IJMPR, 2026, 10(5), 47-57 ISSN: 2319-5878

INTERNATIONAL JOURNAL OF MODERN PHARMACEUTICAL RESEARCH

www.ijmpronline.com

Research Article

Impact Factor: 7.533

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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF PANTOPRAZOLE SODIUM AND ACOTIAMIDE HYDROCHLORIDE HYDRATE IN SYNTHETIC MIXTURE

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Article Received on: 04/04/2026 **Article Revised on: 25/04/2026** **Article Published on: 01/05/2026**

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https://doi.org/10.5281/zenodo.19883916

How to cite this Article: Yash V. Patel[1], Dr. Bhumi R. Patel[2]*, Dr. Jaymin G. Patel[2], Ms. Pooja Soni[3], Ms. Janki A. Patel[4], Mr. Ronak N. Patel[4]. (2026). RP-Hplc Method Development And Validation For Simultaneous Estimation of Pantoprazole Sodium and Acotiamide Hydrochloride Hydrate In Synthetic Mixture. International Journal of Modern Pharmaceutical Research, 10(4),

ABSTRACT

A simple, precise, accurate, and stability-indicating RP-HPLC method was successfully developed and validated for the simultaneous estimation of Pantoprazole Sodium and Acotiamide Hydrochloride Hydrate in a synthetic mixture. Chromatographic separation was achieved using a Hibar ODS C18 column with a gradient mobile phase of buffer and acetonitrile, at a flow rate of 0.8 mL/min and detection at 221 nm. The developed RP-HPLC method showed excellent performance with good chromatographic separation of Pantoprazole Sodium and Acotiamide Hydrochloride Hydrate. The method exhibited strong linearity in the ranges of 4-20 µg/mL, 30-150 µg/ml, respectively. The LOD values were 0.169%, 0.689%, while LOQ values were 0.513%, 2.087%. Assay studies showed of 99.02±0.77, 100.38±0.98 respectively, indicating high reliability of the method. Overall, the method proved to be reliable and robust, making it suitable for routine quality control and analytical applications in pharmaceutical formulations. Therefore, the proposed method is suitable for routine quality control analysis and stability studies.

KEYWORDS: RP-HPLC, Pantoprazole Sodium and Acotiamide Hydrochloride Hydrate, method validation, Stability-Indicating Method.

INTRODUCTION

Refractory gastroesophageal reflux disease (GERD) is the persistence of symptoms such as heartburn and regurgitation despite adequate proton pump inhibitor (PPI) therapy, often due to non-acid reflux, oesophageal hypersensitivity, or poor treatment adherence.

Management includes lifestyle modifications, optimizing PPI use, and considering adjuncts like prokinetics, H2 blockers, or surgical options.[1]

Functional dyspepsia (FD), by contrast, is a chronic disorder of upper abdominal discomfort without structural disease, classified into postprandial distress syndrome (early satiety, fullness) and epigastric pain syndrome (pain, burning).[2]

Refractory GERD is typically defined as the persistence of typical reflux symptoms such as heartburn and regurgitation despite at least 8 weeks of optimized, twice-daily PPI therapy. Importantly, this definition requires confirmation that the patient is adherent to therapy and is taking the medication appropriately (i.e., before meals). Diagnostic evaluation often includes upper endoscopy, esophageal pH monitoring (with or without impedance), esophageal manometry, and sometimes gastric emptying studies. These tests help differentiate between ongoing reflux, functional disorders, and structural or motility abnormalities.[1-3]

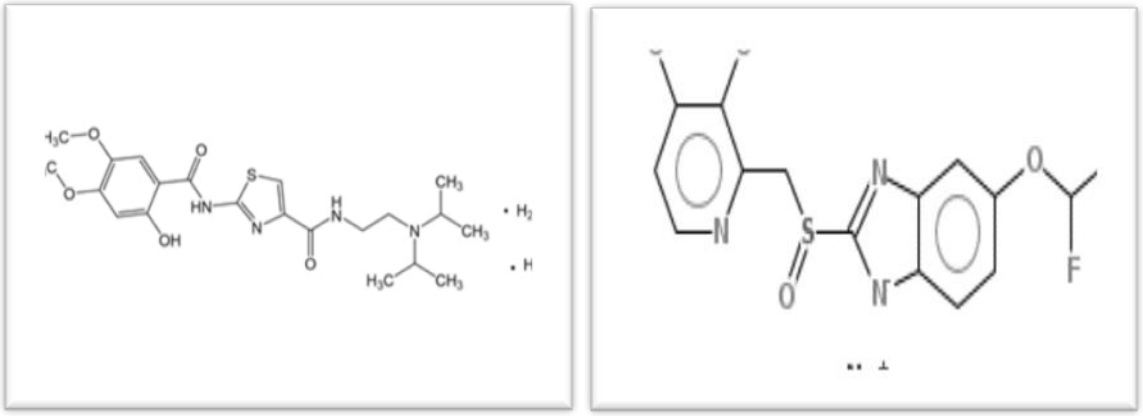

Figure 1: Structure of Acotiamide Hydrochloride Hydrate and Pantoprazole Sodium.

The literature review reveals that few analytical methods were reported like RP-HPLC methods[4], Spectrophotometric method[5], LC/MS[6] and Assay[7] in single or in Combination with other drug in bulk and pharmaceutical dosage form. But no method is reported. So, it is valuable to develop and validate a RP-HPLC method for determination of Acotiamide Hydrochloride Hydrate and Pantoprazole Sodium in synthetic mixture.

MATERIALS AND METHOD

Chemicals and Reagents

Sunij Pharma Pvt. Ltd. (Gujarat, India) provided Pantoprazole Sodium, while Sun Pharmaceutical Industries Ltd provided a gift sample of Acotiamide Hydrochloride Hydrate. Other common reagents such as methanol, orthophosphoric acid, hydrochloric acid and sodium hydroxide provided as a Anachem and acetonitrile and HPLC grade water provided in a Astron chemicals.

Preparation of Solutions

Pantoprazole Sodium stock solution (400 µg/ml)

Accurately weigh 40 mg of drug, dissolve in 100 ml acetonitrile which will give a stock solution of 400 µg/mL.

Acotiamide Hydrochloride Hydrate Stock Solution (3000 µg/mL)

Accurately weigh 300 mg of drug, dissolve in 100 ml acetonitrile which will give a stock solution of 3000 µg/mL.

Mixed standard solution (40 µg/ml Pantoprazole Sodium + 300 µg/ml Acotiamide Hydrochloride Hydrate)

1 ml Pantoprazole Sodium stock solution + 1 ml Acotiamide Hydrochloride Hydrate stock solution into 10 ml volumetric flask. Volume made with diluent.

Preparation of synthetic mixture[8]

The mixture contained Pantoprazole Sodium and Acotiamide Hydrochloride Hydrate along with commonly used pharmaceutical excipients, including mannitol, microcrystalline cellulose, hydroxypropyl methylcellulose and magnesium stearate.

Forced Degradation Studies

To evaluate the stability of the drugs stress studies were performed on a synthetic mixture confirm the separation of Pantoprazole Sodium and Acotiamide Hydrochloride Hydrate from potential degradation products. Stress testing involved exposing the mixture to acidic and alkaline conditions using 0.1 M HCl and 1 M NaOH at room temperature for 30 min, followed by neutralization. Oxidative stress in a 10ml volumetric flask 2ml of synthetic mixture solution was added from 300 µg/mL and 40 µg/mL of ACT and PAN concentration respectively and add 5 ml of 1 % H_2O_2. Heat the solution in water bath at 50°c for 20 minutes. Make up volume with mark with ACN, while thermal degradation was assessed by subjecting the sample to a conical flask and was kept in preheated oven at 60 °C for 3 hours. In all stress conditions, the degraded samples were neutralized or diluted with the mobile phase.

Method Validation[9]

Adhering to the ICH Q2(R2) framework, the study assessed vital performance metrics, specifically robustness, sensitivity, accuracy, precision, Working range, specificity, and system suitability.

Specificity

Different samples, including mobile phase, diluent, individual drug standards, and a synthetic mixture with excipients, were prepared and analyzed under optimized chromatographic conditions. Chromatograms were examined to identify analyte retention times and to check for any interfering peaks from the mobile phase, diluent, or excipients. The results confirmed that there was no interference, demonstrating the specificity of the method.

Range and Linearity

The linearity of the analytical method was established by preparing standard solutions at various concentration levels from the stock solution. Different aliquots were diluted to obtain a range covering the working concentrations of the analytes. These solutions,

containing both drugs in specified ratios, were prepared, mixed, and analyzed under optimized chromatographic conditions. Calibration curves were plotted by relating peak area to concentration, demonstrating the linear response of the method over the selected range.

Precision

The intermediate precision of the RP-HPLC method was evaluated by analyzing Acotiamide (ACT) and Pantoprazole Sodium (PAN) at three concentration levels: **30:4 µg/mL, 90:12 µg/mL, and 150:20 µg/mL**. For intra-day precision, each level was analyzed in triplicate (**n = 3**) within the same day, while inter-day precision was assessed by analyzing the same concentrations over **three consecutive days** in triplicate. The results were quantified using a calibration curve, and precision was expressed as mean, standard deviation (SD), and %RSD, confirming the method's reproducibility and reliability.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

These parameters are useful for understanding the sensitivity of the developed analytical method. Hence, LOD and LOQ were evaluated for Pantoprazole Sodium and Acotiamide Hydrochloride Hydrate. The LOD & LOQ were calculated on basis of formula

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma / S$

Accuracy

The accuracy of the developed HPLC method was evaluated using the standard addition method at three levels: 50%, 100%, and 150%. A placebo matrix of 210 mg containing common excipients was spiked with standard solutions of Acotiamide (ACT) and Pantoprazole Sodium (PAN). The spiking was done using 1 mL, 2 mL, and 3 mL of standard solution to obtain final concentrations of 30:4 µg/mL, 60:8 µg/mL, and 90:12 µg/mL for ACT and PAN, respectively. Each level was analyzed in triplicate (n = 3) under optimized chromatographic conditions. The accuracy was determined by calculating the percentage recovery using calibration curves, confirming the method's reliability and accuracy across the tested concentration range.

Robustness

Robustness evaluates the reliability of an analytical method under small and deliberate variations in method parameters. The robustness of the developed RP-HPLC method was assessed by introducing slight variations in mobile phase composition and flow rate.

Assay of synthetic mixture

The synthetic mixture was prepared by accurately weighing 275 mg of formulation blend containing 150 mg of Acotiamide (ACT) and 20 mg of Pantoprazole Sodium (PAN) along with excipients. This was transferred to a 100 mL volumetric flask, dissolved in acetonitrile with sonication, and the volume was made up to the mark to obtain a stock solution of 1500 µg/mL (ACT) and 200 µg/mL (PAN). The solution was centrifuged at 5000 rpm for 5 minutes and filtered through a 0.45 µm membrane filter to remove insoluble excipients. For further dilution, 2 mL of stock solution was diluted to 10 mL to get 300 µg/mL (ACT) and 40 µg/mL (PAN). Then, 2 mL of this solution was again diluted to 10 mL, producing the final working solution of 60 µg/mL (ACT) and 8 µg/mL (PAN). The final solution was analyzed using RP-HPLC under optimized conditions, and peak areas were recorded for quantification.

RESULT AND DISCUSSION

Analytical wavelength detection

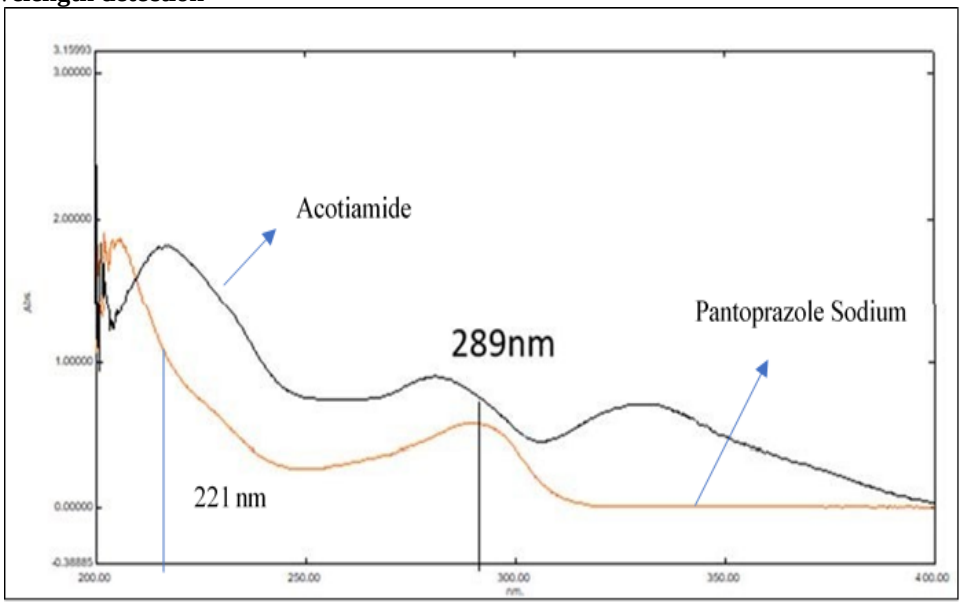

Figure 2: Overlay spectra of PAN and ACT.

During method optimization, detector responses were evaluated at **221 nm and 289 nm**. Both Acotiamide (ACT) and Pantoprazole Sodium (PAN) showed maximum absorbance and better sensitivity at **221 nm** compared to 289 nm. Based on the observed results and literature support, **221 nm** was selected as the optimal wavelength for method development and validation.

Chromatographic conditions optimization

After conducting multiple trials aimed at refining the chromatographic conditions for the estimation of ACT and PAN, a reliable RP-HPLC procedure was successfully developed. The optimized method will subsequently undergo validation to confirm its accuracy, precision, and other essential analytical performance parameters.

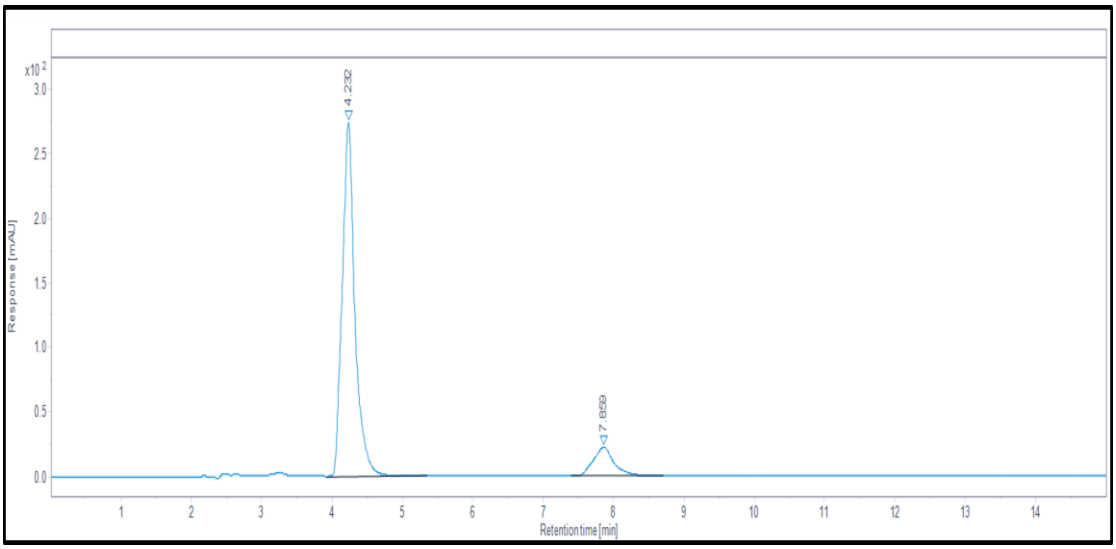

Figure 3: Acotiamide 60 µg/mL: Pantoprazole 8 µg/mL.

Table 1: Optimized chromatographic conditions.

Stationary Phase	**Hibar ODS C 18 (250 cm×4.6 mm, 5µm)**
Mobile Phase (v/v)	Acetonitrile: Phosphate buffer (40:60 % v/v) pH- 5.5
Diluent	Acetonitrile
Mode of elution	Isocratic
Flow Rate (mL/min)	0.8 ml/min
Detection Wavelength (nm)	221 nm
Temperature	30 ℃
Injection Volume (µL)	15 µL
Run Time (minutes)	15 min
Retention time (minutes)	ACT (4.2), PAN (7.8)

System suitability parameters

A mixed standard solution containing PAN (8 µg/mL) and ACT (60 µg/mL) was subjected to system suitability evaluation prior to analysis (n=5).

The SST parameters passed the test with acceptance criteria of %RSD ≤2 for Rt, Tailing factor and Area, and theoretical plates ≥2000.

Table 2: System Suitability parameters.

Parameters	**Results**			
	PAN ± SD	% RSD	ACT ± SD	% RSD
Retention time	7.733 ± 0.051	0.667	4.255 ± 0.005	0.128
Tailing factor	1.91 ± 0.021	1.147	1.753 ± 0.025	1.472
Theoretical Plates	3635.167 ± 54.557	1.500	3564.333 ± 17.783	0.498
Area	465.948 ± 0.589	0.126	3461.648 ± 6.028	0.174

Forced Degradation studies

1. Acid hydrolysis

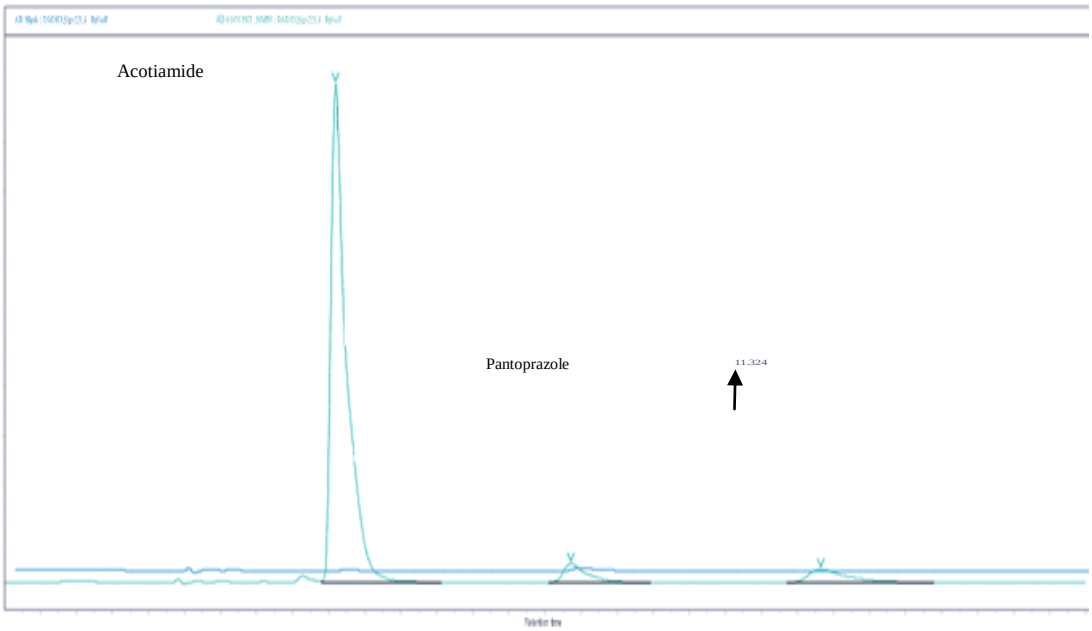

Figure 4: Blank and Synthetic mixture overlay for Acid degradation (30 min)

2. Base Hydrolysis

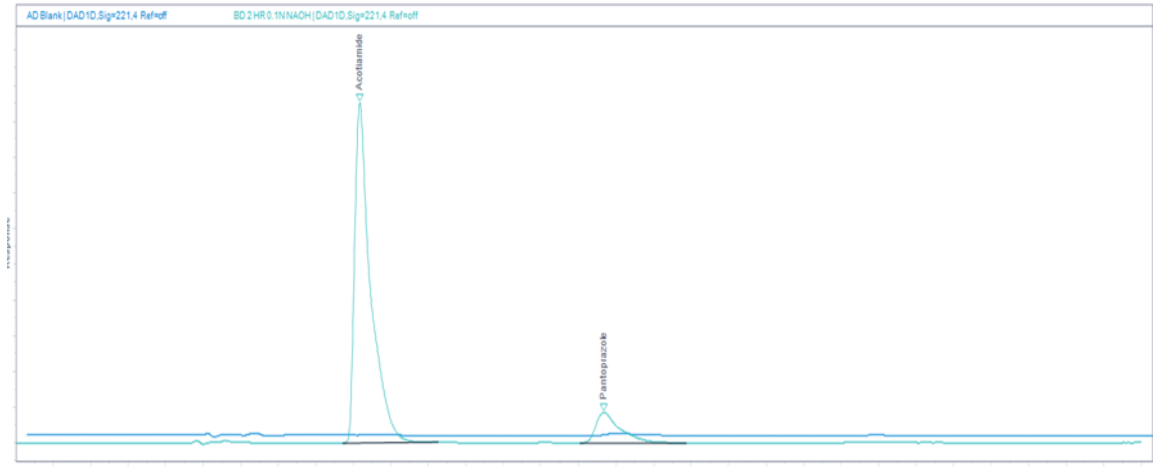

Figure 5: Blank and Synthetic mixture overlay for Base degradation.

3. Oxidative Hydrolysis

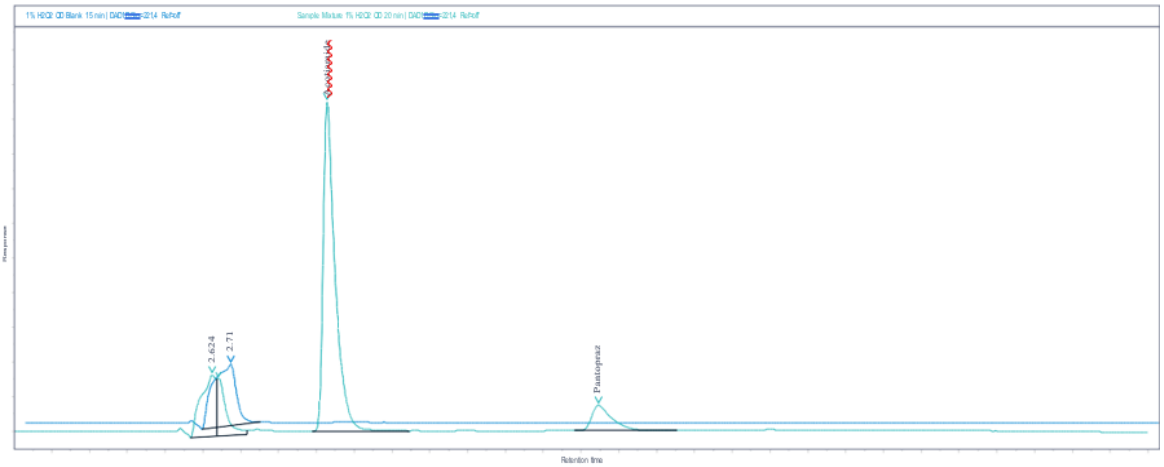

Figure 6: Blank and Synthetic mixture overlay for Peroxide degradation (20min).

4. Thermal Degradation

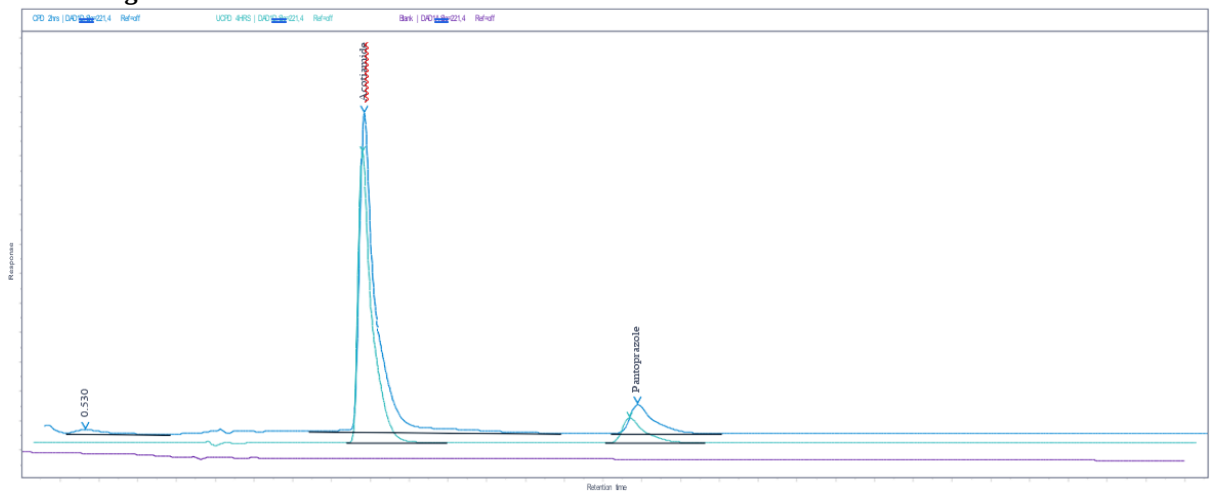

Figure 7: Blank, Control and Synthetic mixture overlay for Thermal Degradation.

Table 3: Summary of Forced Degradation.

Stress Conditions	Area	ACT	PAN	% Degradation (ACT)	% Degradation (PAN)
Acid Degradation	Std. area	3463.19	466.32	0.063	21.640
	Obs. Area	3461	365.41		
Base Degradation	Std. area	3456.21	465.53	1.262	13.670
	Obs. Area	3412.58	401.89		
Oxidative stress	Std. area	3467.85	466.42	6.044	37.938
	Obs. Area	3258.24	289.47		
Thermal degradation	Std. area	3466.40	465.58	5.102	11.386

Validation of RP-HPLC method

Specificity

The specificity of the developed HPLC method was evaluated by analyzing chromatograms of the mobile phase, diluent, individual standard solutions, and synthetic mixture. No peaks were observed at the retention times of ACT and PAN in the blank chromatograms, indicating no interference from solvents. The peaks were symmetrical and free from co-eluting components.

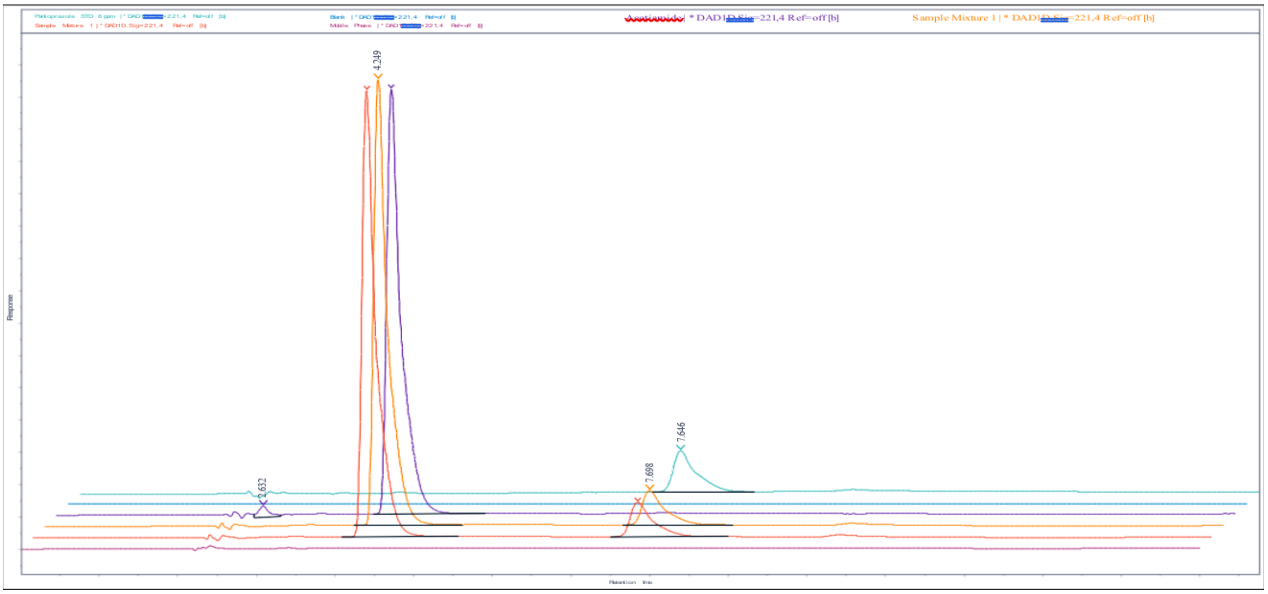

Figure 8: Overlay of (A) Mobile Phase, (B) Standard Solution, (C) Synthetic mixture, (D) Acotiamide, (E) Blank, (F) Pantoprazole Sodium.

Linearity and Range

The developed HPLC method exhibited good linearity over the concentration range of 30–150 µg/mL for Acotiamide HCl Hydrate and 4–20 µg/mL for Pantoprazole Sodium. The calibration curves were constructed by plotting peak area versus concentration for both analytes.The correlation coefficients (R^2) were found to be 0.9999 for Acotiamide HCl Hydrate and 0.9981 for Pantoprazole Sodium, indicating excellent linear relationships between concentration and peak area within the studied range.

Table 4: Linearity Data for ACT.

Sr. No.	Concentration (µg/mL)	Peak Area (Mean ± S. D.)	% RSD
1.	30	1739.58 ± 22.02	1.27
2.	60	3427.15 ± 36.40	1.06
3.	90	5254.74± 37.92	0.72
4.	120	6957.67± 37.29	0.54
5.	150	8743.00± 44.19	0.51
Linear Regression equation			y=58.533x - 31.6
Linear Regression Coefficient			R^2 =0.9999

Table 5: Linearity Data for PAN.

Sr. No.	Concentration (µg/mL)	Peak Area (Mean ± S. D.)	% RSD
1.	4	68450.33 ± **96**	0.14
2.	8	100577.00 ± **87.43**	0.09
3.	12	137406.33 ± **278.99**	0.20
4.	16	169586.00 ± **314.50**	0.19
5.	20	199353.67 ± **223.51**	0.11
Linear Regression equation			y=2646.5x +2748.4
Linear Regression Coefficient			R^2 =0.9988

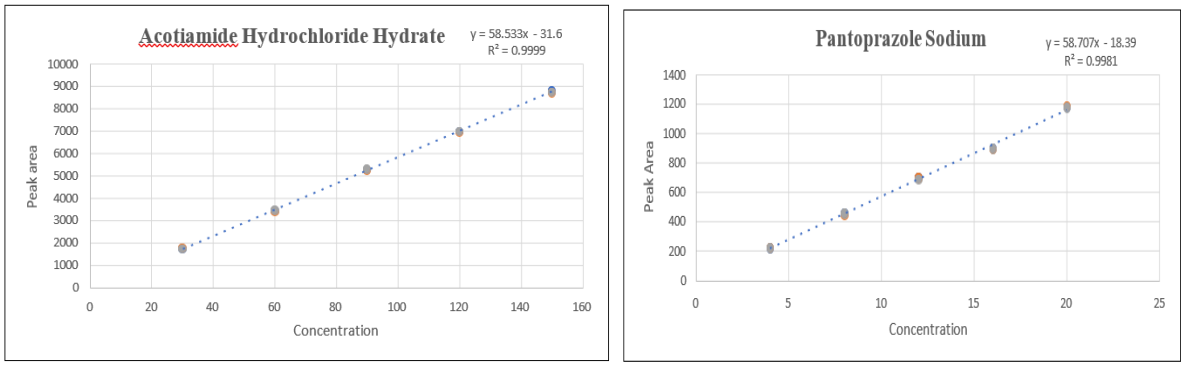

Figure 12: Calibration curve for Linearity of ACT and PAN.

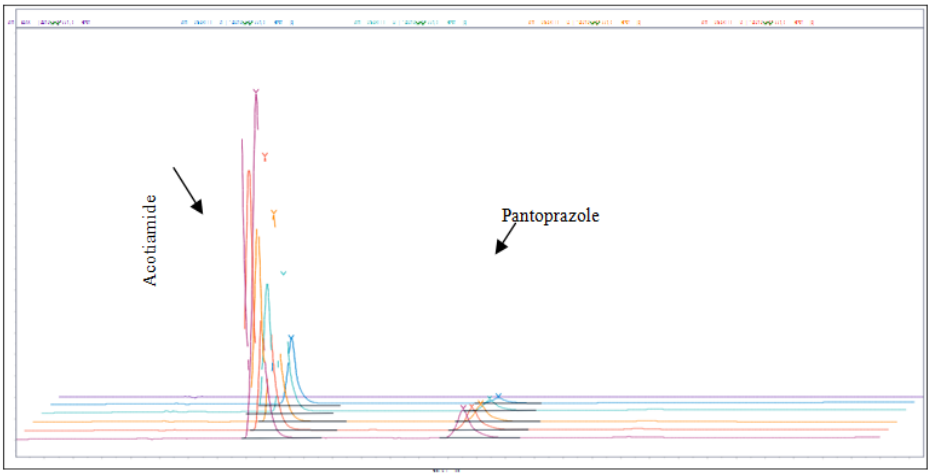

Figure 13: Linearity overlay for ACT and PAN.

Precision

Table 6: Data of Intraday and Inter day precision of ACT.

Concentration (µg. mL^{-1})	Intraday (Mean Area ± SD)	% RSD	Inter-day (Mean Area ± SD)	% RSD
30	1737.86 ± 14.02	0.81	1737.47 ± 23.24	1.34
90	5230.31 ± 57.30	1.10	5186.82 ± 81.42	1.57
150	8675.67 ± 100.06	1.15	8631.67 ± 137.60	1.59

Table 7: Data of Intraday and Inter day precision of PAN.

Concentration (µg. mL^{-1})	Intraday (Mean Area ± SD)	% RSD	Inter-day (Mean Area ± SD)	% RSD
4	218.58 ± 3.15	1.44	222.33 ± 3.02	1.36
12	706.09 ± 7.75	1.10	677.46 ± 8.65	1.28
20	1170.78 ± 14.35	1.23	1153.17 ± 16.40	1.42

Repeatability

Table 8: Repeatability Data for ACT and PAN.

Area	Concentration of ACT (90 µg/mL)	Concentration of PAN (12 µg/mL)
1.	5260.21	698.54
2.	5262.12	699.41
3.	5263.01	699.78
4.	5261.12	700.84
5.	5263.84	701.65
6.	5264.87	697.1
Mean	5262.528	699.5533
SD	1.729779	1.622907
%RSD	0.03287	0.231992

Accuracy

Table 9: Accuracy data for ACT and PAN.

Accuracy Data for ACT						
Level of Spiking	**Quantity of placebo(mg)**	**Amount of drug Added (µg/mL)**	**Amount of drug Recovered (µg/mL)**	**Std area**	**Test area (Mean)**	**% Mean recovery ± SD**
Un-spiked	210	-	-	-	-	-
50%	210	30	29.94	1751.2	1747.52	99.77 ± 0.30
100%	210	60	60.11	3474.2	3480.55	100.17 ± 0.11
150%	210	90	90.10	5153.8	5159.47	100.12 ± 0.12
Accuracy data for PAN						
Un-spiked	210	-	-	-	-	-
50%	210	4	4.05	214.2	216.67	100.94 ± 0.27
100%	210	8	7.93	435.1	431.25	99.16 ± 0.68
150%	210	12	11.98	650.2	649.34	99.83 ± 0.31

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD & LOQ were calculated on basis of formula

$LOD = 3.3 \times \sigma / S$

$LOQ = 10 \times \sigma / S$

σ for Acotiamide HCl Hydrate found to be 12.216 on the basis of calibration curve.

S for the Acotiamide HCl Hydrate was found to be 58.457.

LOD of Acotiamide HCl Hydrate found to be 0.689.

LOQ of Acotiamide HCl Hydrate found to be 2.087.

σ for Pantoprazole Sodium found to be 3.028 on the basis of calibration curve.

S for the Pantoprazole Sodium was found to be 59.059.

LOD of Pantoprazole Sodium found to be 0.169.

LOQ of Pantoprazole Sodium found to be 0.513.

Robustness

Table 10: Robustness data for ACT.

PARAMETER	LEVEL	AREA	MEAN	%RSD
pH	5.25	3471.44	3480.82	0.276
		3480.35		
		3490.67		
	5.65	3565.55	3567.55	0.049
		3568.21		
		3568.88		
Flow Rate (ml/min)	0.6	3305.62	3307.31	0.104
		3299.43		
		3316.88		
	1	3664.61	3669.03	0.267
		3670.84		
		3671.65		
Wavelength(nm)	219 nm	3633.95	3630.41	0.086
		3627.99		
		3629.3		
	223 nm	3366.96	3363.96	0.078
		3361.97		
		3362.94		
Temp °C	28 °C	3495.84	3496.6	0.148
		3491.84		
		3502.13		
	32 °C	3493.7	3482.57	0.276
		3477.08		
		3476.94		

Table 11: Robustness data for PAN.

PARAMETER	LEVEL	AREA	MEAN	%RSD
pH	5.25	419.66	418.86	0.177
		418.74		
		418.19		
	5.65	432.13	424.12	1.83
		417.85		
		416.62		
Flow Rate (ml/min)	0.6	406.11	403.51	0.996
		405.54		
		398.88		
	1	466.43	465.96	0.122
		466.13		
		465.33		
Wavelength(nm)	219 nm	448.71	450.14	0.278
		451.05		
		450.67		
	223 nm	372.91	374.48	0.428
		376.12		
		374.41		
Temp °C	28 °C	405.04	406.92	0.443
		407.08		
		408.64		
	32 °C	426.31	422.81	1.006
		418.07		
		424.04		

Assay

The percentage assay of Acotiamide HCl Hydrate and Pantoprazole Sodium was found to be 100.38% and 99.02%, respectively. The obtained values are within acceptable limits, indicating accurate quantification of both analytes in the sample.

Table 12: Synthetic Mixture Analysis Results.

%Assay ± SD		**%RSD**	
Acotiamide	**Pantoprazole Sodium**	**Acotiamide**	**Pantoprazole Sodium**
100.38±0.98	99.02±0.77	0.99	0.79

CONCLUSION

A robust and precise RP-HPLC method was developed for simultaneous estimation of Acotiamide HCl Hydrate and Pantoprazole Sodium. The method showed high specificity with no interference from excipients. Excellent linearity was achieved with **correlation coefficient ($R^2 > 0.999$)** for both drugs. Precision studies (repeatability, intra-day, inter-day) showed **%RSD < 2.0%**, confirming reproducibility. Accuracy studies demonstrated **recovery within 98–102%**. The method was also found to be robust against small variations and stability-indicating, as it effectively separated analytes from degradation products under stress conditions. Overall, the method complies with ICH Q2(R2) guidelines and is suitable for routine QC and stability studies.

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