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DEVELOPMENT AND VALIDATION OF A REVERSED PHASE HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CLOMIPRAMINE AND FLUVOXAMINE

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ABSTRACT

A new method was established for simultaneous estimation of Clomipramine and Fluvoxamine by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Clomipramine and Fluvoxamine by using Inertsil C18 5µm (4.6*250mm) column, flow rate was 1ml/min, mobile phase ratio was Phosphate buffer (0.05M) pH 4.6: ACN (30:70%v/v) (pH was adjusted with orthophosphoric acid), detection wave length was 255nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, PDA Detector 996, Empower-software version-2.The retention times were found to be 2.399 mins and 3.907mins. The % purity of Clomipramine and Fluvoxamine was found to be 100.7% and 101.4% respectively. The system suitability parameters for Clomipramine and Fluvoxamine such as theoretical plates and tailing factor were found to be 1.3, 5117.5and 1.4, 3877.3 the resolution was found to be 8.0.The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for Clomipramine and Fluvoxamine was found in concentration range of 100µg500µg and 1µg-5µg and correlation coefficient (r2) was found to be 0.997 and 0.999, % mean recovery was found to be 100% and 100.5%, %RSD for repeatability was.0.2 and 0.4, % RSD for intermediate precision was 0.5 and 0.1 respectively. The precision study was precise, robust, and repeatable. LOD value was 2.95 and 3.04, and LOQ value was 9.87 and 10 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Clomipramine and Fluvoxamine in API and Pharmaceutical dosage form.

INTRODUCTION

Clomipramine, a tricyclic antidepressant, was the first medication approved by the Food and Drug Administration (FDA) for OCD (obsessive-compulsive disorder). It is a strong, but not completely selective serotonin reuptake inhibitor (SSRI), as the active main metabolite demethyl-clomipramine acts preferably as an inhibitor of noradrenaline reuptake. α1-receptor blockage and β-down regulation have been noted and most likely play a role in the short term effects of clomipramine. A blockade of sodium-channels and NDMA-receptors might, as with other tricyclics, account for its effect in chronic pain, in particular the neuropathic type [1, 2].

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Fluvoxamine is an FDA approved SSRI for the treatment of OCD in children and adults. It is a potent and selective serotonin reuptake inhibitor with approximately 100-fold affinity for the serotonin transporter over the nor-epinephrine transporter. It has negligible affinity for the dopamine transporter or any other receptor, with the sole exception of the σ1 receptor. It behaves as a potent agonist at this receptor and has the highest affinity of any SSRI for doing so. This may contribute to its antidepressant and anxiolytic effects and may also afford it some efficacy in treating the cognitive symptoms of depression [1, 3].

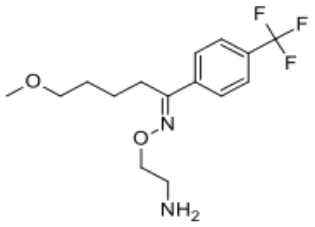

Figure 1: Structure of Fluvoxamine

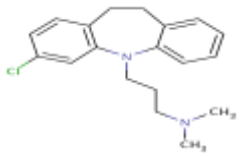

Figure 2: Structure of Clomipramine

Literature review reveals that the HPLC determination of pure clomipramine hydrochloride has been reported for identification and quantification [4-10]. A far reaching survey of the literature on Clomipramine and Fluvoxamine revealed no HPLC method for determining these compounds in pharmaceutical formulations and bulk substances. In the current study, a new, simple, sensitive and reliable RP-HPLC method for determination of Clomipramine and Fluvoxamine has been proposed.

MATERIALS AND METHODS:

Chemicals and Reagents:

The pharmaceutical working standards of Clomipramine and Fluvoxamine were procured as a gift sample from spectrum pharma research solutions, Hyderabad. Marketed samples of Clomipramine and Fluvoxamine were got from local markets. HPLC grade Acetonitrile, KH_2PO_4, Ortho phosphoric Acid, Methanol and HPLC grade distilled water procured from Finer chemical Ltd, Merck, India.

HPLC Equipment and Chromatographic conditions:

The chromatographic separation was carried out by Waters Acquity HPLC 2695 with binary solvent manager, equipped with PDA detector and auto sampler. The Empower 2 software was used for signal monitoring, data collection and data processing. In addition, an electronic balance (BL-220H; Shimadzu Corporation), a pH meter (ELICO® LI 120), a sonicator (PCi, Mumbai), pipettes, Burettes and Beakers of borosil has been employed in this study.

Mobile Phase Optimization:

Initially the mobile phase tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized to potassium dihydrogen phosphate with buffer (pH 3.0), Methanol in proportion 30: 70 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, hypersil column, lichrosorb, and inertsil ODS column. Inertsil ODS (4.6 x 150mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 0.8ml/min flow.

Preparation of Phosphate buffer:

Accurately weighed 6.8 grams of KH_2PO_4 was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with HPLC water and the volume was adjusted to pH 3.0 with Orthophosphoric acid.

Preparation of mobile phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml of Methanol HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluents.

Preparation of Standard Stock Solution:

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Accurately weigh and transfer 10 mg of Clomipramine and Fluvoxamine 10mg of working standard into a 10 ml & 10 ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (100 µg/ml of Clomipramine and Fluvoxamine).

Preparation of Standard Working Solution:

Pipette 3ml& 0.3ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent.

Preparation of Sample Stock Solution:

Accurately weigh 20 tablets crush in mortor and pestle and transfer equivalent to 10 mg of Clomipramine and Fluvoxamine (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of Sample Working Solution:

Pipette 1 ml of Clomipramine and Fluvoxamine of the above sample stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

The above prepared standard, sample working solutions were injected (10 µL) into the chromatographic system and measured the areas for Clomipramine and Fluvoxamine peaks.

Method Validation

The optimized method was validated as per ICH guidelines for the following parameters.

Precision:

The standard solution was injected for five times and measured the area for all five Injections in HPLC.

Intermediate Precision:

To evaluate the intermediate precision (also known as Reproducibility) of the method, Precision was performed on different day by using different make column of same dimensions. The standard solution was injected for five times and measured the area for all five injections in HPLC.

Accuracy:

Preparation of Standard stock solution:

Accurately weigh and transfer 100 mg of Clomipramine and 25mg Fluvoxamine of working standard into a 100ml & 100 ml clean dry volumetric flask add about 70mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 1 ml& 0.25 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):

Accurately weigh and transfer 10mg of Clomipramine and 2.5mg of Fluvoxamine working standard into a 10mL and 10 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution). Further pipette 0.05 ml of Clomipramine & 0.014 ml of Fluvoxamine of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately weigh and transfer 10 mg of Clomipramine and 10 mg of Fluvoxamine working standard into a 10mL and 100 ml 0f clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock Solution). Further pipette 0.1 ml of Clomipramine & 0.25 ml of Fluvoxamine of the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent.

For preparation of 150% solution (With respect to target Assay concentration):

Accurately weigh and transfer 10mg of Clomipramine and 25mg of Fluvoxamine working standards into a 10mL and 100ml of clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.15 ml of Clomipramine & 0.39 ml of Fluvoxamine of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

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Procedure: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Clomipramine & Fluvoxamine and calculate the individual recovery and mean recovery values.

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

Linearity:

Preparation of stock solution: Accurately weigh 20 tablets crush in mortor and pestle and transfer equivalent to 10 mg of Clomipramine and Fluvoxamine (marketed formulation) sample into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (100ppm of Clomipramine&25ppm of Fluvoxamine): 1ml and 0.1 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (200ppm of Clomipramine &50ppm of Fluvoxamine): 2ml and 0.2 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (300ppm of Clomipramine &75ppm of Fluvoxamine): 3ml and 0.3 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV (400ppm of Clomipramine&100ppm of Fluvoxamine): 4ml and 0.4 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluents.

Preparation of Level – V (500ppm of Clomipramine&150ppm of Fluvoxamine): 5ml and 0.5 ml of stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Procedure: Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Acceptance Criteria: Correlation coefficient should be not less than 0.999.

Limit of Detection:

Preparation of 300µg/ml solution of Clomipramine: Accurately weigh and transfer 10 mg of Clomipramine working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

Preparation of 0.12µg/ml solution of Clomipramine: Pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. Pipette 0.4mL of 1µg/ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Preparation of 3µg/ml solution of Fluvoxamine: Accurately weigh and transfer 10mg of Fluvoxamine working standard into a 100ml clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).

Preparation of 0.015µg/ml solution of Fluvoxamine: Pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 0.5ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Limit of Quantification:

Preparation of 300µg/ml solution of Clomipramine: Accurately weigh and transfer 10 mg of Clomipramine working standard into a 10mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.42µg/ml solution of Clomipramine: Pipette 1.0mL of above

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solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Pipette 1.4 mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Preparation of 3µg/ml solution of Fluvoxamine: Accurately weigh and transfer 10mg of Fluvoxamine working standard into a 100mL clean dry volumetric flask add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.05µg/ml solution of Fluvoxamine: Pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Pipette out 1.7mL of above solution into a 10 ml of volumetric flask and dilute up to the mark with diluent.

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

RESULTS AND DISCUSSION

Wavelength Detection: The overlay spectrum of Clomipramine and Fluvoxamine was obtained and the isobestic point of Clomipramine and Fluvoxamine showed absorbance's maxima at 260 nm. The spectrums are shown in Figs.

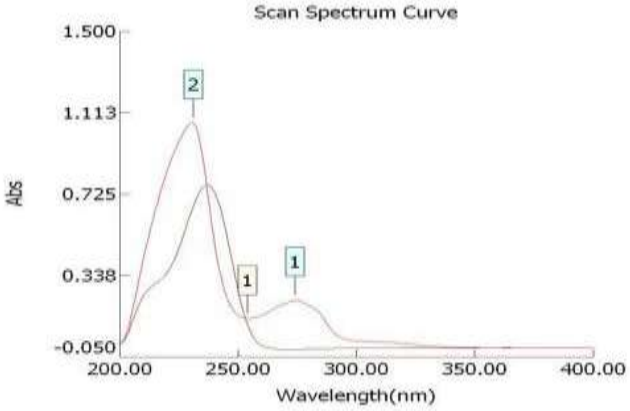

Figure 3: Overlay spectrum of Clomipramine and Fluvoxamine

The UV spectra of individual drugs are as follows:

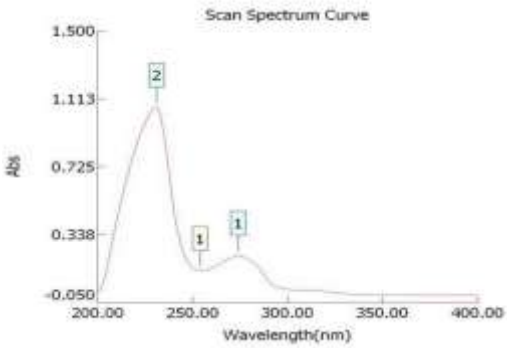

Figure 4: Spectrum of Clomipramine

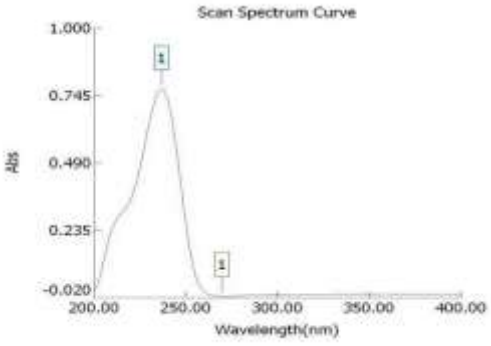

Figure 5: Spectrum of Fluvoxamine

Method Development: The chromatographic method development for the simultaneous estimation of Clomipramine and Fluvoxamine were optimized by several trials for various parameters as different column, flow rate and mobile phase, finally the optimized chromatographic method was selected for the separation and quantification of Clomipramine and Fluvoxamine in API and pharmaceutical dosage form by RP-HPLC method.

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Optimized Chromatographic conditions:

Column : InertsilC18 5µm (4.6*250mm)
Mobile phase ratio : Phosphate buffer (0.05M) pH4.6: ACN (30:70%v/v)
Detection wavelength : 255nm
Flow rate : 1ml/min
Column temperature : Ambient
Injection volume:20µl

Tailing factor for the peaks due to Clomipramine and Fluvoxamine in Standard solution should not be more than 2.0. Theoretical plates for the Clomipramine and Fluvoxamine peaks in Standard solution should not be less than 2000.

Observation:

The chromatogram is perfect with clear separation of components. The peak symmetry and system suitability parameters are within the limits.

Validation Parameters

Accuracy:

The accuracy study was performed for 50%, 100% and150 % for Clomipramine and Fluvoxamine. Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery**.**

Specificty

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The study was performed by injecting blank. The chromatograms are shown in Figure 7.

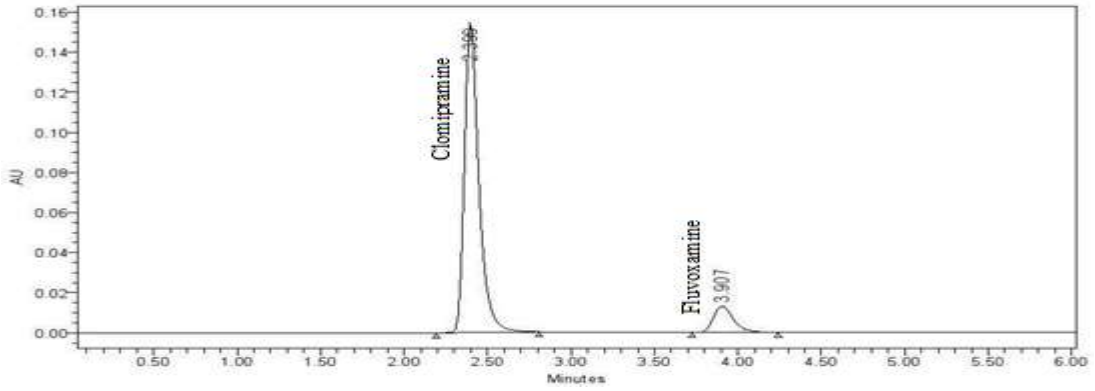

Figure 6: Optimized Chromatogram of Clomipramine and Fluvoxamine

Table 1: System Suitability data of Clomipramine and Fluvoxamine

S.No.	Peak name	Rt	Area	Height	USP Plate count	USP Tailing	USP Resolution
1	Clomipramine	2.399	946124	155429	5105	1.3	8.0
2	Fluvoxamine	3.907	111541	13239	3788	1.4	

Table 2: Accuracy results of Fluvoxamine

% Level	Area	Amount added(mg)	Amount found(mg)	% Recovery	Mean Recovery
50%	2332744	5	5.10	101.8%	100.5%
100%	3132697	10	9.99	99.9%	
150%	3918997	15	14.9	99.1%	

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Table 3: Accuracy results of Clomipramine

% level	Area	Amount Added(mg)	Amount Found(mg)	%Recovery	Mean Recovery
50%	353867	5	5.0	101.3%	100.0%
100%	4735088	10	9.94	99.4%	
150%	5911798	15	14.8	99.2%	

Acceptance Criteria:

The %Recovery for each level should be between 98.0 to 102.0%.

Table 4: Repeatability results of Clomipramine &Fluvoxamine.

S.No.	Fluvoxamine		Clomipramine	
	Rt	Area	Rt	Area
1	4.304	1501417	2.321	2235319
2	4.300	1486940	2.317	2240678
3	4.308	1490656	2.323	2249490
4	4.310	1487329	2.322	2245822
5	4.314	1490384	2.324	2251694
Mean		1491345		2244601
Std .dev		5881.4		6656.8
% RSD		0.39		0.30

The % RSD for the area of five standard injections results should not be more than 2%. The Method precision study was performed for the %RSD of Clomipramine and Fluvoxamine was found to be 0.3 and 0.3(NMT2).

Intermediate Precision

Table 5: Intermediate Precision results of Clomipramine and Fluvoxamine

S.No.	Clomipramine		Fluvoxamine	
	Rt	Area	Rt	Area
1	2.328	2194758	4.335	1456296
2	2.326	2195700	4.336	1457422
3	2.327	2196191	4.334	1456513
4	2.326	2195326	4.337	1454579
5	2.331	2200951	4.340	1451483
Mean		2196585		1455259
Std .dev		2496.0		2347
% RSD		0.11		0.16

The % RSD for the area of five standard injections results should not be more than2%. The intermediate precision was performed for %RSD of Clomipramine and Fluvoxamine was found to be 0.1 and 0.1 respectively (NMT2).

Table 6: System Suitability data of Standard Injection

S.NO	Peak name	Rt	Area	Height	USP Plate count	USP Tailing	USP Resolution
1	Clomipramine	2.237	7913799	394185	2632	1.8	
2	Fluvoxamine	4.342	1853381	162758	2614	1.6	5.23

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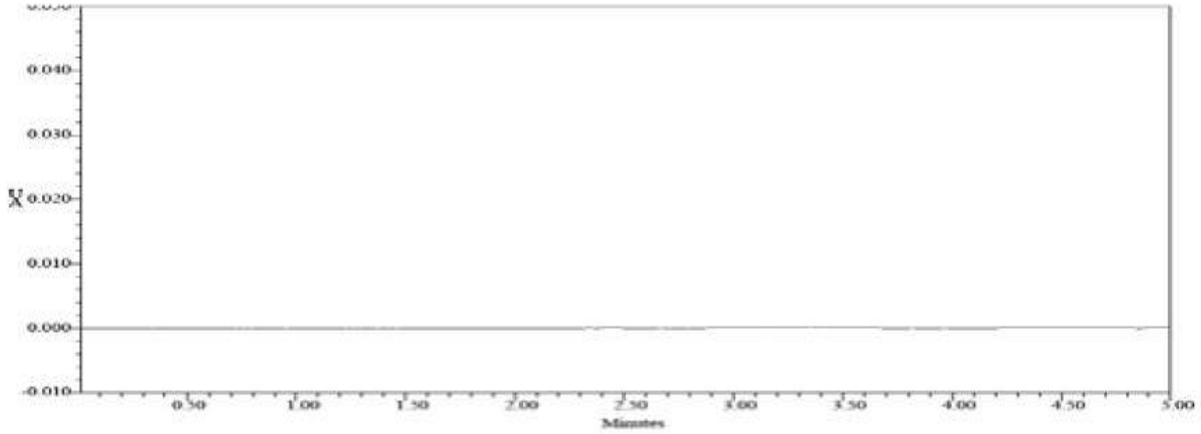

Figure 7: Chromatogram of blank Injection

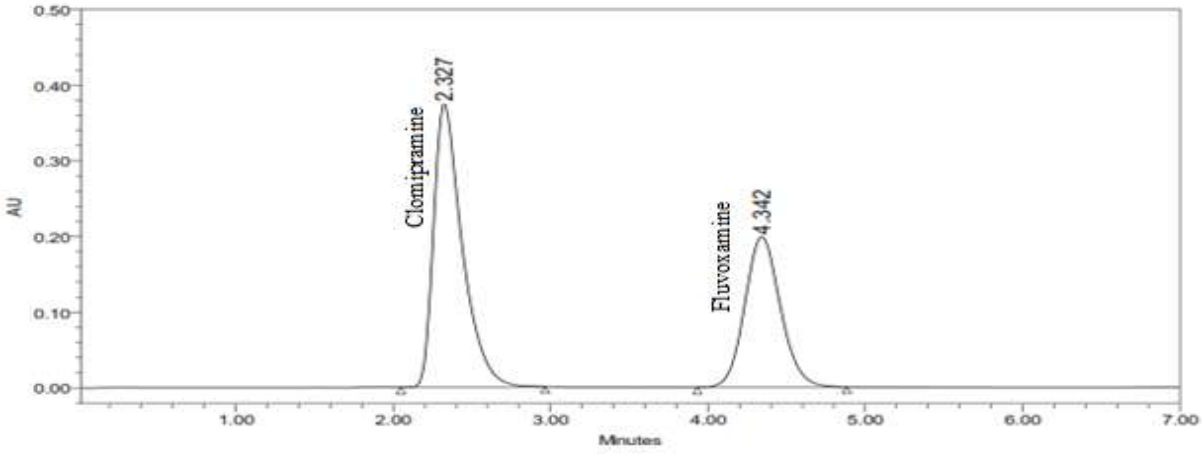

Figure 8: Chromatogram of Standard Injection

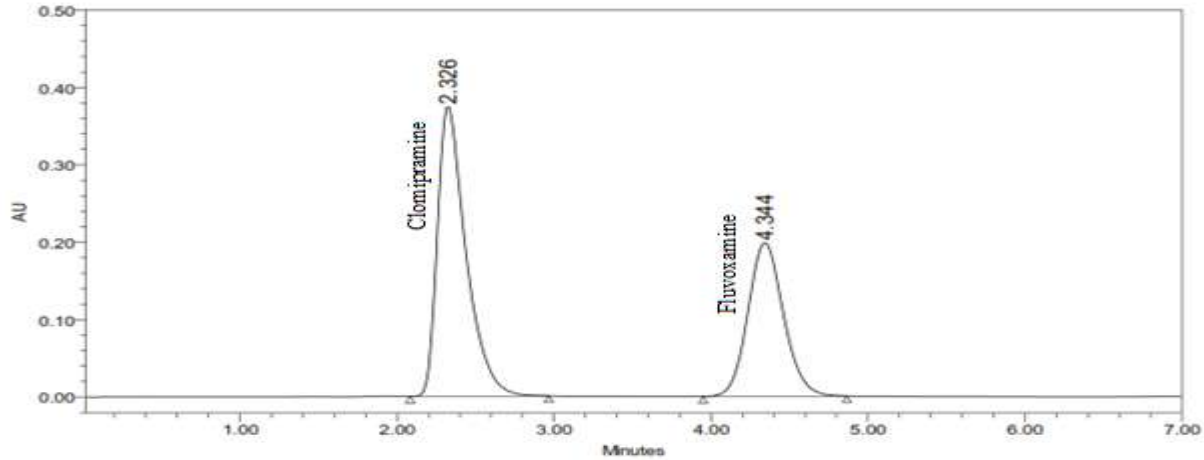

Figure 9: Chromatogram of Sample Injection

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Table 7: System Suitability data of Sample Injection

S.No	Peak name	R_t	Area	Height	USP Plate count	USP Tailing	USP Resolution
1	Clomipramine	2.32	472635	376488	2455	1.60	
2	Fluvoxamine	4.34	312257	198418	2614	1.11	5.52

The specificity test was performed for Clomipramine and Fluvoxamine. It was found that there was no interference of impurities in retention time of analytical peak.

Limit of Detection: LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve(S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y intercepts of regression lines.

Formula:

$$\mathbf{LOD = 3.3\ X\ \frac{\sigma}{S}}$$

Where

σ- Standard deviation (SD)

S–Slope

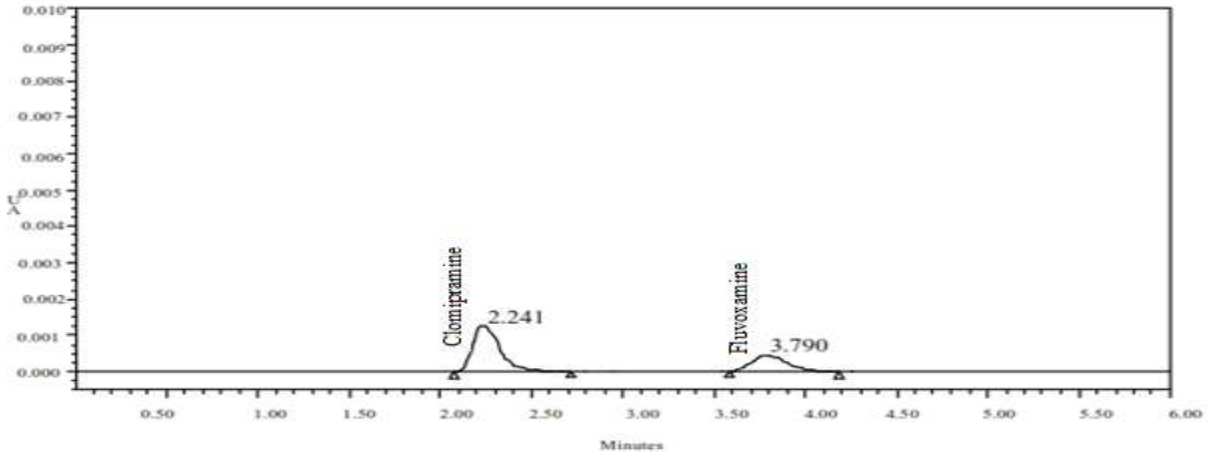

Figure 10: Chromatogram of LOD Injection

Calculation of S/N Ratio

		Clomipramine	Fluvoxamine
Average Baseline Noise obtained from Blank		41µV	41µV
Signal obtained from LOD solution		121µV	125µV
S/N		121/41= 2.95	125/41= 3.04
Acceptance Criteria:	S/N Ratio value shall be 3 for LOD solution		

The LOD was performed for Clomipramine and Fluvoxamine was found to be 2.95 and 3.04 respectively.

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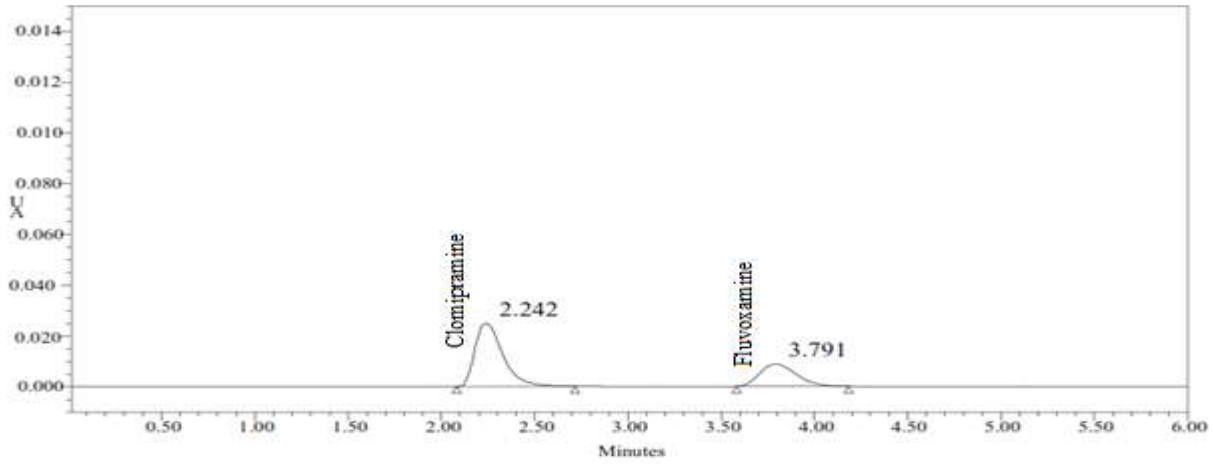

Figure 11: Chromatogram of LOQ Injection

Calculation of S/N Ratio

	Clomipramine	**Fluvoxamine**
Average Baseline Noise obtained from Blank	41µV	41µV
Signal obtained from LOD solution	405µV	412µV
S/N	405/41=9.87	412/41=10.0
Acceptance Criteria:	S/N Ratio value shall be 10 for LOQ solution	

The LOQ was performed for Clomipramine and Fluvoxamine was found to be 9.87 and 10 respectively.

Linearity: The linearity study was performed for the concentration of 10 µg/ml to 50 µg/ml and 20 µg/ml to 100 µg/ml level. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. The results are tabulated in Table 8. Calibration graph for Clomipramine and Fluvoxamine are shown in Figure 12 & 13.

Table 8: Linearity results of Clomipramine and Fluvoxamine

Clomipramine		**Fluvoxamine**	
Concentration (µg/ml)	**Peak area**	**Concentration (µg/ml)**	**Peak area**
10	1810101	20	1164173
20	2044287	40	1342535
30	2367133	60	1555931
40	2602279	80	1777973
50	2869778	100	1942319

Acceptance Criteria: Correlation coefficient should be not less than 0.999

Plotting of calibration graphs:

The resultant areas of linearity peaks are plotted against Concentration

Range- The linearity study was performed for concentration range of 10µg -50µg and 20µg-100 µg of Clomipramine and Fluvoxamine and the correlation coefficient was found to be 0.9988 and 0.999.(NLT 0.9988).

Robustness- As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, temperature variation was made to evaluate the impact on the method.

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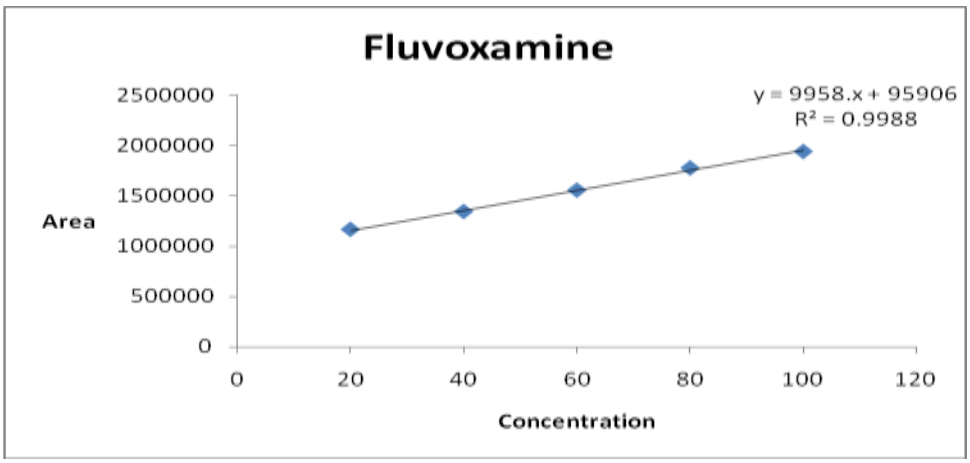

Figure 12: Calibration curve of Fluvoxamine

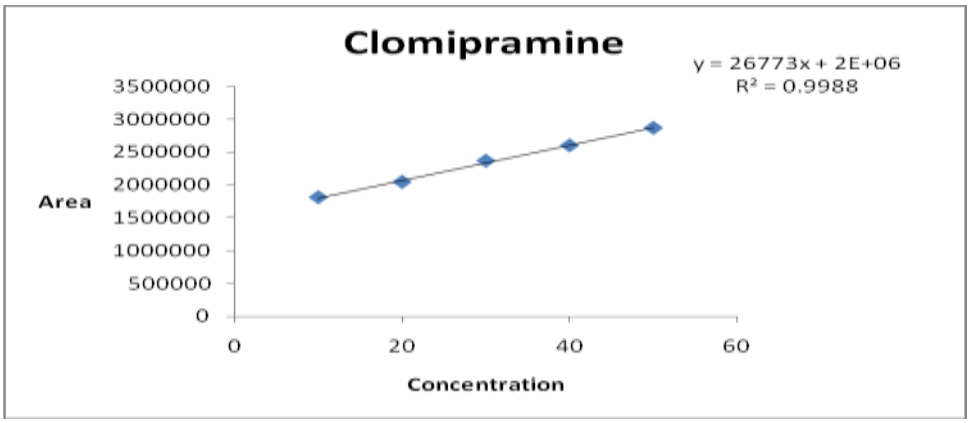

Figure 13: Calibration curve of Clomipramine

Table 9: Robustness Data

Parameter	%RSD	
	Clomipramine	Fluvoxamine
Flow Minus	0.8	0.7
Flow Plus	0.3	0.5
Mobile phase Minus	0.2	0.4
Mobile phase Plus	0.6	0.8
Temperature Minus	0.5	0.6
Temperature plus	0.4	0.7

SUMMARY AND CONCLUSION:

Chromatographic conditions used are stationary phase Inertsil C18 5µm (4.6×250mm) column, Mobile phase ratio was Phosphate buffer (0.05M) pH 4.6: ACN (30:70%v/v) (pH was adjusted with orthophosphoric acid) and flow rate was maintained at 1.0ml/min, detection wave length was 255nm, column temperature was set to 30°C and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. The retention times were found to be 2.399mins and 3.907mins. The linearity study for Clomipramine and Fluvoxamine was found in concentration range of 10µg-50 µg and 20

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µg -100 µg and correlation coefficient (r2) was found to be 0.997 and 0.999, % mean recovery was found to be 100% and 100.5%, %RSD for repeatability was 0.2 and 0.4, % RSD for intermediate precision was 0.5 and 0.1 respectively. LOD value was 2.95 and 3.04, and LOQ value was 9.87 and 10 respectively. From the above experimental results it was concluded that, this newly developed method for the estimation of Clomipramine and Fluvoxamine was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department and approved testing laboratories.

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