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Analytical Separation and Characterisation of Degradation Products and the Development and Validation of a Stability-Indicating Method for the Estimation of Impurities in Ipratropium Bromide Respules Formulation

AnasRasheed*¹, Dr. Osman Ahmed²

1. Research Scholar, Faculty of Pharmacy, Pacific Academy of Higher Education and Research University, Udaipur.
2. Research Supervisor, Faculty of Pharmacy, Pacific Academy of Higher Education and Research University, Udaipur.

Corresponding Author:

Anas Rasheed,
Research Scholar, Faculty of Pharmacy,
Pacific Academy of Higher Education and Research University, Udaipur.
E-mail: anasrasheed6500@gmail.com

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Abstract:

A short selective, precise, accurate and sensitive stability-indicating gradient LC-MS/MSⁿ method was developed for the quantitative determination of process-related impurities and degradation products of Ipratropium bromide in pharmaceutical respules formulations. During the stress study, the degradation products of Ipratropium bromide were well-resolved from Ipratropium bromide and its impurities and the mass balances were found to be satisfactory in all the stress conditions, thus proving the stability-indicating capability of the method. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection and quantification, accuracy, precision, ruggedness, and robustness. During the stability analysis of the drug product, one unknown impurity was detected by the above stability-indicating method. The flow rate was 0.5 ml/min and effluent was monitored at 242nm. Retention time was found to be 5.015±0.15 min. The LOD and LOQ values for were found to be 0.20996 (µg/ml) and 0.63624 (µg/ml) respectively.

1. Introduction

Ipratropium bromide is a muscarinic antagonist structurally related to atropine but often considered safer and more effective for inhalation use. It is used for various bronchial disorders. It opens up the medium and large airways in the lungs. Chemically, it is (1R,3R,5S,8R)-3-[(3-hydroxy-2-phenylpropanoyl)oxy]-8-methyl-8-(propan-2-yl)-8-azabicyclo[3.2.1]octan-8-ium bromide.

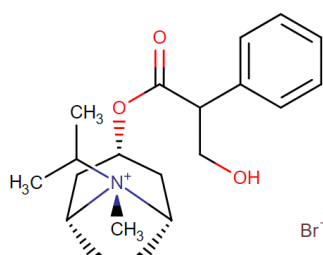


Figure.1: Molecular Structure of Ipratropium bromide

Chemical stability of Ipratropium bromide is a matter of great concern as it affects the safety and efficacy of the finished drug product. Forced degradation studies provide data to support identification of possible degradants; degradation pathways and intrinsic stability of the Ipratropium bromide molecule and validation of stability indicating analytical procedures. (ICH Q2 (R1), 2005)

A detailed literature revealed that several analytical methods have been reported for the determination of Ipratropium bromide in pharmaceutical pressurized inhalation dosage forms. (S.V Erram et al., 2006, N.A. Epshtein, 2004, E.M. Hassan, 2000)

In our present knowledge, there is no method reported for the estimation forced degradation studies of Ipratropium bromide in pharmaceutical pressurized inhalation dosage form by UPLC (Michael E Swartz, 2005).

As per the stringent regulatory requirements recommended by the ICH and regulatory agencies, it is

mandatory and important to identify and structurally characterize any impurity formed during production and stability testing, exceeding the identification threshold. Various analytical instruments and advanced hyphenated techniques are routinely used to carry out the impurity profile study.

The present work aims with the development of a method to separate the degradation product by preparative UPLC and subjected to ESI-MS/MS. The present study describes the separation of different impurities of Ipratropium bromide, as well as the development and validation of a stability-indicating RP-UPLC method for the estimation of degradation and process-related impurities of Ipratropium bromide. Forced degradation studies were performed on the drug product to show the stability-indicating nature of the method. These studies were performed in accordance with established ICH guidelines.

2. Experimental

2.1 Materials

Ipratropium bromide (99.99 % purity) used as analytical standard was procured from Active Pharma Labs (Hyderabad).

HPLC grade methanol, Acetonitrile (HPLC grade) was purchased from Qualigens fine chemicals, Mumbai, India. Distilled, 0.45 μm filtered water was used for UPLC quantification and preparation of buffer. Buffers and all other chemicals were analytical grade. The aqueous suspension for nebulizer (IpraventRespules) labeled to contain 20mcg/aem of Ipratropium bromide. All chemicals used were of pharmaceutical or special analytical grade.

2.2 Instrumentation

Acquity, Waters UPLC system consisting of a Water 2695 binary gradient pump, an inbuilt auto sampler, a column oven and Water 2996 wavelength absorbance detector (PDA) was employed throughout the analysis.

The data was collected using Empower 2 software. The column used was Hypersil BDS C18 (100 mm x 2.1 mm, 1.7 μm). A Band line sonerexsonicator was used for enhancing dissolution of the compounds. A Labindia pH System 362 was used for pH adjustment.

The electrospray ionization and MS-MS studies were performed on the triple quadrupole mass spectrometer PE Sciex Model: API 3000.

2.3 Chromatographic conditions

In order to achieve the separation under the optimized condition after experimental trials that can be summarized. Stationary phase like Hypersil BDS C18 (100 mm x 2.1 mm, 1.7 μm) column was most suitable one, since it produced symmetrical peaks with high resolution, very good sensitivity and with good resolution. The flow rate was maintained 0.5 mL min⁻¹ which shows good resolution. The PDA detector

response of Ipratropium bromide was studied and the best wavelength was found to be 242 nm showing highest sensitivity.

Table 1: Chromatographic Conditions of the validating method

Parameter	Value
Column	Hypersil BDS C18 (100 mm x 2.1 mm, 1.7 μm)
Mobile Phase	Ammonium formate and Acetonitrile in the ratio of 45:55% v/v
Flow rate	0.5 mL/min
Run time	8 Min.
Column Temperature	Maintained at ambient temperature
Injection volume	10 μL
Detection wavelength	242nm
Diluent	Mobile Phase

2.4 Preparation of Standard Stock Solution:

2.4.1 Preparation of Diluent:

The mixture of two solutions Ammonium formate and Acetonitrile in the ratio of 45:55%v/v with gradient programming was used as mobile phase at 0.5mL/min, which was found to be an appropriate mobile phase for separation of Ipratropium bromide. The column was maintained at ambient temperature.

2.4.2 Preparation of internal standard solution:

Weighed accurately about 10 mg of D-Phenylalanine working standard and transfer to 100 ml volumetric flask, add 50 ml of mobile phase and sonicate to dissolve it completely and then volume was made up to the mark with mobile phase to get 100 $\mu\text{g/ml}$ of standard stock solution of working standard. Then it was ultrasonicated for 10 minutes and filtered through 0.20 μ membrane filter.

2.4.3 Preparation of Ipratropium bromide standard solution:

Weighed accurately about 10 mg of Ipratropium bromide and transfer to 100 ml volumetric flask, add 50 ml of mobile phase and sonicate to dissolve it completely and then volume was made up to the mark with mobile phase to get 100 $\mu\text{g/ml}$ of standard stock solution of working standard. Then it was ultrasonicated for 10 minutes and filtered through 0.20 μ membrane filter. Linearity was determined in the range of 2- 10 $\mu\text{g mL}^{-1}$.

2.5 Stability Indicating Studies

Stability Indicating studies like acid hydrolysis, basic hydrolysis, wet heat degradation and oxidative degradation were carried out.

2.6 Mass Spectrometry Conditions for MS/MS

The samples (10 µL) is injected directly into the source by the flow injection method using Ammonium formate and Acetonitrile in the ratio of 45:55%v/v as mobile phase at a flow rate of 0.5 mL/min. The mass spectra were recorded in ESI negative mode. Ultra-high purity nitrogen and helium were used as curtain and collision gas, respectively. The typical ion source conditions were: nebulizer gas, 60 psi; dry temperature, 325°C; dry gas, 5.0 mL/min; capillary voltage, 5kV; capillary current, 80.243 nA; vapourizer temperature, 400°C; dwell time, 200 ms. For the collision-induced dissociation (CID) experiments, the precursor ion was selected using the quadrupole analyzer and product ions were analyzed by the time-of-flight analyzer. HRMS data acquisition was performed by the following source conditions: capillary voltage, 5 kV; declustering potential (DP) and collision energy (CE) were -60 V and -10 V, respectively; focusing potential, 220 V; resolution 40,000 (FWHM).

3. Results and discussions

3.1 Validation

The analytical method was validated with respect to parameters such as linearity, precision, specificity and accuracy, limit of detection (LOD), limit of quantitation (LOQ) and robustness in compliance with ICH guidelines.

3.2 Linearity and Range:

The linearity of an analytical procedure is the ability to obtain test results that are directly proportional to the concentration of an analyte in the sample.

The calibration curve showed good linearity in the range of 2 – 10 µg/ml for Ipratropium bromide (API) with correlation coefficient (r^2) of 0.9967. A typical calibration curve has the regression equation of $y =$

$3376.75x + 12447.9$ for Ipratropium bromide. Results are given in Table 2.

3.3 Limit of detection (LOD) and limit of quantitation (LOQ):

The LOD and LOQ of Ipratropium bromide were calculated by mathematical equation. $LOD = 3.3 \times \text{standard deviation} \div \text{slope}$ and $LOQ = 10 \times \text{standard deviation} \div \text{slope}$. The LOD of Ipratropium bromide was found to be 0.20996 (µg/ml) and the LOQ of Ipratropium bromide was found to be 0.63624 (µg/ml). Results are given in Table 2.

3.4 Precision

The precision of the method was studied in terms of intraday and interday precision of sample injections (4 µg/ml). Intraday precision was investigated by injecting six replicate samples of each of the sample on the same day. The % RSD was found to be 0.05%. Interday precision was assessed by analysis of the 6 solutions on three consecutive days. The % RSD obtained was found to be 0.04%. Low % RSD values indicate that the method is precise. The results are given in Table 3.

3.5 Accuracy

To study the accuracy of method, recovery studies were carried out by spiking of standard drug solution to pre-analyzed sample at three different levels i.e., at 50, 100, and 150%. The resultant solutions were then re-analyzed by the proposed method. At each level of the amount, six determinations were performed. From the data obtained, the method was found to be accurate. The % recovery and %RSD were calculated and presented in Table 4.

3.6 Robustness

Small deliberate changes in chromatographic conditions such as change in temperature ($\pm 2^\circ\text{C}$), flow rate ($\pm 0.1\text{ml/min}$) and wavelength of detection ($\pm 2\text{nm}$) were studied to determine the robustness of the method. The results were in favour of (% RSD < 2%) the developed UPLC method for the analysis of Ipratropium bromide. The results are given in Table 5.

Table 2: Summary of validation parameters for the proposed method

PARAMETER	IPRATROPIUM BROMIDE
Linearity	2 – 10 µg/ml
Intercept (c)	12447.9
Slope (m)	3376.75
Correlation coefficient	0.9967
LOD	0.20996 (µg/ml)
LOQ	0.63624 (µg/ml)

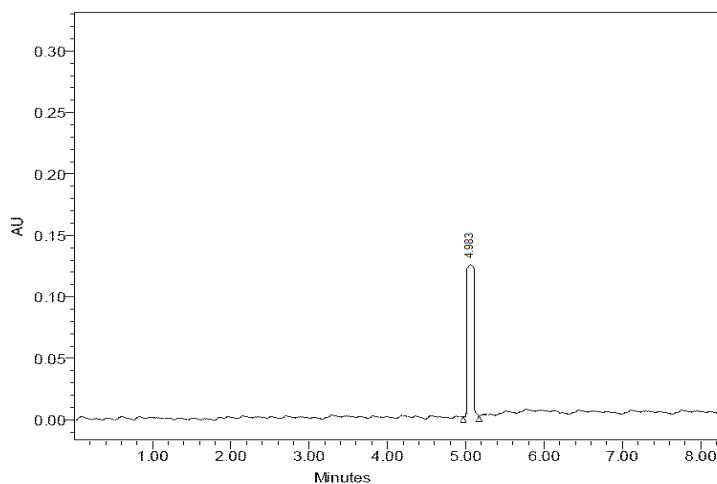


Figure 2: Standard Chromatogram of Ipratropium bromide, using mobile phase of Ammonium formate and Acetonitrile in the ratio of 45:55%v/v

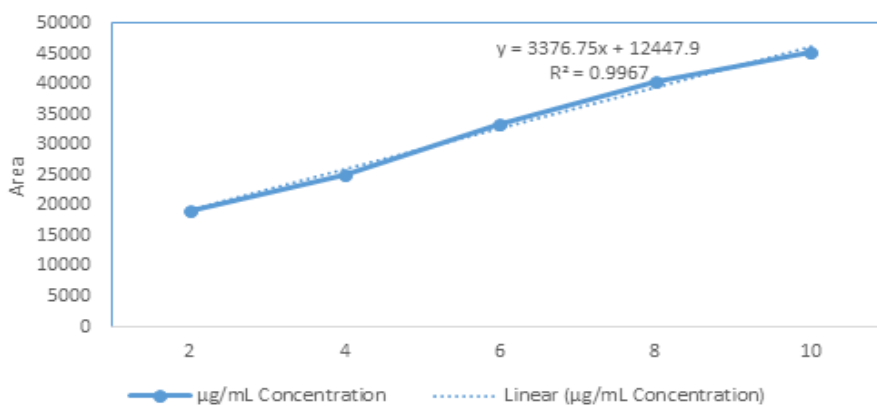


Figure 3: Calibration curve of Ipratropium bromide

Table 3: Results of Precision Studies

Replicate		Ipratropium bromide	
S.No.	Concentration Taken (µg/ml)	Area	%LC
1	04.00	25185	99.98%
2		25184	99.98%
3		25178	99.95%
4		25182	99.97%
5		25181	99.96%
6		25184	99.98%
Average			99.97%
Std.Dev			0.012649
% RSD			0.01%
Standard weight			4mcg
Standard potency			99.99%

Table 4: Results of accuracy study

Ipratropium bromide						
Level %	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	Mean recovery (%)	Std.Dev	% RSD
50	02.09	02.08	99.52	99.13	0.34947	0.35%
100	04.18	04.14	99.04			
150	06.29	06.28	98.84			

Table 5: Results of Robustness Studies

Robustness Studies			
Parameter	Value	Peak Area	% RSD
Flow Rate	Low	25237	0.20%
	Actual	25256	
	Plus	25332	
Temperature	Low	25583	0.21%
	Actual	25649	
	Plus	25688	
Wavelength	Low	25548	0.18%
	Actual	25585	
	Plus	25639	

3.7 Results of stability indicating studies

According to Singh and Bakshi 2000, the stress testing suggests a target degradation of 20-80% for establishing stability indicating nature of the method. UPLC study of samples obtained on stress testing of Ipratropium bromide under different conditions using mixture of Ammonium formate and Acetonitrile in the ratio of 45:55%v/v as a mobile solvent system suggested the following degradation behaviour.

3.7.1 Acid hydrolysis

An accurate 10 ml of pure drug sample solution was transferred to a clean and dry round bottom flask (RBF). 30 ml of 0.1 N HCl was added to it. It was refluxed in a water bath at 60°C for 4 hours. Drug became soluble after reflux which was insoluble initially. Allowed to cool at room temperature. The sample was then neutralized using 2N NaOH solution and final volume of the sample was made up to 100ml with water to prepare 100ppm solution. It was injected into the UPLC system against a blank of Ammonium formate and Acetonitrile in the ratio of 45:55% v/v after optimizing the mobile

phase composition, chromatogram was recorded and shown in Figure 4.

3.7.2 Basic hydrolysis

An accurate 10 ml of pure drug sample solution was transferred to a clean and dry RBF. 30 ml of 0.1N NaOH was added to it. It was refluxed in a water bath at 60°C for 4 hours. Drug became soluble after reflux which was insoluble initially. It was allowed to cool at room temperature. The sample was then neutralized using 2N HCl solution and final volume of the sample was made up to 100ml with water to prepare 100ppm solution. It was injected into the UPLC system against a blank of Ammonium formate and Acetonitrile in the ratio of 45:55%v/v after optimizing the mobile phase composition, chromatogram was recorded and shown in Figure 5.

3.7.3 Wet heat degradation:

Accurate 10 ml of pure drug sample was transferred to a clean and dry RBF. 30 ml of HPLC grade water was added to it. Then, it was refluxed in a water bath at 60°C for 6 hours uninterruptedly. After the completion of

reflux, the drug became soluble and the mixture of drug and water was allowed to cool at room temperature. Final volume was made up to 100 ml with HPLC grade water to prepare 100 ppm solution. It was injected into the UPLC system against a blank of Ammonium formate and Acetonitrile in the ratio of 45:55% v/v after optimizing the mobile phase composition, chromatogram was recorded and shown in Figure 6.

3.7.4 Oxidation with (3%) H₂O₂:

Approximately 10 ml of pure drug sample was transferred in a clean and dry 100 ml volumetric flask. 30 ml of 3% H₂O₂ and a little methanol was added to it to make it soluble and then kept as such in dark for 24 hours. Final volume was made up to 100 ml using water to prepare 100ppm solution. The above sample was injected into the UPLC system. The chromatogram was recorded and shown in Figure 7.

In all degradation studies, there was a significant formation of degradation products when compared to that of a standard. This indicates that, the drug may be degraded to low molecular weight non-chromophoric compounds.

3.8 Structure and separation of the known/ unknown impurities by LCMS/MS

An unknown impurity with a relative retention time (RRT) of 1.55 with respect to Ipratropium bromide was observed during the stability study of the drug product and we tried to enhance the impurity by using the forced degradations to separate it. But the impurity was not increased in any trial. So the impurity was separated by preparative UPLC from stability samples with a purity of > 97% and used for its characterisation by LC-MS -MSⁿ studies.

The positive ESI-MS spectrum of the unknown impurity showed a peak at m/z 111.4 amu [M+H]⁺ (Figure 8) which was 300.6 amu less than that of Ipratropium bromide (m/z 412). The comparison of MS/MS studies of the unknown impurity and Ipratropium bromide showed common fragment ions at m/z 302.42. The common fragment ion peak suggests that 3-Hydroxy was intact and changes were at the 8-azoniabicyclo atom.

3.9 Impurity profile

The impurity profile shows us the amount of respective impurities obtained in the studied formulations.

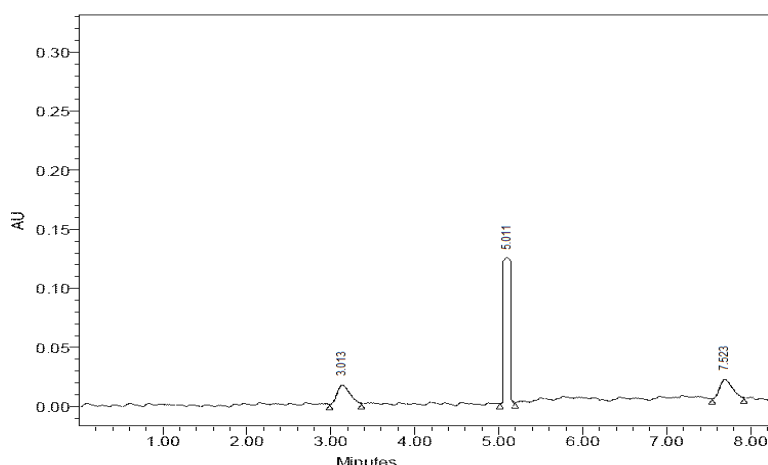


Figure 4: Chromatogram showing the degraded products in acidic degradation

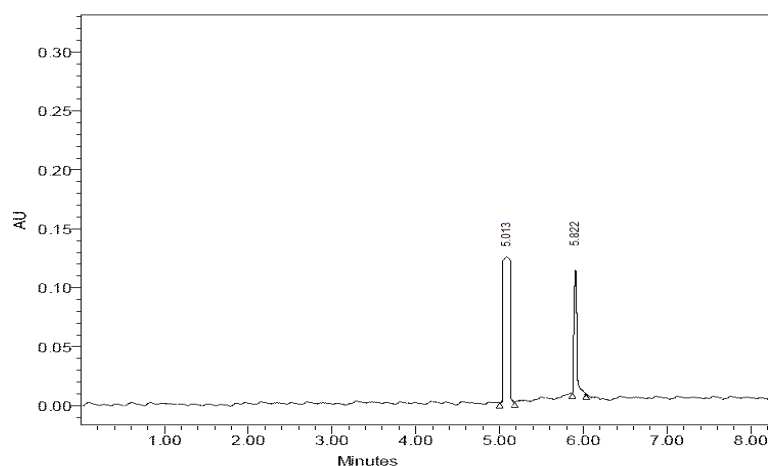


Figure 5: Chromatogram showing the degraded products in basic degradation

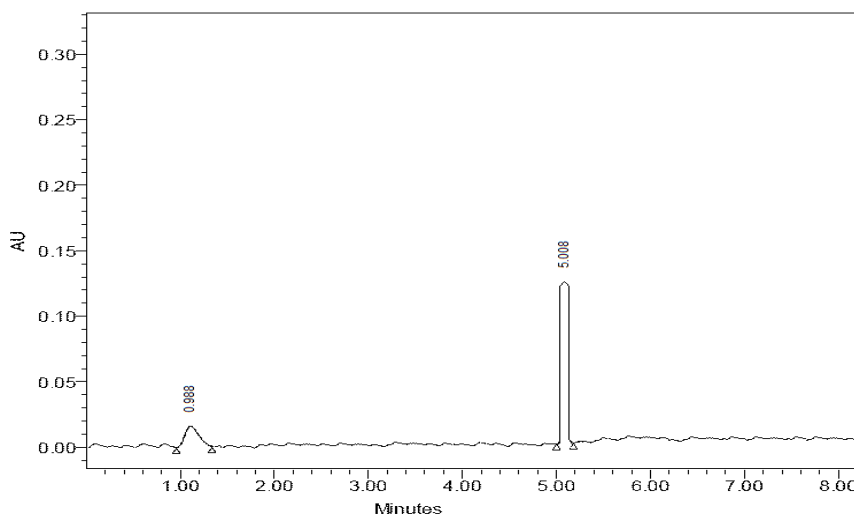


Figure 6: Chromatogram showing the degraded products in wet heat degradation

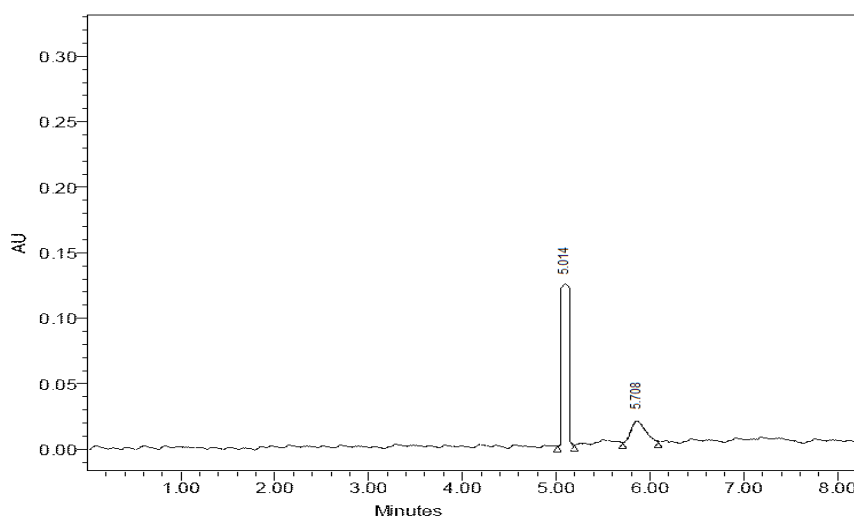


Figure 7: Chromatogram showing the degraded products in H₂O₂

Table 6: Stability indicating study for the developed method

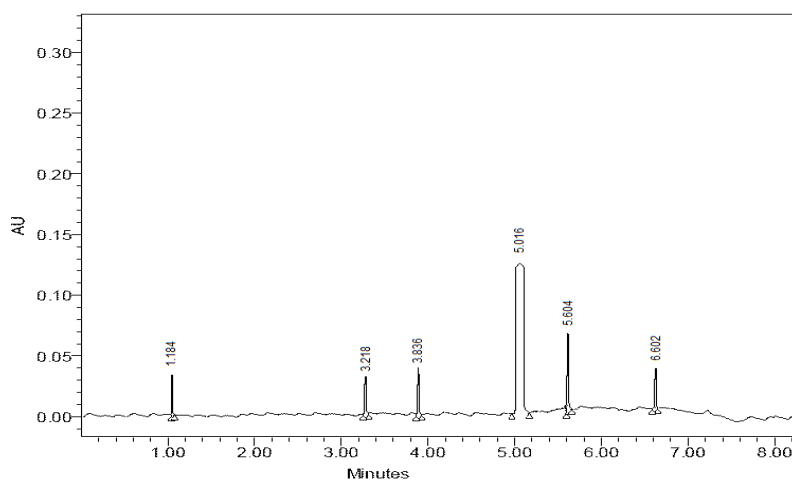
Nature of Stress	Degradation condition	Time(h)	Number of degradation products (Rt)
Acidic	60°C	3	2 (3.013, 7.523)
Basic	60°C	9	1 (5.822)
Oxidative	RT	48	1 (5.708)
Wet Heat	105°C	24	1 (0.988)

Table 7: Impurity Profile of Ipratropium bromide

S.NO	IMPURITY NAME	ACTIVE PHARMACEUTICAL INGREDIENT	RELATIVE RETENTION TIME
1	Ipratropium related compound C	Ipratropium bromide	0.89
2	Ipratropium related compound B [(8 <i>s</i>)-ipratropium bromide]		0.75
3	<i>N</i> -isopropylnoratropinium bromide		1.30
5	Any individual unknown impurity		1.55
4	Apo-ipratropium bromide		4.23

Table 8: Compositions of Ipratropium bromide in MS/MS spectra

Analyte	Observed ion mass (Da)	Proposed formula	Calculated mass (Da)	Error (ppm)
Ipratropium bromide	412.17	C ₂₀ H ₃₀ BrNO ₃	412.10	-2.02
	302.42	C ₂₀ H ₂₈ NO ₂	302.37	-3.05
	284.37	C ₁₉ H ₂₇ NO ₃	284.31	-3.04
	272.08	C ₁₁ H ₂₂ BrNO	271.89	2.29
	204.23	C ₉ H ₁₀ O ₃	204.16	-3.07
	111.40	C ₉ H ₈ O ₂	111.32	-1.08

**Figure 8:** Chromatogram showing the impurities of Ipratropium bromide

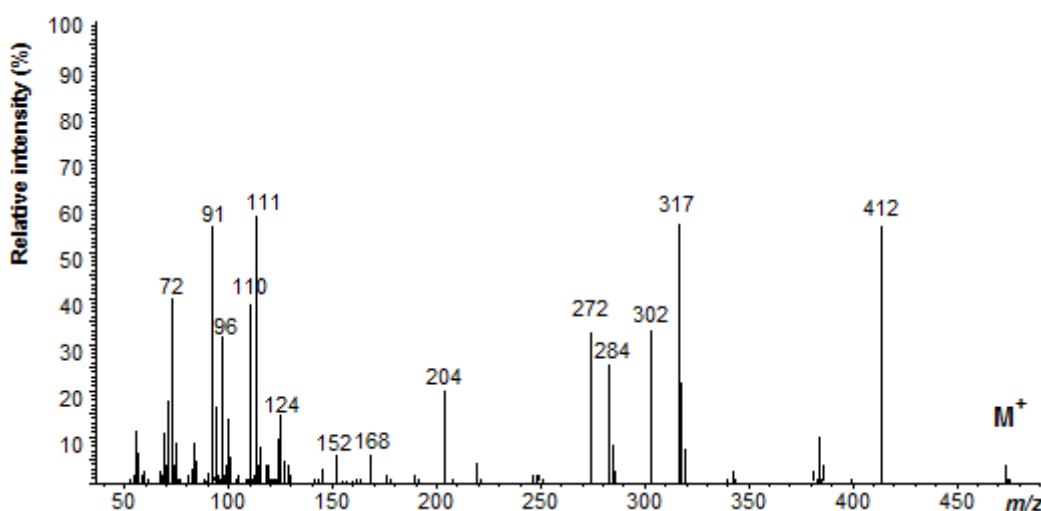


Figure 9: MS/MSⁿ characterisation of impurities in Ipratropium bromide

4. Conclusion

This research paper describes the separation and characterization of impurities in Ipratropium bromide pharmaceutical respules formulations. The impurities were isolated by preparative liquid chromatography and characterized by using spectroscopic techniques. A simple and efficient RP-UPLC method development and validation were discussed. The degraded products were formed during the study and was well-resolved from Ipratropium bromide by the proposed RP-UPLC method. The proposed structure of Ipratropium bromide was characterized by MS/MSⁿ analysis and was further confirmed to be accurate mass measurements.

Conflict of interest

None declared

5. References

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