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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF NEOSTIGMINE METHYL SULFATE INJECTION

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ABSTRACT

The current proposed methodology of the research is to determine the related substances present in Neostigmine by using high-performance liquid chromatographic method. the developed method was validated for their accuracy and reproducibility. Reversed-phase chromatography was performed on Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower2 photodiode array detector using Ace C18 (250 mm × 4.6 mm, 5 µm particle size) column with pH 6.4 buffer: methanol : acetonitrile in the ratio of 75:10:15 as mobile phase at a flow rate of 1.0 mL/min. by isocratic elution with UV detection at 220 nm. Recovery and Linearity was observed well within the limits (R2 = more than 0.99 for concentration range of LOQ to 150% level for linearity and the % recovery was within the ICH acceptance limits of 85-115%) for all the impurities. The limit of quantitation (LOQ) and limit of detection (LOD) were found to be less than 0.05%. The method was validated as per ICH guidelines. The RSD for intra-day and inter-day (<3.0% RSD) precision were found to be less than 1 %. The percentage recovery was in good agreement with the labeled amount in the pharmaceutical formulations. From the method validation data, it can be concluded that the method is simple, specific, precise and accurate for the determination of Neostigmine in pharmaceutical formulations.

KEYWORDS: Neostigmine, Estimation of related substances, HPLC.

INTRODUCTION

Neostigmine methyl sulfate (Figure 1.1) chemically known as [3

(dimethylcarbamoyloxy)phenyl]-trimethylazanium;methyl sulfate. Molecular formula C13H22N2O6S, molecular weight 334.40.

Neostigmine Methyl sulfate is an anticholinesterase quaternary ammonia. Neostigmine Methyl sulfate injection is associated with less initial tachycardia and better protection against the subsequent cholinergic effects of Neostigmine methyl sulfate compared to a mixture of atropine sulfate and Neostigmine methicillin. Neostigmine is mainly used for its effects on skeletal muscle in severe myasthenia and in aesthesia for the cessation of the effects of competitive neuromuscular blocking drugs.

Neostigmine methyl sulfate is extensively hydrolyzed in the blood. In one study, after intravenous administration, plasma concentration decreased to approximately 8% of its initial value after 5 minutes with a distribution Half- Life of less than one minute. The elimination Half-Life ranged from 15 to 30 minutes. Traces of Neostigmine methyl sulfate could be detected in the plasma after an hour. In a study in non-myasthenic patients, the plasma half-life was 0.89 hours.

There are several methods reported in the literature to estimate the content of neostigmine in the bulk drug and formulations using different methods such as titrimetry, UV spectroscopy and HPLC. However, no method for estimating impurities in Neostigmine tablets has been published. the author sought to develop a simple robust stability indicating the analytical method using HPLC for the estimation of impurities in the pharmaceutical substance Neostigmine. In this work, a simple analytical method was developed and validated to estimate impurities in Neostigmine through the use of reverse phase liquid chromatography in accordance with the ICH guidelines. In this work we develop a simple, fast and accurate reverse phase liquid chromatic method for the determination of Neostigmine and its impurities.

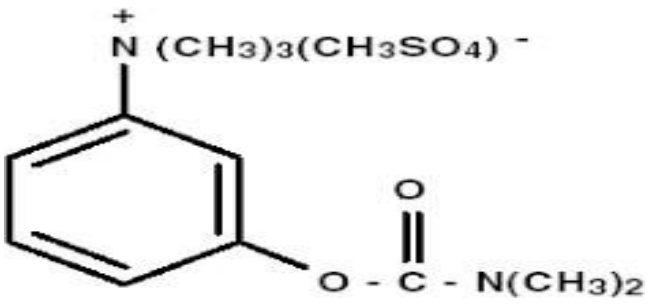


Figure 1.1: Chemical Structure of Neostigmine Metilsulfate.

1.1 Related Substance Structures

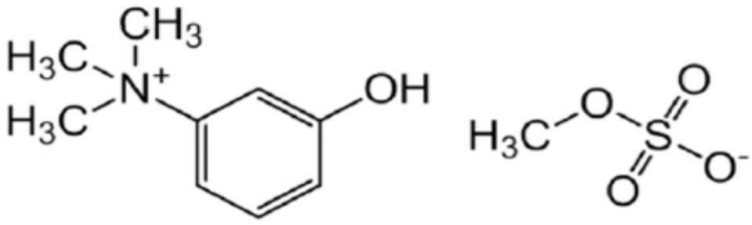


Figure 1.2: Chemical Structure of Imp-A.

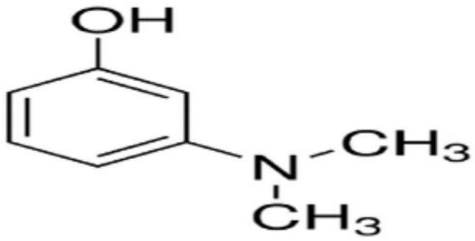


Figure 1.3: Chemical Structure of Imp-B.

II. EXPERIMENTAL

2.1 Reagents & Chemicals

Potassium dihydrogen Phosphate, Methanol, Acetonitrile (HPLC grade), Potassium hydroxide, were obtained from Merck (India). All chemicals were of an analytical grade and used as received.

2.2 Chromatographic conditions

Chromatographic separation was achieved by using a Waters 2489 UV 2695 pump, Waters 2998 PDA 2695 pump Software Empower² photodiode array detector using ACE C18 (250 mm × 4.6 mm, 5 µm particle size) column with pH 6.4 buffer: methanol: Acetonitrile (75:10:15) as mobile phase by using isocratic mode of elution at a flow rate of 1.0 mL/min. with UV detection at 220 nm. Column maintained at temperature 35 ºC, sample temperature 5ºC. The overall run time was 60 min. and the flow rate were 1.0 mL/min. 50 µl of sample was injected into the HPLC system. Retention times of impurities were 7.8 for Imp-A, 24 min for Imp-B and about 26 min for Neostigmine.

III.METHOD VALIDATION

3.1 System Suitability

Performed the system suitability by injecting the standard solution for six times as per recommendations from US pharmacopeia. Calculate the theoretical plates and tailing for main peak.

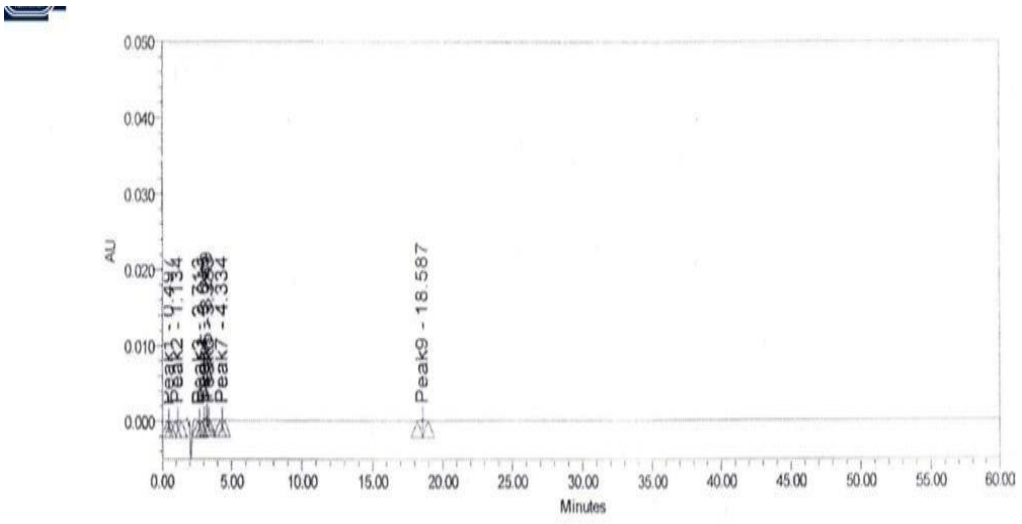


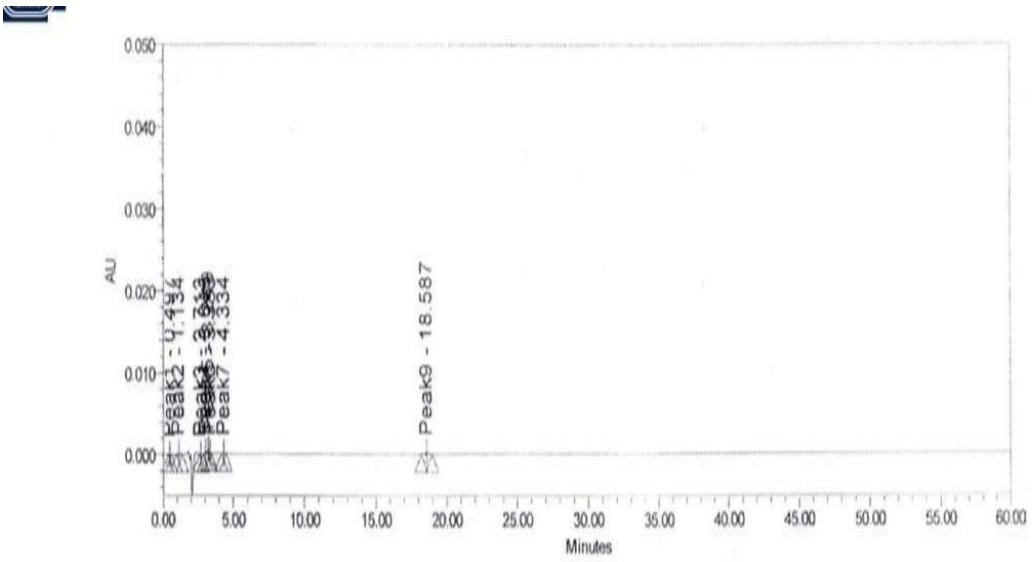
Figure: 1.5 Standard chromatogram of Neostigmine by proposed method.

Table: 1.1 Summary of system suitability.

Retention time of Neostigmine	Tailing factor for Neostigmine peak	Theoretical plates for Neostigmine peak	S/N Ratio Neostigmine sensitivity solution
26.14	1.1	16780	10.8

3.2 Specificity[8-14]

All the individual impurity solutions were prepared and injected at a specification level along with a spiked sample into the chromatograph by using the optimized chromatographic conditions along with diluent as blank.



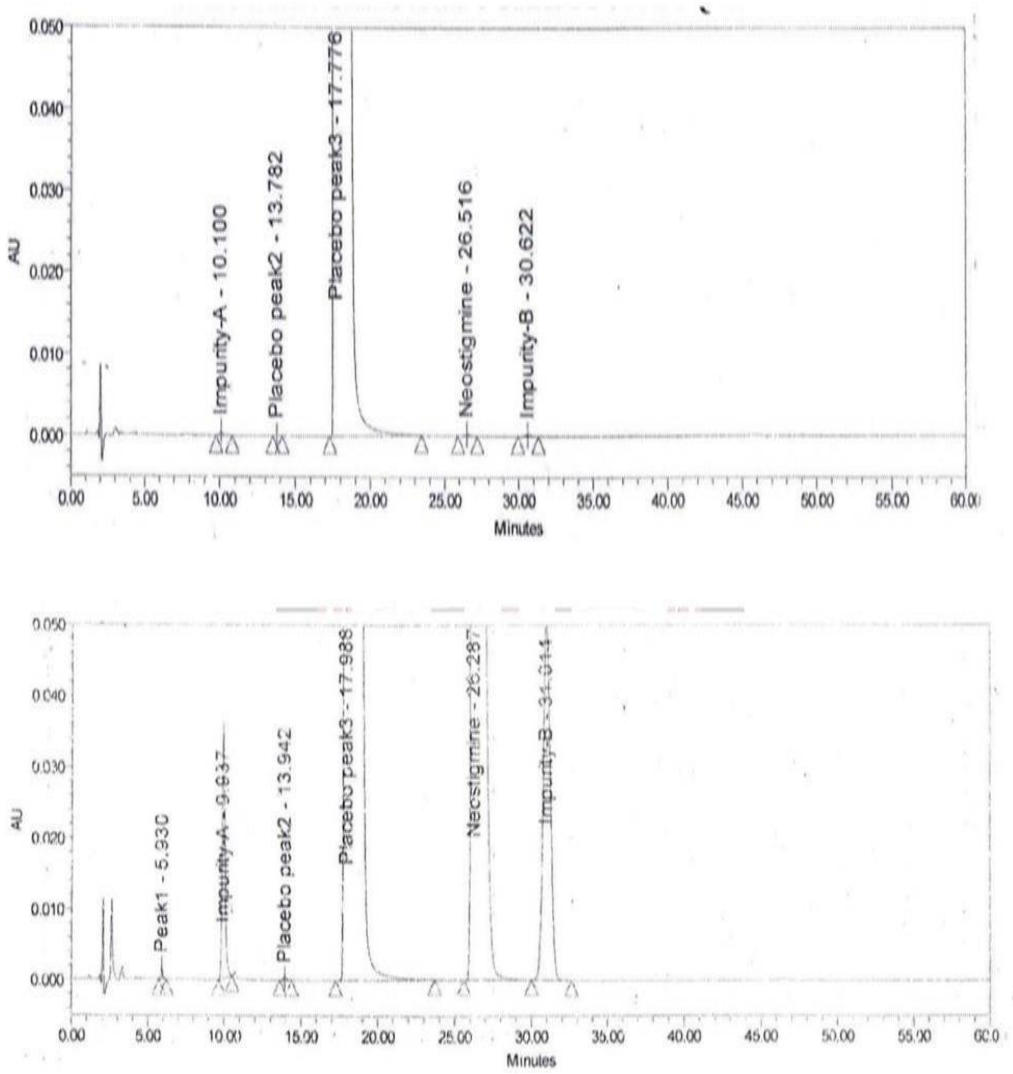


Figure 2.0: Specificity chromatogram of Spiked Solution and Forced Degradation summary.

Table: 1.3 summary of retention time, and relative retention time for known impurities.

Peak Name	Retention Time	Relative retention Time (RRT)
RC-A	7.85	0.30
RC-B	30.72	1.17
Neostigmine	26.16	1.00

The study showed that all the known impurities of Neostigmine are adequately resolved. there is no blank interference in at the retention time of impurities and main peak. hence it can be concluded that the method is selective for the determination of related substances in Neostigmine.

3.3 Limit of detection[8-14]

Table: 1.4 Limit of detection (LOD) for Neostigmine and impurities.

Component	Concentration (mg/ml)	Signal to noise	LOD (%)
Imp-A	0.06	3.2:1	0.001
Imp-B	0.02	2.9:1	0.004
Neostigmine	0.01	3.4:1	0.002

The limit of detection values obtained for each impurity and Neostigmine are within the acceptance criteria as per US Pharmacopeia.

3.4 Limit of Quantitation

Table: 1.5 Limit of Quantitation for Neostigmine and impurities.

Component	Concentration (mg/ml)	Signal to noise	LOQ (%)
IMP-A	0.16	10.9:1	0.02
Imp-B	0.28	9.9:1	0.04
Neostigmine	0.24	9.5:1	0.05

Limit of quantitation values obtained for each impurity and Neostigmine are within the acceptance criteria as per US Pharmacopeia.

3.5 Precision at LOQ

The precision at LOQ was performed by analysing six replicate injections of the standard solution containing all known impurities and Neostigmine at LOQ level. Determine the percentage relative standard deviation of peak areas of each impurity and Neostigmine. Results of peak area of impurities and Neostigmine are summarized in table 9.

Table: 1.6 Summary of peak areas for precision at LOQ.

Inj. No	IMP-A	IMP-B	Neostigmine
1	6500	6117	6536
2	6597	5958	7124
3	6461	6247	6267
4	6334	6214	6646
5	6658	5857	7130
6	6534	5913	5875
Mean	16468	5981	6582
%RSD	2.4	1.4	7.2

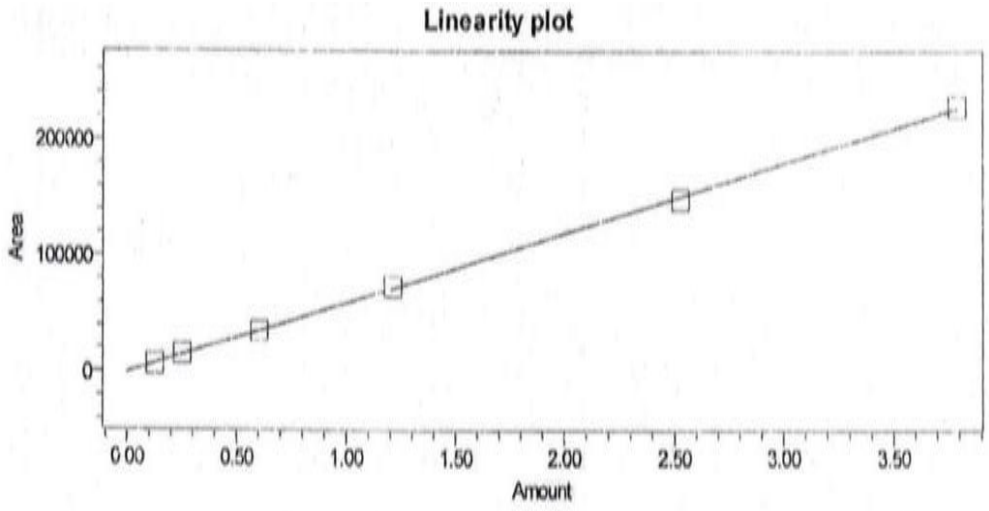


Fig. Typical Chromatogram at LOQ level for Neostigmine and its impurities.

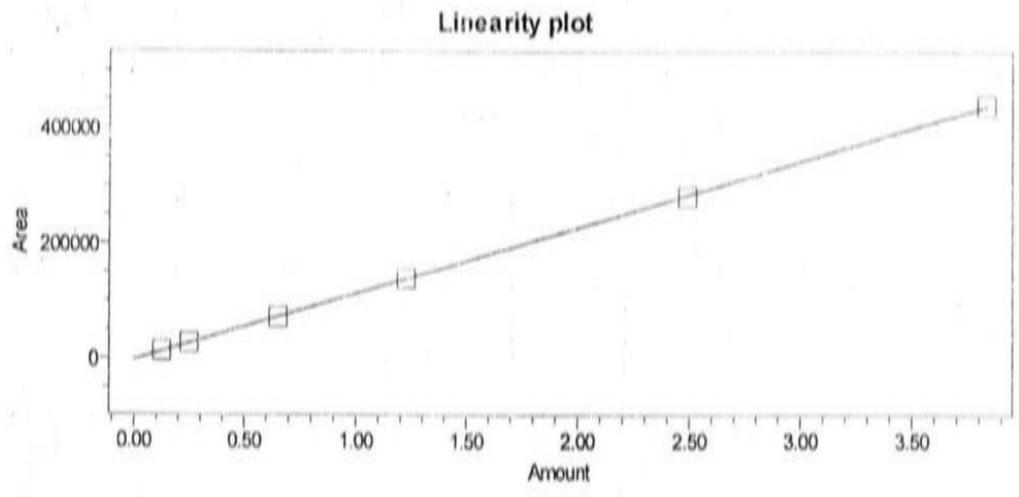
3.6 Linearity and Range[8-14]

The linearity was determined by injecting the solutions in duplicate containing known impurities and Neostigmine ranging from LOQ to 150% (LOQ, 10%, 25%, 50%, 100%, and 150%) of the specified limit. Performed the regression analysis and determined the correlation coefficient and residual sum of squares. Determine the response factor for each impurity with respect to Neostigmine.

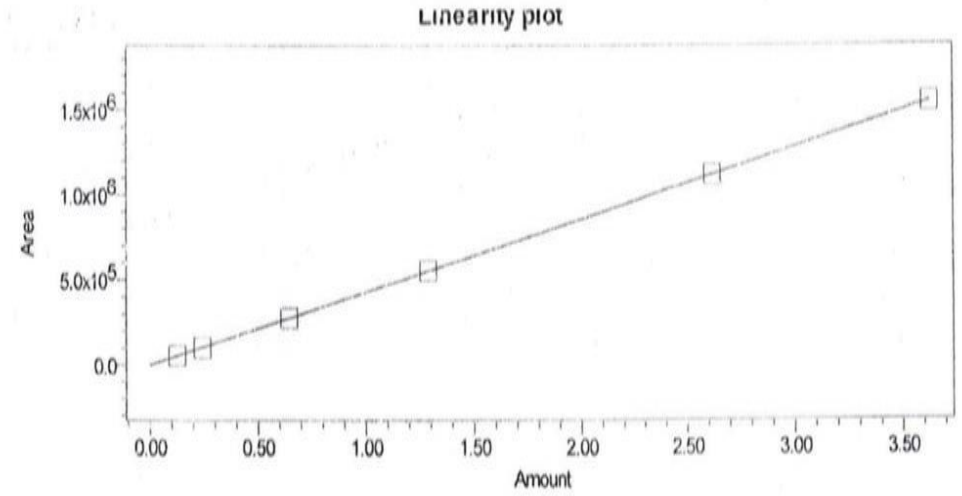
Reported the linearity range as the range for determining the impurities. Results obtained are in the tables & figures show the line of best fit for peak area versus concentration for each impurity.

Linearity Graph For Neostigmine

Linearity Graph For Imp-A



Linearity Graph For Imp-B



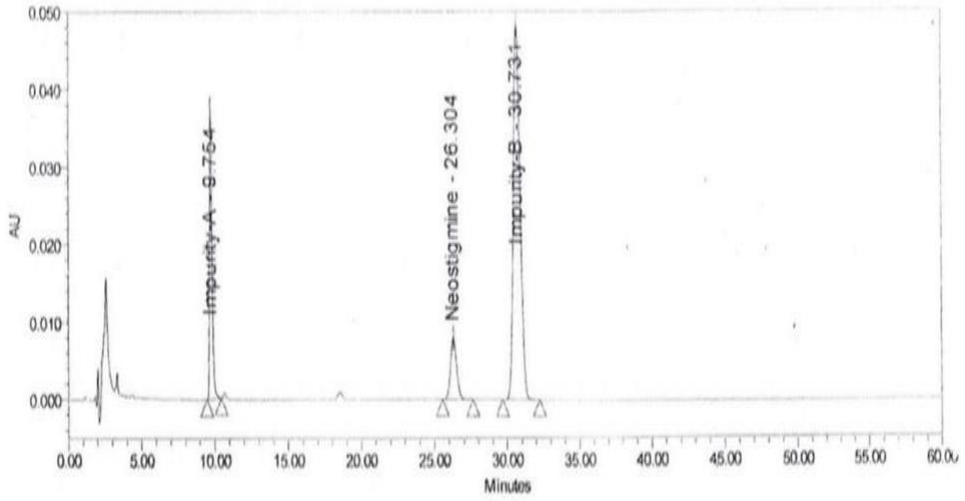


Table: 1.7 Linearity of Neostigmine and Its Impurities

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

3.7 Accuracy[8-14]

Prepared Neostigmine solution spiked with a known amount of each impurity at three levels each in triplicate (in total 12 determinations) and analysed as per the method. The impurities are to be spiked at LOQ, 100% and 150% of the specified limit. the results are summarized in table 2.1 and 2.2.

Table 2.1: Summary of % recoveries for IMP-A.

Recovery Level	Sample No.	µg\mL added	µg\mL added	% Recovery	Average %Recovery
LOQ	1	0.0613	0.0570	93.0	93.2
	2		0.0578	94.3	
	3		0.0565	92.2	
50%	1	1.2461	1.1658	93.6	93.5
	2		1.1650	93.5	
	3		1.1633	93.4	
100%	1	2.4922	2.3855	95.7	95.9
	2		2.4020	96.4	
	3		2.3843	95.7	
150%	1	3.8342	3.7603	98.1	98.3
	2		3.7830	98.7	
	3		3.7648	98.2	

Table 2.2: Summary of % recoveries for IMP-A and IMP-B.

Recovery Level	Sample No.	µg\mL added	µg\mL added	%Recovery	Average %Recovery
LOQ	1	0.0141	0.0145	102.8	101.2
	2		0.0140	99.3	
	3		0.0143	101.4	
50%	1	1.2491	1.2515	100.2	100.2
	2		1.2523	100.3	
	3		1.2500	100.1	
100%	1	2.4176	2.4430	100.1	101.2
	2		2.4495	101.3	
	3		2.4483	101.3	
150%	1	3.8279	3.8618	100.9	100.8
	2		3.8550	100.7	
	3		3.8633	100.9	

The percentage recovery values obtained for each impurity are in the range of about 92.2-102.8, which are within the specified criteria as per US pharmacopeia and ICH Q3 guideline. The relative standard deviation values of recoveries obtained for all impurities are found less than 2%.

3.8 Precision[8-14]

3.8.1 System precision

Performed the analysis of reference solution six times and determine the percentage relative standard deviation of peak area of replicate injections of each impurity and Neostigmine.

Table 2.2: Summary of peak areas of the Neostigmine standard.

Injection No	Neostigmine
1	151173
2	151046
3	150406
4	150996
5	150816
6	150180
Mean area	150976
%RSD	**0.2**

The relative standard deviation observed for Neostigmine and impurities are less than 10%. The results comply with the acceptance criteria and indicate acceptable precision of the system.

3.8.2 Method precision

The precision of the method is determined by analysing a sample of Neostigmine solution spiked with impurities at 100% of the specification limit.

Table 2.3: Summary of results for precision of the method.

Injection No	% of IMP-A	% of IMP-B
1	1.045	1.051
2	1.050	1.024
3	1.049	1.071
4	1.020	1.058
5	1.053	1.062
6	1.055	1.055
Mean (%)	1.050	1.058
% RSD	**0.3**	**0.6**

Similarly, solution stability and robustness also established and found that the method is robust enough for the estimation of related substances in Neostigmine.

IV. CONCLUSION

A simple, economic, accurate and precise HPLC method was successfully developed. In this method it was carried out by using ACE C18, (250× 4.6mm) with 5µm particle size. Injection volume of 50µl is injected and eluted with the mobile phase as Acetonitrile: Methanol and buffer of KH2PO4 pH 6.4 with potassium hydroxide over isocratic program, which is pumped at a flow rate of 1.0 ml/min. Detection, was carried out at 220 nm. all impurities are well resolved from the main peak and there is no interference from blank and placebo. The results obtained were accurate and reproducible. The method developed was statistically

validated in terms of Selectivity, accuracy, linearity, precision, robustness, and stability of solution.

For Selectivity, the chromatograms were recorded for standard and sample solutions of Neostigmine and its related substances. Selectivity studies reveal that the peak is well separated from each other and there is no interference of blank at the retention time of main peak and impurities. Therefore, the method is selective for the determination of related substances in Neostigmine.

The limit of detection (LOD) and limit of quantitation (LOQ) was found to be for IMP-A 0.014µg/ml, 0.045 µg/ml, for IMP-B 0.005 µg/ml, 0.01 µg/ml, for Neostigmine 0.007 µg/ml, 0.08 µg/ml, respectively. The linearity results for Neostigmine and all the impurities in the specified concentration range are found satisfactory, with a correlation coefficient greater than 0.99. Calibration curve was plotted and correlation co-efficient for Neostigmine and its impurities found to be more than 0.99.

The accuracy studies were shown as % recovery for Neostigmine and its impurities at LOQ, 100% and 150%. The limit of % recovered shown is in the range of 90 and 110% and the results obtained were found to be within the limits. Hence the method was found to be accurate. The accuracy studies showed % recovery of the Neostigmine and its related substances in the range 92.2-102.8 respectively. this indicates that the developed method is more accurate and reproducible over the range specified.

For Precision studies six (6) replicate injections were performed. %RSD was determined from the peak areas of Neostigmine and its impurities. The acceptance limit should be not more than 10, and the results were found to be within the acceptance limits. For intermediate precision the bias is not more than ± 0.03, the bias observed for individual impurities are within the acceptance criteria.

Hence, the chromatographic method developed for Neostigmine and its related substances are rapid, simple, sensitive, precise, and accurate. Therefore, the proposed method can be successfully applied for the routine analysis of the active pharmaceutical ingredients for assurance of its quality in active pharmaceutical ingredients and different formulation.

Further as part of future course of extended research, the method can be further applied directly for estimating impurities in pharmaceutical substances and formulation in

commercial labs as well as can be extended for identifying the impurities in the drug substances.

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