**RESEARCH ARTICLE**

# A Sustainable Chromatographic Method for Simultaneous Analysis of Fluoxetine and Olanzapine Antidepressants: Assessment of Greenness with Green Metric Tools

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**ABSTRACT**

The combination of Fluoxetine HCL and Olanzapine treats depression in bipolar disorder and patients unresponsive to other antidepressants. The research aims to optimize a sensitive, simple, rapid, reliable, eco-friendly liquid chromatographic method by minimizing feedstock's, reagents, energy, and waste to protect the environment and conserve resources, additionally assessed the environmental impact of a method using various green metric tools, and applied the method to pharmaceuticals. The separation was conducted using isocratic RP-HPLC with a DAD at 227nm. The mobile phase used was a 40:40:20 (v/v/v) ratios of buffer, acetonitrile, and ethanol. An L1 column (150 x 4.6mm, 5µm) was utilized with a flow rate of 1.5mL/min and injection volume of 5µL. The column oven and auto-sampler were both maintained at 45°C and 25°C respectively. The optimized method was validated as per ICH guidelines and found to be specific, precise, and robust to slight changes in mobile phase flow, column temperature, and buffer pH. Linearity was demonstrated from 0.1-150µg/mL for olanzapine and 0.4-610µg/mL for fluoxetine HCL. The accuracy was tested with recovery ranges of 0.1–157 µg/mL for olanzapine and 0.4–606 µg/mL for fluoxetine HCl in tablet placebo, and 0.1–150 µg/mL for olanzapine and 0.4–605 µg/mL for fluoxetine HCl in capsule placebo, indicating that the method is suitable for both tablet and capsule dosage forms. The method's greenness was evaluated using AES, NEMI, GAPI, and AGREE tools. The optimized method was successfully applied to pharmaceutical analysis. The newly optimized method found simple, sensitive, rapid, eco-friendly, incorporating green analytical concepts, has been successfully achieved for the identification and quantification of Fluoxetine HCL and Olanzapine present in pharmaceuticals.

**Keywords:** Eco-friendly chromatographic method; Green HPLC method; Development and Validation; Green metric tools; RP-HPLC method.

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**Conflict of interest:** None

## INTRODUCTION

Since the rapid development of industries, environmental issues have increasingly become a concern. Chemists and scientists have been working diligently to mitigate undesirable side effects on human health, such as environmental contamination, the use of harmful reagents and solvents, and waste generation. As a result of improvements in analytical techniques, new challenges focusing on practical characteristics such as methods, analysis time, costs, safety considerations, and the side effects of environmental problems have been thoroughly studied[1]. The current research considers a combined drug molecules, Fluoxetine hydrochloride and Olanzapine. This combination is used to treat depression associated with bipolar disorder and depression in patients who have not responded well to other antidepressants. Fluoxetine Hydrochloride is an antidepressant that belongs to the class of selective serotonin reuptake inhibitors (SSRIs) for oral use. Its chemical designation is (±)-N-methyl-3-phenyl-3-[(α,α,α-trifluoro-p-tolyl)oxy]propylamine HCL, with the empirical formula $C_{17}H_{18}F_3NO•HCl$ and a molecular weight of 345.8g/mol. Hydrochloride is a white to off-white crystalline solid that is soluble in water. Olanzapine, an atypical antipsychotic, belongs to the thienobenzodiazepine class. Its chemical designation is 2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5]benzodiazepine, with the molecular formula $C_{17}H_{20}N_4S$ and a molecular weight of 312.4g/mol. Olanzapine is a yellow crystalline solid that is practically insoluble in water. Due to the differing solubilities of these compounds, developing a combined method for their identification and quantification is somewhat challenging. Based on a literature review, several

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methods have been identified which are available on USP NF[2-8] and other resources[9-14] for estimation of Fluoxetine alone and in combination with olanzapine for drug substances and drug products, by utilizing reverse phase high performance liquid chromatography (RP-HPLC)[15-18]. A method has been identified using spectrophotometry[19]. These reported methods are associated with longer run times and utilize larger dimension columns. Some of these published methods also involve the use of hazardous chemical reagents such as tetra hydro furan, ion pair reagents, and surfactants like sodium lauryl sulfate, glacial acetic acid, and ethylene diamine tetra acetic acid. Handling these chemicals can lead to toxic effects on individuals, and their use in analysis negatively impacts the environment. None of these HPLC methods align with sustainable practices or follow the concept of green analytical chemistry (GAC)[20] for developing eco-friendly methods. According to a literature study, a method utilizing the principles of green chemistry was observed for different products[21]. However, there is a notable lack of rapid, eco-friendly RP-HPLC methods suitable for analyzing Fluoxetine alone and in combination with Olanzapine in pharmaceutical drugs and products. This observation has led to an attempt to develop a rapid, reliable, and eco-friendly liquid chromatographic method. This method aims to be more environmentally benign and safer for humans, suitable for multicomponent analysis, and its greenness was assessed using green metric tools[22, 23] such as the Analytical Eco-Scale (AES), National Environmental Methods Index (NEMI), Green Analytical Procedure Index (GAPI), and Analytical Greenness metric approach (AGREE), in accordance with the principles of GAC[24, 25]. Refer figure 1 for chemical structure of Active analytes.

## MATERIAL AND METHODS

**Standard, Sample, Excipients and Reagents:**
Standard Fluoxetine HCL (100% potency) and Olanzapine (99.26% potency) were received from Chemclues laboratory, Mumbai, India. Tablet samples containing 5mg Olanzapine and 20mg Fluoxetine HCL, along with FDA-approved excipients, was provided by the pharmaceutical industry. For tablet dosage forms, the excipients included mannitol, microcrystalline cellulose, maize starch, povidone, hypromellose, magnesium stearate, titanium dioxide, sucrose, glycerol, and polysorbate. For capsule dosage forms, pregelatinized starch, gelatin, dimethicone, titanium dioxide, sodium lauryl sulfate, red iron oxide, and yellow iron oxide were utilized in the study. Analytical grade reagents Potassium dihydrogen phosphate ($KH_2PO_4$), Orthophosphoric acid (OPA), Triethyl amine (TEA) and acetonitrile were utilized for mentioned chromatographic method.

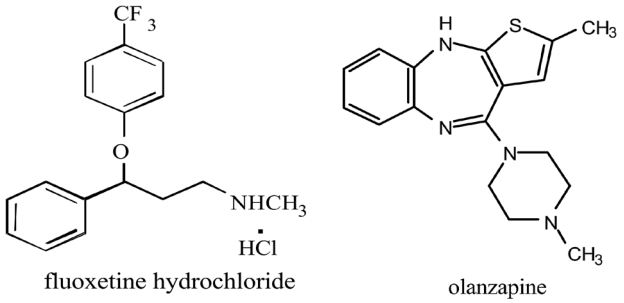



**Figure 1:** Chemical structure of Fluoxetine HCL and Olanzapine

**Instrumentation and Chromatographic condition:**
A Thermo Scientific Dionex Ultimate-3000 liquid chromatograph system with an autosampler, quaternary pump, column compartment, and diode array detector (DAD), operated using Chromeleon software. Active analytes were separated on a BDS Hypersil L1 (150 x 4.6mm, 5µm) column with an isocratic mode at a flow rate of 1.5mL/min. The mobile phase consisted of $KH_2PO_4$ buffer (pH 7.6), acetonitrile, and ethanol (40:40:20v/v/v). The column and autosampler temperatures were set at 45°C and 25°C, respectively. A 5µL sample solution was injected, and analyte responses were monitored at 227nm.

**Mobile phase preparation:**
A mixture of phosphate buffer, acetonitrile, and ethanol in the ratio of 40:40:20v/v/v, degassed by sonication. The phosphate buffer was prepared by dissolving 3.4g of $KH_2PO_4$ in 500mL of $H_2O$, adding 0.5mL of OPA, and adjusting the pH to 7.6 with diluted TEA using calibrated pH meter, then filtered through a 0.45µm membrane filter.

*Diluent*
Mixture of $H_2O$, Acetonitrile, and Ethanol (40:40:20 v/v/v).

**Preparation of standard solution**
Prepared mixture of 100µg/mL of Olanzapine and 400µg/mL of Fluoxetine HCL using diluent and used for analysis.

**Preparation of Test solution**
Weighed 10-tablets, determined their average weight, and crushed them into a powder. Weighed powdered sample equivalent to the average weight and transferred it into a 50mL volumetric flask. Added 30mL of diluent and sonicated for 15-minutes with intermittent shaking. Cooled the solution to room temperature, diluted it to the mark, mixed well, and filtered through a 0.45µm nylon filter, discarding the initial 3mL of filtrate. (Conc. 100µg/mL Olanzapine and 400µg/mL of Fluoxetine HCL)

## RESULTS AND DISCUSSION

**Experimental Design of Chromatographic method** During optimization, an attempt was made to develop a rapid, reliable, and eco-friendly method that is more environmentally benign, safer for humans, and suitable for multicomponent estimation. The standard solution was analyzed from 200-400nm using a DAD detector, with both molecules showing maxima around 227nm.Therefore, 227nm was selected. In the initial stage, a trial was conducted using a mixture of phosphate

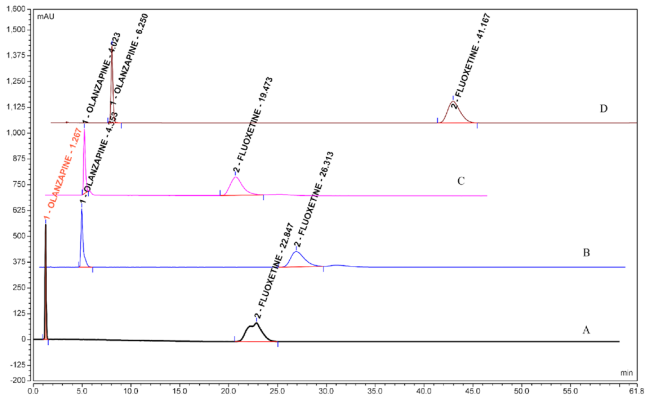
buffer at different pH levels, methanol (MeOH), and green solvents like ethanol (EtOH) by varying the composition of the mobile phase (M.P.), column temperature (Col. Temp.), and flow rate of the M.P. while utilizing different stationary phases. It was observed that increasing ethanol resulted in increased column pressure. This initial trial produced improper peak shapes and higher retention times (RT), but using a more non-polar (L1) column improved the peak shapes, refer **igure 2** representative chromatogram of different trials using flow 1mL. Hence, for further study, the L1 column was finalized. Later, acetonitrile was introduced with a buffer pH close to the pKa of the analyte, resulting in proper peak shapes with lower RT; refer to **igure 3** for representative chromatogram of different trials. Fluoxetine HCL is water-soluble, whereas Olanzapine is not, making it challenging to select a proper diluent also to incorporate green solvents, a mixture of water, acetonitrile, and ethanol in a 40:40:20 v/v ratios were chosen as a diluent. Since the selected molecule is Polar as per pKa value, a more non-polar stationary phase is needed to minimize interaction with the stationary phase and achieve a shorter run time. Based on previous trials, the L1 column provided better peak shapes, so it was finalized for the method. Due to multiple pKa values and wide ionic strength, $KH_2PO_4$ was chosen as a suitable reagent for buffer with a pH of 7.6 (pH 7.6 is close to pK of Fluoxetine & Olanzapine). Previous trials showed better peak shapes with the addition of acetonitrile, so the buffer was mixed with acetonitrile. To reduce the amount of acetonitrile as the organic phase, ethanol was added as a green solvent. Finally, the mixture of buffer (pH 7.6), acetonitrile, and ethanol was selected as the final mobile phase (MP) since it produced well-defined peaks with shorter RT. At a lower column temperature, analyte interaction with the stationary phase was strong, so the column temperature was increased to 45°C to expedite elution. The solution at 25°C showed no impact on system suitability and remained stable, so the autosampler was kept at room temperature. Using a flow rate less than 1.5mL resulted in long RT, while a flow rate of 1.5mL provided well-separated, properly resolved peaks with short RT, 1.5mL was finalized. Injection volumes greater than 5µL caused column overloading and decreased efficiency, risking system suitability failure; therefore, a 5µL injection volume was finalized. Initially, the standard was run with a longer run time, and no other peaks were observed after the peak of fluoxetine HCL. To avoid the overuse of solvents and to develop a greener method that produces less waste, a very short run time of 4min was implemented.

Considering above trials, a new, short, eco-friendly method was fine-tuned; refer to **Figure 4** for chromatogram.

**Note:** The optimized method is very short method with run time of 4-min against various published method including USP NF method[4] refer **Figure 5** for representative chromatogram of USP published method.

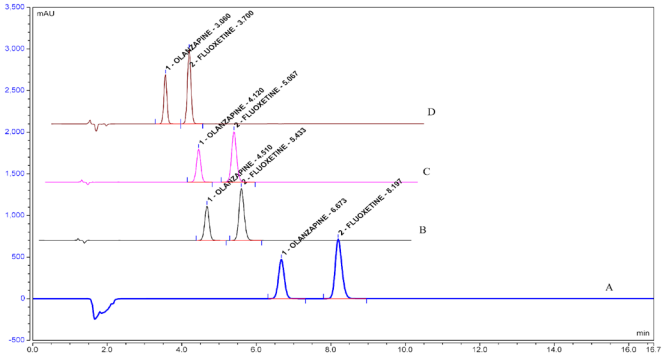
### Assessment of Greenness of Proposed Method

The ideal green analysis reduces reagents, energy use, and waste. To assess a method's greenness, the AES tool, based




**A:** M.P.-Buffer pH2.5: MeOH: EtOH (70:10:20v/v), Col.temp.-25°C, Col.-L7
**B:** M.P.-Buffer pH6: MeOH: EtOH (70:10:20v/v), Col.temp.-25°C, Col.-L7
**C:** M.P.-Buffer pH6: MeOH: EtOH (70:10:20v/v), Col.temp.-40°C, Col.-L7
**D:** M.P.-Buffer pH6.0: EtOH (70:30v/v), Col.temp.-40°C, Col.-L1

**Figure 2:** A representative chromatogram of different trials,




**A:** M.P.-Buffer pH 7.5: ACN: EtOH (55:25:20v/v), flow 1mL, Col.temp.-40°C,
**B:** M.P.-Buffer pH 7.5: ACN: EtOH (55:25:20v/v), flow 1.5mL, Col.temp.-40°C,
**C:** M.P.-Buffer pH 7.5: ACN: EtOH (55:25:20v/v), flow 1.6mL, Col.temp.-45°C,
**D:** M.P.-Buffer pH 7.85: ACN: EtOH (55:30:20v/v), flow 1.5mL, Col.temp.-45°C.

**Figure 3:** A representative chromatogram of different trials using L1 column,

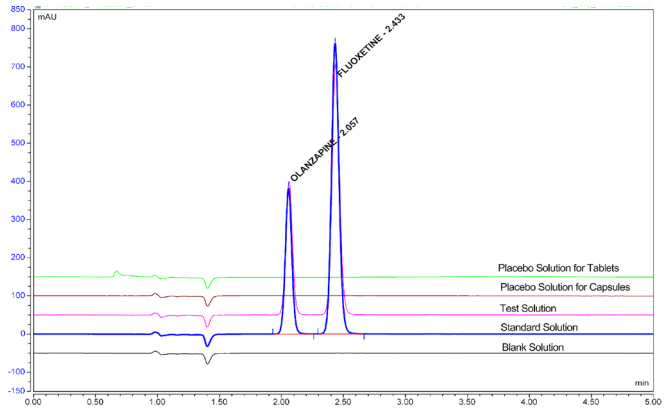
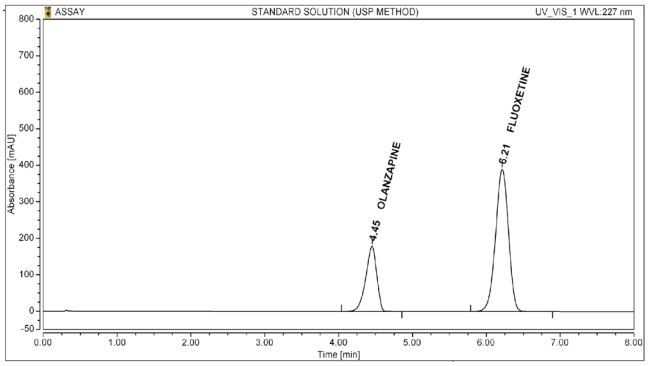



**Figure 4:** Representative chromatogram for Blank, Placebo, standard and Test solution




(Run time =4.45min RT of Olanzapine X 2.5 =11min)

**Figure 5:** Representative chromatogram of standard solution for USP NF method

on Van Aken et al.'s Eco-Scale for organic synthesis[26] is used. AES assigns penalty points for factors like reagent quantity, hazards, energy, and waste. The total penalty score is subtracted from a base of 100, representing an ideal method. In this study, the optimized method scored 77, indicating excellent greenness.

The second metric, NEMI, uses a pictogram with four sections to assess an analytical method's environmental impact: 1- PBT solvents (persistent, bio accumulative, toxic), 2- hazards, 3- corrosiveness, and 4- waste. For the proposed methods, three sections were green, indicating non-PBT solvents, non-corrosive conditions (pH 2-12), and waste under 50 grams. However, the hazards section was blank due to the use of hazardous solvents like acetonitrile and triethylamine, as per the Code of Federal Regulations and Environmental Protection 2016[27].

The third metric, GAPI, uses five pentagrams to assess the green aspects of an analytical method, from sample collection to final analysis. It employs a color code—red, yellow, green—representing high, medium, and low environmental impact. The proposed HPLC method has 4 green, 8 yellow, and 3 red sections, indicating a low to medium environmental impact.

The fourth metric, AGREE, is based on the 12 principles of green analytical chemistry and uses downloadable software. Results are displayed on a 0-1 scale in a 12-section colored pictogram. The result 0.65 indicate acceptable greener method. AGREE is simple, user-friendly, and comprehensive. The proposed methods show a color range from light to dark green, reflecting their overall greenness. Detailed results obtained from all the green metric tools are shown in figure 6.

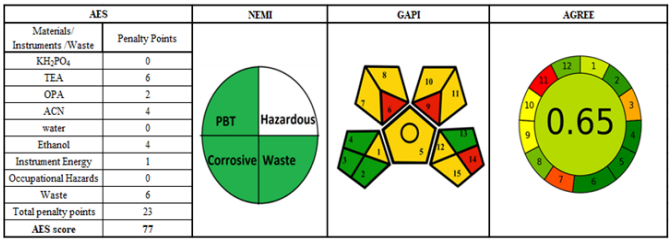



**Figure 6:** Results showing assessment of greenness of method using different metric tools.

## Validation of Proposed Method

The developed methods were validated in compliance with the ICH guidelines to ensure accuracy, reliability, regulatory compliance, consistency, data integrity, quality control, cost efficiency, customer confidence, and early problem identification in the analytical testing process. Following are the parameters considered during analytical method validation as per ICH Q2 (R2)[28] .

*System suitability criteria*

System suitability parameters, including Resolution, Area % RSD, Asymmetry, and Theoretical Plates, were monitored to ensure reliable and reproducible results. All values met the acceptance criteria (see Table 1).

*Specificity*

The specificity test showed no interference with Fluoxetine HCL and Olanzapine in blank and placebo solutions. Peak purity was within acceptable limits; confirming specificity and selectivity (see Table 2 for details and Figure 6 for the chromatogram).

*LOQ, linearity and range*

The linearity test used six concentrations (0.1% to 150%) for Fluoxetine HCL and Olanzapine. S/N for LOQ, correlation coefficient, y-intercept, and slope were calculated, confirming the method's sensitivity and consistency across concentrations. See Table 2 for detailed results, Figures 7-8 for the linearity plot, and Figure 9 for the LOQ chromatogram.

*Accuracy*

The recovery study was conducted in the presence of excipients commonly used in both capsule and tablet dosage forms, ensuring that the method is suitable for estimating both types of dosage forms. The recovery of the current method was performed in triplicate at three levels ranging from LOQ to 150%, and the results obtained were well within the acceptance

**Table 1:** Results of System Suitability Criteria

| *Mean Area(n=6) And % RSD (Limit: % RSD ≤ 2%)* | | *Mean Asymmetry (n=6) (Limit: ≤ 2.0)* | | *Mean Theoretical plates (n=6) (Limit: ≥2000)* | | *Resolution (Limit: ≤ 2.0)* |
|---|---|---|---|---|---|---|
| Olanzapine | Fluoxetine HCL | Olanzapine | Fluoxetine HCL | Olanzapine | Fluoxetine HCL | The observed Resolution between both the analyte is 3.57 |
| 1426.9, 0.1 | 3269.3, 0.1 | 1.03 | 1.08 | 6997 | 7422 | |

(SD-Standard deviation, RSD-Relative standard deviation)

**Table 2:** Results of Specificity, Linearity and range parameter (RT-Retention time, NA-Not applicable, LOQ-Limit of quantification)

| *Parameter* | *Olanzapine* | *Fluoxetine HCL* | *Limit* |
|---|---|---|---|
| RT for standard solution | 2.057 | 2.433 | NA |
| Mean S/N for LOQ level (n=3) | 10.1 | 15.5 | 10:1 |
| Peak Purity of analyte in standard solution | 997 | 994 | Not less than 950 |
| Peak Purity of analyte in Test solution | 994 | 996 | |
| Linearity Range (µg/mL) | 0.1-150 | 0.4-610 | NA |
| Coefficient of correlation | 0.9998 | 0.9999 | ≥ 0.999 |
| Slope of regression line | 13.69 | 7.12 | For Information |
| Y- intercept | 40.06 | 21.96 | |
| Residual sum of square | 498.75 | 80.22 | |

limits, refer table 3 for detail results, indicating that the method is suitable for accurate estimation of Fluoxetine HCL and Olanzapine.

*Precision*

Precision was assessed at three levels: repeatability, reproducibility, and intermediate precision. Repeatability involves injecting six replicates of a standard solution and checking its system suitability criteria (refer to Table 1 for details). Reproducibility and intermediate precision were achieved by analyzing six different samples on different days, respectively. The closeness between the results of each set and the average difference between both sets were calculated, with the results well within the limits, indicating method provides precise results, refer table 4.

*Robustness*

Robustness is assessed through intentional variations in analytical procedure parameters. In the current method the study was performed by deliberate changes in column

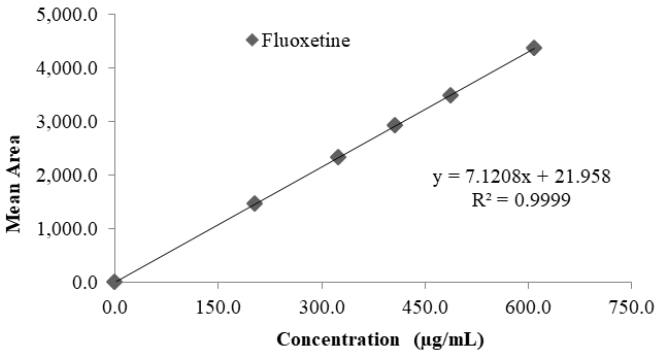



**Figure 7:** Linearity plots of Fluoxetine HCL

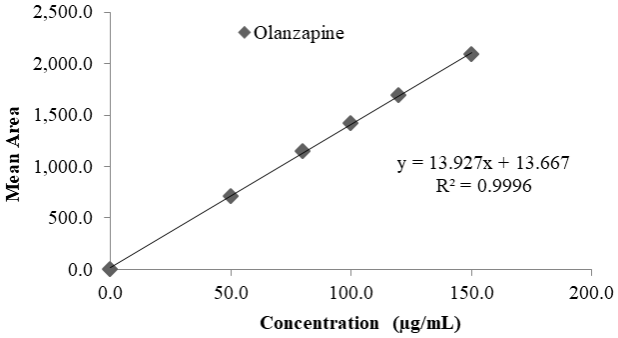



**Figure 8:** Linearity plots of Olanzapine

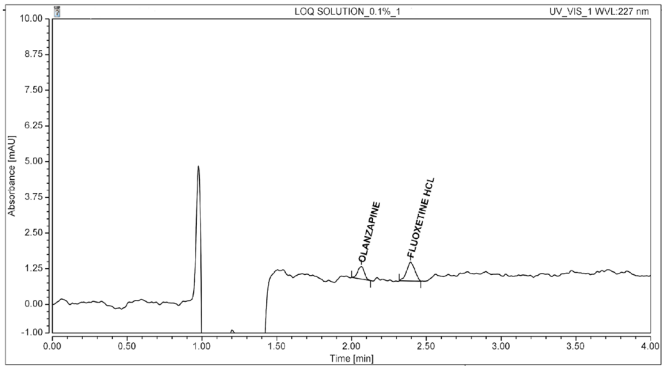



**Figure 9:** Representative chromatogram of LOQ solution

**Table 3:** Results of Accuracy for Capsules and Tablets dosage forms

| *Dosage forms* | *Analyte* | *Accuracy range (µg/mL)* | *Mean accuracy for LOQ & %RSD (n=3)* | *Mean accuracy for 50% & %RSD (n=3)* | *Mean accuracy for 100% & %RSD (n=3)* | *Mean accuracy for 150% & %RSD (n=3)* | *Overall mean accuracy& %RSD (n=9)* |
|---|---|---|---|---|---|---|---|
| For Capsules | Olanzapine | 0.1 -150 | 97.8, 9.7 | 100.9, 0.3 | 100.8, 0.9 | 99.0, 0.1 | 100.6, 0.9 |
| | Fluoxetine HCL | 0.4 - 605 | 108.3, 10.0 | 101.2, 0.3 | 99.7, 0.7 | 99.1, 0.1 | 100.2, 1.0 |
| For Tablets | Olanzapine | 0.1 -157 | 99.1, 7.8 | 100.2, 0.5 | 101.1, 0.2 | 100.8, 0.2 | 100.7, 0.5 |
| | Fluoxetine HCL | 0.4 - 606 | 103.3, 7.8 | 100.7, 0.5 | 100.3, 0.8 | 99.7, 0.2 | 100.4, 0.6 |

(**Limit:** For LOQ – accuracy 90 to 110, % RSD NMT 10% and for other level accuracy 98 to 102, %RSD ≤ 2%)

**Table 4:** Results of Precision parameters (MP-Method precision, IP-Intermediate precision)

| *Parameter* | *Olanzapine* | *Fluoxetine HCL* | *Limit* |
|---|---|---|---|
| Mean MP % assay results, %RSD (n = 6) | 96.6, 0.23 | 99.5, 0.07 | %RSD ≤ 2% |
| Mean IP% assay results, %RSD (n = 6) | 96.8, 0.39 | 99.3, 0.26 | |
| overall Mean % assay results of MP and IP, %RSD (n = 12) | 96.7, 0.32 | 99.4, 0.30 | |
| Mean %Assay Difference between MP and IP | 0.2 | 0.2 | ≤ 2% |

**Table 5:** Results of Robustness parameters

| *Parameter* | % assay of *Olanzapine* | % assay of *Fluoxetine HCL* | *Limit* |
|---|---|---|---|
| Col. Temp.-43°C(Low) | 96.5 | 98.9 | % difference of each against mean MP results is NMT 2% |
| Col. Temp.-47°C (High) | 96.8 | 99 | |
| Buffer pH 7.5 (Low) | 97.3 | 99.7 | |
| Buffer pH 7.7 (High) | 97.6 | 101.3 | |
| M.P. Flow 1.4mL (Low) | 98 | 100.5 | |
| M.P. Flow 1.6mL (High) | 98.3 | 100.4 | |

**Table 6:** Results of Filter study parameters

| *Parameter* | % assay of *Olanzapine* | % assay of *Fluoxetine HCL* | *Limit* |
|---|---|---|---|
| Centrifuged test Solution | 98.6 | 101.1 | % difference of result obtained by each filtered sample against Centrifuged solution NMT 2% |
| Test filtered through 0.45µ nylon | 97.5 | 99.8 | |
| Test filtered through 0.45µ PVDF | 97.3 | 99.9 | |
| Test filtered through 0.45µ PTFE | 97.8 | 100.3 | |

temperature, mobile phase flow and pH of buffer solution and % assay of analyte was determined and compared with precision set, the observed result was within the acceptance criteria, refer table 5 for detail results.

**Table 7:** Results of solution stability parameters

| *Parameter* | *% Assay of Olanzapine* | *% Assay of Fluoxetine HCL* | *Limit* |
|---|---|---|---|
| Test solution Initial | 96.9 | 99.5 | % difference of each against Initial results NMT 2% |
| Test solution 18 Hrs | 98.6 | 101.2 | |
| Test solution 24 Hrs | 98.4 | 101.1 | |
| Test solution 48 Hrs | 98.8 | 101.6 | |

**Table 8:** Results of analyzed test solution for active pharmaceutical drug and tablet dosage

| *Test* | *Wt. taken (mg)* | *%Assay of Olanzapine* | *%Assay of Fluoxetine HCL* |
|---|---|---|---|
| Olanzapine API | 5.362 | 102.7 | NA |
| Fluoxetine HCL API | 20.292 | NA | 99.2 |
| Test solution (Tablet) | 154.49 | 96.2 | 99.7 |

*Filter study*

The impact of the filter on the results during analysis was confirmed by performing a filter study test. In this study, a sample was filtered using different filters, and the results were compared with an unfiltered sample. It was observed that the filters had no impact on the results of the active analytes. Refer to table 6.

*Solution stability*

Solution stability studies, a key parameter in method validation, assess the stability of analytes in solution over time under specific conditions. The sample was analyzed at different time intervals from initial time after preparation and observed that standard solution and test solution was stable upto 48Hrs, this ensures consistent and reliable results throughout the method's application, refer table 7.

**Application of Proposed Method**

Applying a validated method to drug substances as well as drug product analysis is essential for ensuring the accuracy, quality, and safety of pharmaceutical products, while also meeting regulatory standards. The newly developed and validated eco-friendly method was analyzed using active pharmaceutical ingredients as well as tablet dosage forms of Fluoxetine HCl and Olanzapine. It was observed that the method is suitable for identification and quantification of drugs and drug products, for detail results refer table 8.

## CONCLUSION

A new, sensitive, rapid, and eco-friendly analytical method has been successfully developed and validated for estimating individual and multicomponent analytes in pharmaceutical drugs and dosage forms. This method utilizes a green solvent, ethanol, in the mobile phase, significantly reducing the amount of acetonitrile, thereby lowering the environmental impact.

One notable advantage of this method is its very short run time of 4-min. such short run times are possible using UPLC, which is an expensive instrument requiring a costly column that operates at high pressure. This short run time reduces the consumption of solvents, time, and energy, and also minimizes waste generation, ultimately making the method more cost-effective and sustainable. The newly developed methods were validated according to ICH guidelines, demonstrating selectivity, high sensitivity (up to 0.1%), accuracy, linearity, precision, and robustness, even with very short run times. The solution remained stable for up to 48hours after preparation. The greenness of the method was evaluated using various green metric tools such as AES, NEMI, GAPI, and AGREE, showing that the optimized method is environmentally friendly. It can serve as an alternative to many published methods, including the USP-NF method, for the estimation of Fluoxetine HCl and Olanzapine, both alone and in combination, during the research and development of new formulations, as well as for quality and stability testing.

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