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Spectrophotometric Quantification of Atomoxetine Hydrochloride Based on Nucleophilic Substitution Reaction With NQS

Short title: Spectrophotometric Quantification of Atomoxetine Hydrochloride

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ABSTRACT
Objectives:
A simple, sensitive, selective and cost effective colorimetric method has been entrenched for the quantitative estimation of Atomoxetine hydrochloride in bulk and formulation.
Materials and methods:
It was established based on the visible reaction between Atomoxetine hydrochloride and 1,2-naphthoquinone-4-sulfonic acid sodium salt in basic medium (potassium hydroxide). The resulting orange colored chromogen exhibited absorption maximum at 474 nm.
Results
Based on the optimization studies, distilled water as solvent, 1% w/v of potassium hydroxide (2 mL) and 0.3% w/v of 1,2-naphthoquinone-4-sulfonic acid sodium salt (2 mL) were utilized in the method. The developed method was validated as per International Council for Harmonization guidelines. The linearity was found to be in concentration of 10-50 µg/mL. The method appeared to show a good correlation between concentration of Atomoxetine hydrochloride and its absorbances. The correlation coefficient (r^2) of 0.999 evidenced the same. The limit of detection and quantification were observed as 0.20 and 0.606 µg/mL, respectively for Atomoxetine hydrochloride. Accuracy and precision of the method were also evaluated, the results obtained were within the acceptance criteria (% relative standard deviation < 2.00). The % assay of Atomoxetine hydrochloride was proved to be 101.52, which is in accord with its label claim.
Conclusion
The developed method was non-complex and can be effectively employed in the analytical practices of Atomoxetine hydrochloride in pharmaceutical dosage forms.
Keywords: Colorimetry, Atomoxetine hydrochloride, Accuracy, Precision, Linearity.

Introduction
Chemically Atomoxetine hydrochloride is known as (R)-N-methyl-3-phenyl-3-(*o*-tolyloxy) propylamine hydrochloride (**Fig. 1**).[1] Being a norepinephrine reuptake inhibitor, it is used in the management of attention/deficit hyperactivity disorder. Atomoxetine hydrochloride is the subject of a monograph in Indian Pharmacopoeia. Its titration with acetous perchloric acid in the presence of acetous mercuric acetate and further potentiometric determination at the end-point was described in Indian Pharmacopiea.[2]
Several methods have been presented in literature for the determination of atomoxetine hydrochloride. A few of them include visible spectrometric methods involving oxidative-coupling with sodium per-iodate and folic reagent[3], nucleophilic substitution with 1,2-naphthoquinone-4-sulfonic acid sodium salt (NQS) in alkaline medium[4] and condensation with vanillin/*para*-dimethylamino benzaldehyde[5], Ultraviolet spectrophotometric methods using solvents, such as double distilled water[6], acetonitrile[7], 0.05 N hydrochloric acid[8], spectrofluorimetric methods based on emergence of binary complex with eosin Y in Teorell-Stenhagen buffer[9] and reaction with 4-chloro-7-nitro-2,1,3-

benzoxadiazole in chloroform[4]. Furthermore, reverse phase high performance liquid chromatographic methods with combinations of stationary and mobile phases[10-15], ultra-performance liquid chromatographic method[16] and liquid chromatography-tandem mass spectroscopic method[17] were also mentioned in the literature.

Literature survey revealed that even though there are sophisticated instrument techniques available for atomoxetine hydrochloride (reverse phase high performance liquid chromatography, ultra-performance liquid chromatography and liquid chromatography-tandem mass spectroscopy), their use is limited as they require costly instruments, solvents/reagents, skilled operators and tedious extraction protocols. It was identified that the colorimetric method reported used borate buffer-chloroform-NQS in alkaline medium and the color was developed with the aid of heat (70 °C) after 40 min of addition of the reagents.[4] NQS is one of the extensively used chromogenic reagent for the determination of primary or secondary amine containing drugs. [18] Colorimetry is persistently competitive with chromatographic techniques due to its simplicity, sensitivity and selectivity, cost effectiveness, fair accuracy, precision and easy access in most quality control laboratories for pharmaceutical analysis.[19]

Keeping these facts in view, the present work was attempted by dissolving the analyte in the most economical and easily available solvent i.e., distilled water and using NQS as reagent. Further the reaction was performed at room temperature. The details of the methods and materials adopted and the results obtained were discussed in the next chapters.

MATERIALS AND METHODS

Materials and Instrumentation

Atomoxetine hydrochloride standard (Hetero Drugs Pvt. Ltd., India) and marketed tablets (Axepta-10, contains 10 mg of Atomoxetine hydrochloride per tablet) from a local drugstore were used as received. We have used analytical grade chemicals and reagents in the establishment of analytical method. Colorimetric measurements were carried out using double–beam Shimadzu Ultraviolet–Visible Spectrophotometer 1800. The standard statistical functions were computed using options available in MS-EXCEL.

Reagents and standard solutions

NQS reagent (0.3% w/v)

An accurately weighed NQS (0.30 g) was transferred into a 100 mL volumetric flask and dissolved in appropriate volume of distilled water.

Potassium hydroxide solution (1% w/v)

In a 100 mL volumetric flask, accurately measured potassium hydroxide (1.00 g) was transferred and the volume was made with distilled water.

Standard stock solution of Atomoxetine hydrochloride

Atomoxetine hydrochloride (1000 µg/mL) was produced by solubilizing precisely weighed substance (10.00 mg) in distilled water contained in a volumetric flask (10 mL). Transferred 1 mL in to another 10 mL volumetric flask from the above stock and the volume was finally made with distilled water to acquire 100 µg/mL as end concentration.

Analysis of Atomoxetine hydrochloride

Aliquots of standard drug solution of Atomoxetine hydrochloride (100 µg/mL) ranging from 1 - 5 mL were transferred to a group of 10 mL volumetric flasks. They were shaken vigorously after adding potassium hydroxide solution (1% w/v, 2 mL) and NQS reagent (0.3% w/v, 2 mL). The volume was adjusted with water to draw up a batch of analytical solutions comprising 10 - 50 µg/mL of Atomoxetine hydrochloride. The absorbance of the resulting colored complex was recorded after 20 min at λ_{max} 474 nm against corresponding reagent blank. Beer - Lambert's plot was utilized to compute the amount of Atomoxetine hydrochloride.

Analytical method optimization

The optimum concentration of potassium hydroxide and NQS reagent, reaction time and mole ratio were studied during the course of the method development and particulars were provided in results and discussions.

Analytical method validation

Validation of the method was attempted by checking linearity, accuracy, precision, sensitivity and robustness by adopting the procedures stated in International Council for Harmonization (ICH) guidelines.[20]

Linearity

Aliquots of stock solution (100 µg/mL) of Atomoxetine hydrochloride were conveyed to a set of volumetric flasks in order to get final concentrations in the span of 10 - 50 µg/mL. Potassium hydroxide solution (1% w/v, 2 mL), NQS reagent (0.3% w/v, 2 mL) were prepended to above analyte solution and the volume was made using distilled water. Utilizing appropriate blank, the absorbance of colored chromogen was recorded after 20 min at λ_{max} 474 nm. The calibration curve was developed by plotting the absorbances against the drug concentrations.

Accuracy

Standard solutions of Atomoxetine hydrochloride were added at 80, 100 and 120% levels to pre-quantified sample solutions of Atomoxetine hydrochloride (20 µg/mL). Each sample was made triple at individual level. The

Atomoxetine hydrochloride quantity was estimated using acquired absorbance values to the regression equation.

Precision

The intra-day precision and the inter-day precision of the proposed colorimetric technique were established by estimating the responses in six replicates of Atomoxetine hydrochloride (30 µg/mL) on the corresponding day and on three distinct days in a week, respectively. The outcomes are described in terms of % RSD.

Sensitivity and robustness

The lowest detectable amount (Limit of detection, LOD) and the lowest quantifiable amount (Limit of quantitation, LOQ) in the method were determined using samples containing very low concentrations of Atomoxetine hydrochloride as stated in the ICH guidelines. The LOD was calculated by 3.3 multiplied by the ratio of standard deviation and slope. Similarly, LOQ was calculated by 10 multiplied by the ratio of standard deviation and slope. Sandell's sensitivity of the Atomoxetine hydrochloride was computed from the quotient of molecular weight and molar absorptivity. Further, the method was also tried with minor modifications in the concentration of NQS and potassium hydroxide to ensure robustness and the resulting responses were recorded.

Assay of Atomoxetine hydrochloride in marketed tablets

The marketed tablets (Axepta-10 containing 10.00 mg Atomoxetine hydrochloride) were precisely measured and grounded to fine powder. The powder analogous to 10.00 mg analyte was dispersed in distilled water (10 mL) to produce 1000 µg/mL and then it was sonicated for 5 min. Resulting mixture was then shaken vigorously and filtered using Whatman's filter paper (No: 41). Clear filtrate (1 mL) from this was shifted into a 10 mL volumetric flask and diluted with distilled water up to the mark. From this 100 µg/mL solution, 3 mL portion was moved to another 10 mL volumetric flask, 2 mL of 1% w/v of potassium hydroxide and 2 mL of 0.3% w/v of NQS were added. Final fabrication was done with distilled water. This solution was used for the estimation of Atomoxetine hydrochloride after 20 min. The absorbance was recorded at 474 nm using appropriate reagent blank. The amount of Atomoxetine hydrochloride was computed by incorporating responses into the regression equation, with correction for dilution and their results were statistically validated.

RESULTS AND DISCUSSION

Development and optimization of Analytical method

The present colorimetric method was developed based on the reaction between NQS and Atomoxetine in basic conditions at ambient temperature. The reaction yielded orange colored chromogen, which has shown maximum absorbance at 474 nm after 20 min of addition of the reagent (**Fig. 2**). The probable reaction of NQS with Atomoxetine hydrochloride in basic medium was depicted in **Fig. 3**.

Selection of solvent

The UV absorbance of Atomoxetine hydrochloride was determined by dissolving the analyte in different solvents like distilled water, methanol, ethanol and acetonitrile (Supplementary 1). Distilled water was selected as solvent among all, based on its high absorbance, low cost and ease of availability.

Optimization of concentration of potassium hydroxide and NQS

Atomoxetine hydrochloride solution (100 µg/mL, 1 mL) was shifted to a volumetric flask (10 mL), mixed with various concentrations of potassium hydroxide (2 mL, 0.5, 1.0, 1.5 and 2.0 % w/v) and NQS reagent (0.3 and 0.5 % w/v) and finally diluted with distilled water. Each analyte solution was produced by changing only one variable (either concentration of potassium hydroxide or NQS) at a time and the response of the resulting solutions was recorded at 474 nm after 20 min (**Fig. 4** and **5**). The results indicated that 0.3% w/v concentration of NQS and 1% w/v concentration of potassium hydroxide showed constant and highest absorbance among all the combinations. Therefore, the same were selected for further investigation.

Selection of wavelength

The analyte solution (10 µg/mL in distilled water) along with above mentioned reagents was scanned under visible range (400 - 800 nm) and the spectrum obtained was shown in **Fig. 2**. From the spectrum, the λ_{max} was found to be 474 nm for Atomoxetine hydrochloride. The absorbance of the analyte solutions at the same wavelength was recorded throughout this investigation.

Optimization of reaction time

The color development was monitored at discrete time interims (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min) to optimize the reaction time. Maximum absorbance was noted after 20 min (**Fig. 6**). Stable color development was noticed up to 6 h under optimized conditions.

Stoichiometry of the reaction

Continuous variation method was used to investigate the stoichiometry of the reaction. Samples were prepared with equimolar amounts of Atomoxetine hydrochloride (3.43×10^{-5} M) and NQS, while other reaction conditions were the same as mentioned earlier. The drug and reagent (NQS) were assorted to produce different mole ratios (0.2:0.8,

0.4:0.6, 0.5:0.5, 0.6:0.4 and 0.8:0.2, respectively). The stoichiometric relationship between the two variables was shown in **Fig. 7** and a mole ratio of 0.5:0.5 accorded the highest absorbance value.

Analytical method validation

The response of Atomoxetine hydrochloride at 474 nm was deliberated in 10 - 50 µg/mL concentration range. The data for calibration curve and regression analysis of calibration curve was shown in **Fig. 8**. The study resulted with a correlation coefficient (r^2) value of 0.999. From the results, it was observed that with increase in concentration of Atomoxetine hydrochloride, the absorbance was also increased linearly.

The exactness of the method was confirmed by spiking the standard analyte in 80, 100 and 120% level to the fixed concentration of Atomoxetine hydrochloride in formulation (20 µg/mL). The details were conferred in **Table 1**. They were found to be significant under specification limits, with % recovery 98.00 – 101.13 % and % RSD was found to < 2.00 for the drug. The % recovery of Atomoxetine hydrochloride for three levels was found to be satisfactory.

Atomoxetine hydrochloride at concentration 30 µg/mL was utilized to determine the repeatability (intra-day precision) and intermediate precision of the method. The data obtained in the precision studies was provided in **Table 2**. The precision of the method was confirmed by observing % RSD value less than 2.00 in both the studies.

The sensitiveness of the methodology was resolute in terms of LOD and LOQ and they were calculated as 0.2 and 0.606 µg/mL, respectively. The Sandell's sensitivity of the method was determined and it was calculated as 0.0553 µg/cm^2. The influence of minor dissimilarities in the concentration of potassium hydroxide and NQS (1.0±0.1 and 0.3±0.1% w/v, respectively) was studied to ensure robustness in the method. The results revealed that these dissimilarities did not affect the absorbance of the formed colored complex at a large extent.

Assay of atomoxetine hydrochloride in marketed tablets

The assay of commercial tablets was done to determine the reliability of the proposed method. The results obtained were compared against corresponding labeled claim. The amount of Atomoxetine hydrochloride was determined as 10.152±0.049 mg and the %assay was computed as 101.52%. The results denoted that the assay results were agreed with the respective labeled claim and there was no interference of excipients from formulation at the determined wavelength (**Table 3**).

The details of current and literature colorimetric and UV spectrophotometric methods developed for Atomoxetine hydrochloride were provided in **Table 4**. The colorimetric method described by Ulu (2011) utilized borate buffer and chloroform as solvents, the color was developed at 70 °C after 40 min. In the current method, distilled water was used as solvent and NQS in potassium hydroxide was used as chromogenic reagent. The color was developed at room temperature after 20 min. Thus the temperature and time required for the analysis of Atomoxetine hydrochloride was found to be minimal in the present investigation. Lower LOD and LOQ values established in the current colorimetric method in comparison with the literature methods, further evidences the high sensitivity of the method (**Table 4**).

CONCLUSION

The present nucleophilic addition-based spectrophotometric method developed for the quantification of Atomoxetine hydrochloride using NQS in potassium hydroxide was found to be a simple, expeditious and extraction-free strategy. Use of universal solvent, such as distilled water makes it a non-pollutant methodology (as no organic solvent was used). Further, less reaction time and analysis at room temperature will be more promising features of this method. The proposed analytical method was passed through validation parameters as per ICH specifications. The %assay was in congruent as stated on the label. Additionally, mediation of the formulation excipients was found to be nil in the estimation. With these benefits, the suggested methodology can be adopted in routine quality control testing of Atomoxetine hydrochloride in its pharmaceutical dosage forms.

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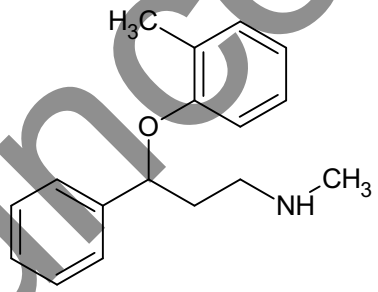

Fig. 1 Structure of Atomoxetine hydrochloride

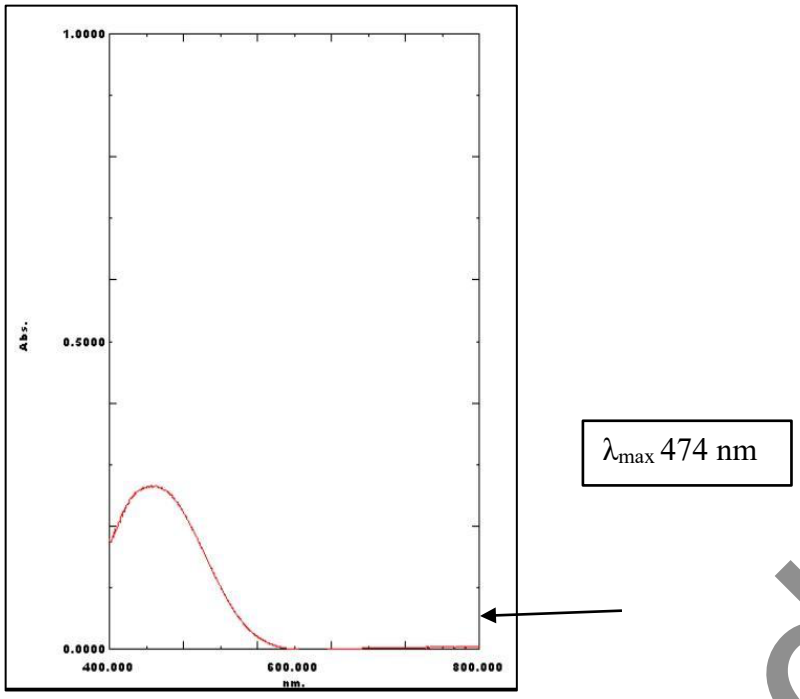

Fig. 2 UV absorption spectrum of Atomoxetine hydrochloride at λ_{max} 474 nm in distilled water (10 µg/mL)

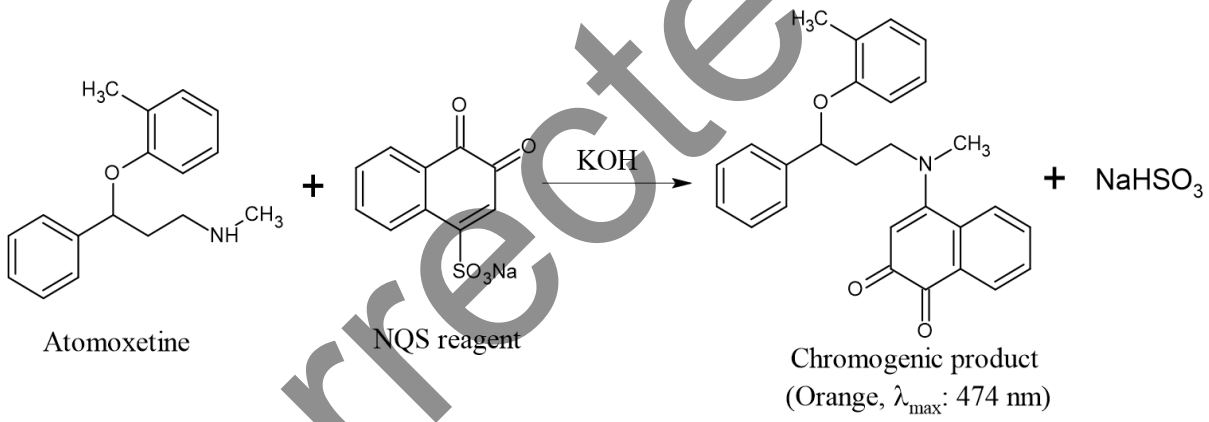

Fig. 3 Probable reaction between Atomoxetine hydrochloride and NQS

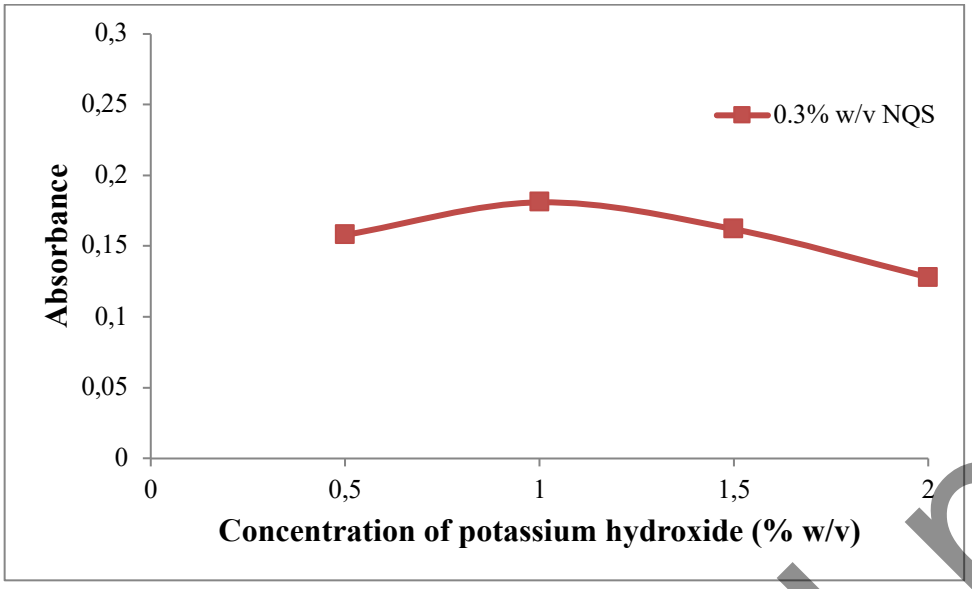

Fig. 4 Effect of concentration of potassium hydroxide and 0.3% w/v NQS

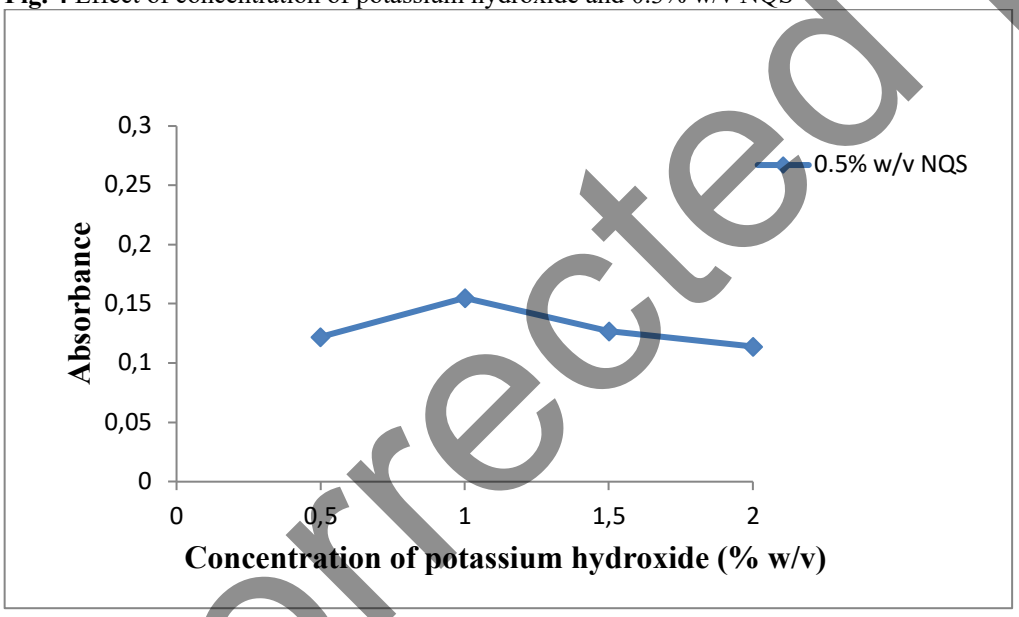

Fig. 5 Effect of concentration of potassium hydroxide and 0.3% w/v NQS

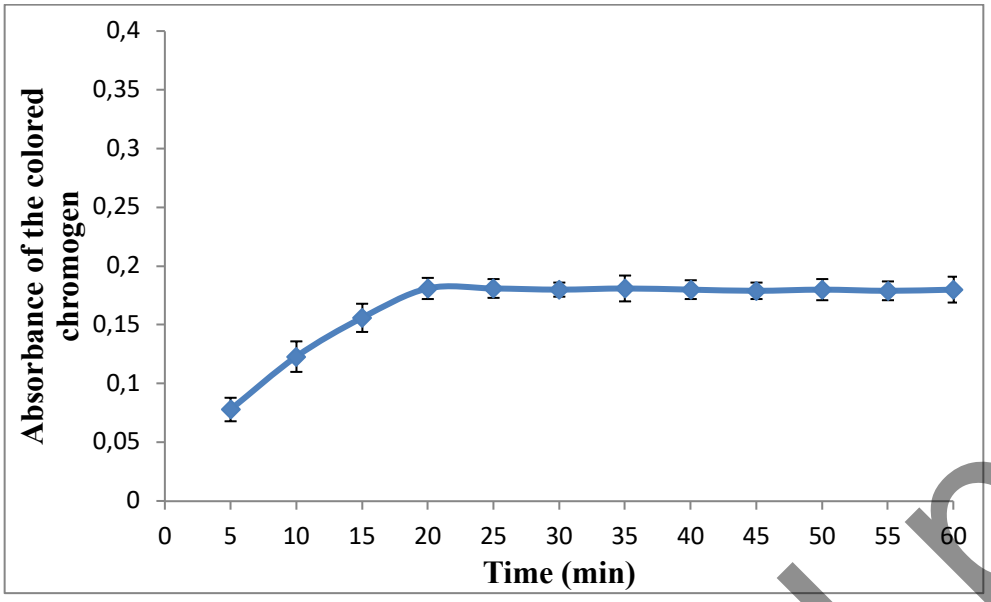

Fig. 6 Effect of time on the stability of colored complex at 474 nm

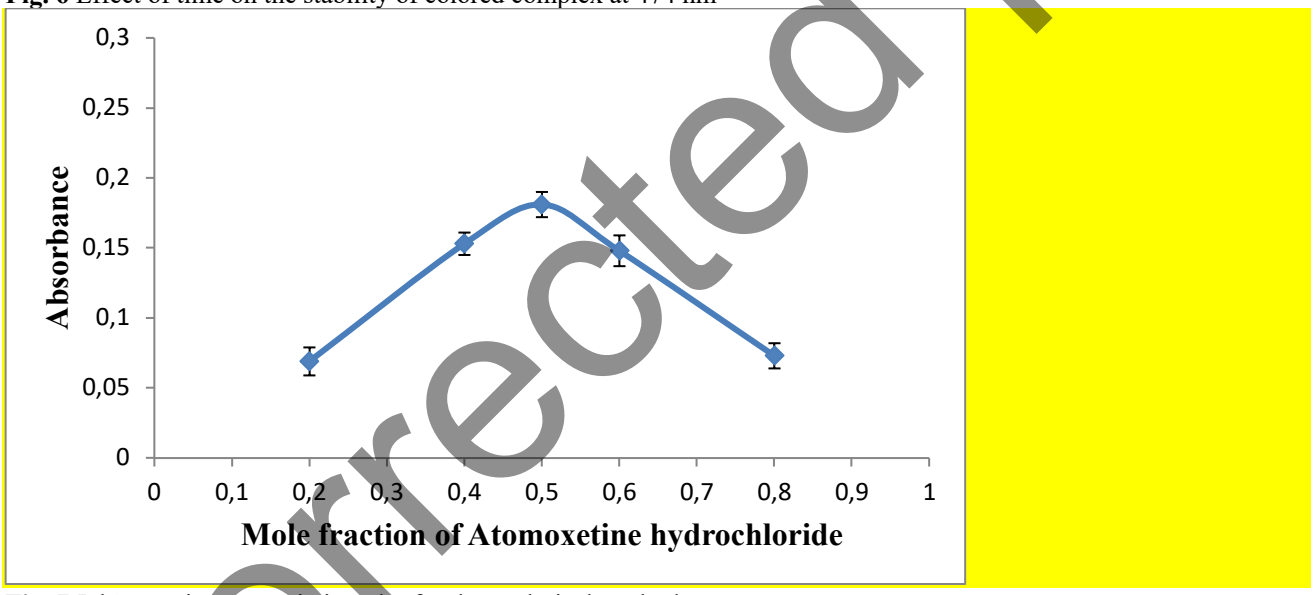

Fig. 7 Job's continuous variation plot for the analytical method

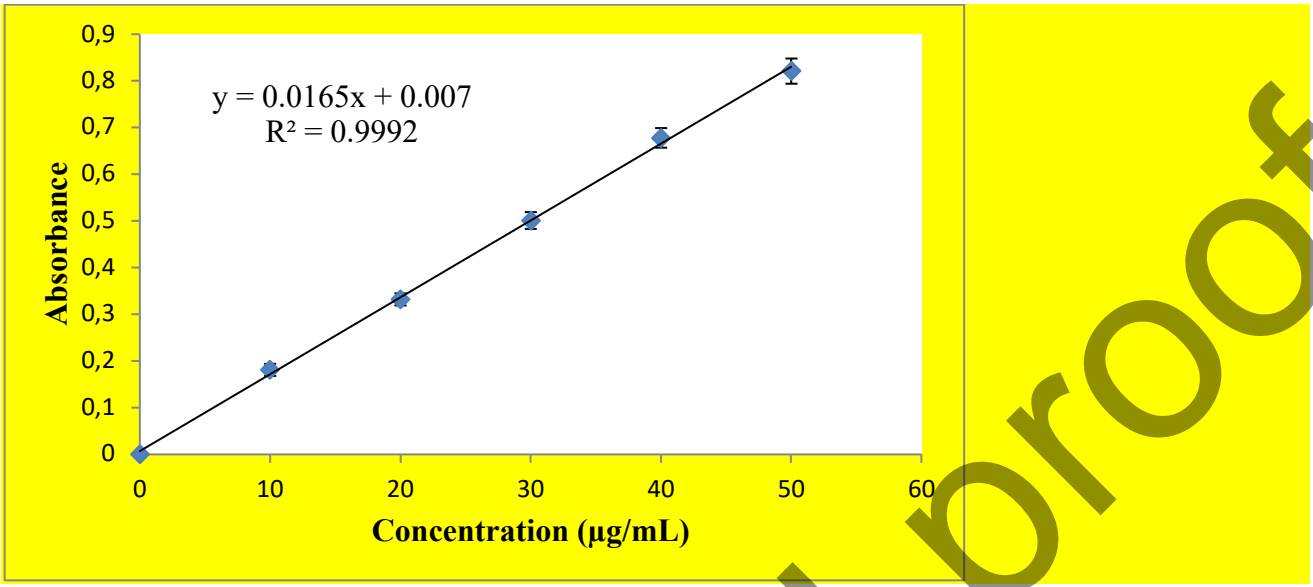

Fig. 8 Calibration plot of Atomoxetine hydrochloride (10 - 50 µg/mL) in distilled water

Table 1 Accuracy data of the analytical method

% level	Formulation (µg/mL)	Conc of std spiked (µg/mL)	Amount found (AM±SD, n=3)	Bias	% Recovery	% RSD*
80	20	16	35.283±0.301	0.174	98.00	0.85
100	20	20	40.451±0.313	0.181	101.13	0.77
120	20	24	43.524±0.675	0.389	98.92	1.55

AM: Arithmetic Mean, SD: Standard Deviation, Bias = standard deviation/square root of sample size.
*Acceptance Criteria: % RSD should not be more than 2.00

Table 2 Precision data of the analytical method

Statistical variables	Concentration estimated (µg/mL)[b]	
	Intra-day precision	Inter-day precision
AM ± SD	30.514 ± 0.548	30.646 ± 0.359
% RSD[a]	1.79	1.17

AM: Arithmetic Mean, SD: Standard Deviation.
[a]Acceptance Criteria: % RSD should not be more than 2.00
[b] Concentration taken = 30 µg/mL

Table 3 Assay data of Atomoxetine hydrochloride in tablets using developed method

Formulation	Label Claim (mg)	Amount found (mg) (AM±SD, n=3)	Bias	% Assay	% RSD*
AXEPTA-10	10	10.152±0.049	0.028	101.52	0.48

AM: Arithmetic Mean, SD: Standard Deviation, Bias = standard deviation/square root of sample size.

*Acceptance Criteria: % RSD should not be more than 2.00

Table 4 Method conditions in current and literature methods

S. No.	Method conditions	Validation parameters	Reference
1	Solvent: Borate buffer and chloroform Reagent: NQS in an alkaline medium, Temperature: 70 °C, Time for development of colour: 40 min, λ_{max}: 449 nm.	Rectilinear: 5 - 40 µg/mL LOD: 0.02 µg/mL LOQ: 0.06 µg/mL	4
2	Method A: Solvent: Methanol Reagent: Vanillin in Sulphuric acid λ_{max}: 560 nm	Linearity: 1 - 5 µg/mL	5
	Method B: Solvent: Methanol Reagent: *Para*-dimethyl amino benzaldehyde in Sulphuric acid λ_{max}: 600 nm	Linearity: 10 - 50 µg/mL	
3	Solvent: double distilled water, λ_{max}: 270 nm	Linearity: 20 - 180 µg/mL LOD: 4.04 µg/mL LOQ: 12.25 µg/mL	6
4	Solvent: Acetonitrile λ_{max}: 271 nm.	Linearity: 20 - 140 µg/mL LOD: 3.5 µg/mL LOQ: 10.62 µg/mL	7
5	Solvent: 0.05 N Hydrochloric acid λ_{max}: 225 nm.	Linearity: 5 - 40 µg/mL LOD: 0.154 µg/mL LOQ: 0.467 µg/mL	8
6	Solvent: **Distilled water**, Reagent: NQS in potassium hydroxide, Temperature: **room temperature**, Time for development of colour: **20 min** λ_{max}: 474 nm.	Linearity: 10 - 50 µg/mL LOD: 0.20 µg/mL LOQ: 0.606 µg/mL	Current method

Supplementary 1 Data for selection of solvent

S. No.	Solvent	Absorbance* (AM±SD)
1	Distilled water	0.181±0.001
2	Methanol	0.117±0.003
3	Ethanol	0.148±0.006
4	Acetonitrile	0.073±0.004

AM: Arithmetic Mean, SD: Standard Deviation

* Measured using 10 µg/mL analyte solution