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Development and Validation of a RP-HPLC Method for the Determination Atropine and its Impurities in Pharmaceutical Dosage Form as per ICH Guidelines

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Abstract

The analysis of improved RP-HPLC method for the separation and quantification of Atropine sulfate and its impurities are described. Samples are analysed by means of reverse phase (RP-HPLC) using a Phenomenex Kinetex C18 (250x4.6mm, 5µm), and the mobile phase consists of two Channels A and B. Channel-A pH 2.50 buffer: acetonitrile (950:50 %v/v) and Channel-B: pH 2.50 buffer: acetonitrile (200:800 %v/v). The flow rate is 2.0 ml/min. The column temperature was maintained at 50°C and sample temperature was maintained at (5°C) and wavelength fixed at 210nm UV-detection. It is found that the method of RP-HPLC with UV-detection system for the analysis of Atropine sulfate impurities are straight forward and applied in qualitative and quantitative analysis. The developed LC method was validated with respect to specificity, precision, linearity, ruggedness and robustness. Validation study compared as per ICH guideline.

Key words: Atropine sulfate, estimation of related substances, liquid chromatography.

Introduction

Atropine is a tropane alkaloid and anticholinergic medication used to treat certain types of nerve agent and pesticide poisonings as well as some types of slow heart rate, and to decrease saliva production during surgery (2). It is typically given intravenously or by injection into a muscle. Eye drops are also available which are used to treat uveitis and early amblyopia (3-4). The intravenous solution usually begins working within a minute and lasts half an hour to an hour (1). Large doses may be required to treat some poisonings. Chemically it is (RS)-(8-Methyl-8-azabicyclo[3.2.1]oct-3-yl) 3-hydroxy-2-phenylpropanoate. The chemical structure of Atropine sulfate shown in Figure: 1.01.

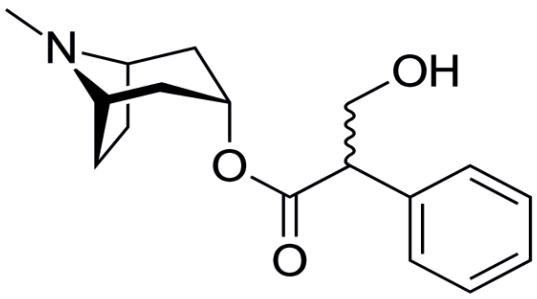

Figure: 1.01. Chemical Structure of Atropine sulfate

A few analytical methods have been reported for the determination of Atropine sulfate in pure drug, pharmaceutical dosage forms and biological samples using TLC (5-6), gas chromatography (7), LC-MS (8-9), high performance liquid chromatography (10-15), capillary zone electrophoresis (16-17), chiral separation (18),

with fluorescence detection (19), with conductometric detection (20), cation exchange (21), ion-pair high performance chromatography (22).

The objective of the present work is to develop a stability indicating HPLC method and validated as per ICH and USP validation guidelines for the estimation of Atropine sulfate in applied for routine analysis in laboratories and is suitable for the quality control of the raw materials.

Materials and Methods

Reagents and chemicals

Potassium dihydrogen orthophosphate, Sodium 1-Pentane sulfonate, Orthophosphoric acid, Acetonitrile was procured from Merck. Water (Milli-Q). All chemicals were of an analytical grade and used as received.

Instrumentation

Chromatographic separation was achieved by using an Agilent-1200, Open-lab software using, Phenomenex Kinetex C18 (250x4.6mm, 5µm), and the mobile phase consists of two Channels A and B. Channel-A pH 2.50 buffer: acetonitrile (950:50 %v/v) and Channel-B: pH 2.50 buffer: acetonitrile (200:800 %v/v). The flow rate is 2.0 ml/min. The column temperature was maintained at 50°C and sample temperature was maintained at 5°C and wavelength fixed at 210nm UV-detection. The overall run time was 25 minutes. 5 µL of sample was injected into the HPLC system.

Preparation of mobile phase and standard and sample solution

Preparation of Buffer

Accurately weighed and transferred 1.8 gm of potassium dihydrogen phosphate and 2.5 gm of Sodium 1-pentane sulfonate monohydrate into a 1000 mL of water and sonicated to dissolved the contents. Adjusted the pH of solution to 2.50 with ortho phosphoric acid. Filtered the solution through 0.45 µm membrane filter paper.

Mobile phase-A

Transfer 950 mL of pH 2.50 buffer and 50mL of Acetonitrile into 1000mL beaker mixed well. Filtered through 0.45µ membrane filter and sonicated to degas.

Mobile phase-B

Transfer 200 mL of pH 2.50 buffer and 800mL of Acetonitrile into 1000mL beaker mixed well. Filtered through 0.45µ membrane filter and sonicated to degas.

Diluent preparation

Prepared a mixture of 800 mL of Buffer with 200 mL of Acetonitrile and mixed well sonicated for 10 minutes.

Preparation of Hyoscyamine Related compound-A Stock solution

Accurately weighed and transferred 2.5 mg of Hyoscyamine Related compound-A into 50mL volumetric flask. Added 40.0 mL of diluent and sonicated to dissolved the contents. Diluted the volume with diluent and mixed well.

Preparation of System suitability solution

Accurately weighed and transferred 50.0 mg of Atropine sulfate standard into 100.0 mL volumetric flask. Added 80.0 mL of diluent and sonicated to dissolved the contents. Added 2.0 mL of Hyoscyamine Related compound-A stock solution and diluted the volume with diluent and mixed well.

Preparation of Sensitivity solution

Accurately weighed and transferred 25.0 mg of Atropine sulfate standard into 100.0 mL volumetric flask. Added 80.0 mL of diluent and sonicated to dissolved the contents. Dilute the volume with diluent and mixed well. Diluted 1.0 mL of this solution to 100.0 mL with diluent and mixed well. Further diluted 1.0 mL of the above solution to 10 mL with diluent and mixed well.

Preparation of Placebo solution

Inject as such Placebo solution.

Preparation of Sample solution

Inject as such sample solution (0.4mg/mL).

Results and Discussion

Method optimization parameters

An understanding of the nature of API (functionality, acidity, or basicity), the synthetic process, related impurities, the possible degradation pathways and their degradation products are needed for successful method development in reverse-phase HPLC. In addition, successful method development should result a robust, simple and time efficient method that is capable of being utilized in manufacturing setting.

Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 210 nm from the absorption spectrum.

Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C18 columns could be appropriately used for the separation of related substances for Atropine sulfate.

Selection of mobile phase

Different mobile phase and stationary phases were employed to develop a suitable LC method for the quantitative determination of impurities in Atropine sulfate. A number of column chemistries supplied by different manufacturers and different mobile phase composition were tried to get good peak shapes and selectivity for the impurities present in Atropine sulfate.

Poor peak shape and resolution was observed when Hypersil BDS C18 (250 x 4.6mm, 5µ) and gradient mobile phase programmed of Mobile Phase: A pH 2.50 buffer Mobile Phase: B Acetonitrile. There was no proper resolution of impurities and analyte peak and efficiency of the peak is also not achieved and peak interferences are present.

Further trial was taken column changed from Hypersil BDS C18 to Phenomenex Kinetex C18 (250x4.6mm, 5µm), there was no proper resolution of impurities and analyte peak. Way forward, the resolution between impurity peaks and analyte peak. Next trial was taken introduce the organic solvent acetonitrile into mobile phase ‘A’ pH 2.50 buffer and acetonitrile in the ratio of 950:50 v/v and mobile phase ‘B’ pH 2.50 buffer and acetonitrile in the ratio of 200:800 v/v. The resolution of both analyte peak and impurities were well separated. These chromatographic conditions were selected for validation studies.

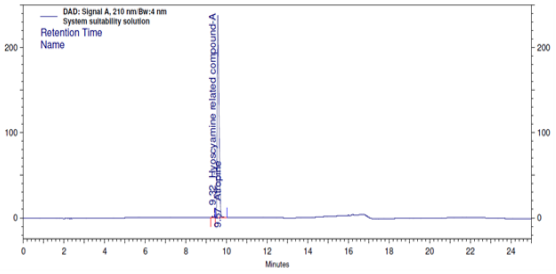

Figure: 1.02 typical chromatogram of system suitability \

Method Validation

Specificity

Specificity was demonstrated by injected blank solution, placebo solution, standard solution, sample solution and individual impurities and analyzed as per the test method.

Table: 1.01 Impurity interference data (Specificity results)

S .No	Sample	Retention time (min)	Blank	Placebo
1	Blank	ND	NA	NA
2	Placebo	ND	NA	NA
3	Standard solution	8.83	No	No
4	Sample solution	8.83	No	No
5	Tropic acid	4.71	No	No
6	7-Hydroxyhyoscyamine	5.77	No	No
7	Scopolamine	6.29	No	No
8	6-Hydroxyhyoscyamine	6.53	No	No
9	Hyoscyamine related compound-A	8.55	No	No
10	Littorine	9.84	No	No
11	Apoatropine	14.67	No	No

It was observed that known impurities are not co eluting with each other and main analyte peak

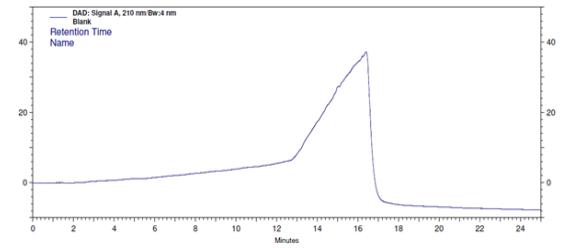

Figure: 1.03 typical chromatogram of Blank

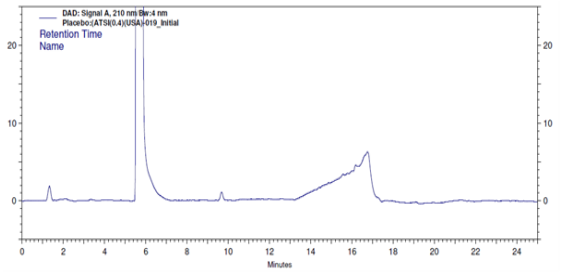

Figure: 1.04 typical chromatogram of Placebo

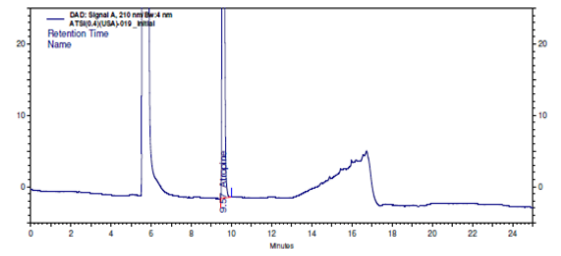

Figure: 1.05 typical chromatogram Sample

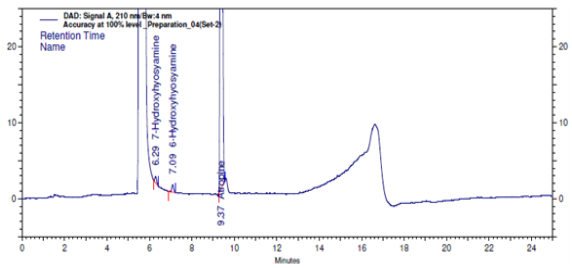

Figure: 1.06 typical chromatogram Spiked Sample (Set-I)

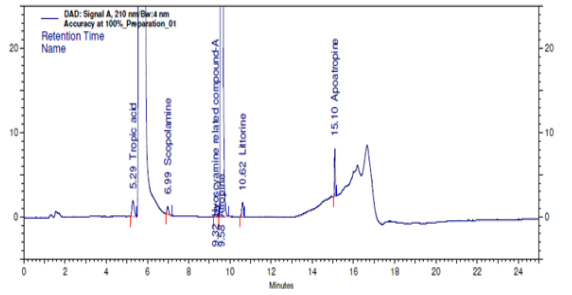

Figure: 1.07 typical chromatogram Spiked Sample (Set-II)

Interference from degradation products

A study was conducted to reveal the effective separation of degradants/impurities from Atropine. sample solutions and placebo solutions were exposed to the following stress conditions to degradation. Stressed and unstressed samples were injected into the

HPLC system with photo diode array detector by following test method conditions. All degrading peaks were resolved from Atropine peak in the chromatograms of all samples and placebo did not show any interference at the retention time of Atropine and impurities under the above conditions.

Table: 1.02 Forced Degradation results

S. No.	Degradation	Assay (%)	Total Impurities (%)	Mass Balance (%)	Purity Index	Peak Purity
01	Control sample	100.6	0.07	NA	1.0	Pass
02	Acid degradation (0.025N HCl/80°C/15hr)	99.1	0.51	98.95	1.0	Pass
03	Base Degradation (0.0025N NaOH/RT/1hr)	90.7	5.13	95.19	1.0	Pass
04	Peroxide Degradation (0.075% H_2O_2/80°C/15hrs)	100.3	0.04	99.67	1.0	Pass
05		101.10	0.10	100.53	1.0	Pass

There is no interference of Blank peak at the retention time Atropine sulfate and its impurities.
There is no interference of placebo peak at the retention time of Atropine sulfate and its impurities.
There is no interference of impurities at the retention time of Atropine sulfate.
Peak purity of analyte peak meeting the acceptance criteria.
Mass balance of each condition is within the acceptance criteria (Mass balance should be 95.0% to 105.0%). From the above observations, it is found that method is specific.

Precision

System Precision

System precision was demonstrated by prepared standard solution as per the test method and injected for six times into HPLC system. The retention time and area response of analyte peak were recorded.

Table: 1.03 System Precision data for Atropine sulfate

System Precision		
	Area response	**Retention time**
1	3040948	8.83
2	3044775	8.83
3	3015064	8.83
4	3028604	8.85
5	3040706	8.84
6	3045353	8.83
Average	**3035908**	**8.835**
STDV	**11866.5**	**0.01**
% RSD	**0.4**	**0.09**

The %RSD of peak area for Atropine sulfate was

Table 4: Effect of Ethanolic Extract of *Corchorus trilocularis* on Ulcer Index in Ethanol induced Gastric Ulceration in Rats

Groups	Treatment	Dose	Ulcer Index (Mean ± SEM)	% Protection
I	Control	--	5.66±0.802	--
II	Standard (Lansoprazole 8mg/kg)	8mg/kg	1.416±0.3***	75.08
III	70% Ethanolic extract of Corchorus trilocularis	250mg/kg	3.33±0.27**	41.16
IV	70% Ethanolic extract of Corchorus trilocularis	500mg/kg	3.08±0.35**	45.58

found to be 0.4% which is below 5.0% indicates that the system gives precise result.

Method Precision

Method precision was demonstrated by prepared six samples by spiking of impurities at specification level and analyzed as per the test method. The samples were prepared as per the method and the result for precision study is tabulated in **Table: 1.04 & 1.05.**

The results were well within the limits. From the above results, it is concluded that method is precise.

Table:1.04 Results of method precision

S.No.	Sample Name	Tropic acid	7-Hydroxyhyoscyamine	Scopolamine
1	Sample -01	0.59	0.40	0.46
2	Sample-02	0.58	0.44	0.46
3	Sample -03	0.58	0.43	0.44
4	Sample -04	0.58	0.43	0.44
5	Sample -05	0.59	0.44	0.44
6	Sample -06	0.58	0.44	0.44
Average		0.58	0.43	0.45
Std. Dev		0.01	0.02	0.01
% RSD		0.89	3.60	2.31

Table: 1.05 Results of method precision

S.No.	Sample Name	6-Hydroxy Hyoscyamine	Hyoscyamine RC-A	Littorine	Apoatropine
1	Sample -01	0.52	0.53	0.61	0.65
2	Sample-02	0.51	0.52	0.61	0.64
3	Sample -03	0.51	0.54	0.60	0.64
4	Sample -04	0.50	0.53	0.62	0.65
5	Sample -05	0.51	0.53	0.61	0.64
6	Sample -06	0.50	0.53	0.63	0.64
Average		0.51	0.53	0.61	0.64
Std. Dev		0.01	0.01	0.01	0.01
% RSD		1.48	1.19	1.68	0.80

Table: 1.06 LOQ for Atropine sulfate and impurities

S.No	Tropic acid	7-Hydroxy Hyoscyamine	Scopolamine	6-Hydroxy Hyoscyamine	Hyoscyamine related compound-A
1.0	8920	3367	1805	2972	5456
2.0	8914	3274	1744	3102	5349
3.0	9826	2656	1858	2666	5326
4.0	8312	3120	2174	2956	5308
5.0	8625	3195	1840	2976	4897
6.0	9184	3060	1877	3080	5814
Avg.	8963.50	3112.00	1883.00	2958.67	5358.33
Std	516.90	248.54	150.06	155.82	294.50
RSD	5.77	7.99	7.97	5.27	5.50

Limit of Quantitation (LOQ)

A solution containing 0.2880 µg/mL of Atropine, 0.6821µg/ml of Tropic acid, 0.4675 µg/mL of 7-Hydroxy Hyoscyamine, 0.4615 µg/mL Scopolamine, 0.4930 µg/mL 6-Hydroxyhyoscyamine, 0.8916 µg/mL Hyoscyaminerelated compound-A, 0.3370 µg/mL Littorine and Apoatropine 0.2106 µg/mL was injected six times. The %RSD of areas, deviations of each six replicates from the linear regression curve and average deviation for each standard were calculated. The results are presented in the following tables:

Table: 1.07 LOQ for Atropine sulfate and impurities

S.No	Littorine	Apoatropine
1.0	1867	2472
2.0	2339	2262
3.0	1932	2096
4.0	2290	2161
5.0	2213	2110
6.0	2132	2256
Avg.	2128.83	2226.17
Std.Dev	192.11	139.49
%RSD	9.02	6.27

The limit of limit of quantitation values obtained for each impurity and Atropine sulfate are within the acceptance criteria.

Linearity

The linearity of detector response for atropine and its impurities was demonstrated by preparing solutions over the range of LOQ level to 150 % level of target concentration level. The above solutions were injected into the HPLC system and the responses of the same were recorded. A plot of concentration vs. area response of peak was done. The correlation co-efficient between concentration and area response was evaluated. The results are tabulated below.

Table: 1.08 Linearity of detector response Atropine

Atropine		
% Level	Concentration (ppm)	Area
LOQ	0.2880	1914
50	1.1285	6428
80	1.8056	8659
100	2.2570	12347
120	2.7084	15462
150	3.3855	18175
R^2		0.9882
Slope		5372.2
Intercept		135.49

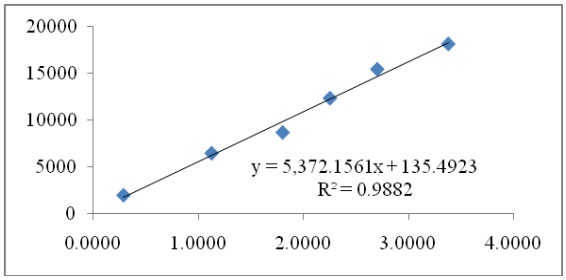

Figure: 1.08 linearity graph of Atropine

Table: 1.09 Linearity of detector response Tropic acid

Tropic acid		
% Level	Concentration (ppm)	Area
LOQ	0.6821	8807
50	1.0398	14027
80	1.6636	19746
100	2.0796	28440
120	2.4955	34617
150	3.1193	43034
R^2		0.9913
Slope		14185.2
Intercept		-1416.67

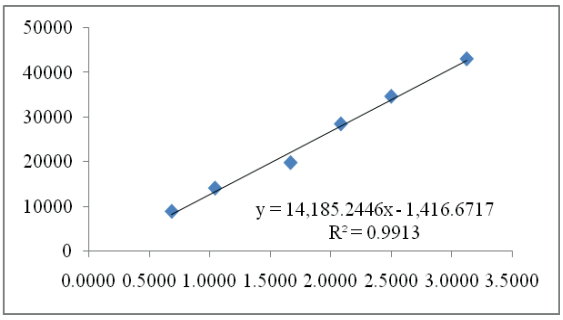

Figure: 1.09 linearity graph of Tropic acid

Table: 1.10 Linearity of detector response 7-Hydroxy Hyoscyamine

7-Hydroxy Hyoscyamine		
% Level	Concentration (ppm)	Area
LOQ	0.4675	2920
50	1.0227	6746
80	1.6363	8552
100	2.0454	13310
120	2.4544	15445
150	3.0680	20102
R^2		0.982
Slope		6556.3
Intercept		-506.60

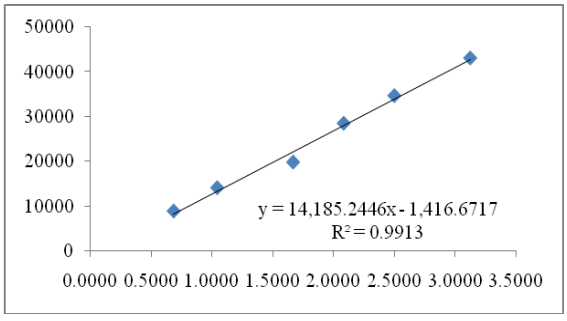

Figure: 1.10 linearity graph of 7-Hydroxy Hyoscyamine

Table: 1.11 Linearity of detector response Scopolamine

Scopolamine		
% Level	Concentration (ppm)	Area
LOQ	0.4615	1805
50	1.0438	3855
80	1.6701	6902
100	2.0876	10657
120	2.5052	12081
150	3.1314	15060
R^2		0.986
Slope		5193.4
Intercept		-1040.98

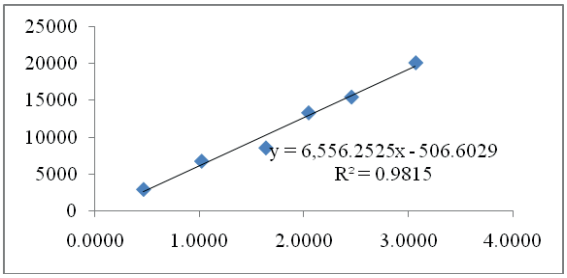

Figure: 1.11 linearity graph of Scopolamine

Table: 1.12 Linearity of detector response 6-Hydroxyhyoscyamine

6-Hydroxyhyoscyamine		
% Level	Concentration (ppm)	Area
LOQ	0.4930	3073
50	1.0043	6914
80	1.6069	11293
100	2.0087	13727
120	2.4104	16071
150	3.0130	20712
R^2		0.9983
Slope		6876.2
Intercept		-110.01

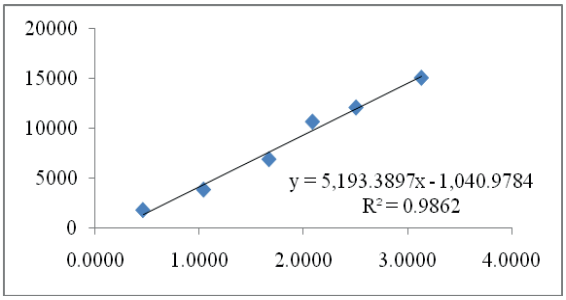

Figure: 1.12 linearity graph of 6-Hydroxyhyoscyamine

Table: 1.13 Linearity of detector response Hyoscyaminerelated compound-A

Hyoscyaminerelated compound-A		
% Level	Concentration (ppm)	Area
LOQ	0.8916	5456
50	1.0062	6648
80	1.6099	9409
100	2.0124	12636
120	2.4149	15244
150	3.0186	19298
R^2		0.995
Slope		6418.0
Intercept		-268.30

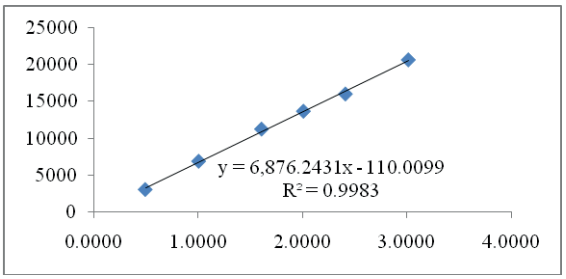

Figure: 1.13 linearity graph of Hyoscyaminerelated compound-A

Table: 1.14 Linearity of detector response Littorine

Littorine		
% Level	Concentration (ppm)	Area
LOQ	0.3370	1762
50	1.0298	6691
80	1.6477	10831
100	2.0597	14365
120	2.4716	17540
150	3.0895	22223
R^2		0.999
Slope		7459.5
Intercept		-987.17

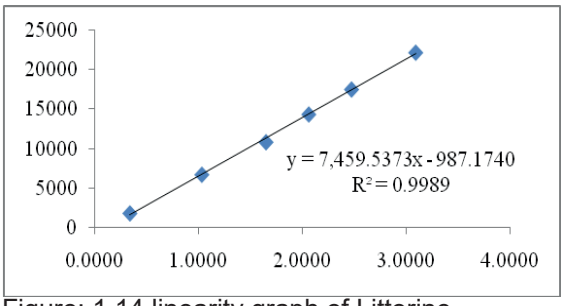

Figure: 1.14 linearity graph of Littorine

Table: 1.15 Linearity of detector response Apoatropine

Apoatropine		
% Level	Concentration (ppm)	Area
LOQ	0.2106	2405
50	1.0049	12836
80	1.6079	19558
100	2.0099	25888
120	2.4119	31260
150	3.0148	39315
R^2		0.999
Slope		13162.2
Intercept		-630.35

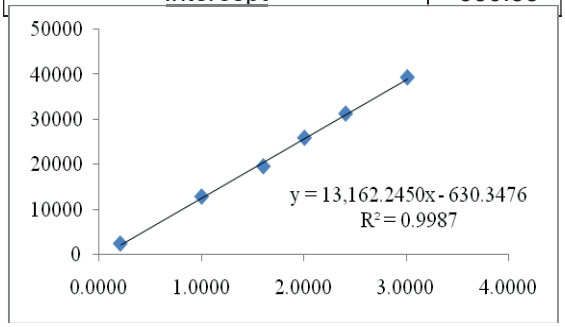

Figure: 1.15 linearity graph of Apoatropine

The linearity results for Atropine sulphate and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

Accuracy

The accuracy of the test method was demonstrated by preparing recovery samples LOQ level to 150% of level. The recovery samples were prepared in triplicate for each concentration level. The above samples were chromatographed and evaluated the percentage recovery and mean recovery.

Table: 1.16 Accuracy study of Atropine sulfate

S.No.	Theoretical (%)	% Mean Recovery			
		Tropic acid	7-Hydroxy Hyoscyamine	Scopolamine	6-Hydroxy Hyoscyamine
1	LOQ	97.9	NA	NA	81.7
2	50	106.2	88.8	83.6	96.0
3	100	108.6	85.2	86.1	98.0
4	150	104.1	84.4	83.4	99.6

Table: 1.17 Accuracy study of Atropine sulfate

S.No.	Theoretical (%)	% Mean Recovery		
		Hyoscyamine related compound-A	Littorine	Apoatropine
1	LOQ	90.5	97.9	107.6
2	50	99.8	106.2	113.8
3	100	101.8	108.6	113.2
4	150	96.3	104.1	108.4

Accuracy at LOQ level for 7-Hydroxyhyoscymine and Scopolamine is not meeting the acceptance criteria as they are eluting at shoulder of Benzyl alcohol. Therefore, range for 7-Hydroxyhyoscymine and Scopolamine, from 50 % level to 150% level is established. Range for Tropic acid, 6-Hydroxyhyoscymine, Hyoscymine related compound A, Littorine and Apoatropine is from LOQ level to 150% level. From the above results, it is concluded that method is accurate.

Solution stability

A study to establish the analytical solution stability of the standard and test preparations on bench top and refrigerator was conducted over a period of 24 hours. Standard preparation and test preparation with impurities spiked at target concentration were injected at initial and 24hours. The difference in the % of known individual impurities and the % of total impurities from initial to 24 hours was within the limits. From the above study, it was established that the standard and sample solutions were stable for 24 hours on bench top and in refrigerator.

Results and Discussion

A simple, economic, accurate and precise HPLC method was successfully developed. In this method it was carried out by using Phenomenex Kinetex C18 (250x4.6mm, 5µm)**.** Injection volume of 5µl is injected and eluted with the mobile phase eluent-A: pH 2.50

buffer: acetonitrile (950:50 %v/v) and pH 2.50 buffer: acetonitrile (200:800 %v/v), which is pumped at a flow rate of 2.0 ml/min. Column temperature 50°C and sample temperature 5°C. Detection was carried out at 210 nm. The results obtained were accurate and reproducible. The method developed was statistically validated in terms of Selectivity, accuracy, linearity, precision, and stability of solution. For Selectivity, the chromatograms were recorded for standard and sample solutions of Atropine and its related substances. Selectivity studies reveal that the peak is well separated from each other. Therefore the method is selective for the determination of related substances in Atropine. There is no interference of blank and placebo at Atropine and impurities peaks. The elution order and the retention times of Impurities and Atropine obtained from individual standard preparations and mixed standard Preparations are comparable.

The limit of quantitation (LOQ) for 0.2880 µg/mL of Atropine, 0.6821µg/ml of Tropic acid, 0.4675 µg/mL of 7-Hydroxy Hyoscyamine, 0.4615 µg/mL Scopolamine, 0.4930 µg/mL 6-Hydroxyhyoscyamine, 0.8916 µg/mL Hyoscyaminerelated compound-A, 0.3370 µg/mL Littorine and Apoatropine 0.2106 µg/mL respectively.

The linearity results for Atropine and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99.

The accuracy studies were shown as % recovery for Atropine and its impurities at specification level. The limit of % recovered shown is in the range of 80 and 120% and the results obtained were found to be within the limits. Hence the method was found to be accurate.

The relative standard deviation values of recoveries obtained for all impurities are in the range of 0.80%-3.60%. For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Atropine and its impurities. The acceptance limit should be not more than 10, and the results were found to be within the acceptance limits.

Conclusion

The new HPLC method developed and validated for determination of Quetiapine Hemi fumarate pharmaceutical dosage forms and assured the satisfactory precision and accuracy and also determining lower concentration of drug in its solid dosage form by RP-HPLC method. The method was found to be simple, accurate, economical and rapid and they can be applied for routine analysis in laboratories and is suitable for the quality control of the raw materials.

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Conflict of interests

The authors claim that there is no conflict of interest.

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