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Development and Validation of RP-HPLC Method for the Determination of Atomoxetine Capsules

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Abstract: The objective of the present study was to develop and validate a novel RP-HPLC method for determination of Atomoxetine hydrochloride (ATX) in pharmaceutical dosage form. Chromatographic separation was conducted on Shimadzu-2010 with the quaternary pump, Symmetry-C_8 column (4.6 mm I'd. X 150 mm, 5 µm particle sizes) and with photodiode array detector. Mobile phase consisted of Buffer and Methanol were mixed in the ratio of 40:60 v/v, was used at a flow rate of 1.0 ml/min and detection wavelength was set at 271 nm. The retention time for ATX was found to be 3.20 min. The calibration was linear (r^2= 1) in the concentration range of 15 to 105 µg/ml. The limit of detection and the limit of quantitation were found to be 0.595 µg/ml and 1.805 µg/ml respectively. Recovery of ATX in tablet formulation was observed in the range of 99.25 - 100.91 %. Percentage assay of ATX was found to be 99.69 % w/w. Thus the novel proposed method for ATX was found to be feasible for the estimation of ATX in bulk as well as a pharmaceutical dosage form.

Key words: Atomoxetine hydrochloride, RP-HPLC, Validation, ICH guidelines.

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I. INTRODUCTION

The chemical name of ATX is (-)-N-Methyl-3-phenyl-3-(o-tolyloxy)-propyl amine). ATX is indicated for the treatment of Attention-Deficit/Hyperactivity Disorder (ADHD). Mechanism of action of ATX by which ATX products its therapeutic effects in ADH is unknown, but is thought to be related to selective inhibition of the pre-synaptic nor epinephrine transporter, as determined through in vitro studies. ATX appears to have minimal affinity for the noradrenergic receptors or for other neurotransmitter transporters or receptors. Atomoxetine is metabolized primarily through the CYP2D6 enzymatic pathway[1].

Literature Survey shows that the ATX has been estimated by Ultra violet spectrophotometric method [2], HPLC-UV by liquid liquid extraction [3], HPTLC [4], Stability indication RP-HPLC [5] , Impurity method development [6],RP-HPLC[7-8], LC-MS/MS [9-10]. Infact there is no method for the estimation of atomoxetine in capsules and their degradation studies. This novel proposed method contributes quick estimation, correct peak shape, precise, simple, and quick, use of smaller sample volumes when compared with other existing methods. So it is necessary to develop a simple, precise and rapid RP-HPLC method for quantitative determination of ATX in capsules. This work describes the validation parameters stated by the International Conference on Harmonization guidelines. Fig 1 shows the chemical structure of ATX.

However no stability indicating High Performance LC method has been reported for the estimation of ATX in bulk and pharmaceutical dosage forms thus far. Hence the prominent important objective of the present research is to develop and validate a precise, sensitive, robust and simple liquid chromatography method for ATX in its bulk and pharmaceutical dosage form and stress degradation studies of ATX as per International Conference on Harmonization (ICH) Q2 (R2) guidelines. Fig 1 shows the chemical structure of ATX.

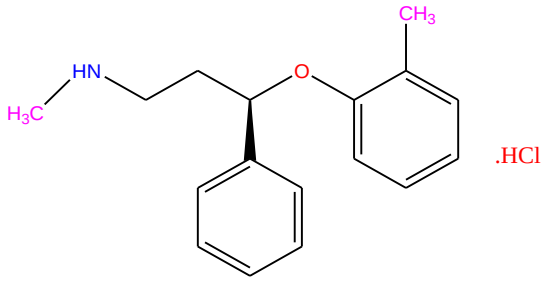


Figure 1: Chemical structure of Atomoxetine hydrochloride (ATX).

II. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The above said the standard drug was gifted from Aurobindo Pharma Ltd, Hyderabad, India. All the chemicals used in this method were of high-grade purity and purchased from Merck Chemical Division Ltd., Mumbai. HPLC grade acetonitrile, water, methanol and triethylamine were obtained from Merck Pharmaceuticals private Ltd., Mumbai, India. Commercial tablets of the above said formulation was obtained from a local pharmacy.

2.2 Instrumentation and conditions

The high pressure liquid chromatographic system utilized was a Shimadzu high-pressure liquid chromatograph with quaternary pump, Symmetry-C_8 column (5 µm particle size x 4.6 × 150 mm) and a diode array detector. LC-Solutions software was used for chromatography data acquisition, processing and control of HPLC chromatograph. Digital pH meter (systronics model-802), an electronic balance (Sartorius), a sonicator (spectral lab, model UCB 40) and UV-Visible spectrophotometer (Shimadzu) were used in this study.

2.3 Preparation of reagents and standards

2.3.1 Preparation of buffer: Weigh accurately and dissolve 2.72 g of potassium dihydrogen orthophosphate in 1000 ml of water. Adjust pH to 3.0 ± 0.05 with Ortho-phosphoric acid. Filter the solution through 0.45 µm membrane filter.

2.3.2 Preparation of mobile phase: Prepared a required volume of degassed mixture of buffer and methanol in the ratio of 60: 40 v/v.

2.3.3 Preparation of buffer: Utilized mobile phase as diluent.

2.4 Preparation of standard stock and sample solutions

2.4.1 Standard stock solution: Accurately weigh and transfer 20 mg of ATX working standard into a 20 mL clean, dry volumetric flask , Add about 10 mL of diluent and sonicated to dissolve, make up to the volume with same diluent and mix.

2.4.2 Standard solution: 2.4 mL of standard stock solution is diluted to 20 mL with diluent and mix well. Filtered the solution through 0.45 µ membrane filter.

2.4.3 Sample solution: Take out as completely as possible the contents of not less than10 capsules (100 mg) and mix decorously. Weighed and taken an precise quantity of sample equivalent to about 20 mg of ATX into a 20 mL clean, dry volumetric flask. Add about 10 mL of diluent and sonicated for about ten minutes at room temperature with intermittent shaking every 3 minutes. Allow it to cool to room temperature. Diluted to volume with diluent up to mark of volumetric flask. Centrifuge the solution at 5000 RPM for about 10 minutes. 2.1 mL of the supernatant solution to 20 mL with diluent is added and mix. Filtered through 0.45 µ membrane filter. (Concentration of ATX was about 105 ppm).

2.5 Analytical method validation

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters such as system suitability, linearity, specificity, precision, accuracy, robustness, limit of detection and limit of quantitation as per guidelines.

2.5.1 System suitability parameters: The chromatographic systems used for analysis must pass system suitability before going to start the experiment. At first HPLC system is stabilized for thirty minutes. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters such as tailing factor (not more than 0.2 to 2.0), theoretical plate count (not less than 2000) and retention time ± 10 %. The % RSD for the peak area of six replicate injections of

ATX standard (not more than 2.0). The parameters such as tailing factor, % RSD and theoretical plates were studied.

2.5.2 Linearity: A standard stock solution of the ATX (1000 µg/ml) was prepared with the mobile phase. To study the linearity range of drugs, serial dilutions were made from standard stock solution in the range of 15 -105 µg/ml.

2.5.3 Specificity: Specificity of an analytical method is its ability to measure accurately and specifically the analyte of interest without interference from placebo and degradation products. The specificity of the method was established by injecting blank, placebo and standard solution in triplicate and recording the chromatograms.

2.5.4 Precision: The precision of the method was estimated by repeatability (interday) and intermediate precision (intraday). Repeatability was determined by performing six repeated analysis of the same working solution of ATX on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on different days and also by another analyst performing the analysis in the same laboratory (between-analysts).

2.5.5 Accuracy: The accuracy of a method is defined as the closeness of a measured value to the true value. The recovery studies were carried out at 50 %, 100 %, and 150 % of the target level in the tablet in triplicate each in the presence of placebo.

2.5.6 Robustness: The robustness was determined by analyzing the same sample under a variety of conditions. The factors considered to be: the organic ratio of mobile phase, variations in the flow rate, and pH. There were no significant changes in the chromatographic pattern when the above modifications were made in the experimental conditions, showing thus that the method is robust. The % RSD of ATX should be not more than 2.0 %.

2.5.7 Limit of detection and Limit of quantitation: Limit of detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated based on using following formulas, LOD = 3.3 x σ/S and LOQ = 10 x σ/S, where σ is the deviation response. S is the slope of the calibration curve.

III. RESULTS AND DISCUSSION

3.1 Method development and optimization

The current study was aimed at developing a sensitive, rapid and accurate reversed-phase HPLC method for the analysis of ATX in bulk drug and in pharmaceutical dosage form. In order to get decorous retention time, sharp and well-resolved peak, the parameters such as different flow rates, detection wavelength, and a choice of mobile phases containing acetonitrile, methanol, and HPLC grade water were studied. Good quality symmetrical sharp peak, minimum tailing factor in short run time was obtained with C_8 column and mobile phase composed of methanol: buffer in the ratio of 60:40 v/v, at a flow rate of 1.0 ml/minute with maximum lambda max at 271 nm. All the system suitability parameters were computed at the optimized chromatographic conditions. The obtained values of the entire system suitability parameters are within the limits of the agreeable range, which shows that the proposed method is fit for detection of ATX in the tablet form. The optimum chromatographic conditions are shown in Table1.

Table 1: Optimized chromatographic conditions

Parameter	**Chromatographic conditions**
Instrument	Shimadzu-2010 with quaternary pump
Column	Symmetry C_8 column (150 × 4.6 mm, 5 µm particle size)
Detector	Photo diode array detector
Mobile phase	Methanol: Buffer (60: 40 % v/v)
Flow rate	1.0 ml/minute
Detection wavelength	UV at 271 nm
Runtime	10 minutes
Temperature	40 °C
Volume of injection loop	10 µl
Retention time	3.2 minutes
Run Time	6.0

3.1.1 System suitability tests (SST)

SST are used to verify the resolution and reproducibility of the chromatographic system, because whether the system is adequate for the analysis or not. These tests are based on the concept that the equipment, electronics, analytical operations and samples which are to be analyzed and which constitute an integral system that can be evaluated as such**.** Retention time (*R*T), number of theoretical plates (*N*), tailing factor (*T*), and peak asymmetry (*A*S), resolution (RS) were identified for five replicate injections of the drug. These SST are performed using five replicate injections of standards before analysis of samples, 10 µL of resolution solution was injected into the chromatograph and the chromatogram was recorded. Table 2 represents the SST data of ATX.

Table 2: SST data of ATX

Title	Sample ID	Retention time	Peak area	Tailing factor	Theoretical Plates
Average*	Standard-1 to standard-5	3.16	160989	1.26	3512
% RSD		0.12	0.20	0.18	0.40

Conclusion: The % RSD of 5 Replicate injection is 0.20, Average of 5 determinations.

3.1.2 Linearity

The linearity of an analytical method is its ability to obtain test results which has a definite mathematical relation to the concentration of analyte. Figures 2a to 2f shows the different linearity levels and the Fig 3 represents the calibration graph of ATX. The linearity data is represented in Table 3. Table 4 shows the summary out put of anova regression statistics of ATX.

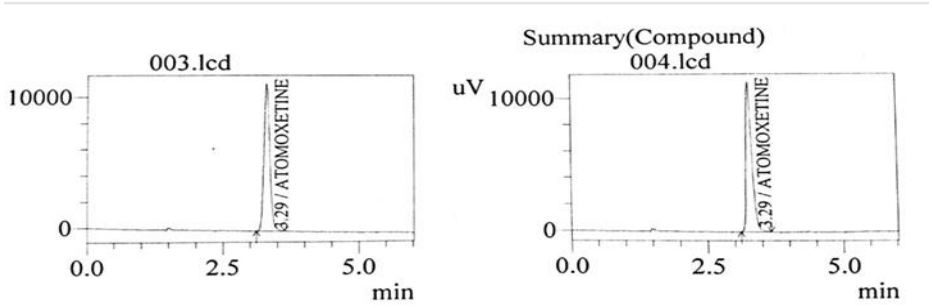


Figure 2a: 25 % level of ATX

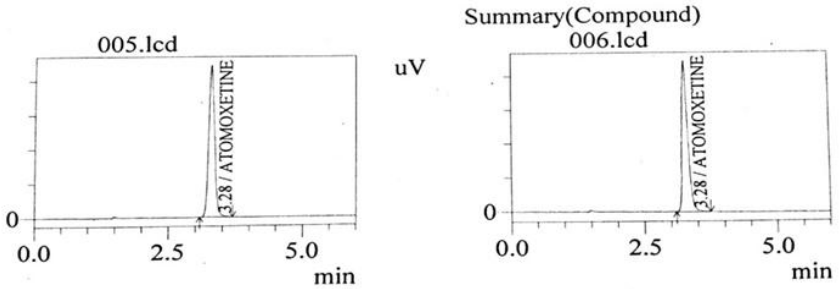


Figure 2b: 50 % level of ATX

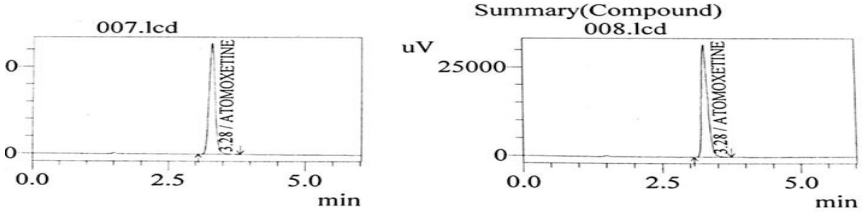


Figure 2c: 75 % level of ATX

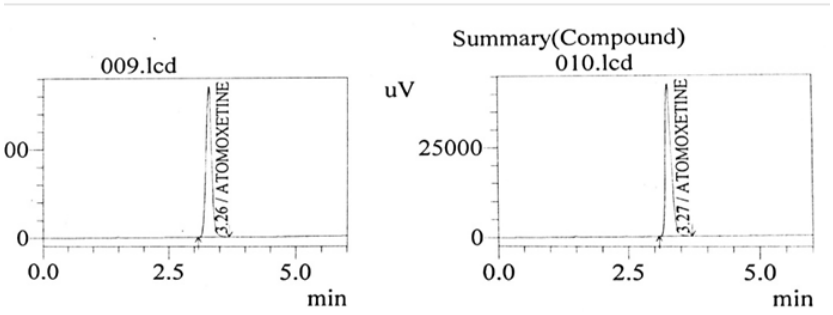


Figure 2d: 100 % level of ATX

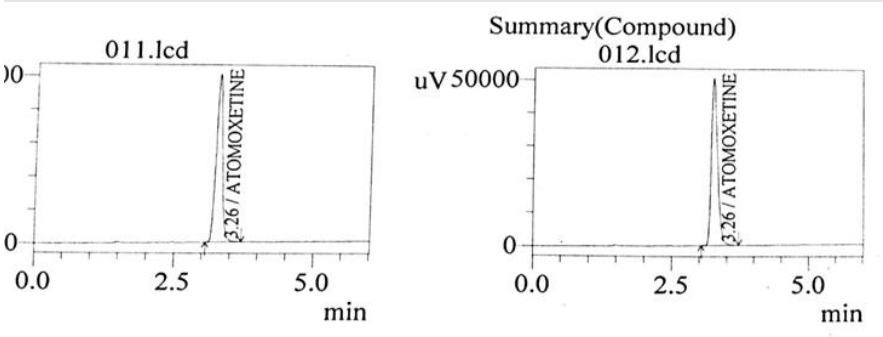


Figure 2e : 125 % level of ATX

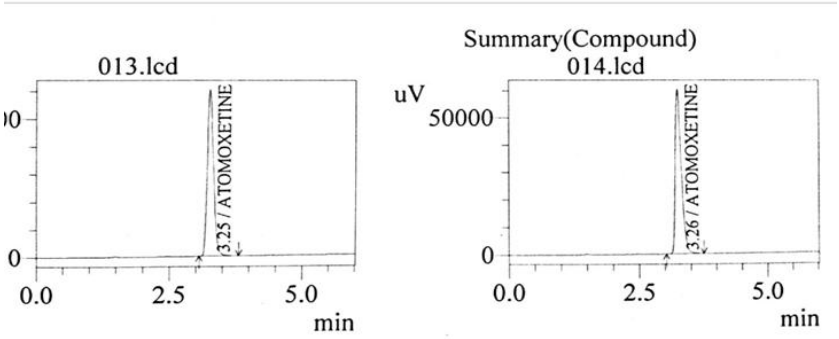


Figure 2f: 150 % level of ATX

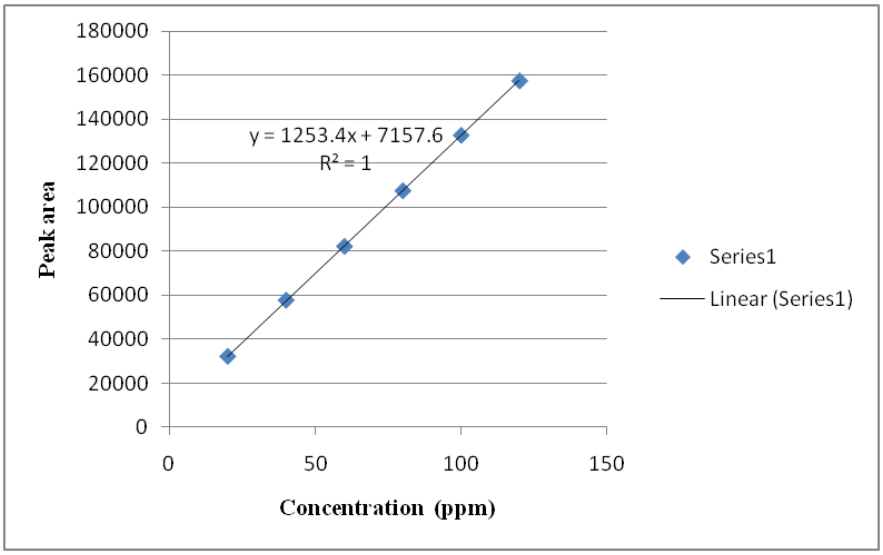


Figure 3: Calibration graph of ATX

Table.3: Linearity results with different concentration

Level std. concentration	**Volume of Stock taken**	**Dilution (mL)**	**Con. (µg/mL)**	**Area-1**	**Area-2**	**Mean area**
25	0.20	10	17.50	32994	31122	32058
50	0.40	10	35.00	57034	58257	57646
75	0.60	10	52.50	82113	82090	82102
100	0.80	10	70.00	107322	107562	107442
125	1.00	10	87.50	132123	133235	132679
150	1.20	10	105.00	157123	157771	157447
Statistical analysis	R^2					1

Observation:

The linearity of response for ATX standard was determined in the range of 15 to 105 µg/mL. The calibration curve of analytical method was assessed by plotting concentration versus peak area and represented graphically. The correlation coefficient was found to be 1. Therefore the HPLC method was found to be linear.

Table 4: Summary out put of anova regression statistics of ATX

	A	B	C	D	E	F	G	H	I
1	SUMMARY OUTPUT								
2									
3	*Regression Statistics*								
4	Multiple R	0.99998787							
5	R Square	0.99997574							
6	Adjusted R Square	0.999969676							
7	Standard Error	258.2600941							
8	Observations	6							
9									
10	ANOVA								
11		*df*	*SS*	*MS*	*F*	*Significance F*			
12	Regression	1	10997181192	1.0997E+10	164879.541	2.207E-10			
13	Residual	4	266793.1048	66698.2762					
14	Total	5	10997447985						
15									
16		*Coefficients*	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
17	Intercept	7157.266667	240.4270632	29.7689726	7.5829E-06	6489.73412	7824.79921	6489.734124	7824.799209
18	X Variable 1	1002.724571	2.469438825	406.053619	2.207E-10	995.86831	1009.58083	995.8683101	1009.580833

3.1.3 Specificity

Commonly used tablet excipients did not interfere with this method. It shows that the method is specific. Furthermore, the well-shaped peaks also indicate the specificity of the method. The specificity results are tabulated.

3.1.4 Precision

It was noted that the percentage RSD values of precision for Intra-day and inter-day precision was 0.19 and 0.23 respectively. Intra-day and inter-day % RSD values lower than 2 % clearly assuring that this method was found to be fairly precise and reproducible.

3.1.5 Accuracy (% recovery):

Sample solutions were prepared using an equivalent amount of placebo and ATX API and analyzed as per methodology. The recovery at 50 %, 100 % and 150 % levels of test concentration were prepared and injected into HPLC as per methodology. Table 5 to table 7 shows the accuracy results of ATX. Figure 4 to figure 6 shows the chromatograms of recovery. Accuracy results are represented in table 8.

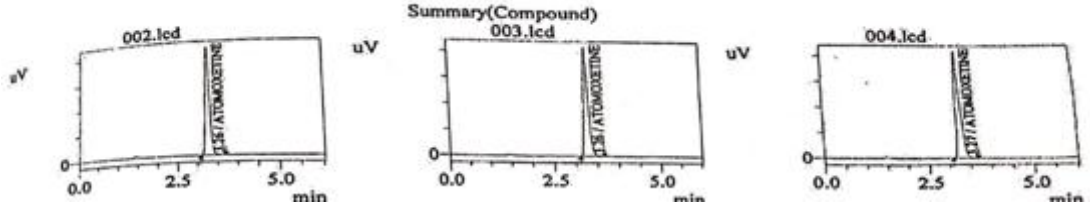


Figure 4: 50 % recovery chromatograms of ATX

Table 5: 50 % accuracy results table of ATX

Title	Sample ID	Retention Time	Area	Theoretical plates	Tailing factor
02	Std. 50 % level 1	3.26	155358	3848	1.25
03	Std. 50 % level 2	3.26	155411	3817	1.25
04	Std. 50 % level 3	3.27	155183	3811	1.25
Average		3.26	155318	3825	1.25
% RSD		0.06	0.08	0.51	0.17

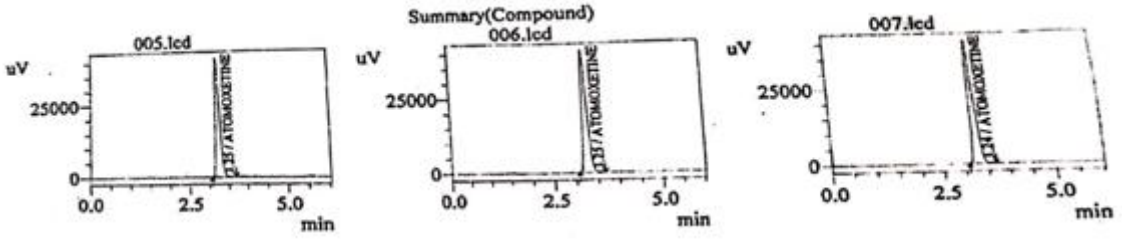


Figure 5: 100 % Recovery chromatograms of ATX

Table 6: 100 % accuracy result table

Title	Sample Id	Retention Time	Peak Area	Theoretical plates	Tailing factor
05	Std. 100 % level 1	3.25	309029	3662	1.35
06	Std. 100 % level 2	3.25	308416	3648	1.35
07	Std. 100 % level 3	3.24	309623	3625	1.35
Average		3.25	309023	3645	1.35
% RSD		0.04	0.20	0.52	0.11

Figure.6: 150 % Recovery chromatograms of ATX

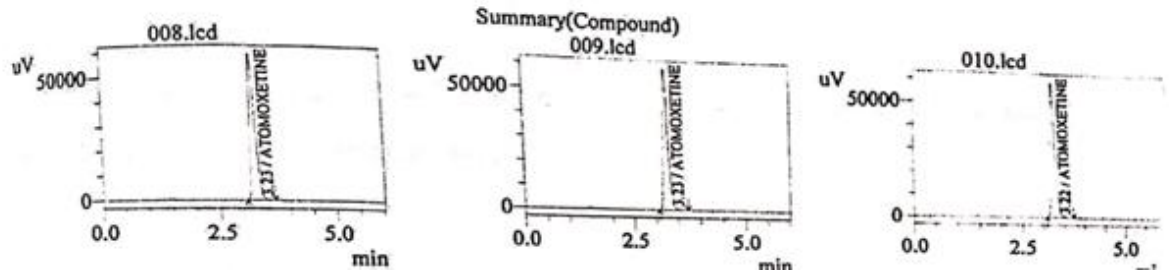


Table 7: 150 % accuracy result table

Title	Sample ID	Retention Time	Area	Theoretical plates	Tailing factor
08	Std. 50 % level 1	3.23	456823	3447	1.46
09	Std. 50 % level 2	3.23	457085	3429	1.46
10	Std. 50% level 3	3.22	455153	3474	1.46
Average		3.23	456353	3450	1.46
% RSD		0.15	0.23	0.67	0.27

Table 8: Accuracy table results

Concentration	Amount added (mg)	Amount Recovered (mg)	% recovery
50 % level	10.11	10.18	100.69
100 % level	20.30	20.25	99.75
150 % level	30.13	29.90	99.25
Statistical analysis			Mean= 99.896 SD = 0.7311 % RSD=0.731

Acceptance criteria: % Recovery values should be in the range of 98 % - 102 % with % RSD NMT.2.0.

Conclusion

The Recovery results indicated that the method had an acceptable level of accuracy for the assay of ATX at 50 %, 100 % and 150 % of test concentration.

3.1.6 Precision:

The system precision is checked by using standard chemical substance to ensure that the analytical system is working properly. The retention time and area of six determinations was measured and RSD was calculated. Blank and Standard solutions were injected six times into the HPLC and the chromatograms were recorded to obtain RSD. Fig 7 to Fig 8 shows the intra-day and inter-day chromatograms and the intra and inter day precision results are represented in table 9 and table 10 respectively.

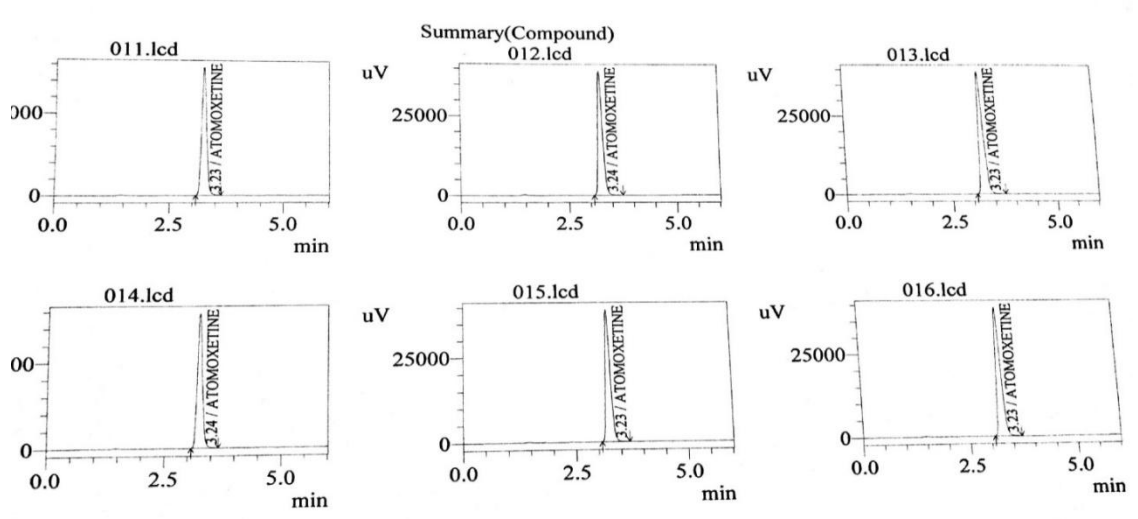


Figure 7: Chromatograms for intraday precision

Table 9: Intraday precision results of ATX

INTRA DAY PRECISION						
S.NO	**Sample name**	**Ret. Time**	**Area**	**Theoretical plate**	**Tailing Factor**	**Assay**
Average*		3.20	293818	3666	1.33	99.521
% RSD*		0.12	0.19	0.58	0.29	0.13

*Average of 6 determinations.

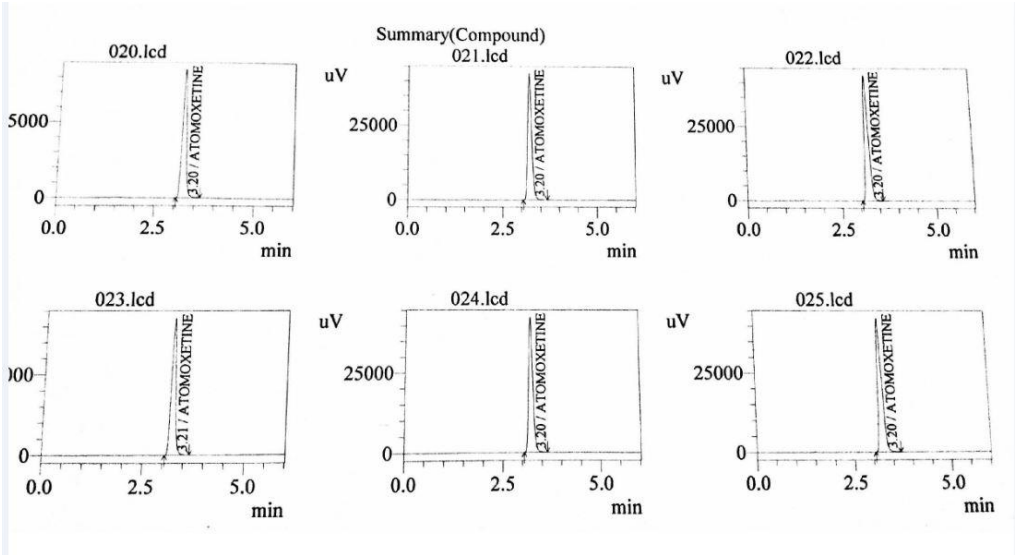


Figure 8: Chromatograms for inter day precision

Table 10: Inter-day precision results

Inter day precision						
S.NO	**Sample name**	**Ret. Time**	**Area**	**Theoretical plate**	**Tailing Factor**	**Assay**
Average*	ATX	3.23	293670	3666	1.35	100.12
%RSD*	ATX	0.10	0.23	0.41	0.28	0.13

* Average of 6 determinations.

Conclusion: The % RSD of intraday and interday precision was found to be 0.19 % and 0.23 % respectively. As per ICH guidelines % RSD should be less than 2 % is accepted. Hence the method was found to be precise.

3.1.7 LOD and LOQ

Limit of detection and limit of quantification

LOD (also called detection limit) – the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero. LOQ the lowest concentration of analyte that can be determined with an acceptable repeatability and trueness. LOD and LOQ results are shown in the table 11.

$$LOD = 3.3 \times \sigma / S$$

$$LOD = \frac{3.3 \times 258.2}{1253.4} = 0.6799\ \mu g/mL$$

$$LOD = 10 \times \sigma / S$$

$$LOQ = \frac{10 \times 258.2}{1253.4} = 2.05\ \mu g/mL$$

σ = Standard deviation of areas from the calibration curve

S = Slope of the calibration curve

Table 11: LOD and LOQ results of ATX

Limit of Detection (LOD)	0.6799 µg/ml
Limit of Quantitation (LOQ)	2.05 µg/ml

3.1.7: Robustness

The robustness of the developed method was evaluated by small deliberate changes in method parameters such as flow rate (± 0.2 ml/min), detection wavelength (± 5 nm) and mobile phase composition (± 0.5 %). The % RSD values of robustness which is less than 2 % reveals that the proposed method is robust. The results of robustness study results are shown in table 12. Even though the small changes in the conditions did not significantly effect on the peak asymmetry, plate count and retention time of ATX. Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small deliberate variation in method parameters. The robustness of a method is evaluated by varying method parameters such as percent organic content, ionic strength, pH, flow rate, and temperature. The robustness of the method was checked by varying flow rate (± 0.1 mL/min), buffer composition (± 1 %), and temperature (± 5 ˚C) and Detection wavelength (± 0.2 nm), system suitability parameters should be within the limits at all variable conditions. According to ICH guidelines tailing factor should be NMT 2.0 and theoretical plate count should be NLT 2000

Table 12: Robustness studies of ATX

S. No	Parameter	Optimized	Variation	Retention time (Rt), min	Plate count	Tailing factor	% RSD
1	Flow rate (± 0.1 ml/min)	1.0 ml/min	0.9 ml/min	2.84	3240	1.12	0.11
			1.0 ml/min	3.20	3568	1.06	0.12
			1.1 ml/min	2.86	3209	1.11	0.13
2	Column temperature variation (±5^{0}C)	40^0 C	35^0 C	3.35	3658	1.1	0.12
			40^0 C	3.20	3568	1.06	0.12
			45^0 C	2.60	3128	1.2	0.13
3	Detection wavelength (± 2 nm)	271 nm	269 nm	3.20	3562	1.12	0.13
			271 nm	3.20	3568	1.06	0.12
			273 nm	3.19	3563	1.07	0.22
3	Buffer pH (± 1)	Buffer (pH 3.0)	pH 2.99	3.18	3513	1.2	0.12
			pH 3.0	3.20	3568	1.06	0.12
			pH 3.1	3.24	3688	1.23	0.25

Acceptance criteria: Plate count NLT 2000, tailing factor NMT 1.5, % RSD NMT 2.0.
Conclusion: The robustness of the method flow rate (±0.1 mL/min), buffer composition (±1 %), and temperature (±5 ˚C), Detection wavelength (±0.2 nm), tailing factor (NMT 2.0) and theoretical plate count (NLT 2000) are in limits, hence the method was robust.

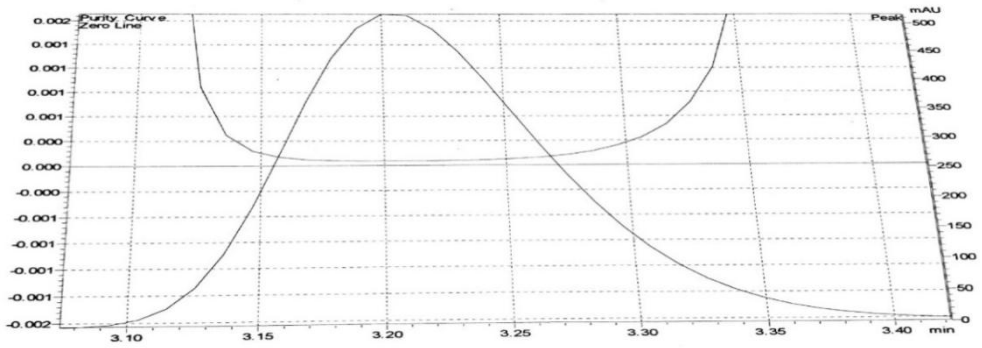
3.2 Forced degradation studies

ATX capsules and placebo were subjected to following degradation procedures and solutions were prepared. Each degraded sample was injected into HPLC as per methodology; peak purity was also established.

Degradation conditions:

a) **Acid degradation** : 5N HCl, 2.5 mL for 60 minutes at 50˚C temperature.
b) **Base degradation** : 5N NaOH, 2.5 mL for 60 minutes at 50˚C temperature.
c) **Peroxide degradation** : 30 % H_2O_2, 2.5 mL for 60 minutes at room temperature.
d) **Thermal degradation** : Heated at 70˚C for 12 hours.
e) **Humidity degradation** : 90 % RH for 12 hours.

Figure 9: peak purity plot of ATX



Impurity not detected, Peak purity index is 0.999997, Single point threshold 0.999996 and Minimum peak purity index 1. Degradation table of ATX is shown in the table 14. peak purity plot of ATX is shown in Fig 9.

Table 13: Degradation table of ATX

Degradation condition	Degradation condition	ATX %)	% degradation	Min. peak Purity index
Control	Under graded	99.42	NA	1
Acid	5N HCl, 2, 5mL, for 60 min at 50 ˚C temperature	94.93	4.47	1
Base	5 N NaOH, 2.5 mL for 60 min at 50 ˚C	97.42	1.99	1

	temperature			
Hydrogen peroxide	30 % H_2O_2, 2.5 mL for 60 min at room temperature	97.99	1.41	3
Thermal	70 ˚C for 12 hours	98.49	0.92	2
Humidity	90 % RH for 12 hours	98.38	1.02	2

Result: No interference was observed due to the placebo solution at the retention time of ATX peak in all the stress conditions. Hence, the assay method was found to be specific with respect to diluents and placebo, and the Atomoxetine peak should be pure and homogenous. There should not be any interference. Peak purity should pass as per the acceptance criteria. (Minimum peak Purity should be positive.)

Analysis of tablet formulation

The developed method was fruitfully applied for the estimation of ATX in their capsule dosage form. The assay result (table14) shows that the amount of the drug present and was outstanding agreement with the labelled value of the formulation. The representative sample chromatogram of ATX is shown in figure.

TABLE 14. Results of analysis of ATX

S. No	Formulation (Capsules)	Labelled amount mg/tablet	Amount found mg/tablet	Mean % Assay ± SD	% RSD*
1	Strattera 100 mg (ATX-100mg)	100	99.69	99.69±1	1.007

*Average of six determinations; SD: standard deviation; RSD: relative standard deviation

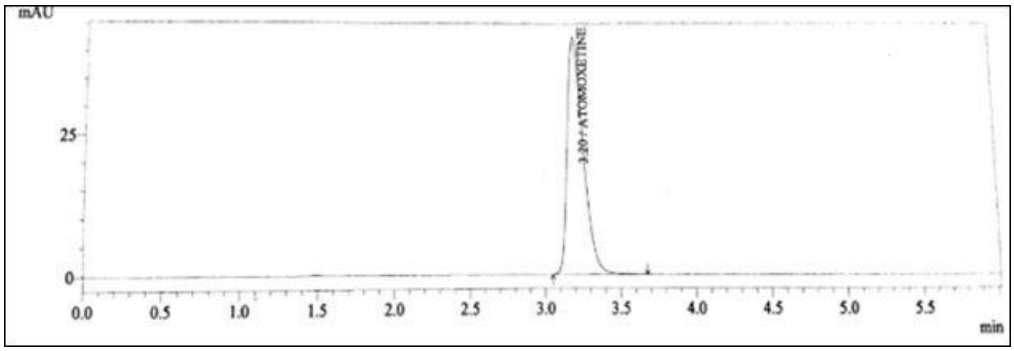


Figure 10. Sample chromatogram of ATX

Table.15: Summary of validation parameters

Validation parameters	Results
Detection wavelength (λ_{max})	271 nm
Regression equation	y = 1253.4x + 7157.6
Correlation coefficient (r^2)	1
Flow rate	1.0 ml/minute
Retention time (R_t)	3.20 minutes
Intra-day Precision (% RSD)	0.19
Inter-day Precision (% RSD)	0.23
Accuracy (% recovery)	100.31 % w/w
Limit of Detection (µg/ml)	0.6799 µg/ml
Limit of Quantitation (µg/ml)	2,05 µg/ml
Assay (% w/w)	99.69 % w/w

IV. CONCLUSION

In conclusion, a simple, accurate, sensitive, rapid and precise RP-HPLC method was developed and validated for the estimation of ATX in pharmaceutical dosage form. Statistical analysis for the above said results clearly demonstrates that the method is fit for the determination of ATX in tablet forms without any interference. This method can be helped for research studies, quality control and routine analysis with lesser

resources available. The results of the assay of pharmaceutical dosage forms of the developed method are highly reliable and reproducible and also high-quality agreement with the label claim of the drug. Hence the method can be utilized for the usual analysis of ATX in tablet dosage form without any interference of excipients.

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