

**Original Article**

**DEVELOPMENT AND VALIDATION OF STABILITY INDICATING METHOD RP-HPLC METHOD OF ACOTIAMIDE**

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**ABSTRACT**

**Objective:** The objective of present work was to develop and validate simple, precise, accurate and specific stability indicating method for determination of acotiamide in presence of its degradation products.

**Methods:** An isocratic RP-HPLC method has been developed using C-8 Thermo Hypersil BDS Column (250 x 4.6 mm i.d., 5µparticle size) with the mobile phase composition of acetonitrile: 0.1 % triethylamine in 0.2% formic acid (30: 70) at column oven temperature of 40 °C. The flow rate was 1.0 ml min<sup>-1</sup> and effluent was detected at 282 nm. The method was validated in terms of linearity, accuracy, precision, LOD (Limit of Detection), LOQ (Limit of Quantification) and robustness as per ICH guidelines.

**Results:** The method was found to be linear in the range of 10-60µg/ml. Limit of detection and limit of quantification was found to 0.36µg/ml and 1.10 µg/ml.% Recovery was found to be in the range of 99.45%-99.75%and precision less than 2%. The developed method was successfully applied for estimation of Acotiamide in marketed tablet formulation and percentage assay was found to be 100.45%. Acotiamide was subjected to stress degradation under acid, base, neutral hydrolysis, oxidation, dry heat, photolysis conditions. Significant degradation was observed in acid and base degradation.

**Conclusion:** The developed RP-HPLC method was simple, rapid, accurate, precise and stability indicating for the estimation of Acotiamide in bulk and tablet dosage form.

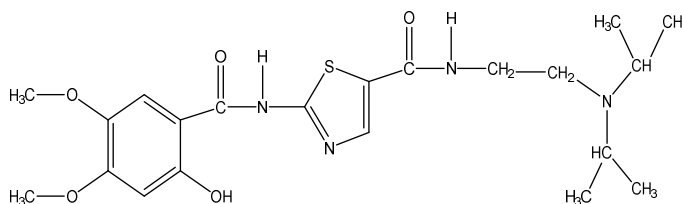
**Keywords:** Acotiamide, Reverse Phase High Performance Liquid Chromatography(RP HPLC), Stability Indicating Assay Methods (SIAM's), Stress Degradation, ICH Q1A(R2), Q2R1

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**INTRODUCTION**

Acotiamide (ACOT) is N-[2-[di(propene-2-yl)amino]ethyl]-2-[(2-hydroxy-4,5-dimethoxybenzoyl)amino]-1,3-thiazole-4-carboxamide; trihydrate; hydrochloride. Acotiamide is a new prokinetic agent [1]. It is approved in Japan in 2013 [2]. It causes an increase in the release of acetylcholine thereby it exerts gastroprokinetic activity

through acting as an antagonist on the M1 and M2 muscarinic receptors in the enteric nervous system. It inhibits anticholinesterase activity. It may also act directly on the gut and indirectly on the central nervous system by way of brain-gut axis. Studies have shown that acotiamide could enhance gastric emptying and gastric accommodation. Acotiamide could be a promising agent in the treatments with functional dyspepsia [3, 4].



**Fig. 1: Structure of acotiamide**

The presence of impurities can have a significant impact on the product quality, safety and efficacy, hence the percentage level of impurities need to control in the drug substance as well as a drug product. Information on the stability of molecule aids in the selection of proper storage conditions which is the pre-requisite of documentation of drug profile. Force degradation study is an important parameter in pharmaceutical research and development to predict stability of drug. Stress testing helps in identification of degradation products and degradation behaviour of drug [5, 6]. The literature reveals there were few analytical methods available for determination of acotiamide by HPLC [7], LC-MS-MS [8], UPLC-Q-TOF-MS [9, 10], stability indicating the method by UPLC-Q-TOF-MS (gradient mode) [11], this reported method based on UPLC method.

In the present study, a simple, rapid, precise, isocratic, accurate, cost-effective stability indicating liquid chromatographic method was developed by HPLC for the determination of Acotiamide in tablet dosage forms and validated as per ICH guidelines [12, 13].

**MATERIALS AND METHODS**

**Chemicals, reagents and solutions**

Acotiamide hydrochloride trihydrate (ACOT) bulk drug was provided by Hetero Drugs Pvt. Ltd. Acogut tablets 100 mg were purchased from local pharmacy. HPLC grade methanol and acetonitrile were purchased from Rankem Ltd, Mumbai. HPLC grade triethylamine and formic acid were purchased from Loba Chemie

Pvt. Ltd, Mumbai. Hydrochloric acid (HCl), sodium hydroxide (NaOH) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were purchased from SD Fine Chem. Ltd, Mumbai, India.

#### Preparation of mobile phase buffer

The buffer was prepared by adding 1 ml of triethylamine in 1000 ml of double distilled water and adjusting the pH with 2 ml of formic acid.

#### Mobile phase composition

The mobile phase was prepared using buffer and acetonitrile in the ratio of 70:30. Before use, the mobile phase was filtered through 0.2µ nylon membrane filter and sonicated for 5 min.

#### Instrumentation and chromatographic conditions

##### HPLC-PDA

Analytical Chromatography was performed on Waters Alliance 2695 separation module, Waters 2996 Photo Diode Array Detector (PDA). Data acquisition and integration were performed using Emchem 2 software. Chromatographic separation of ACOT was achieved at column oven temperature of 40 °C using C-8 Thermo Hypersil BDS column (250 X 4.6 mm i.d., 5 µ particle size) with mobile phase composition of acetonitrile: 0.1% triethylamine with 0.2% formic acid (30:70). The flow rate was at 1.0 ml min<sup>-1</sup> and effluent was detected at 282 nm.

#### Preparation of standard stock solution

ACOT stock solution (1000 µg/ml) was prepared by dissolving 25 mg of ACOT in 25 ml of double distilled water and acetonitrile (70:30) in 25 ml of volumetric flask.

#### Preparation of working standard solution

ACOT working solution (100 µg/ml) was prepared by transferring 2.5 ml from a stock solution to 25 ml volumetric flask and diluted up to the mark with mobile phase.

#### Construction of calibration curve

Aliquots ranging from 0.5 ml to 3.0 ml were taken, from standard stock solution in 10 ml volumetric flask and diluted to 10 ml with mobile phase to give a final concentration of 5, 10, 15, 20, 25, 30µg ml<sup>-1</sup>. 20 µl of each concentration was injected in the chromatographic system and the chromatogram was recorded. A calibration curve was constructed by plotting peak area versus concentration of drug and regression equation was calculated.

#### Preparation of test sample solution

20 tablets were crushed using mortar-pestle, the powder was weighed and quantity of powder equivalent to 25 mg of ACOT was transferred in 25 ml volumetric flask, dissolved in a solvent (water: acetonitrile 70:30) and sonicated for 30 min. The solution was filtered through 0.45 µ Nylon 6,6 syringe filter. 2 ml was transferred in 10 ml volumetric flask and volume were adjusted upto the mark with mobile phase to give a concentration of 20µg/ml of ACOT.

#### Method validation

The proposed method was validated according to the ICH guidelines [12] for system suitability, linearity, precision, recovery, limit of detection (LOD) and limit of quantitation (LOQ), robustness, specificity.

#### System suitability

System suitability test should be carried out to verify that the analytical system is working properly and can give accurate and precise results. Working standard solutions were prepared and injected six times into the chromatographic system. The system suitability parameters were evaluated for peak area, retention time, tailing factor and theoretical plates of the standard chromatogram.

#### Linearity and range

The linearity of the method was evaluated thrice by analyzing the prepared concentration of ACOT in the range of 5-30 µg/ml from the

stock solution. 20 µl of each solution was injected into HPLC system and the peak area of chromatogram was noted. Linear regression was obtained over the concentration range ( $y=mx+c$ ).

#### Precision

The intra-day precision of the assay method was evaluated at six concentration levels (5-30µg/ml) (n=6) against a qualified reference standard. % RSD of the three obtained assay values at six different concentration levels was calculated. The inter-day precision study was performed in three different days at six different concentration levels (5-30µg/ml) and each value is the average of the three determinations (n=3). The % RSD of the three obtained assay values on three different days was calculated.

#### Accuracy

The % recovery was performed by standard addition method. In this method, fixed amount of sample solution of ACOT and increasing amount of its working standard solutions were added at 80,100 and 120% level of pure drug solution. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantitation of ACOT in the drug product.

#### Limit of detection and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) were calculated by the following formula:

$$\text{Limit of detection} = 3.3 \cdot \sigma / S$$

$$\text{Limit of quantification} = 10 \cdot \sigma / S$$

Where  $\sigma$  is the standard deviation of y-intercept and S is the slope of the calibration curve

#### Robustness

The robustness of the assay method was calculated by introducing small changes in the HPLC condition which included pH of the buffer (2.8,3.2), flow rate (0.9 and 1.1 ml/min), the percentage of acetonitrile in the mobile phase (28 and 32). Robustness of the method was studied using three replicates at a concentration level of 15µg/ml of ACOT.

#### Specificity

Specificity is the ability of the method to measure an analyte in presence of its degradation products. ACOT was subjected to various stress conditions like hydrolytic, oxidative, thermal and photolytic. Degraded samples were injected into HPLC system and developed chromatograms were observed for resolution of degraded products.

#### Force degradation study [14-16]

A stock solution of ACOT was prepared by dissolving 25 mg of ACOT in 25 ml of double distilled water. This stock solution was used for degradation studies.

#### Hydrolytic conditions

##### Acid degradation

5 ml of stock solution of ACOT was taken in 25 ml of volumetric flask, 1 ml of 1N HCl was added and the solution was heated in a water bath at 100 °C for 3 h. The solution was cooled and neutralized with 1N NaOH. Volume was made up to 25 ml with water to make the concentration of 200µg/ml. The solution was filtered through 0.45 µ Nylon 6,6 membrane syringe filter.

##### Base degradation

5 ml of stock solution of ACOT was taken in 25 ml of volumetric flask, 1 ml of 0.5 N NaOH was added and the solution was heated in a water bath at 100 °C for 3 h. The solution was cooled and neutralized with 0.5 N HCl. Volume was made up to 25 ml with water to make the concentration of 200µg/ml. The solution was filtered through 0.45 µ Nylon 6,6 membrane syringe filter.

##### Water degradation

5 ml of stock solution of ACOT was taken in 25 ml of volumetric flask, 1 ml of water was added and the solution was heated in a

water bath at 100 °C for 6 h. The solution was cooled. Volume was made upto 25 ml with water to make the concentration of 200µg/ml. The solution was filtered through 0.45µ membrane syringe filter.

#### Oxidizing conditions

Hydrogen peroxide-induced degradation 5 ml of stock solution of ACOT was taken in 25 ml of volumetric flask, 1 ml of 6% hydrogen peroxide was added. The solution was kept at room temperature for 48 h. The solution was made upto volume with water. The solution was filtered through 0.45 µ Nylon 6,6 membrane syringe filter.

#### Thermal conditions

Dry heat 25 mg of ACOT was placed in an oven at 80°C for 8 d under dry heat condition in the dark and then cooled to room temperature. Volume was made with water to get a concentration of 200µg/ml.

#### Photolytic degradation

For the photochemical study, ACOT equivalent to 25 mg was spread in 1 mm thickness on a petridish and exposed to 5383 LUX and 144 UV/cm<sup>2</sup> for 8 d and volume was made up to 25 ml with water. 5 ml of the solution was taken and further diluted upto 25 ml with water to make the concentration of 200µg/ml.

**Table 1: Stress conditions for forced degradation study of ACOT**

Stress condition	Solvent	Temperature	Time
Hydrolytic			
Acid	1 N HCl	100°C	3 h
Base	0.5 N NaOH	100°C	3 h
Neutral	Water	100°C	6 h
Oxidative	6% H <sub>2</sub> O <sub>2</sub>	RT	48 h
Thermal			
Dry Heat	---	80°C	8 d
Photolytic	---	5382LUX and 144UV/cm <sup>2</sup>	10 d

## RESULTS

### Method development and optimization

A detection wavelength 282 nm was selected from the range UV spectral data due to its high sensitivity for all degradation products and minimal difference in response factors. ACOT having pKa 10.24, pH 3.0 buffer was selected to enable better column performance. Isocratic run was accessed using mobile phase 0.1% triethylamine adjusted to pH 3 with 0.2 % formic acid and acetonitrile (70:30) on C-8 and C-18 column. Column C-8 with column oven temperature of 40 °C provided the highest

number of peaks and better resolution. Thus, further experiments were carried out using thermo hypersil BDS C8 column.

### Method validation

#### System suitability method

The developed method has produced theoretical plates more than 2000 with tailing factor less than 2. The % RSD of peak area was less than 2 which ensured the suitability of the developed method. The results of the system suitability study are summarised in table 2.

**Table 2: System suitability parameters of ACOT**

Parameters	mean±SD
Retention Time	9.387±0.057
Theoretical Plates	12098±21.36
Tailing factor	1.047±0.043
Peak Area	762504±0.495

### Linearity and range

In the proposed validation method, the retention time of ACOT was 9.3 min. The calibration curve for ACOT was constructed by plotting area versus their corresponding concentrations, linearity was found over the range 5-30µg/ml with a coefficient of relation (R<sup>2</sup>). Results of linearity are shown in fig. 2 and table 3.

### Detection and quantification limits

According to the pharmaceutical recommendations, limit of detection (LOD) and limit of quantification (LOQ) were determined, at the signal to noise ratios of 3:1 and 10:1, respectively. Limit of detection of ACOT was found to be 0.36µg/ml. Limit of quantification of ACOT was found to be 1.10µg/ml.

**Table 3: Calibration curve of ACOT**

Level	Concentration (µg/ml)	Area
1	5	150914
2	10	361779
3	15	568471
4	20	771652
5	25	945829
6	30	1180668

### Precision and accuracy

The within-day (intra-day) precision and accuracy for the proposed method were studied at three concentration levels of ACOT using three replicate determinations for each concentration within one day. Similarly, the between-day (inter-day) precision and accuracy were tested by analyzing the same three concentrations using three

replicate determinations repeated on three days. Recoveries were calculated using the regression equation and they were satisfactory. The percentage of relative standard deviation (%RSD) was less than 1.5% providing the high repeatability, intermediate precision and accuracy of the developed method for the estimation of ACOT in bulk form. Results of precision are shown in table 4, 5. Results of recovery are shown in table 6.

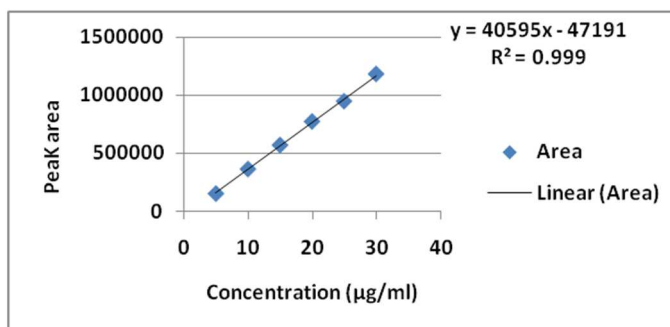


Fig. 2: Calibration curve of ACOT

Table 4: Intra-day precision of ACOT (n=3)

Concentration (µg/ml)	Area (mean±SD)	%RSD	Retention time (mean±SD)	%RSD
10	352540±4129.327	1.171	9.608±0.085	0.891
20	764518.3±3789.857±	0.495	9.644±0.040	0.417
30	1189078±14035.98	1.180	9.652±0.022	0.232

n= number of determinations, SD =Standard Deviation, %RSD = % Relative Standard Deviation

Table 5: Inter-day precision of ACOT (n=3)

Concentration (µg/ml)	Area (mean±SD)	% RSD	Retention time (mean±SD)	%RSD
10	366767±6371.719	1.737	9.613±0.078	0.817
20	772256±8524.635	1.103	9.609±0.043	0.453
30	1200857±16000.6	1.332	9.637±0.040	0.421

n= number of determinations, SD =Standard Deviation, %RSD = % Relative Standard Deviation

Table 6: Recovery studies of ACOT (n=3)

Concentration (µg/ml)	Amt of ACOT added (µg/ml)	Amt of ACOT recovered (µg/ml)	mean±SD	% RSD	% recovery	mean±SD	% RSD
20	10	9.972	9.93±0.04	0.40	99.72	99.3±0.40	0.40
20	20	19.938	19.8±0.12	0.63	99.69	99.3±0.29	0.29
20	30	29.847	29.6±0.15	0.57	99.49	98.9±0.51	0.52

n= number of determinations, SD =Standard Deviation, %RSD = % Relative Standard Deviation

### Robustness

The robustness was examined by evaluating the influence of small variations in different conditions such as flow rate,

detection wavelength and organic ratio. The average value of %RSD for determination of ACOT less than 2% revealed robustness of the method. Results of robustness studies are shown in table 7.

Table 7: Robustness studies of ACOT (n=3)

Flow rate	Area (mean±SD)	% RSD	Retention time (mean±SD)	%RSD
0.9	571144.3±7238.28	1.267	11.39333±0.06	0.566
1	570358.3±8351.74	1.464	9.606333±0.02	0.282
1.1	573424.3±7510.62	1.309	8.342667±0.02	0.306
Organic ratio acetonitrile	Area (mean±SD)	%RSD	Retention Time (mean±SD)	%RSD
28	577713.7±4829.15	0.835	11.41633±0.10	0.911
30	578040.3±5985.97	1.035	9.631667±0.01	0.176
32	567522.3±5833.48	1.027	8.592333±0.07	0.852
Wavelength	Area (mean±SD)	% RSD	Retention Time (mean±SD)	%RSD
281	578040.3±7076.25	1.224	9.521±0.008	0.084
282	581313.7±7879.20	1.355	9.505±0.072	0.760
283	568682±5850.65	1.028	9.510±0.017	0.179

n= number of determinations, SD =Standard Deviation, %RSD = % Relative Standard Deviation

### Stability of solutions

Solutions containing different concentrations of ACOT were prepared from standard solution and stored at room temperature for 24 h. They were then injected into LC system. No additional peaks were found in chromatogram and area under the peak comes within 2%.

### Filter compatibility

Filter compatibility was performed on 0.45 µNylon 6,6 syringe filter, 0.45µ PTFE hydrophilic syringe filter and 0.45 µ PTFE hydrophobic syringe filter. Filter compatibility study was done by % recovery method. The sample was filtered through syringe filters

and percentage assay was calculated and variation in the assay value was compared against the centrifuged sample. Results are shown in

table 8. After analysis, it was found that Nylon 6,6 syringe filter and PTFE hydrophilic syringe filter were suitable for filtration.

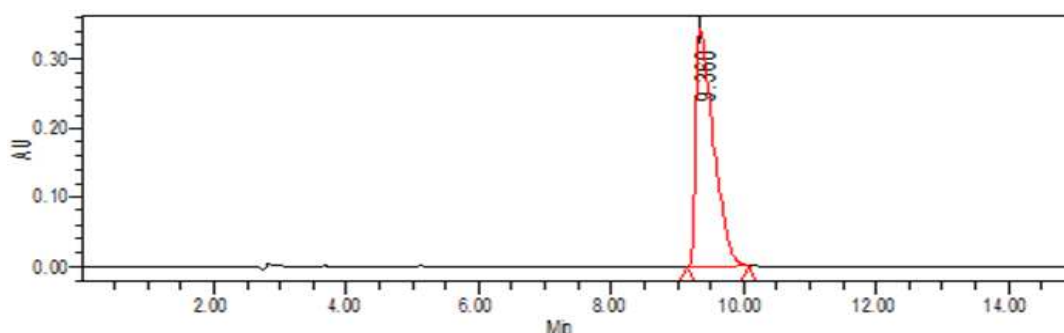
**Table 8: Filter compatibility study**

Sample name	Peak area	% difference
Standard sample	771427	---
Sample filtered through 0.45 $\mu$ Nylon 6,6 syringe filter	770936	0.2
Sample filtered through 0.45 $\mu$ PTFE hydrophilic syringe filter	768234	0.5
Sample filtered through 0.45 $\mu$ PTFE hydrophobic syringe filter	793456	2.8

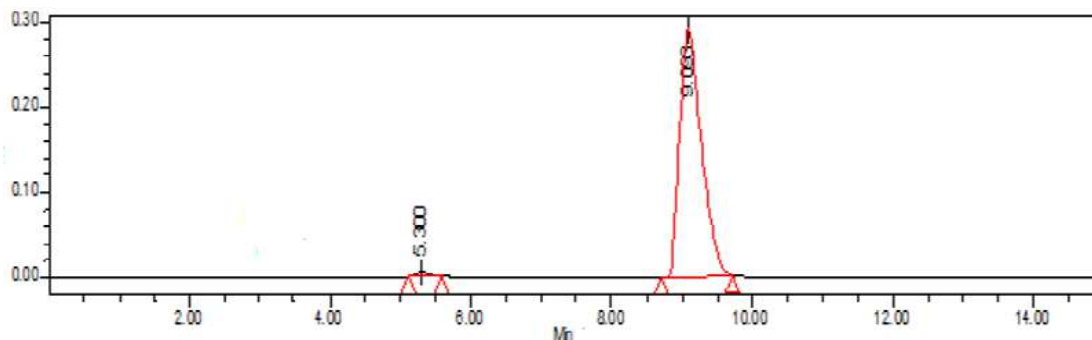
### Force degradation study

The results from the stress testing studies indicate that the method is highly specific for ACOT. Degradation products were completely distinguishable from the parent compound. The drug undergoes significant degradation under acid, alkaline conditions. Alkaline degradation was faster than acidic degradation. The drug

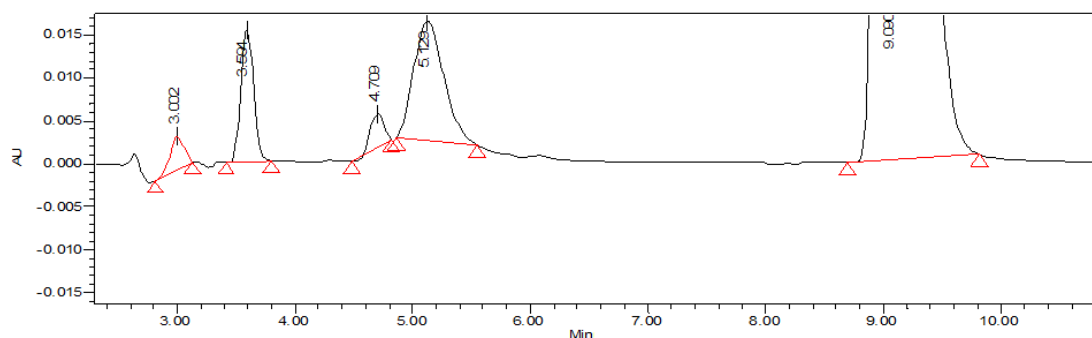
was relatively stable in oxidative neutral, dry heat and photolytic degradation. Three degradation products were obtained in alkaline hydrolysis. In alkaline hydrolysis, degradation peaks were obtained at RT of 3.7, 4.5 and 6.8 min on heating. One degradation product was obtained in acid hydrolysis at RT of 5.3 min. The typical chromatogram of ACOT and stressed samples are shown in fig. 3-10.



**Fig. 3: Chromatogram of ACOT (200 ppm)**



**Fig. 4: Degradation of ACOT in 1 N HCl at 100 °C for 3 h**



**Fig. 5: Degradation of ACOT in 0.5 M NaOH at 100°C for 3 h (Zoomed view)**

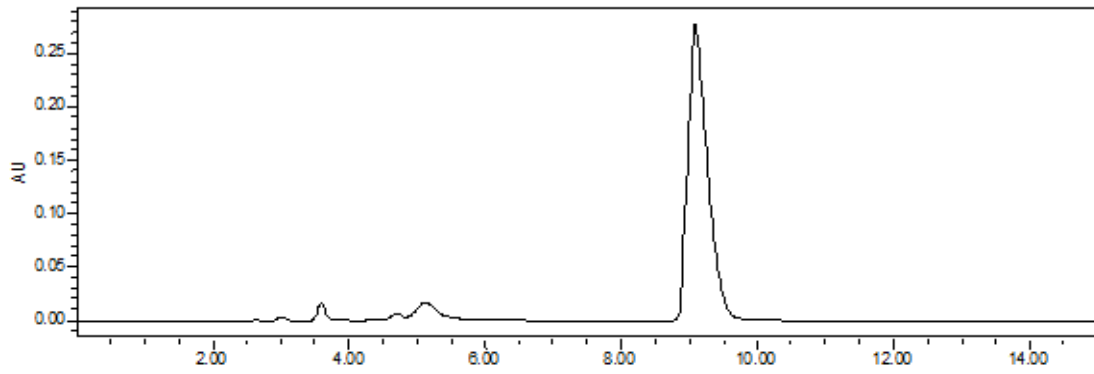


Fig. 6: Degradation of ACOT in 0.5 M NaOH at 100°C for 3 h (Full view)

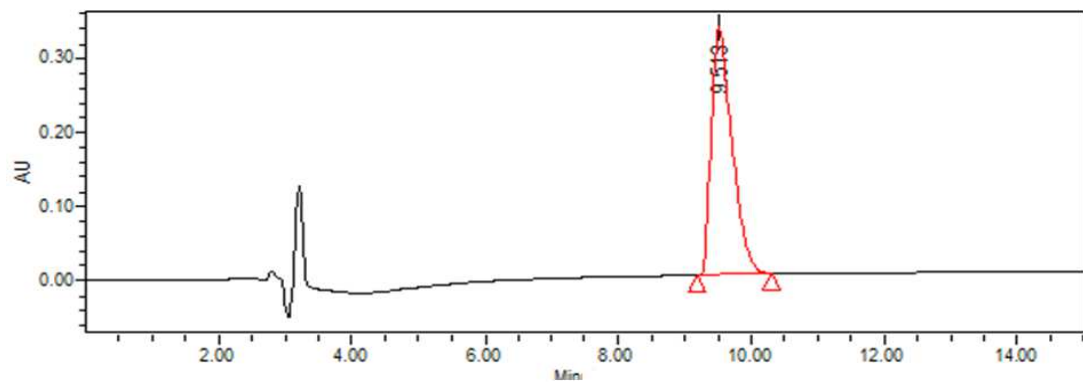


Fig. 7: Degradation of ACOT in 6% H<sub>2</sub>O<sub>2</sub> at room temperature for 48 h

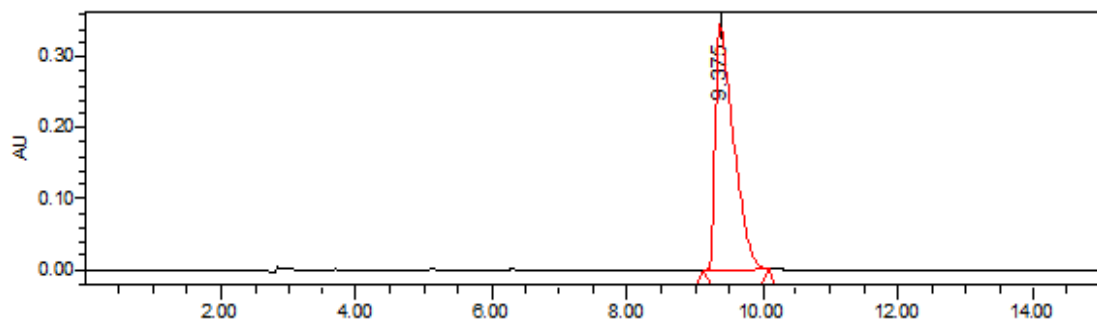


Fig. 8: Degradation of ACOT in thermal at 80°C for 8 d

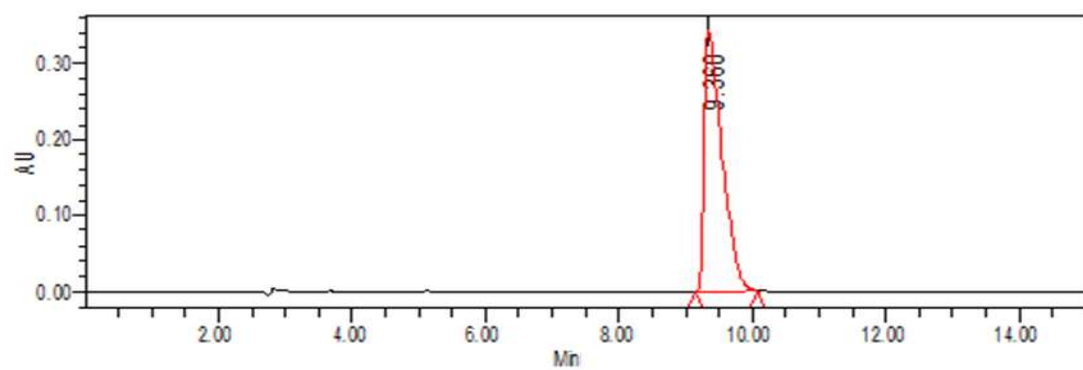


Fig. 9: Degradation of ACOT in photolytic condition dry for 11 d

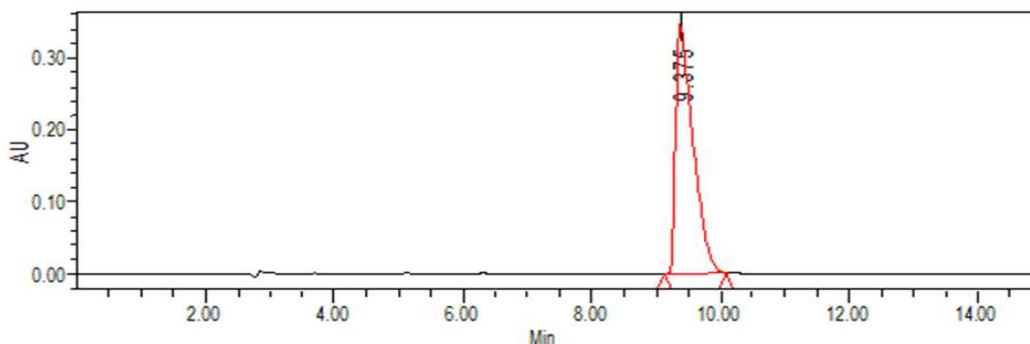


Fig. 10: Degradation of ACOT in photolytic solution for 11 d

Table 9: Data of forced degradation studies of ACOT

Conditions		% degradation in API	% API	% degradation in formulation	% API in formulation	No. of degradation products	RT of degradation products
Acidic	1N HCl at 100 °C for 3 h	0.96%	99.3%	0.7%	99.3%	1	5.300
Alkaline	0.5 N NaOH at 100 °C for 3 h	15.2%	84.7%	14.7%	85.3%	3	3.591 4.709 5.129
Oxidation	6% H <sub>2</sub> O <sub>2</sub> for 48 h at RT	No degradation	99.2 %	No degradation	99.5 %	-	-
Thermal	Dry at 80 °C for 8 d	No degradation	99.8%	No degradation	99.7%	-	-
	Solution at 80 °C for 8 d	No degradation	99.5 %	No degradation	99.7 %	-	-
Photolytic	Dry for 11 d	No degradation	99.5 %	No degradation	99.8 %	-	-
	Solution for 11 d	No degradation	99.7 %	No degradation	99.8 %	-	-

## DISCUSSION

The ICH Q1A [13] guideline suggests that stability indicating method must be performed to monitor the properties of drug substance which are likely to change during storage and which are likely to affect quality, the efficacy of the formulation.

During development of stability indicating method, in alkaline degradation conditions for separation of degradation products from ACOT, trials were taken with mobile phase 0.1 % formic acid: methanol, 0.1 % formic acid: acetonitrile on C-18 column and C-8 column. Degradation peaks were resolved from ACOT on C-8 column with column oven temperature of 40 °C with a mobile phase of 0.1 % triethylamine in 0.2 % formic acid: acetonitrile in the ratio of 70:30. The retention time of ACOT was 9.387 min. Detection was done at 282 nm. The method was validated according to ICH guidelines. The linearity of ACOT was found to be in the range of 5-30 µg/ml with a correlation coefficient of 0.999. Recovery was found to be 98.9-99.3%. Significant degradation was observed in alkaline conditions with three degradation products and in acidic conditions, one degradation product is formed. The reported method [11] is developed on UPLC, with cyano column. Those facilities as UPLC instrument and cyano column both are rarely available in University or institute. The degradation conditions in the reported methods are harsh conditions. Our method is developed by HPLC method on C-8 column. The developed method is simple, precise, isocratic, accurate, robust, cost-effective and with available facilities in common laboratories in absence of advanced facilities and instrument.

## CONCLUSION

A simple, specific, accurate, precise stability indicating method has developed. The proposed method presented the ability to separate ACOT from all its degradation products and therefore can be applied instability testing of the commercially available formulation.

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## AUTHORS CONTRIBUTIONS

All authors have contributed equally to the manuscript.

## CONFLICTS OF INTERESTS

Authors declare no conflicts of interest

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