HPLC: METHOD DEVELOPMENT, VALIDATION AND FORCED DEGRADATION STUDY FOR SIMULTANEOUS ESTIMATION OF HYDROCORTISONE ACETATE AND ATROPINE SULPHATE IN MARKETED FORMULATION

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***Abstract*- The objective of this study was to develop and validate a method for simultaneous estimation of Hydrocortisone acetate and Atropine sulphate in Marketed Formulation. An Isocratic HPLC analysis method using a reverse phase Inertsil 3V ODS (250 mm × 4.6 mm, 5 µm) and Mobile phase 0.1M 1-Heptane sulfonic acid buffer (pH 4 adjusted with 0.025% OPA): Methanol: Acetonitrile (35:45:20 v/v) was developed, optimized and validated. Analysis were carried out at flow rate 1 ml/min and monitored at 224 nm. Retention time of Hydrocortisone acetate, Atropine sulphate, is about 8.2316 and 4.575 min respectively. Linearity of Hydrocortisone acetate 7.5-30 µg/mL and Atropine sulphate was 15-60 µg/mL. The developed methods have been validated statistically as per ICH guidelines. The method showed good reproducibility and recovery with %RSD less than 2. So, the proposed methods were found to be simple, specific, precise, accurate and linear. Forced degradation study was carried out and there is no interference of excipients or degradation products. Hence it can be applied for routine analysis of Hydrocortisone acetate, Atropine sulphate in Marketed formulation.**

***Keywords*: Hydrocortisone acetate, Atropine sulphate, Method development and Validation, Forced degradation.**

INTRODUCTION:

HPLC is an analytical technique used to separate, identify and quantify the component. It finds its use for research, manufacturing, medical, legal purposes. The development of an analytical method for the identification and quantification of drugs by HPLC has received considerable attention in recent years because of their importance in quality control of drugs and drug products. In the present study attempt is made to estimate the following three drugs simultaneously. Hydrocortisone acetate IUPAC [2-[(8S,9S,10R,11S,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-2,6,7,8,9,11,12,14,15,16-decahydro-1H cyclopenta[a]phenanthren-17-yl]-2-oxoethyl] acetate. It is slightly soluble in methanol; soluble in ethanol; practically insoluble in distilled water; It is used for relieving inflammatory condition, and it is a synthetic corticosteroid. Atropine sulphate IUPAC name (8-methyl-8-azabicyclo [3.2.1] octan-3-yl) 3-hydroxy-2-phenylpropanoate; sulfuric acid it is very soluble in distilled water and freely soluble in methanol. It is used to treat bradycardia, decrease salivation, dilated pupil of eye, pesticide poisoning, and antidote of overdose of anticholinergic.

MATERIALS AND METHODS:

- **Chemicals:** HPLC grade Acetonitrile, HPLC grade Methanol from Merck (India) Ltd., Mumbai, India. Mili Q Water was used, Orthophosphoric acid (HPLC grade, Thermo Fischer Scientific India Pvt. Ltd.), Potassium dihydrogen orthophosphate (HPLC grade, RANKEM), 1-Heptane sulphonic acid Sodium Salt (anhydrous) (HPLC grade, Thermo Fischer Scientific India Pvt. Ltd.). Hydrocortisone acetate, Atropine sulphate pure drugs procured from Selvok Pharmaceuticals and Gaurang International respectively and Eye drops (PINO CORT) was manufactured and marketed by BELL PHARMA, Mumbai.
- **Instrumentation:** Analysis was performed on HPLC (WATERS Alliance 2695) instrument equipped with PDA detector Inertsil 3V ODS (250 mm × 4.6 mm, 5 µm), Auto injector, Software: EMPOWER, Other equipments used in the study were Electronic analytical balance (Microbalance- Mettler Toledo), and Digital pH meter (Lab India – Pico pH Meter- PICO$^+$) and ultra sonicator (Telesonic).

➢ **Selection of wavelength:**

Aliquots of 1ml from working solution of HCA (250 µg/ml) and 2ml from working solution of AS (250 µg/ml) were pipette out into two separate 10 ml of volumetric flask and volume was made upto the mark with methanol to get 25 µg/ml of HCA and 50 µg/ml of AS. Each Solutions of HCA and AS were scanned between 200-400 nm using PDA Detector. Wavelength 224 nm was selected from the overlay spectra of above solutions.

METHOD DEVELOPMENT:

➢ **Preparation of standard and working solution**

- **Preparation of HCA standard stock solution (1000 µg/ml)**

10 mg of HCA was weighed and transferred to 10 ml volumetric flask. It was dissolved in methanol and volume was made upto the mark with methanol to give a solution containing 1000 µg/ml.

- **Preparation of HCA working solution (250µg/ml)**

Aliquot of 6.25 ml from above standard stock solution was pipetted out into 25 ml of volumetric flask and volume was made upto the mark with methanol: water (50:50).

- **Preparation of AS standard stock solution (1000 µg/ml)**

10 mg of AS was weighed and transferred to 10 ml volumetric flask. It was dissolved in methanol and volume was made upto the mark with methanol to give a solution containing 1000 µg/ml.

- **Preparation of AS working solution (250 µg/ml)**

Aliquot of 6.25 ml from above standard stock solution was pipetted out into 25 ml of volumetric flask and volume was made upto the mark with methanol: water (50:50).

- **Preparation of Binary mixture of HCA and AS**

Aliquots of 2 ml from working solution of HCA (250 µg/ml) and 4 ml from working solution of AS (250 µg/ml) were taken into common volumetric flask and diluted upto 20 ml with mobile phase to make final concentration HCA (25µg/ml) and AS (50 µg/ml). Overlay spectra of Hydrocortisone acetate (25 µg/ml) and Atropine sulphate (50 µg/ml) shown in **Fig.1**

- **Preparation of 1-Heptane Sulfonic acid buffer (0.1 N)**

5.05 gm of 1-Heptane Sulfonic acid Sodium Salt (anhydrous) was weighed and transferred into 250 ml beaker and dissolved in 250 ml of Mili Q water. The solution was sonicated for 3 min and then total volume was made with water. The pH of resulting solution was adjusted to 4 with OPA (Ortho Phosphoric Acid).

- **Preparation of Sample**

5 ml of Sample was taken into 100 ml of volumetric flask. Methanol was added and sonicated for 2-3 minutes and volume was made up to mark with methanol. Solution was filtered through Whatmann filter paper no. 42. Thus, resulting solution gave 5000 µg/ml of HCA and 10000 µg/ml of AS. From the above solution, 2.5 ml was pipette out and transferred to 50 ml volumetric flask and Volume was made upto mark with methanol:water in order to give a solution containing HCA (250 µg/ml) + AS (500 µg/ml). From the above solution, 2.0 ml was pipette out and transferred to 20 ml of volumetric flask and volume was made upto mark with methanol to give a solution containing HCA (25 µg/ml) + AS (50 µg/ml). This solution was used for assay i.e. estimation of HCA and AS in Marketed formulation.

➢ **Selection of Mobile Phase**

The mobile phase selection was based on the observation like best peak separation, theoretical plate, and resolution and peak symmetry. So number of trials was taken for the selection of mobile phase.

➢ **Validation of Proposed HPLC Method**

1 System Suitability studies

Evaluation of system suitability was done by analysing six replicate of HCA and AS in a mixture at concentration of 25 µg/ml of HCA and 50 µg/ml of AS. The column efficiency, peak asymmetry and resolution were calculated for each replicate and its shown in **Table no.1**

2 Specificity

Specificity involves quantitative detection of analyte in the presence of those components that may be expected to be part of sample matrix. Specificity of developed method was established by spiking of HCA and AS in hypothetical placebo (i.e. might be expected to be present) and expressing that analytes peaks were not interfered from excipients.

3 Linearity

The linearity response was determined by analyzing 5 independent levels of concentration in the range of 7.5-30 µg/ml and 15-60 µg/ml for HCA and AS respectively and it is shown in **Table no.2**

- **Preparation of Calibration Curves**

Calibration curve for HCA and AS

Calibration curve for HCA consisted of five different concentrations solution ranging from 7.5-30 µg/ml. Calibration curve of Peak area vs Conc. was plotted and regression equation was determined. And Calibration curve for AS consisted of five different concentrations solution ranging from 15-60 µg/ml. Calibration curve of Peak area vs Conc. was plotted and regression equation was determined.

2 Precision

a) Repeatability

Repeatability of the developed method was assessed by analysing samples from the same batch 6 times with standard solutions containing concentrations 25 µg/ml for HCA and 50 µg/ml for AS and % R.S.D. was calculated and it is shown in **Table no.3**

b) Intraday precision

It was assessed by analyzing samples from the same batch with three standard solutions containing concentrations 12.5, 20, 25 µg/ml for HCA and 25, 40, 50 µg/ml for AS. Solutions were analyzed thrice (n=3) on the same day within short interval of time and % R.S.D. was calculated and it is shown in **Table no.3**

c) Interday precision

It was assessed by analyzing samples from the same batch with three standard solutions containing concentrations 12.5, 20, 25 µg/ml for HCA and 25, 40, 50 µg/ml for AS. Solutions were analyzed thrice (n=3) on the three different day and % R.S.D. was calculated and it is shown in **Table no.3**

3 Accuracy

5ml of sample was taken into 100 ml of volumetric flask. Methanol was added and sonicated for 2-3 mins and volume was made up to mark with methanol. Solution was filtered through Whatmann filter paper no. 42. Thus, resulting solution gave 5000 µg/ml

of HCA and 10000 µg/ml of AS. From the above solution, 2.5 ml was pipette out and transferred to 50 ml volumetric flask and volume was made upto mark with methanol: water (50:50) in order to give a solution containing HCA (250 µg/ml) + AS (500 µg/ml). From the above solution, 1 ml was pipette out and transferred to 20 ml of volumetric flask and volume was made upto mark with mobile phase to give a solution containing HCA (12.5 µg/ml) + AS (25 µg/ml) and its shown in **Table no.4**

4 LOD and LOQ

The LOD (Limit of Detection) was assessed from the set of 5 calibration curves that were used to determine linearity of the method.

LOD = 3.3 × S.D./Slope

The LOQ (Limit of Quantitation) was assessed from the set of 5 calibration curves that were used to determine linearity of the method and it is shown in **Table no.5**

LOQ = 10 × S.D./Slope

Where, S.D. = Standard deviation of the Y – intercepts of 5 calibration curves

Slope = Mean slope of 5 calibration curves.

5 Robustness

Robustness of the method was determined by subjecting the method to slight change in the method condition like, Mobile Phase Ratio, Flow rate, Temperature.

Five replicates were prepared for the same of concentration 25 µg/ml for HCA and 50 µg/ml for AS and % R.S.D. was calculated and it is shown in **Table no.6**

6 Simultaneous estimation of Hydrocortisone acetate and Atropine sulphate in marketed formulation

5ml of Sample was taken into 100 ml of volumetric flask. Methanol was added and sonicated for 2-3 minutes and volume was made up to mark with methanol. Solution was filtered through Whatmann filter paper no. 42. Thus, resulting solution gave 5000 µg/ml of HCA and 10000 µg/ml of AS. From the above solution, 2.5 ml was pipette out and transferred to 50 ml volumetric flask and volume was made upto mark with methanol:water in order to give a solution containing HCA (250 µg/ml) + AS (500 µg/ml). From the above solution, 2.0 ml was pipette out and transferred to 20 ml of volumetric flask and volume was made upto mark with methanol to give a solution containing HCA (25 µg/ml) + AS (50 µg/ml). This solution was used for assay i.e. estimation of HCA and AS in Marketed formulation. Chromatogram was recorded and the concentration of HCA and AS was obtained by solving the regression equation and it is shown in **Table no.7**

7 Forced Degradation Study

Forced degradation study was carried for standard drugs Hydrocortisone acetate and Atropine sulphate to evaluate the effectiveness of the developed method for the separation and identification of known and unknown impurities in the drug. 12.5mg of Hydrocortisone acetate and 25mg of Atropine sulphate standard drug was mixed 50 ml of 0.01N HCL for acid hydrolysis study, 50 ml of 0.01N NaOH in base hydrolysis study and 50 ml of 3% hydrogen peroxide solution for oxidative degradation study. These conditions were carried separately for both the drugs and the solution were incubated 24hrs and then neutralized. The neutralized solutions were analysed in the developed method condition. In thermal degradation conditions, standard drug was kept in oven at 60℃ for 24 hrs. Then the standard drug was diluted to 300µg/ml and was analysed in the developed method condition. The % degradation, potency of drug were observe in the degradation study and it is shown in **Table no.8**

RESULT:

Table no.1: System suitability

Drugs	Parameters	Mean ± S.D. (n=6)	%R.S.D
HCA	Retention Time	8.2316 ± 0.0256	0.3113
	Theoretical Plate	3729.83 ± 59.3681	1.5917
	Tailing Factor	1.75 ± 0.0236	1.3522
	Resolution	7.895 ± 0.0301	0.3820
AS	Retention Time	4.575 ± 0.02345	0.5126
	Theoretical Plate	2921.5 ± 26.0826	0.8927
	Tailing Factor	1.66 ± 0.0172	1.0324

Table no.2: Linearity

Hydrocortisone acetate Concentration (µg/ml)	%RSD n=5	Atropine sulphate Concentration (µg/ml)	%RSD n=5
7.5	0.3108	15	0.3197
12.5	0.3078	25	0.1916
20	0.3270	40	0.2447
25	0.4115	50	0.5782
30	0.5100	60	0.3746

Table no.3: Precision studies

DRUGS	REPEATABILITY n=5 (%RSD)	INTRADAY n=3 (%RSD)	INTERDAY n=3 (%RSD)
Hydrocortisone acetate	0.4095	0.3298-0.5780	0.4226-0.6527
Atropine sulphate	0.5539	0.3517-0.6007	0.4233-0.6984

Table no.4: Accuracy study

Drugs	Level	Amount of sample (µg/ml)	Amount of std spiked (µg/ml)	Total amount	Amount of sample found (µg/ml	Mean %Recovery ± S.D. (n=3)
Hydrocortisone acetate	**0**	12.5	0	12.5	12.52	100.23 ± 0.3459
	80	12.5	10	22.5	22.42	99.68 ± 0.0564
	100	12.5	12.5	25	25.01	100.04 ± 0.4697
	120	12.5	15	27.5	27.43	99.75 ± 0.0629
Atropine sulphate	**0**	25	0	25	24.95	99.83 ± 0.2184
	80	25	20	45	45.02	100.05 ± 0.2016
	100	25	25	50	50.37	100.75 ± 0.8078
	120	25	30	55	54.90	99.83 ± 0.4952

Table no.5: LOD and LOQ

Drugs	Hydrocortisone acetate	Atropine sulphate
LOD	**0.1271**	**0.3092**
LOQ	**0.3853**	**0.9368**

Table no.6: Robustness
HYDROCORTISONE ACETATE (25 µg/ml)

Parameters	Level	Mean Peak Area ± S.D. (n=5)	%R.S.D.	Rt ± S.D. (n=5)	%R.S.D.

Mobile Phase	**33:45:22 v/v/v**	5760884 ± 2384.75	0.4139	7.456 ± 0.01516	0.2034
	37:45:18 v/v/v	566513.2 ± 2034.063	0.3590	9.504 ± 0.0288	0.3031
Flow rate	**0.8 ml/min**	714428.8 ± 3130.598	0.4381	10.154 ± 0.0114	0.1122
	1.2 ml/min	501543 ± 1445.262	0.2881	6.85 ± 0.06928	1.0114
Temperature	**25 ℃**	577377.2 ± 3350.270	0.5802	7.932 ± 0.03563	0.4492
	35 ℃	581206.4 ± 1185.497	0.2039	7.812 ± 0.0083	0.1070
ATROPINE SULPHATE (50 µg/ml)					
Mobile Phase	**33:45:22 v/v/v**	332933.2 ± 1122.06	0.3370	4.106 ± 0.0230	0.5606
	37:45:18 v/v/v	338342 ± 3356.263	0.9919	4.758 ± 0.0130	0.2740
Flow rate	**0.8 ml/min**	430654.6 ± 5070.059	1.1772	5.6936 ± 0.0060	0.1065
	1.2 ml/min	292171.6 ± 2269.984	0.7769	3.86 ± 0.0254	0.6604
Temperature	**25℃**	327395.4 ± 387.7516	0.1184	4.526 ± 0.0089	0.1976
	35 ℃	327772.4 ± 402.7174	0.1228	4.494 ± 0.0054	0.1218

Table no.7: Assay

	Amount taken (µg/ml)		**Amount Obtained (µg/ml)**		**% HCA ± S.D. (n=5)**	**% AS ± S.D. (n=5)**
EYE DROP	**HCA**	**AS**	**HCA**	**AS**	99.19 ± 0.2821	99.69 ± 0.7249
	25	**50**	24.79	49.84		

Table no.8: Forced Degradation study

DRUGS	**CONDITION**	**% DEGRADATION**	**POTENCY**
Hydrocortisone acetate	**Acid**	17.30	82.70
	Alkali	10.89	89.11
	Oxidative	1.08	98.92
	Thermal	0.2	99.80
Atropine sulphate	**Acid**	13.38	86.62

	Alkali	1.01	98.99
	Oxidative	0.8	99.20
	Thermal	11.00	89.00

DISCUSSION:

Based on the results, obtained from the analysis of HCA and AS in their Marketed Formulation using RP-HPLC Method, it can be concluded that the method has linearity in the range of 7.5-30 µg/ml for HCA and 15-60 µg/ml for AS. The regression coefficient (R^2) was found to be 0.9999 and 0.9997 for HCA and AS and correlation coefficient (r) was found to be 0.9999 and 0.9998 for HCA and AS at 224 nm respectively.

Limit of detection for HCA and AS were found to be 0.1271 µg/ml and 0.3092 µg/ml and limit of quantification for HCA and AS were found to be 0.3853µg/ml and 0.9368µg/ml respectively. The % assay was found to be 99.19 % w/w and 99.69 % w/w for HCA and AS respectively. Further % R.S.D. was found to be less than 2% for precision, intraday and interday study.

Thus, overall result obtained for both drugs suggested that all proposed methods are specific for estimation of Hydrocortisone acetate and Atropine sulphate. The degradation studies of Hydrocortisone acetate and Atropine sulphate were shows that maximum degradation in Acidic Condition.

The results of recovery study indicated that all developed methods were accurate. The result of intraday and interday variations with low value of % R.S.D. showed that developed methods were precise.

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ABBREVIATION:

Conc.- Concentration, **UV -** Ultraviolet, **TLC -** Thin Layer Chromatography, **HPLC -** High Performance Liquid Chromatography, **HPTLC -** High Performance Thin Layer Chromatography, **LOD -** Limit of Detection, **LOQ -** Limit of Quantitation, **ml –** Milliliter, **nm –** Nanometer, **R.S.D -** Relative Standard Deviation, **RP -** Reverse phase, **λmax -** Maximum Wavelength

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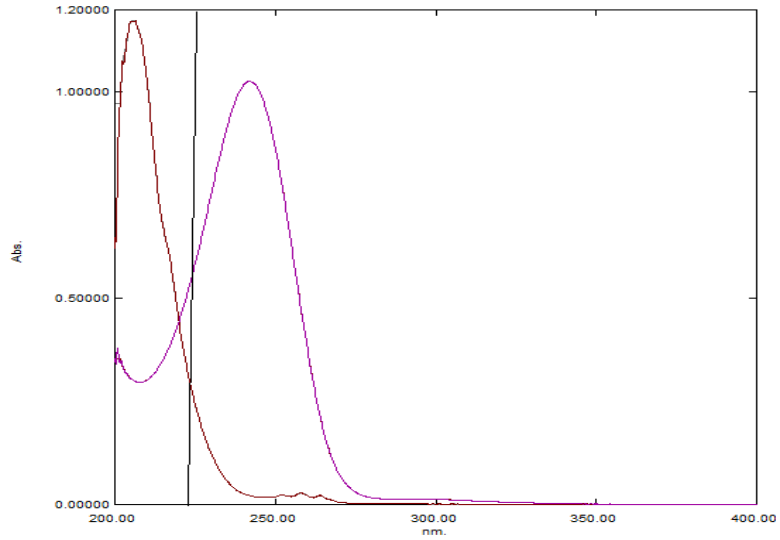

Fig. 1: Overlay spectra of Hydrocortisone acetate (25 µg/ml) and Atropine sulphate (50 µg/ml)

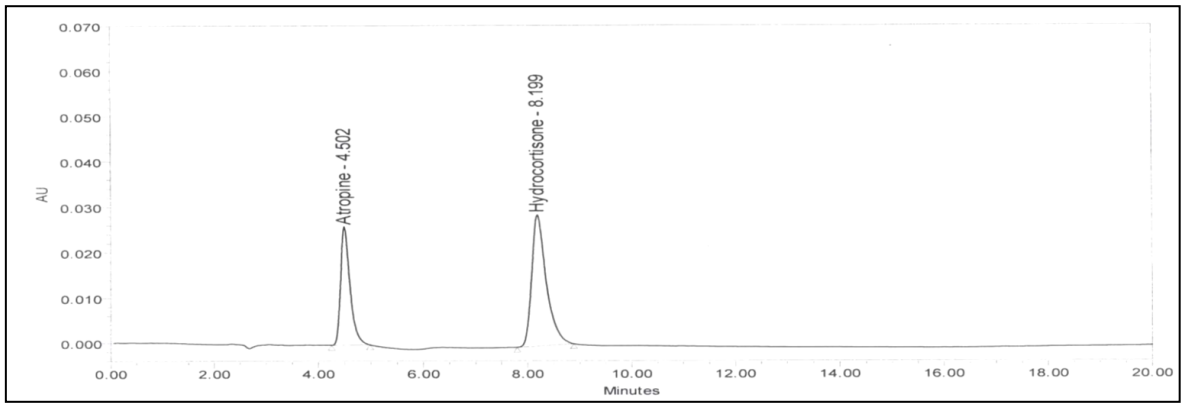

Fig. 2: Standard chromatogram

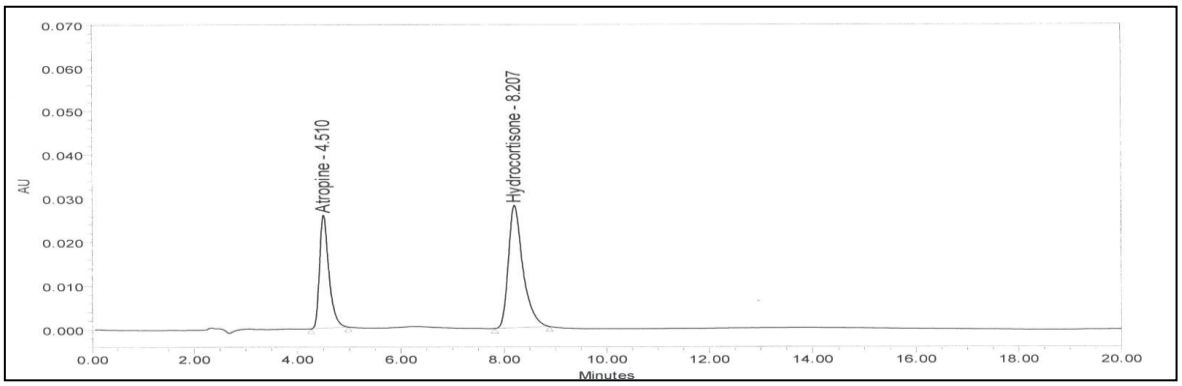

Fig. 3: Formulation chromatogram

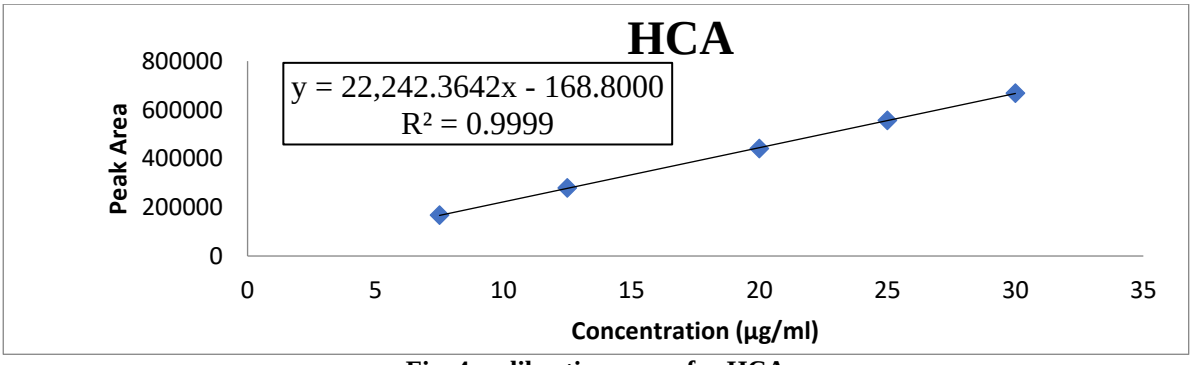

Fig. 4: calibration curve for HCA

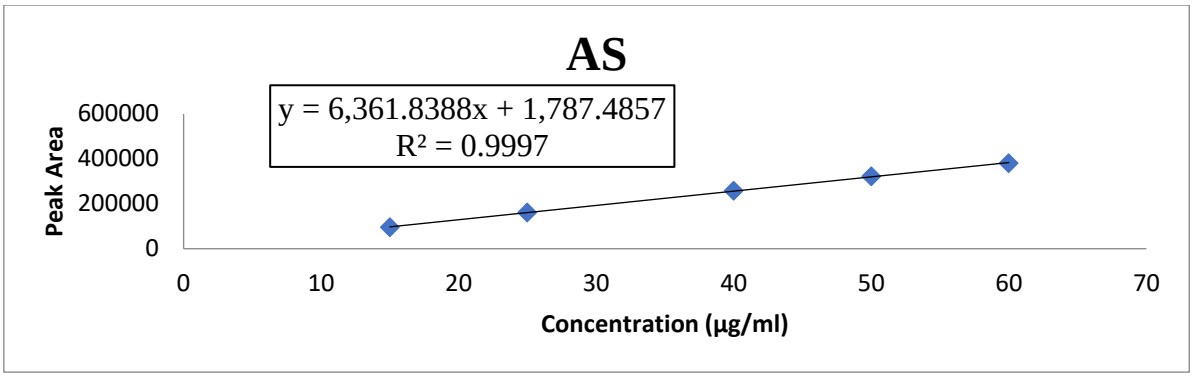

Fig. 5: calibration curve for AS

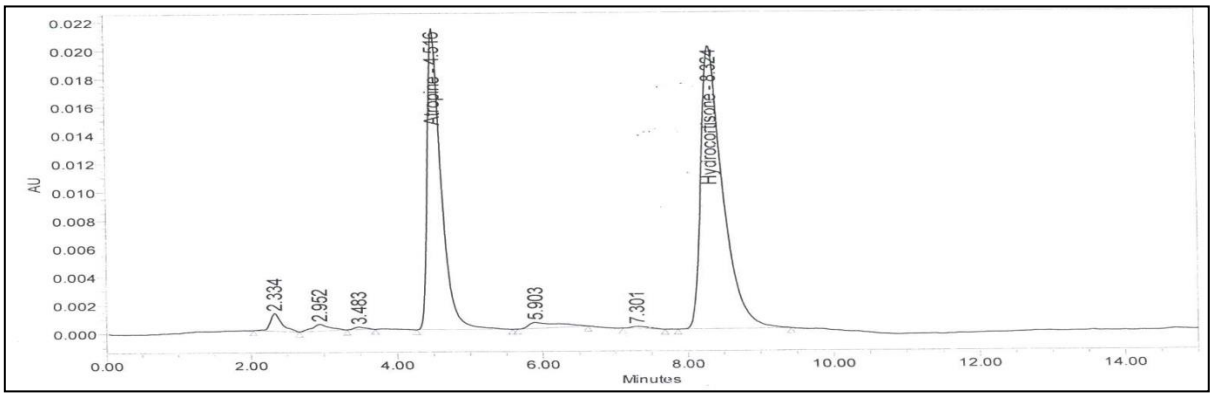

Fig. 6: Acid degradation (0.01N HCl)

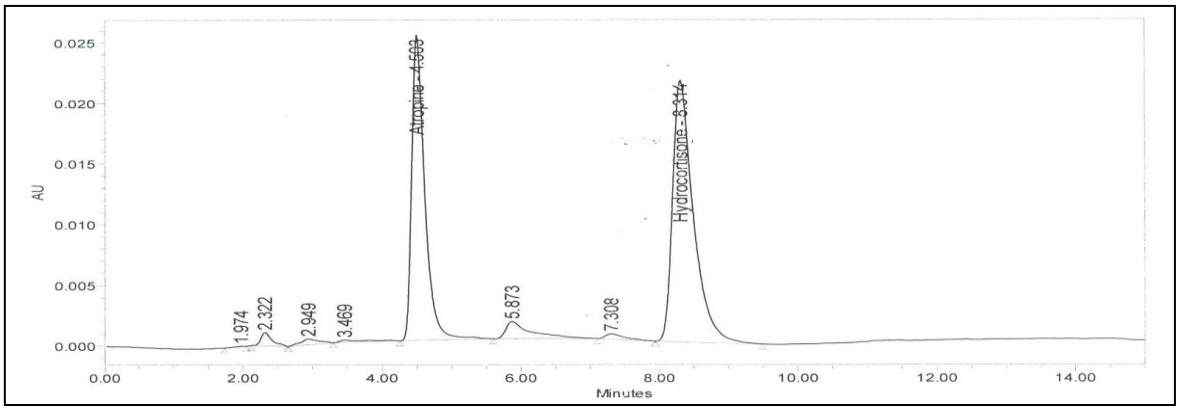

Fig. 7: Base degradation (0.01N NaOH)

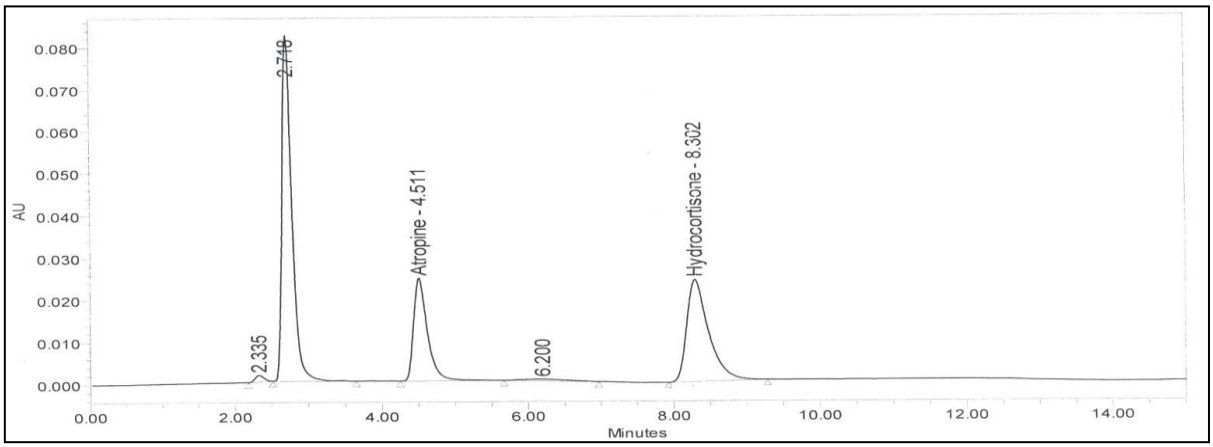

Fig. 8: Oxidative (Peroxide) degradation (3% H_2O_2)

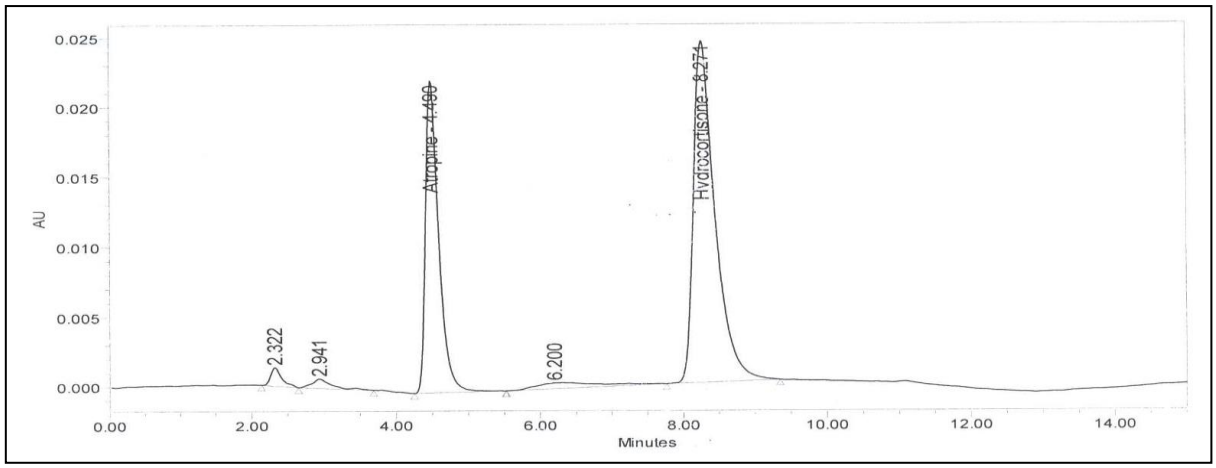

Fig. 9: Thermal degradation