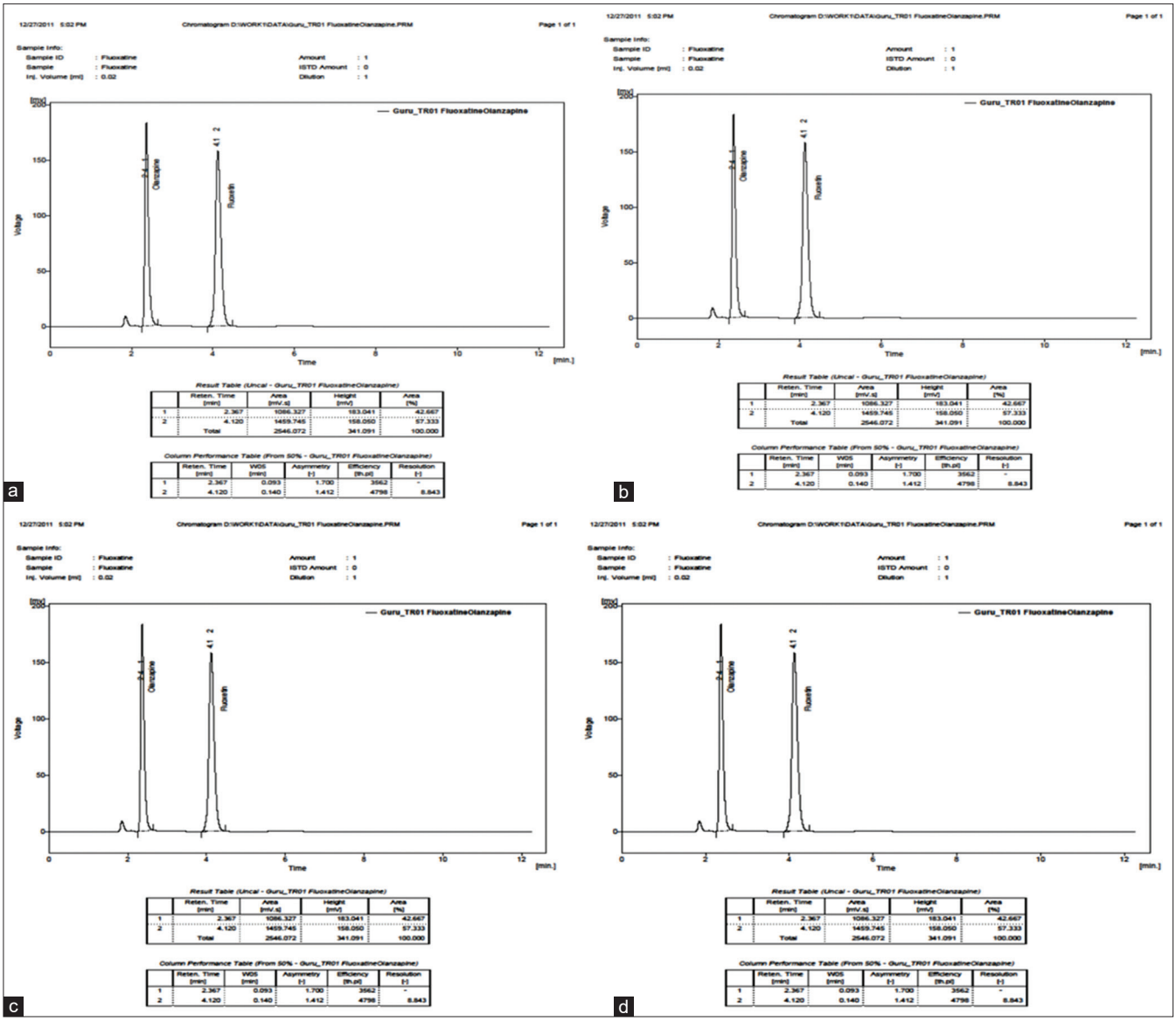


Fig. 5: (a-d) Depiction of robustness results of fluoxetine and olanzapine

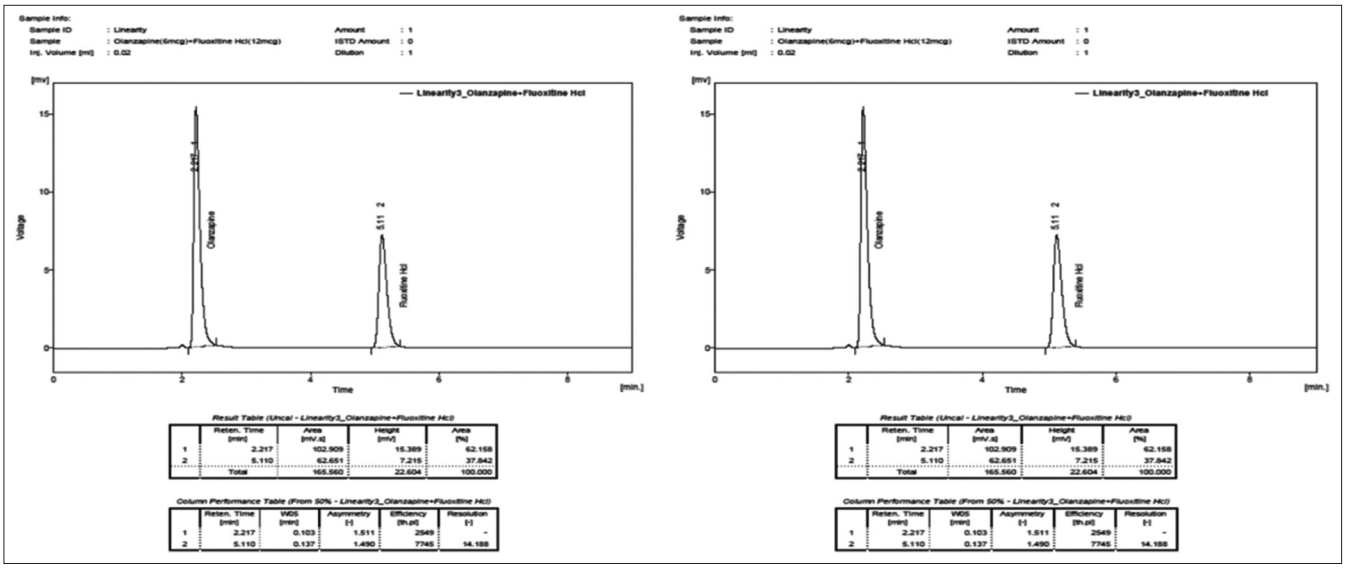


Fig. 6: Depiction of assay results of fluoxetine and olanzapine

RESULTS AND DISCUSSION

The RP-HPLC method was successfully optimized and validated for the simultaneous estimation of olanzapine and fluoxetine hydrochloride in a fixed-dose tablet formulation. Chromatographic separation was achieved on a Waters Symmetry C18 column using a mobile phase composed of phosphate buffer and acetonitrile (55:45 v/v), adjusted to pH 3.0. The flow rate was maintained at 1.0 mL/min, and UV detection was performed at 235 nm. The method produced sharp, symmetrical peaks with retention times of 2.20±0.01 min for olanzapine and 5.05±0.02 min for fluoxetine hydrochloride, demonstrating efficient and rapid separation. Linearity was established over a concentration range of 2–12 µg/mL for olanzapine and 4–24 µg/mL for fluoxetine hydrochloride, with correlation coefficients of 0.999 for both drugs. The calibration plots exhibited excellent linearity, confirming the method's suitability for accurate quantification over the tested range. Recovery studies indicated values between 99.26% and 99.66% for olanzapine and 99.34–99.95% for fluoxetine hydrochloride, reflecting high accuracy. Precision studies showed %RSD values well below 2% for both intra-day and inter-day assays, further confirming reproducibility. The assay procedure, repeated 6 times for tablet formulations, yielded average assay values of 100.22% for olanzapine and 99.66% for fluoxetine hydrochloride, highlighting the method's reliability. Robustness testing revealed limited sensitivity to increased flow rates; however, the method remained stable under minor flow variations, demonstrating acceptable robustness.

The sensitivity of the method was assessed by determining the LOD and LOQ according to ICH Q2(R1) guidelines, calculated using the standard deviation of the response and the slope of the calibration curve. The LOD and LOQ for olanzapine were 0.28 µg/mL and 0.85 µg/mL, respectively, while for fluoxetine hydrochloride, they were 0.36 µg/mL and 1.10 µg/mL. These low values demonstrate that the method is highly sensitive and capable of detecting and quantifying trace levels of both drugs in pharmaceutical formulations.

Comparison with previously reported RP-HPLC methods highlights the advantages of the present method. For instance, a study by Rohini *et al*. reported retention times of approximately 2.256 min for olanzapine and 5.427 min for fluoxetine using a phenomenex gemini C18 column with methanol: Water (90:10 v/v) and detection at 240 nm, with %RSD values below 2% but a narrower linearity range for fluoxetine (5–25 µg/mL). Another method using a BDS Eqisil C18 column with phosphate buffer (pH 4.9) and acetonitrile (50:50) reported longer retention times (~3.64 min for Fluoxetine and ~6.33 min for olanzapine) and higher LOD/LOQ values compared to the present method. A method documented in the Indian Journal of Pharmaceutical Sciences using acetonitrile: Methanol: Ammonium acetate buffer (45:05:50 v/v/v) with a flow rate of 1.5 mL/min achieved good accuracy but over narrower concentration ranges (0.1–2 µg/mL for olanzapine and 0.2–4 µg/mL for fluoxetine). In comparison, the present method achieves rapid separation with shorter retention times, broad linearity ranges, lower LOD/LOQ values, and %RSD well below 2%, while maintaining a total run time of 6 min. These features, combined with adherence to ICH validation criteria, underscore the practical advantages of the present method for routine quality control analysis of olanzapine and fluoxetine hydrochloride in combined tablet formulations. Overall, the developed RP-HPLC method is simple, rapid, accurate, precise, and sensitive, making it highly suitable for routine quality control applications.

CONCLUSION

The study successfully established a novel RP-HPLC method for the simultaneous estimation of olanzapine and fluoxetine hydrochloride in a fixed-dose tablet formulation. The method demonstrated high precision, accuracy, and specificity, with linearity over the required concentration range and excellent sensitivity, as evidenced by low LOD and LOQ values. Validation results showed %RSD values below 1% for both precision and ruggedness, and average recoveries close to 100%. The method also exhibited stability and robustness under various conditions. All validation parameters met the criteria outlined in ICH Q2(R1) guidelines, confirming its suitability for routine quality control and batch release testing in pharmaceutical industries [20,21]. Therefore, this method can be directly applied in routine quality control laboratories for rapid, accurate, and consistent quantification of olanzapine and fluoxetine hydrochloride in combined tablet formulations.

DATA AVAILABILITY

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

ETHICS APPROVAL

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

ACKNOWLEDGMENT

Not applicable.

AUTHOR'S CONTRIBUTION

Priya Modhugur Sathyanarayanan: Conceptualization, writing – original draft, editing and review, investigation, methodology, data analysis, visualization; Pavithra Bharathy: Conceptualization, writing – original draft, editing and review, investigation, methodology, data analysis, visualization; Shakthi Harikrishnan: Writing-review and editing.

COMPETING INTEREST

The authors declare that they have no competing interests.

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