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DEVELOPMENT AND VALIDATION OF THE METHOD FOR THE SIMULTANEOUS ESTIMATION OF ACECLOFENAC AND PANTOPRAZOLE

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ABSTRACT: In the present research work, a new UV spectroscopic and RP-HPLC method was described for simultaneous estimation of Aceclofenac (ACL) and Pantoprazole (PNT). Simultaneous Equation method was applied for the spectrophotometric determination of ACL and PNT. Statistical calculations were done by use of 276 nm and 289 nm wavelengths. In RP-HPLC method, the estimation of ACL and PNT carried out by ACN: Buffer (Phosphate acetate) (80:20 v/v) as mobile phase, pH 6.8 at a flow rate of 1.0 mL/min and YMC Triart C18 column (250 mm × 4.6 mm, 5 μ), column. The detection of ACL and PNT was carried out at 295 nm. The retention time of ACL and PNT was found at 3.739 and 2.991, respectively. The results of analysis in both the methods were validated as per ICH guidelines in terms of linearity, range, accuracy, precision, LOD, LOQ, ruggedness, robustness, and solution stability; from this study, it was concluded that the developed method of RP-HPLC can be successfully used for the estimation of ACL and PNT in their combined tablet formulations.

INTRODUCTION: Aceclofenac: Aceclofenac, chemically a phenylacetic acid derivative, has anti-inflammatory and analgesic properties. It is a potent inhibitor of the cyclo-oxygenase enzyme, which is involved in the production of prostaglandins [1]. ACECLO is practically insoluble in water and soluble in alcohol & methyl alcohol, freely soluble in acetone & dimethylformamide. The chemical structure of ACECLO is **Fig. 1.**

Pantoprazole: Pantoprazole, chemically, 5-Difluoromethoxyl Benzimidazole -2-yl-3, 4-dimethoxy-2-pyridyl methyl sulfoxide.

PANTO is a class of substituted Benzimidazole that belongs to long-acting proton pump inhibitor. It acts by suppressing gastric acid secretion through the inhibition of H+ K+ ATPase at the secretory surface of the parietal cells and blocks the final step of gastric acid secretion [2]. It is well absorbed from the Gastro-Intestinal Tract the chemical structure of PANTO is **Fig. 2.**
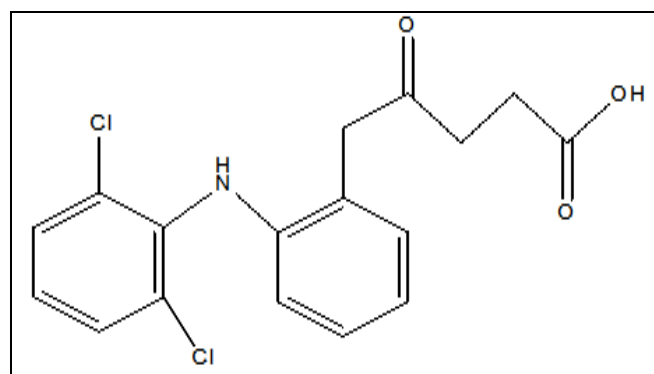
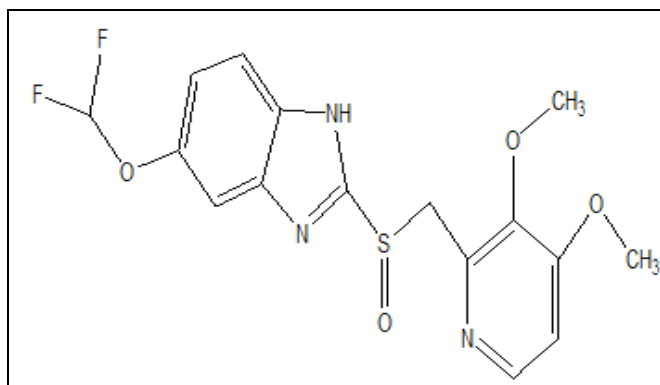**FIG. 1: CHEMICAL STRUCTURE OF ACECLOFENAC**

FIG. 2: CHEMICAL STRUCTURE OF PANTOPRAZOLE

Literature survey revealed that various methods such as UV-spectrophotometry [3, 4,] visible spectrophotometry [5, 6,] RP-HPLC [7, 9,] HPTLC [10, 13] for ACECLO and UV-spectrophotometry [14, 15,] RP-HPLC [16, 17,] HPTLC [18,] UV-spectrophotometry [19, 20,] RP- HPLC [21, 25] for the estimation of PANTO were reported individually and combined with other drugs. But no method was developed and validated for the simultaneous estimation of ACECLO and PANTO in combination by HPTLC.
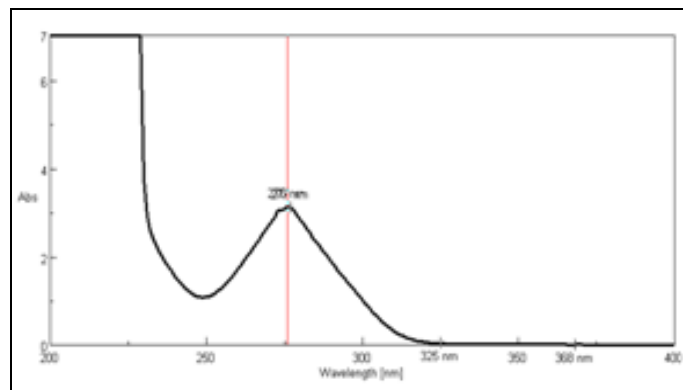
Experimental Section:

Instruments and Apparatus: Electronic analytical balance, micropipette, membrane filtration assembly, pH meter, Shimadzu LC-2010 C, ultra-sonicator and UV-Visible double beam spectrophotometer.

Drugs, Reagents and Chemicals: Drugs, *i.e.*, Aceclofenac and Pantoprazole sodium, were obtained from Vasudha-pharma chemical Pvt Ltd.

Ahmedabad. Chemicals used like ammonium acetate, orthophosphoric acid, potassium dihydrogen orthophosphate, acetone, acetonitrile, deionized water, isopropyl alcohol, triethylamine, and methanol were of AR grade and HPLC grade respectively.

These were obtained from Loba Chem Pvt. Ltd. Mumbai, Moly Chem Pvt. Ltd, Ozone International, Merck Pvt. Ltd, Spectro Chem Pvt. Ltd., J.K. Labs Thane and Thermo Fisher Scientific India. Pvt Ltd. Mumbai.

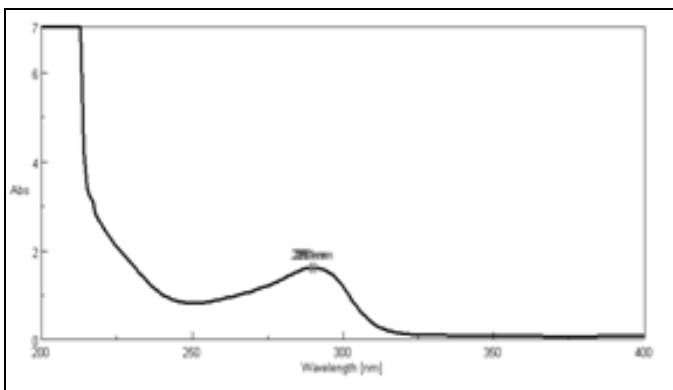

FIG. 3: UV SCAN SPECTRA OF ACL
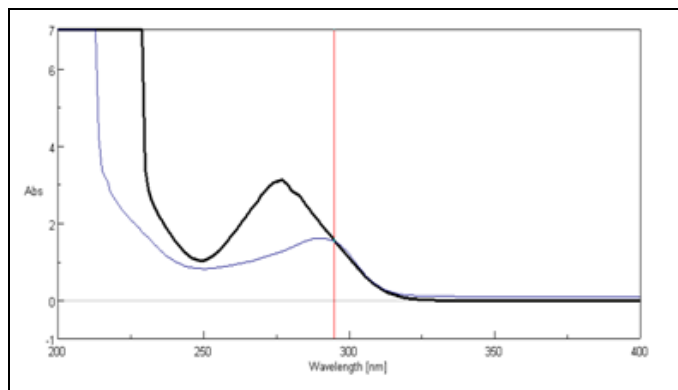

FIG 4: UV SCAN SPECTRA OF PNT

FIG. 5: OVERLAIN SPECTRA OFACL AND PNT SHOWING SELECTED WAVELENGTH (295NM)

Methodology for Uv-spectrophotometry: Selection of Solvent and Wavelength: Solubility of ACL and PNT was checked in solvents like

ethanol, water, methanol and 0.1 N HCl. UV spectrums of the two drugs in these solutions were recorded. The absorbance of the two drugs was

found maximum in methanol: water solvent compared to other solvents, and two wavelengths 276 nm and 289 nm were selected, which were the λ_{max} of ACL and PNT, respectively.

Preparation of Stock Solution of ACL: An accurately weighed quantity of Aceclofenac 10 mg was transferred to the 10 mL volumetric flask and dissolved in methanol: water (80:20). The volume was made up to the mark with the same to make the concentration of 1000 µg/mL.

Preparation of Stock Solution of PNT: An accurately weighed quantity of Pantoprazole Sodium 10 mg was transferred to the 10 mL volumetric flask and dissolved in methanol: water (80:20). The volume was made up to the mark with the same to make the concentration of 1000 µg/mL.

Preparation Standard Stock Solutions: Stock standard solutions of ACL and PNT were prepared separately by dissolving 10 mg in 100 mL Methanol: water (80:20) to obtain a concentration 100 µg/mL of each. From these stock solutions, working standard solutions having concentration 10 µg/mL of ACL and 10 µg/mL of PNT were

prepared by proper dilutions. They were scanned in the UV region, *i.e.*, 400-200 nm. The overlain spectrum was obtained to determine the maximum absorbance (λ_{max}) and iso-absorptive point.

Application of Simultaneous Estimation Method: In the quantitative estimation of two components by the Simultaneous equation method, two wavelengths, *i.e.*, 276 nm ACL, 289 nm PNT were selected as their respective λ_{max} from the overlain spectrum, at which two drugs have maximum absorbance. The concentrations of three drugs in the mixture can be calculated using the following equations.

$$CY = A1ax2\text{-}A2\ ax1/Ax2\text{-} ay1\text{-} ax1\text{-} ay2,\ CX = A2Ay1\text{-}A2\ ay2/Ax2\text{-} ay1\text{-} ax1\text{-} ay2$$

Where, CACL and CPAN are the concentrations of ACL and PNT, respectively in the mixture and in sample solutions. A1 and A2 are the absorbance's of the sample at 276 and 289 nm, respectively, ax1 and ax2 are the absorptivities of ACL at 276 nm and 289 nm respectively, ay1 and ay2 are the absorptivities of PNT at 276 nm and 289 respectively, observations are recorded in **Table 8.**

TABLE 1: DETERMINATION OF ABSORPTIVITY VALUE

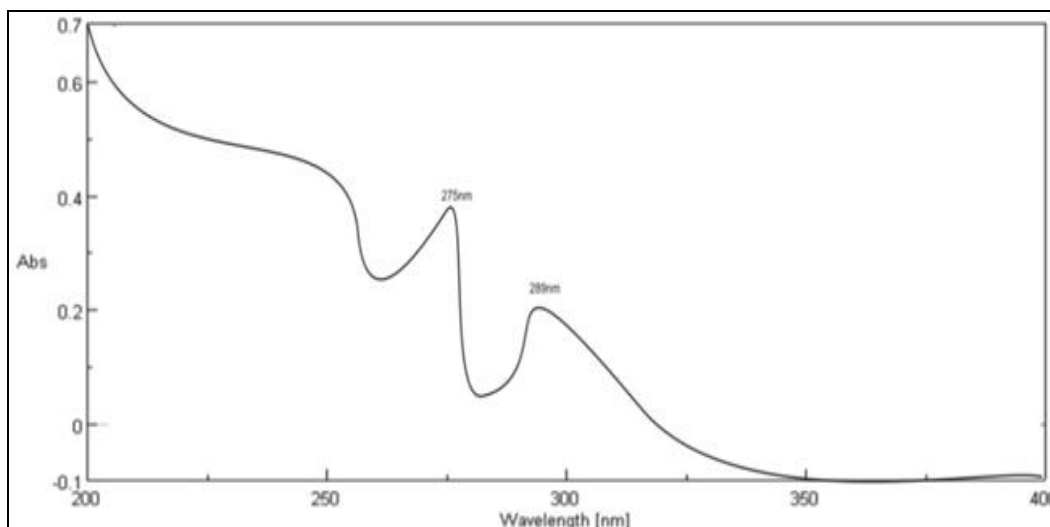| S. no.	Absorptivity of 276 nm		Absorptivity of 289 nm	
	Conc. (µg/mL)	Absorbance	Conc. (µg/mL)	Absorbance
1	5	0.150	0	0.00
2	10	0.316	4	0.172
3	15	0.410	8	0.373
4	20	0.628	12	0.559
5	25	0.755	16	0.761
6	30	0.873	20	0.881
SD		0.0281		0.0187

FIG. 6: SPECTRUM OF FORMULATION CONTAINING ACL AND PNT

Application of the Proposed Method for Estimation of Drugs in Powder: Weigh accurately 10 mg powder transferred to 100 mL volumetric flask and dissolved in 50 mL of methanol: water for 10 min with vigorous shaking. Finally, the volume was made up to the mark with methanol: water.

The solution was then filtered through Whatman filter paper. From this solution, 1 mL was pipette out into a 10 mL volumetric flask and diluted with solvent up to the mark. From this solution, 1 mL was transferred into a 10 mL volumetric flask with and diluted methanol: water up to the mark. The absorbance of the above solution was measured at 276 nm and 289 nm. The concentration of each analyte was determined using the simultaneous equation.

Method Validation: Validation of the proposed spectrophotometric method was carried out as per ICH guidelines by means of the following parameters.

Linearity: As per ICH guidelines, the linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of the analyte in the sample. Linearity study for the proposed method was established by least-square linear regression analysis. An appropriate volume of ACL and PNT in the range of 5-25 µg/mL, 4-20 µg/mL respectively were transferred into series of separate 10 mL volumetric flasks, and volume was made up to mark with methanol-water (80:20) to get final concentrations in the range of 5-25 µg/mL and 4-20 µg/mL respectively.

TABLE 2: LINEARITY DATA OF ACL

S. no	Concentration (µg/mL)	Absorbance at 276 nm
1	5	0.150
2	10	0.316
3	15	0.410
4	20	0.628
5	25	0.755
	Correlation coefficients (r^2)	0.996
	Slope	0.004
	Intercept	0.003

TABLE 3: LINEARITY DATA OF PNT

S. no	Concentration (µg/mL)	Absorbance at 279 nm
1	0	0.00
2	5	0.172
3	10	0.373
4	15	0.559
5	20	0.761
6	25	0.881
	Correlation coefficients (r^2)	0.996
	Slope	0.030
	Intercept	0.028

$$y = 0.045x + 0.003$$
$$R^2 = 0.996$$

FIG. 7: CALIBRATION GRAPH OF ACL

$$y = 0.045x + 0.003$$
$$R^2 = 0.996$$

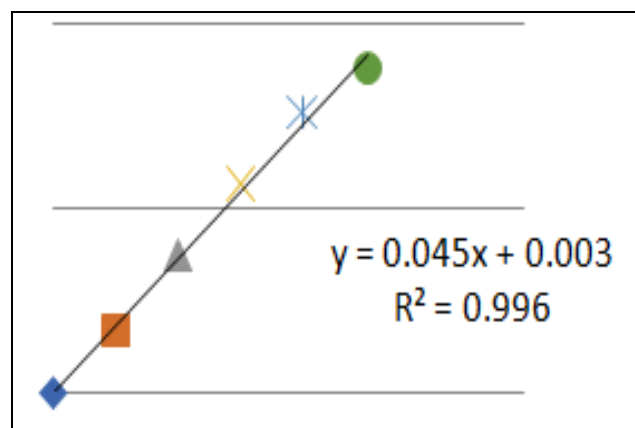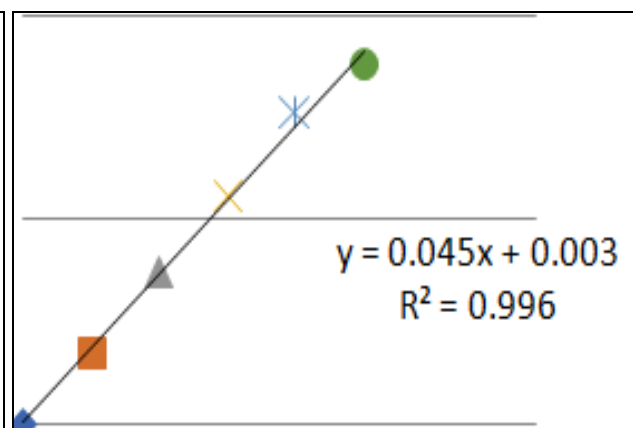**FIG. 8: CALIBRATION GRAPH OF PNT**

Recovery Study (Accuracy Study): The (recovery) accuracy of an analytical procedure expresses the closeness of agreement between the value accepted either as a true conventional value or an accepted reference value and the value found. The accuracy of the proposed methods was checked by recovery studies (standard addition method) by adding standard drug solution to pre-analyzed sample solution at three different concentration levels **Table 5.** Within the range of linearity for all the drugs.

TABLE 4: RECOVERY DATA OF ACL AND PNT

S. no.	Level %	Amount taken (ug/mL)	Amount added (ug/mL)	Amount recovered (ug/mL)	% Recovery ± SD*	RSD*
1	50	5	5	4.984	99.04±0.035	0.3873
	50	5	5	4.987	100.01±0.041	0.0401
	50	5	5	4.982	99.19 ±0.010	0.0206
2	100	10	10	9.979	99.86 ±0.831	0.0835
	100	10	10	9.889	98.33 ±0.567	0.0570
	100	10	10	10.147	99.71±0.813	0.0816
3	150	15	15	15.130	99.65±0.459	0.0465
	150	15	15	14.982	99.43±0.010	0.0336
	150	15	15	14.997	99.69±0.813	0.0815

* indicates determination of three replicates

Precision: The precision of an analytical method expresses the closeness of agreement (degree of scattering) between a series of measurements obtained from multiple sampling of the homogeneous sample under the prescribed conditions. The measurement of precision of an analytical method is performed on three replicates of the standard preparation. The results for the same are usually expressed as the variance, standard deviation or confidence level of a series of measurements. Precision is performed by means of repeatability, reproducibility, and intermediate precision (ruggedness).

Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Reproducibility: Reproducibility expresses the precision between laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from the same homogeneous lots.

Intermediate Precision: Intermediate precision expresses within laboratories variations; different days, different analysts, different equipment, *etc.* The objective of intermediate precision validation is to verify that in the same laboratory, the method will provide the same results once the development phase is over. The precision of the analytical method was checked by repeated scanning and measurement of absorbance of solutions (n=3) for ACL and PNT (5 µg/mL for all two drugs) without changing the parameter of the proposed spectrophotometric method.

TABLE 5: PRECISION DATA FOR ACL

Conc. (µg/mL)	Intraday Mean ± S.D. (n=3)	RSD*	Interday Mean ± S.D. (n=3)	RSD*
5	0.022 ± 0.0572	0.0190	0.024 ± 0.0045	0.0015
10	0.043 ± 0.0558	0.0186	0.044 ± 0.0794	0.0264
15	0.067 ± 0.0719	0.0239	0.068 ± 0.0095	0.0031

* indicates determination of three replicates

TABLE 6: PRECISION DATA FOR PNT

Conc. (µg/mL)	Intraday Mean ± S.D. (n=3)	RSD*	Interday Mean ± S.D. (n=3)	RSD*
4	0.188 ± 0.0502	0.0167	0.189 ± 0.0023	0.00076
8	0.314 ± 0.0597	0.0199	0.315 ± 0.0015	0.00050
12	0.524 ± 0.0664	0.0221	0.525 ± 0.0020	0.00069

* indicates determination of three replicates

Specificity: Specificity is the ability to assess the analyte unequivocally in the presence of components that may be expected to be present. Typically, these might include impurities,

degradants, matrix, *etc.* Comparison of the UV spectrum obtained from the solvent (blank) **Fig. 25** and ACL and PNT tablet shown in **Fig. 26, 27,** and

28. The sample revealed no significant interference, using the same analytical conditions for all samples.
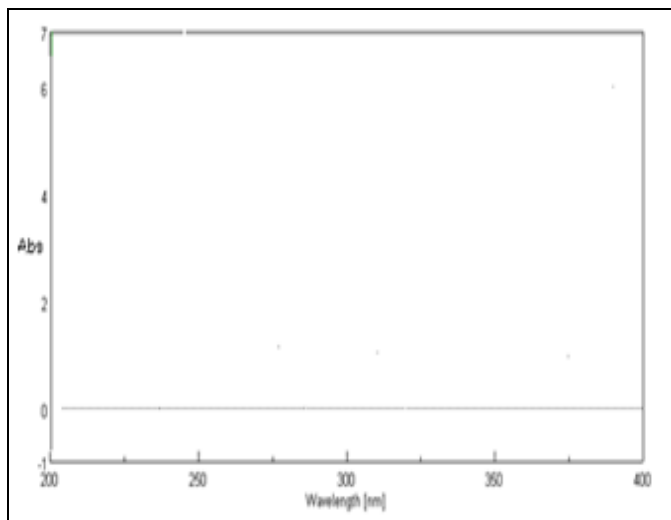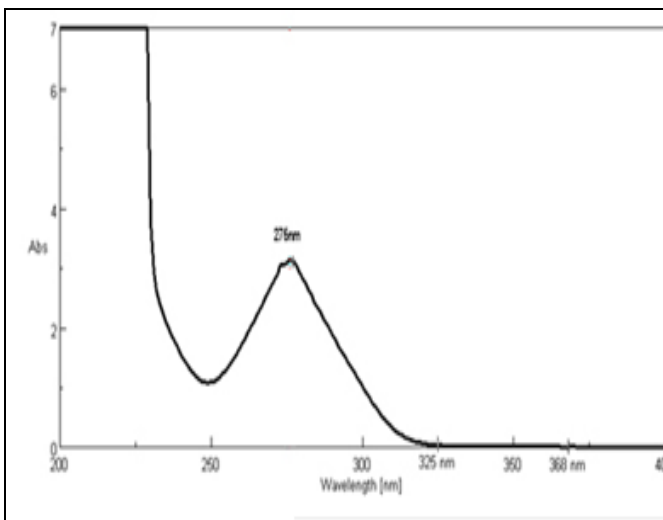

FIG. 9: BASELINE IN METHANOL: WATER

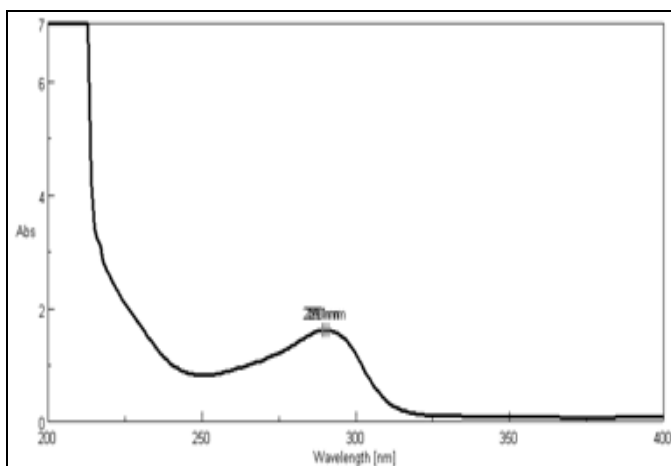**FIG. 10: UV SPECTRA OF ACECLOFENAC (276 NM)**

FIG. 11: UV SPECTRA OF PANTOPRAZOLE CONTAINING
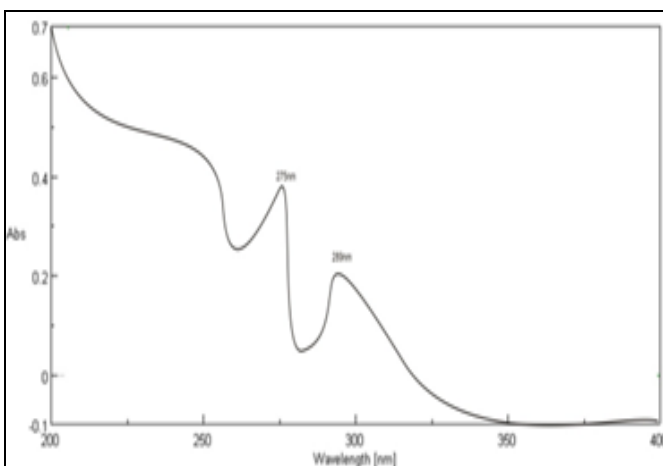
FIG. 12: SPECTRUM OF FORMULATION SODIUM (289 NM) ACL AND PNT

Limit of Detection: According to ICH guidelines, the detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantities as an exact value. The limit of detection can be calculated using the following equation as per ICH guidelines.

$$LOD = 3.3 \times N/S$$

Where, N = Standard deviation of the response, S = Slope of the calibration curve. The detection limit for the proposed method for ACL and PNT was found to be as shown in **Tables 8** and **9**.

Limit of Quantification: The quantitation limit of an individual analytical procedure is the lowest

amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly to determine impurities and/or degradation products. The limit of quantification can be calculated using the following equation as per ICH guidelines.

$$LOQ = 10 \times N/S$$

Where, N= Standard deviation of the response, S = Slope of the calibration curve. The quantitation limit for the proposed method for ACL and PNT was found to be as shown in **Tables 8** and **9**.

TABLE 7: LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION OF ACL

S. no	5 μg/Ml	10 μg/mL	15 μg/mL	20 μg/mL	25 μg/mL	Mean of SD*
1	0.023	0.0421	0.0683	0.0853	0.103	
2	0.0233	0.0423	0.0684	0.0856	0.104	
3	0.0237	0.0425	0.0686	0.0854	0.105	
4	0.0234	0.0427	0.0687	0.0857	0.107	
5	0.0236	0.0428	0.0682	0.0858	0.108	
Mean	0.0235	0.0427	0.0688	0.0857	0.107	
SD*	0.00254	0.00277	0.00277	0.00258	0.00364	0.002866
LOD			0.2053 μg/mL			
LOQ			0.6222 μg/mL			

TABLE 8: LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION OF PNT

S. no	5 μg/mL	10 μg/mL	15 μg/mL	20 μg/mL	25 μg/mL	Mean of SD*
1	0.188	0.314	0.478	0.682	0.786	
2	0.189	0.315	0.477	0.684	0.789	
3	0.190	0.317	0.479	0.685	0.787	
4	0.192	0.318	0.481	0.686	0.788	
5	0.194	0.319	0.482	0.687	0.791	
Mean	0.191	0.316	0.479	0.686	0.790	
SD*	0.00286	0.00207	0.00277	0.00270	0.00319	0.00272
LOD			0.2481 μg/ mL			
LOQ	0.7297μg/Ml					

Ruggedness: Different parameters like different laboratory conditions, different analysts, a different source of reagents and solvents were used for the proposed method of estimation; as a result, there was no significant change in the optimized parameters of the proposed method was observed in **Table 17.**

TABLE 9: RUGGEDNESS DATA FOR ACL AND PNT

Parameter	% Assay		SD*		RSD*	
	ACL	PNT	ACL	PNT	ACL	PNT
Analyst -1st	98.07	98.33	0.6735	0.7043	0.2245	0.2347
Analyst- 2nd	99.60	98.77	0.2886	0.1955	0.0962	0.3985
Lab-1st	98.07	99.08	0.8146	0.7405	0.2715	0.2468
Lab-2nd	99.91	99.43	0.9862	0.9838	0.6583	0.6585
Reagent -1st	99.17	98.19	0.8146	0.7659	0.2713	0.5886
Reagent-2nd	99.27	98.18	0.3569	0.1351	0.4523	0.3783

* indicates average of three determination

Robustness: Robustness is the capacity of a method to remain unaffected by small, deliberate variations in method parameters. The robustness of a method was evaluated by varying method parameters such as percentage organic solvent, ionic strength, or temperature and determining the effect (if any) on the results of the method. The optimized system suitability parameter was unaffected by a small variation in percentage organic solvent, ionic strength, and temperature. Methodology for Rp-Hplc Method: Solubility study of standard drugs: Solubility of ACL and PNT was observed by dissolving them in different polar solvents as indicated in **Table 11.**

Selection of Mobile Phase: For the selection of optimized mobile phase for separation of ACL and PNT various experiments were performed as indicated in **Table 19.** Eventually, the mobile phase with the composition of ACN: Buffer (70:30 v/v) was selected for the analysis and chromatographic study. The buffer preparation was done by dissolving 3.40 gm of potassium dihydrogen phosphate dissolved in 1000 ml of water, pH adjusted to 6.8 by using Potassium Hydroxide.

TABLE 10: SOLUBILITY PROFILE OF ACL AND PNT

Solvents	Solubility	
	ACL	PNT
Acetone	-	+
Acetonitrile	+++	+++
Ethanol	-	+
Methanol	+++	+++
Water	++	++

(+++) Symbolize highly soluble, (++) freely soluble, (+) soluble, (-)

TABLE 11: MOBILE PHASE SELECTION FOR RP-HPLC METHOD OF ACL AND PNT

S. no	Mobile phase Composition	Composition	Remark
1	Acetonitrile+ Ammonium acetate Buffer (pH-3)	80:20	Peak broadening and delay in retention time.
2	Acetonitrile + Ammonium acetate Buffer(pH-5)	70:30	Ghost peak was observed.
3	Acetonitrile + Ammonium acetate Buffer(pH-6.4)	60:40	Retention time very close with less resolution.
4	Acetonitrile + Ammonium acetate buffer (pH-6.8)	50:50	Peak Resolution problem.
5	Acetonitrile + Phosphate Buffer (pH-5)	60:40	Delay in retention time.
6	Acetonitrile + Phosphate Buffer (pH-6.4)	80:20	Delay in retention time.
7	Acetonitrile + Phosphate Buffer (pH-6.8)	70:30	Satisfactory and better results M.P. selected for further analysis.

Selection of Analytical Wavelength: By appropriate dilution of each standard stock solution in the mobile phase, various concentrations of ACL and PNT-were prepared separately. Each solution was scanned in between the range of 200-400 nm **Fig 13, 14** and their overlain spectrum was taken

Fig. 15. The isosbestic point was observed at 295 nm in the overlain spectra of ACL and PNT. The wavelength selected for the HPLC analysis was 295 nm to which these three drugs showed significant absorbance and very good resolution.
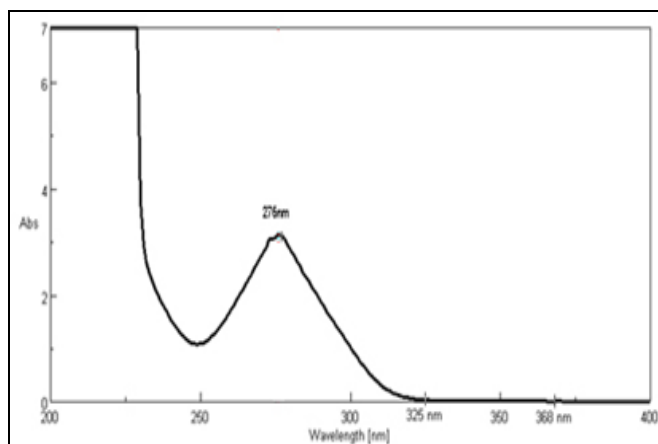
FIG. 13: UV-VIS SPECTRA OF ACL (276 NM) IN MOBILE PHASE
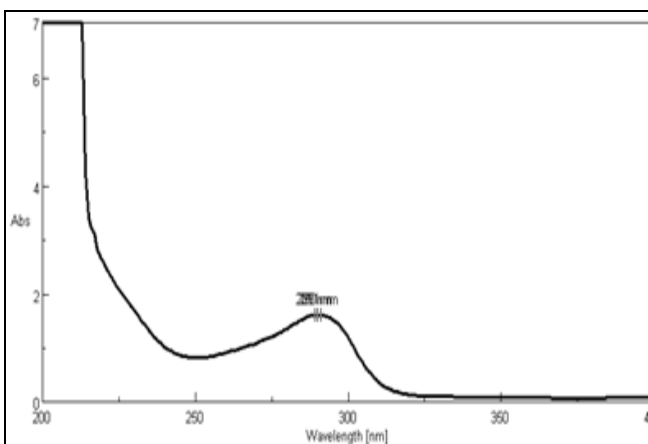
FIG. 14: UV-VIS SPECTRA OF PNT (289 NM) IN MOBILE PHASE
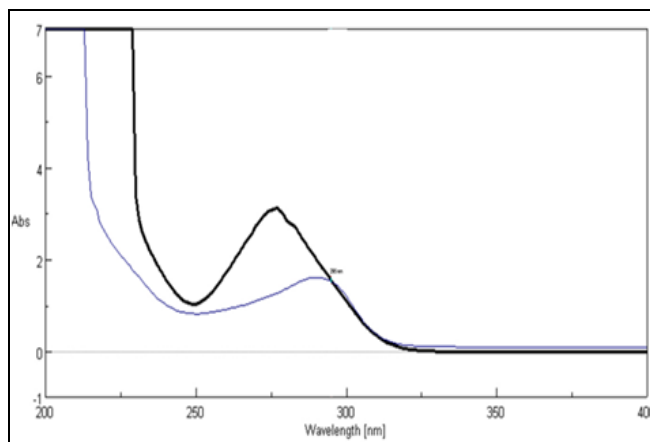
FIG. 15: OVERLAIN SPECTRA OF ACL AND PNT (295 NM) IN MOBILE PHASE

Selection of Retention Time: Prior to HPLC analysis, baseline stabilization has been achieved with the mobile phase. After that, five replicates ACL and PNT were injected in the HPLC column, respectively, and their retention times, a number of theoretical plates, asymmetry factors were

recorded. As the final result has been obtained were same and no significant change has been seen.

Preparation of Buffer Solution: The buffer preparation was done by dissolving 3.40 gm of Potassium dihydrogen phosphate in 1000 mL

water, pH adjusted to 6.8 by using Potassium Hydroxide.

Preparation of mobile phase: Firstly, buffer preparation was done by dissolving 3.40 gm of Potassium dihydrogen phosphate in 1000 mL water, pH adjusted to 6.8 by using Potassium Hydroxide then 20 min ultra-sonication of this buffer solution was done and ACN:

Buffer (70:30 v/v), the prepared mobile phase was degassed by ultra-sonication for about 20 min, lastly, the mobile phase after degassing was filtered through 0.45 μm membrane nylon filter.

Degassing of The Mobile Phase: The mobile phase was prepared degassed by ultra-sonication for about 20 min, so as to avoid the disturbances caused by dissolved gases.

Filtration of Mobile Phase: The mobile phase after degassing was filtered through 0.45 μm membrane nylon filter to remove the smaller particles that may present in the mobile phase.

Preparation of standard stock solutions: ACL 10 mg, PNT 10 mg were accurately weighed on an electronic balance and dissolved in 50 mL of mobile phase separately with shaking. Then the resulting solutions were sonicated, and the volume was made up to 100 mL by addition of the mobile phase to get the conc. 100 μg/mL.

From the standard stock solution of drugs, appropriate dilutions were made with the mobile phase and the sample was filtered through 0.2 μm membrane nylon filter.

Loading of Mobile Phase: Filtered and degassed mobile phase was loaded in the 500 mL reservoir. Priming was done for each freshly prepared mobile phase.

Baseline Stabilization: The detector was turned on for an h before the actual run-in in order to obtain the stable UV light. The mobile phase run was started at desired flow rate, and the run was continued until the stable baseline was obtained.

Loading of Samples: Well prepared and filtered sample ACL and PNT were loaded into the Rheodyne injector port using a 2 mL glass syringe and then the sample was injected.

Washing of The Column: Once the analysis of samples was finished, the column was first washed by flushing with the mobile phase for half an hour, afterward with double distilled water and methanol in 1:1 proportion for another one h.

Selection and Optimization of HPLC Method: After the selection of a suitable mobile phase, it was then optimized for its reproducibility, sensitivity, and accuracy.

Chromatographic Condition: Liquid chromatography was performed on Shimadzu LC-2010HT (Shimadzu, Japan). The system builds with UV-2070 as UV-VIS detector and YMC Triart C18 (4.6 × 250 mm, 5μm) column with a 5 μL by autosampler. The HPLC system was equipped with Chrom-NAV software for data processing.

The ACL and PNT were eluted off the column with a mobile phase consisting of ACN: Buffer (70:30 v/v, pH 6.8) at a flow rate of 1.0 mL/min in isocratic mode. The mobile phase was filtered through a 0.45 μm nylon filter and then ultrasonicated for 30 min. The injection volume was 5 μL, and the eluent was detected at 295 nm, which was selected as the wavelength for further analysis. The retention time of ACL and PNT were around 3.739 and 2.991 min, respectively, and the total run was 10 min.

Procedure: Take the amount of powder equivalent to ACL (10 mg), and PNT (4 mg) weighed accurately and then transferred to 100 mL dried volumetric flask. A sufficient amount of mobile phase was added to dissolve the content, and the resulting solution was shaken for 20 min.

The volume was made up to 100 mL with mobile phase and then filtered through a membrane filter and degassed in a sonicator. From this solution, appropriate dilutions o ACL and PNT were made to get the final concentrations after that sample was injected in to the HPLC system to get a chromatogram.

The chromatogram obtained is shown in **Fig. 16.** and the area obtained in each chromatogram of three replicates was correlated with the regression equation and the amount found was calculated, which was within the limit results are recorded in **Table 12**.

TABLE 12: ANALYSIS OF TABLET FORMULATION

Brand Name of Tablet Formulation	Drug	Label Claim	Peak area (µV/sec)	% of label claim determined	Mean %	SD*	RSD*
Zerodol Zydus	ACL	750	109256	99.89%	73%	0.2192	0.2197
Pantocid Hd (Compound) By-Sunpharma Ltd.	PNT	40	104555	99.22%	99.76%	0.1918	0.1962

* indicates average of three determination
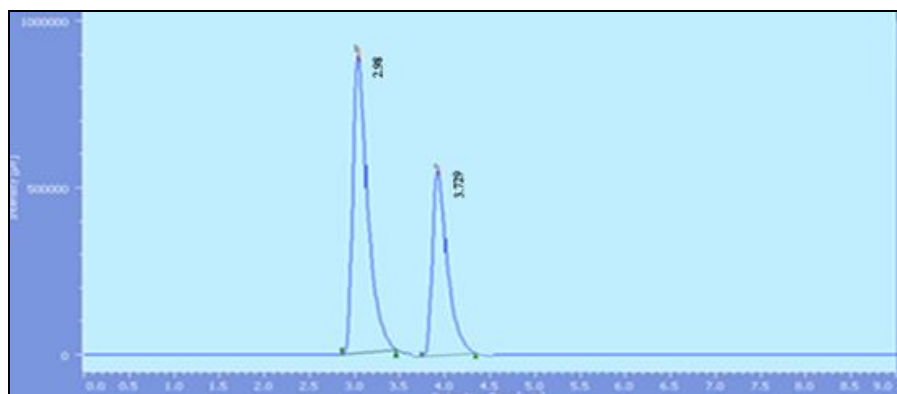
FIG. 16: CHROMATOGRAM OF TABLET SOLUTION OF ACL AND PNT

S. no	Drug	Retention time	Peak Area(µV/sec)	% area	Symmetric factor	NTP
1	ACL	3.739	16393572	100	1.42	3053
2	PNT	2.991	19878508	100	1.36	2535

Analytical method Validation:

Linearity: Suitable dilutions of different concentrations using mobile phase were made from the standard stock solutions. Linearity study for the proposed method was established by least-square linear regression analysis. The linearity is the relationship between peak area and concentration was determined by analyzing over the concentration range of 5-25 µg/mL for ACL and 5-25 µg/ml for PNT. In this the peak area was recorded against the concentration, and the graph of concentration v/s peak area was plotted. Results obtained are shown in **Table 14** and **Table 15.**

TABLE 13: LINEARITY DATA FOR ACL

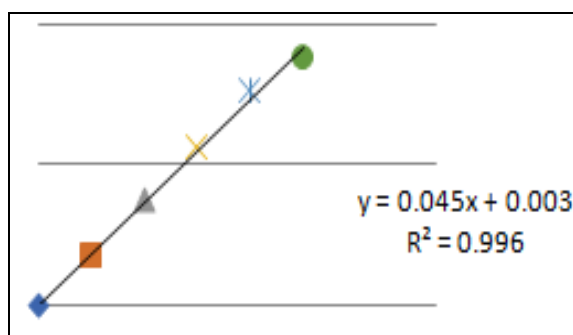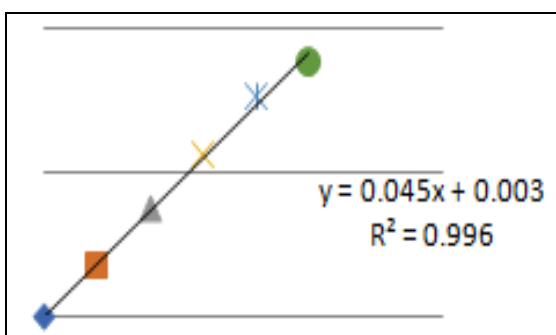| Standard conc	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/Ml	25 µg/mL
Replicates			Peak area		
1	109213	221210	341939	432446	541892
2	109281	221392	342333	432314	542013
3	109301	221991	342415	432610	541916
4	109340	221492	342319	432214	542011
5	109410	222113	342412	432319	542115
Mean	109309	221639	342283	432380	541989
±SD	65.15	395.21	197.60	115.38	88.94
RSD	0.05960	0.1783	0.0577	0.0266	0.01640

FIG. 17: LINEARITY DATA FOR ACL

FIG. 18: LINEARITY DATA FOR PNT

TABLE 14: LINEARITY DATA FOR PNT

Standard conc.	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL
Replicates			Peak area		
1	104550	231644	361268	457984	548588
2	104566	231656	361366	457983	548434
3	104538	231632	361310	457881	548434
4	104432	231522	361322	457921	548510
5	104511	231620	361323	457822	548521
Mean	104519	231614	3613171	457918	548494
±SD	47.25	47.92	79.25	141.18	202.41
RSD	0.0451	0.0206	0.0219	0.0308	0.0036

Accuracy (Recovery study): To check the accuracy of the proposed method, the level of recovery carried out in 50, 100 and 150% of the concentration as per the standard addition method. The standard solution was accessed by the addition of a series of a known amount of standard drug in the tablet sample, and then the contents were estimated by an assay method. The % recovery for ACL and PNT was in the acceptable range shown in Tables **16** and **17.**

TABLE 15: RECOVERY STUDY OF ACL

Recovery level %	Aceclofenac							
	Area (µV/ sec)	Amt. Taken (µg/mL)	Amt. added (µg/mL)	Total amount (µg/mL)	Amt. recovered (µg/mL)	% recovery	Average recovery% ± SD	RSD
50%	109214	5.0	2.5	7.5	7.428	99.04	98.92	0.714
	221211	5.0	2.5	7.5	7.440	98.66	±0.7071	
	341940	5.0	2.5	7.5	7.433	99.06		
100%	109215	5.0	5.0	10	9.803	98.03	98.28	0.527
	221213	5.0	5.0	10	9.901	99.01	±0.5186	
	341942	5.0	5.0	10	9.782	99.82		
150%	109216	5.0	7.5	12.5	12.413	99.58	99.01	0.047
	221215	5.0	7.5	12.5	12.401	99.02	±0.0469	
	341944	5.0	7.5	12.5	12.339	98.43		

TABLE 16: RECOVERY STUDY OF PNT

Recovery level %	Pantoprazole sodium							
	Area (µV/ sec)	Amt. taken(µg/ mL)	Amt. added (µg/mL)	Total amount (µg/mL)	Amt. recovered (µg/mL)	% recovery	Average recovery% ± SD	RSD
50%	104551	5.0	2.5	7.5	7.476	99.68	99.05	0.341
	231645	5.0	2.5	7.5	7.482	99.16	±0.7273	
	361268	5.0	2.5	7.5	7.479	99.06		
	104552	5.0	5.0	10	9.989	99.89	99.60	0.018
100%	231647	5.0	5.0	10	9.982	99.83	±0.4558	
	361269	5.0	5.0	10	9.983	99.15		
150%	104555	5.0	7.5	12.5	11.345	97.12	98.38	0.042
	231648	5.0	7.5	12.5	11.473	98.02	±0.0917	
	361271	5.0	7.5	12.5	11.979	99.24		

TABLE 17: INTER-DAY VARIABILITY OF ACL

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/sec)	± SD[*]	RSD[*]
	Day 1	Day 2	Day 3			
5	109213	109330	109225	109256	97.22	0.0163
15	341910	342235	342307	342150	138.12	0.0185
25	541892	541982	541822	541898	315.61	0.0145

* indicates average of three determination

Precision: Repeatability of method was established by analyzing various replicates of standards. ACL and PNT the solutions, were analysed three times, in order to record any intra-day and inter-day variation in the result. The data obtained for inter-day variations are shown in **Tables 25** and **26.** The result obtained from intra-day variations is shown in **Tables 20** and **21.**

TABLE 18: INTER-DAY VARIABILITY OF PNT

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/sec)	± SD*	RSD*
	Day 1	Day 2	Day 3			
5	104550	104552	104549	104550	526.86	0.0368
15	361261	361460	361663	361461	211.00	0.0581
25	548588	548316	548141	548315	235.24	0.0493

* indicates an average of three determination

TABLE 19: INTRA-DAY VARIABILITY OF ACL

Conc. (µg/mL)	Peak area (µV/ sec)			Mean area (µV/sec)	± SD*	RSD*
	Trial 1	Trial 2	Trial 3			
5	109280	109416	109422	109372	126.62	0.0168
15	341933	341287	341810	341676	58.021	0.0360
25	541882	541922	541983	541929	97.505	0.0311

* indicates an average of three determination

TABLE 20: INTRA-DAY VARIABILITY OF PNT

Conc. (µg/mL)	Peak area (µV/sec)			Mean area (µV/Sec)	± SD*	RSD*
	Trial 1	Trial 2	Trial 3			
5	104650	104552	104754	104652	201.01	0.0975
15	361335	361429	361633	361465	201.53	0.0590
25	548041	548440	548342	548274	222.92	0.0322

* indicates an average of three determination

Limit Detection (LOD): The value for LOD was calculated from the following formula

$$LOD = 3.3\sigma/S$$

Where, σ = Standard deviation of the response, S= Slope of the calibration curve.

Limit of Quantitation (LOQ): The value for LOQ was calculated from the following formula

$$LOQ = 10\sigma/S$$

Where, σ = Standard deviation of the response, S = Slope of the calibration curve.

TABLE 21: LOD AND LOQ ESTIMATION OF ACL

Standard conc.	5 µg/Ml	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL
Replicates			Peak area		
1	102213	221210	341939	432446	541892
2	109281	221392	342333	432314	542013
3	109301	221991	342415	432610	541916
4	109340	221492	342319	432214	542011
5	109410	222113	342412	432319	542115
Mean	109309	221639	342283	432380	541989
±SD	65.15	395.24	197.60	115.38	88.94
LOD			0.02667µg/Ml		
LOQ			0.08084 µg/mL		

The Detection limit for proposed method for ACL was found to be0.02667 µg/mL, The Quantitation limit for proposed method for ACL was found to be0.08084 µg/mL

TABLE 22: LOD AND LOQ ESTIMATION OF PNT

Standard conc.	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL
Replicates			Peak area		
1	104550	231644	361268	457984	548588
2	104566	231656	361366	457983	548421
3	104638	231632	361310	457881	548434
4	104432	231522	361322	457822	548510
5	104511	231620	361333	457819	548521
Mean	104519	231614	361317	457918	548494
±SD	47.25	47.92	79.25	141.18	202.41
LOD			0.0153µg/mL		
LOQ			0.0464 µg/mL		

The Detection limit for proposed method for PNT was found to be0.0153 µg/mL The Quantitation limit for proposed method for PNT was found to be0.0464 µg/mL

Range: The range of analysis for ACL and PNT are as follows ACL: 5-25 µg/mL, PNT: 5-25 µg/mL

Selectivity: After the selection of suitable mobile phase, it was then optimized for its reproducibility, sensitivity and accuracy. The optimized parameters were found to be suitable as well as there was no observation of any peak of the excipients or impurity other than the peak of ACL and PNT during experimental work, hence the proposed method was selected for development. Comparison of the chromatograms obtained from the mobile phase (blank) ACL and PNT standard and the tablet revealed no significant interference, using same chromatographic conditions for all samples. **Fig. 19, 20** are referring to the selective method for the analyte concerned.

Ruggedness: Different parameters like different laboratory condition, different source of reagents and solutions, as a result, there was no any significant change in the optimized parameters were observed in **Table 23.**

TABLE 23: RUGGEDNESS DATA FORACL AND PNT

Parameter	% Assay		SD*		RSD*	
	ACL	PNT	ACL	PNT	ACL	PNT
Analyst -1st	99.48	98.86	0.0339	0.5782	0.0340	0.584
Analyst- 2nd	98.57	98.79	0.0452	0.0904	0.0458	0.0915
Lab-1st	98.74	98.27	0.0603	0.0701	0.0616	0.0713
Lab-2nd	99.15	96.86	0.0413	0.0628	0.0415	0.0648
Reagent -1st	99.43	99.59	0.0405	0.0521	0.0407	0.0523
Reagent-2nd	99.57	99.64	0.0402	0.0903	0.0403	0.0682

* indicates average of three determination

Robustness: The method must be robust enough to with stand slight changes and allow a routine analysis of samples. Variation of organic composition in the mobile phase, pH, wavelength and flow rate were seemed to have no significant impact on resolution, peak area, tailing factor, retention time, and theoretical plate. The observations of robustness are shown in **Table 24, 26.**

TABLE 24: ROBUSTNESS STUDY OF SYSTEM SUITABILITY PARAMETER: CHANGE IN FLOW RATE (ML/MIN)

System suitability parameter*	Drug	Change in flow rate (mL/min)			RSD*		
		0.98	1.0	1.02	0.98	1.0	1.02
Peak area*	ACL	104046	104870	104799	0.0063	0.0016	0.0014
	PNT	104663	104412	104698	0.0143	0.0289	0.0125
Theoretical plates*	ACL	3043	3136	3192	0.6014	0.3668	0.9172
	PNT	2517	2522	2561	0.5186	0.3437	0.3477
Tailing factor*	ACL	1.409	1.412	1.486	0.4622	0.6679	0.3267
	PNT	1.350	1.336	1.311	0.7414	0.4969	0.3078
Retention	ACL	1.923	1.926	1.927	0.4941	0.1454	0.1754
Time*(Min	PNT	3.319	3.326			3.337	0.3060

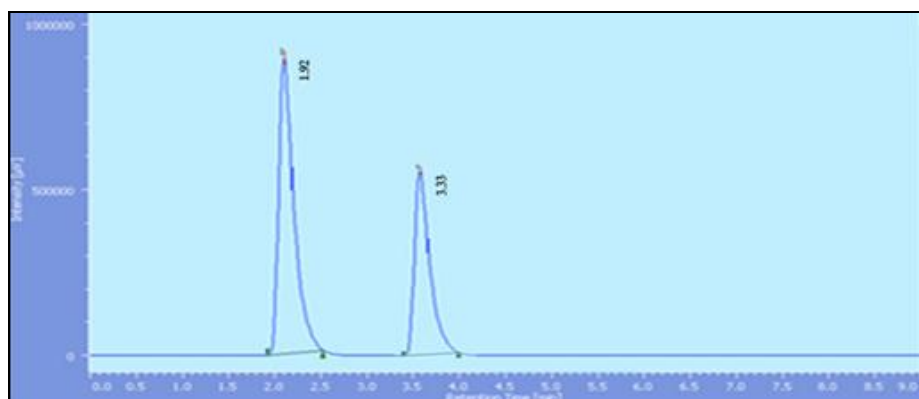* indicates average of three determination

FIG. 19: CHROMATOGRAM OF CHANGE IN FLOW RATE 1.02 (ML/MIN)

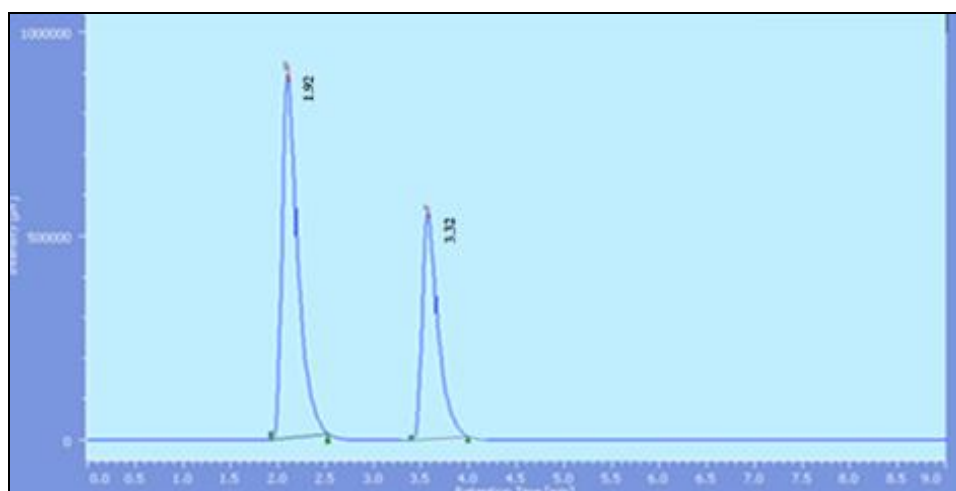| S. no	Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Tailing Factor	NTP
1	ACL	1.927	104799	100	1.486	3192
2	PNT	3.337	104698	100	1.311	2561

TABLE 25: ROBUSTNESS STUDY OF SYSTEM SUITABILITY PARAMETER: CHANGE IN M.P. RATIO

System suitability parameter[*]	Drug	Change in O.C. of M.P. Ratio			RSD[*]		
		65:35	70:30	75:25	65:35	70:30	75:25
Peak area[*]	ACL	104126	104870	104740	0.0158	0.0061	0.0151
	PNT	104321	104314	104654	0.0745	0.0623	0.0360
Theoretical plates[*]	ACL	3044	3137	3180	0.4066	0.3823	0.3432
	PNT	2519	2521	2551	0.2278	0.4308	0.3259
Tailing factor[*]	ACL	1.405	1.416	1.476	0.2178	0.6078	0.1821
	PNT	1.349	1.362	1.316	0.5458	0.1832	0.3788
Retention Time[*] (Min)	ACL	1.943	1.927	1.920	0.1145	0.1245	0.1645
	PNT	3.336	3.322	3.388	0.2359	0.2219	0.3026

* indicates average of three determination
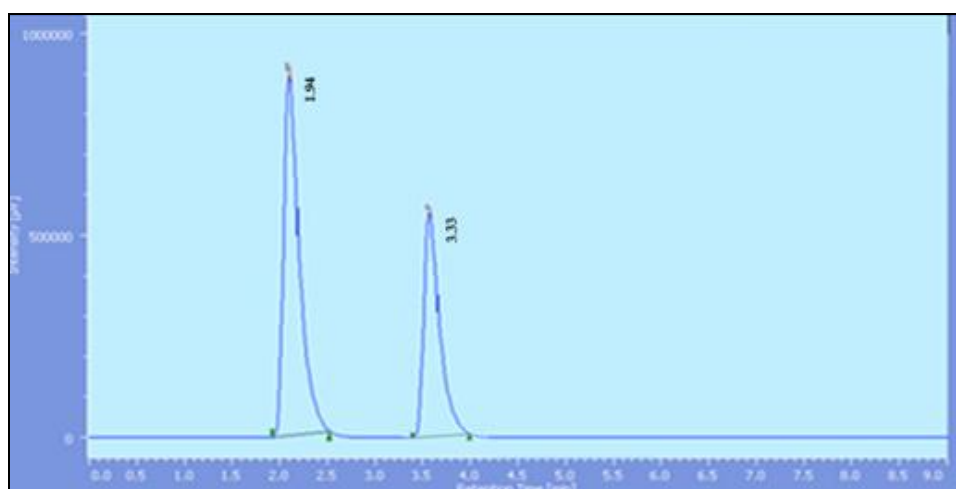
FIG. 20: CHROMATOGRAM OF CHANGE IN O.C. OF M.P. RATIO 65:35 (ACN: BUFFER)

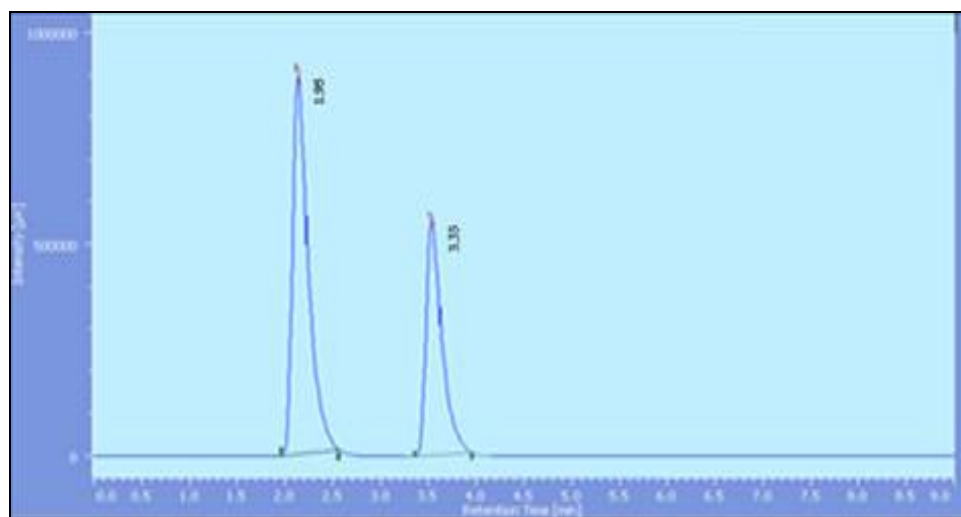| S. no	Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Tailing Factor	NTP
1	ACL	1.943	104126	100	1.405	3044
2	PNT	3.336	104321	100	1.349	2519

FIG. 21: CHROMATOGRAM OF CHANGE IN O.C. OF M.P. RATIO 70:30 (ACN: BUFFER)

S. no	Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Tailing Factor	NTP
1	ACL	1.927	104870	100	1.416	3137
2	PNT	3.322	104314	100	1.362	2521

**TABLE 26: ROBUSTNESS STUDY OF SYSTEM SUITABILITY PARAMETER: CHANGE IN PH**

System suitability parameter*	Drug	Change in pH			RSD*		
		6.6	6.8	7.0	6.6	6.8	7.0
Peak area*	ACL	10465	104879	104423	0.0160	0.0132	0.0187
	PNT	1045571	104423	105565	0.0577	0.0936	0.0573
Theoretical plates*	ACL	3043	3171	3170	0.2329	0.4267	0.3455
	PNT	2518	2527	2554	0.0780	0.2298	0.1459
Tailing factor*	ACL	1.406	1.458	1.468	0.5298	0.5884	0.2763
	PNT	1.359	1.326	1.315	0.9410	0.2824	0.4622
Retention Time*(Min)	ACL	1.953	1.972	1.930	0.1459	0.1506	0.0720
	PNT	3.338	3.323	3.348	0.2655	0.2256	0.3409

* indicates average of three determination

FIG. 22: CHROMATOGRAM OF CHANGE IN pH 6.6

S. no	Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Tailing Factor	NTP
1	ACL	1.953	10465	100	1.406	3043
2	PNT	3.338	1045571	100	1.359	2518
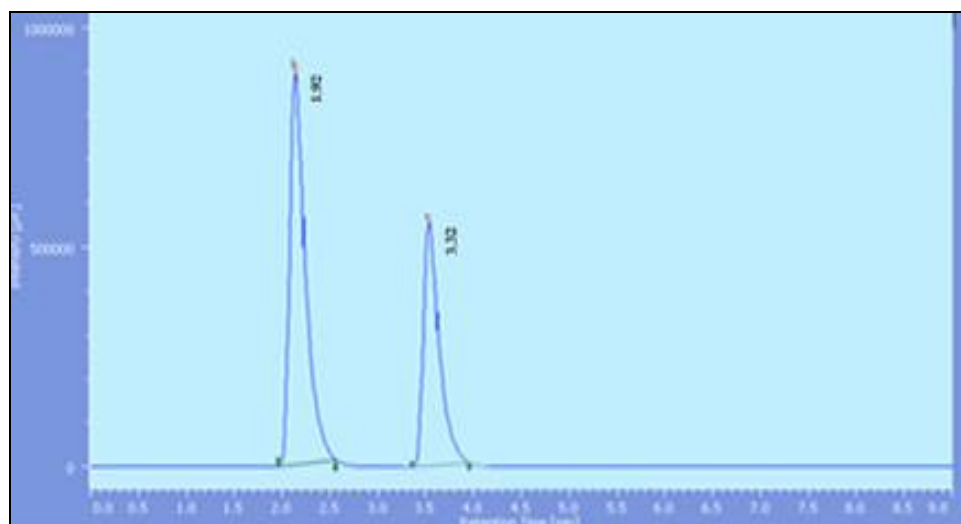
FIG. 23: CHROMATOGRAM OF CHANGE IN PH 6.8

S. no	Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Tailing Factor	NTP
1	ACL	1.972	104879	100	1.458	3171
2	PNT	3.323	104423	100	1.326	2527
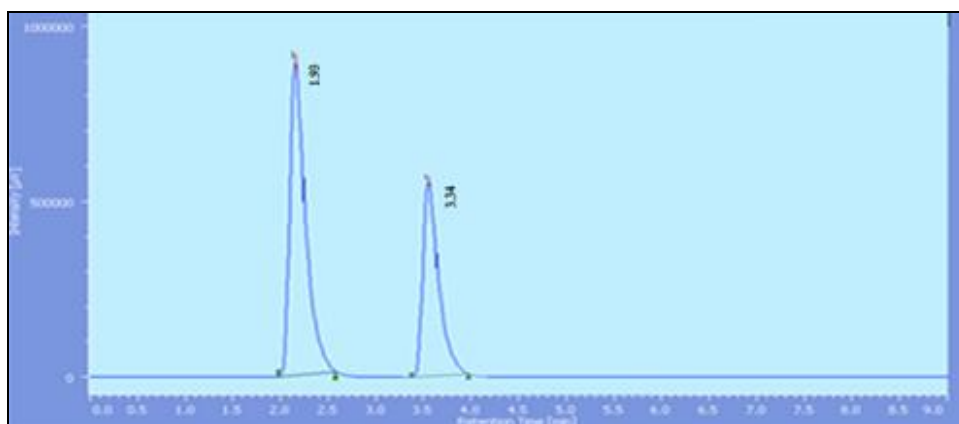
FIG. 24: CHROMATOGRAM OF CHANGE IN PH 6.8

S. no	Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Tailing Factor	NTP
1	ACL	1.930	104423	100	1.468	3170
2	PNT	3.348	105565	100	1.315	2554

TABLE 27: ROBUSTNESS STUDY OF SYSTEM SUITABILITY PARAMETER: CHANGE IN WAVELENGTH (NM)

| System suitability parameter*	Drug	Wavelength (nm)		RSD*	
		240	242	240	242 244
Peak area*	ACL	10478	104838	0.0042	0.0075 0.0189
	PNT	1045681	104524	0.0803	0.0811 0.0678
Theoretical plates*	ACL	3045	3180	0.3432	0.1854 0.3216
	PNT	2522	2530	0.2524	0.1277 0.1826
Tailing factor*	ACL	1.415	1.422	0.5253	0.3867 0.2385
	PNT	1.361	1.378	0.1946	0.7695 0.4487
Retention	ACL	1.968	1.958	0.1721	0.1701 0.2318
Time*(Min)	PNT	3.358	3.392	0.3025	0.3016

* indicates average of three determination
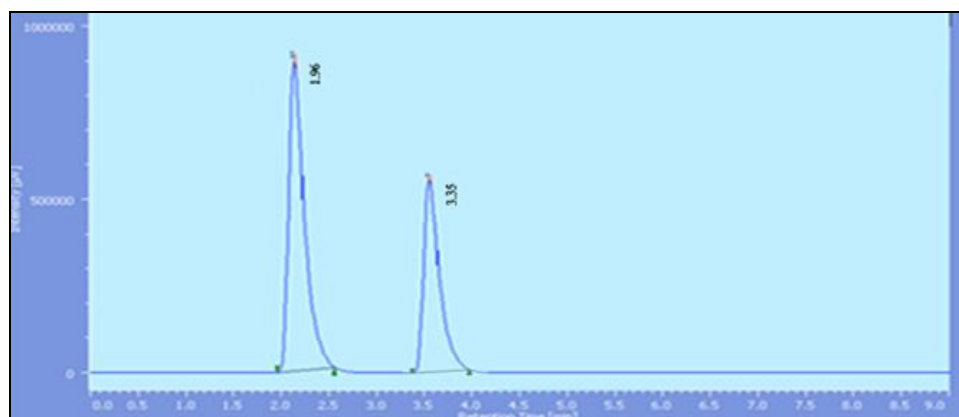
FIG. 25: CHROMATOGRAM OF CHANGE IN WAVELENGTH 294 NM

S. no	Drug	Retention Time (min)	Peak Area(µV/sec)	% area	Tailing Factor	NTP
1	ACL	1.968	10478	100	1.415	3045
2	PNT	3.358	1045681	100	1.361	2522

Solution Stability: Stability in solution was evaluated by the standard solution and the test preparation. The solution was stored at 5 °C ambient temperature without protection from light and tested after 12, 24, 36, and 48 h. The responses for the aged solution were evaluated by comparison with freshly prepared solutions.

The stability study of the stored standard solution and test preparation were performed, and solutions were found to be stable for up to 48 h. The assay values obtained after 48 h. were statistically identical with the initial value without measurable loss shown in **Table 28.**

TABLE 28: SOLUTION STABILITY OF ACL AND PNT

Drug	% Assay Initial	After 12 h	After 24 h	After 36 h	After 48 h
ACL	99.28%	99.12%	99.35%	99.64%	98.52%
PNT	99.68%	99.24%	99.77%	99.34%	99.05%

RESULTS AND DISCUSSION: Analytical method development and validation play an important role in discovering, developing, and manufacturing pharmaceuticals. Pharmaceutical products formulated with more than one drug. These combination products can present a daunting challenge to the analytical chemist responsible for the development and validation of analytical methods. The pharmaceutical dosage form of combinational drugs is very much useful in multiple therapies. The market survey reveals that ACL and PNT are recently introduced in the market as the tablet dosage form. Literature survey reveals that there are assorted methods are available for estimation of in single formulation but, no method has been reported for simultaneous estimation of ACL and PNT in combination. Literature survey also reveals that ACL is official in Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), and PNT in IP. So, attempt has been made to develop and validate new analytical method for the estimation of ACL and PNT in the combine dosage form.

UV-Spectrophotometric Method: From the individual UV spectra and overlain spectra of ACL and PNT in 0.1 N methanol: water respectively at concentration of 5-25 µg/mL and 4-20 µg/mL, two wavelengths 276 nm and 289 nm were selected for simultaneous estimation of drugs respectively. The proposed method applied for the estimation of the drugs in marketed tablet formulation and the amounts of ACL and PNT determined and was found to be 99.57-99.62% and 98.75-99.77% and respectively shown in **Table 2.**

Validation of Proposed Method: It is done to assure the reliability of the proposed method and was performed as per the ICH guidelines for the following parameters.

Linearity and Range: The study of linearity and range was performed as per the ICH recommendation. Linearity study for the proposed method was established by least-square linear regression analysis. ACL and PNT standards were found to be linear in the range of 5-25 µg/mL, 4-20 µg/mL, respectively, with r2> for 0.994, r2> 0.999 for ACL, and PNT found at selected wavelengths. The relation between concentration and absorbance for the individual drugs was studied and shown in **Tables 3** and **4**.

ACL and PNT solution individually follow the Beer-Lambert's law over concentration range 5-25 µg/mL, 4-20 µg/mL, respectively. The absorbances of both the drugs were found to be satisfactorily at the selected wavelength, respectively.

Recovery Study: Accuracy (recovery) of the method is ascertained by recovery studies performed at different levels of concentrations (50, 100 and 150%).

The % recovery was found to be 99.04 to 99.19 for ACL, 99.86 to 99.71 for PNT the value of standard deviation and % RSD was found to be > 2.0%; show in **Table 5.**

Precision: Precision studies were carried out using parameters like intra-day and inter-day variability, the results for precision were obtained within acceptance limit the % RSD > 2.0, indicating high reproducibility of the proposed method.

The Relative Standard Deviation (RSD) for intra-day analysis of ACL and PNT was found in the range of 0.0190- 0.0239 and 0.0167-0.0221, respectively. The RSD for Inter-day analysis of ACL and PNT was found to be0.0015 - 0.0031 and 0.00076-0.00069, respectively as indicated in **Tables 6** and **7.**

Specificity: After recording the UV spectra of ACL and PNT, it was observed that there was no interference of any excipients of formulation presents in solution as shown in **Fig. 11, 15.**

So, MeOH: Water (80:20) is the suitable solvent for spectroscopic evaluation of ACL, and PNT was selected. Hence the proposed method was specific and can be used for regular analysis. The developed method for estimation of ACL and PNT in tablet formulation was found to be simple, accurate, reproducible, sensitive, and economic.

Limit of Detection (LOD): Limit of detection (LOD) for ACL and PNT was found to be 0.2053 µg/mL and 0.2149 µg/mL respectively showed in **Table 10** and **12.**

Limit of Quantitation (LOQ): Limit of Quantitation (LOQ) for ACL and PNT was found to be 0.6222 µg/mL, and 0.797µg/mL, respectively **Table 8** and **9.**

Ruggedness: The ruggedness of the method was studied by two different analysts, different laboratory conditions, and different sources by

means of the same operational and environmental conditions as indicated in **Table 10.**

The results of the ruggedness study suggest that there were no significant changes in the optimized parameters were observed.

Robustness: Robustness is the capacity of a method to remain unaffected by small, deliberate variations in method parameters. The optimized system suitability parameter was unaffected by a small variation in percentage organic solvent, ionic strength, and temperature.

TABLE 29: OPTICAL REGRESSION CHARACTERISTICS AND VALIDATION PARAMETERS OF SIMULTANEOUS EQUATION METHOD FOR THE ANALYSIS OF ACL AND PNT.

Parameters	ACL	PNT
λ *max*	276 nm	289nm
Linear range	5-25µg/mL	4-20µg/mL
Regression coefficient (r^2)	0.992	0.999
Slope (s)	0.004	0.030
Intercept (c)	0.003	0.028
Accuracy	99.04±0.035 to 99.19 ±0.010	99.86 ±0.831 to 99.71±0.813
Precision (intra-day)	0.277 ± 0.0572 to 0.555 ± 0.0719	0.214 ± 0.0502 to 0.524 ± 0.0664
Precision (inter-day)	0.3224 ± 0.0045 to 0.6313 ± 0.0095	0.2733 ± 0.0023 to 0.5663 ± 0.0020
LOD	0.2053µg/mL	0.2481µg/mL
LOQ	0.6222µg/mL	0.7297µg/mL

RP-HPLC Method: A Reverse Phase HPLC Method has been developed for simultaneous estimation of ACL and PNT in the tablet dosage form. The proposed method was validated by using

different parameters like linearity, precision, accuracy, and limit of detection, the limit of quantitation, range and robustness, solution stability as per ICH guidelines [26, 27].

TABLE 30: OPTIMIZED PARAMETERS FOR RP-HPLC METHOD

Parameters	Chromatographic conditions
HPLC System	Shimadzu LC 2010-HT
Pump	PU-2080 plus HPLC pump
Detector	UV-2075 plus as UV-VIS detector
Column	YMC Triart C18 (4.6mm×250mm) column
Column temperature	Ambient (28°c)
Mobile phase	ACN: Buffer (Phosphate Buffer)(70:30v/v), pH 6.8
Detection of Wavelength	295 nm
Flow rate	1 mL/min
Sample volume	5µL
Run time	10 min
Retention time	ACL: 2.987min PNT:3.729 mins

The solubility of the ACL and PNT was checked by using various solvents like methanol, acetonitrile, water and acetone, in that ACL and PNT were freely soluble in methanol, so for the selection of mobile phase of the proposed method, various experiments were carried out, finally mobile phase of ACN: Buffer (70:30 v/v), pH 6.8 has been chosen for the further analysis, which found to be satisfactory for the proposed method.

The wavelength of ACL and PNT in a selected mobile phase was found to be 276 and 289 nm, respectively shown in **Fig. 10 and 11**.

The UV detection of the proposed method was observed at 295 nm wavelength as an isosbestic point of ACL and PNTin overlain spectra shown in **Fig. 15.**

Validation of Proposed Method: Validation of proposed method was performed as per the ICH guidelines.

Linearity and Range: The linearity study for the proposed method was established by least-square linear regression analysis. Linearity was assessed by a plot of concentration versus area **Fig. 23, 24,** and **25.**

The graphs were found to be linear in the range of 5-25 µg/mL and 4-20 µg/mL respectively for ACL and PNT with correlation coefficient values 0.998 and 0.999 respectively as indicated in [Table 17] and [19.]

Recovery Studies: The accuracy (recovery study) was performed by the standard addition method. Three replicate injections, each of three different test concentrations in the range of 50, 100, and 150% were studied.

The accuracy and reproducibility are apparent from the data as results are close to 100%, and the value of standard deviation and % R.S.D were found to be < 2% which shows the method is highly précised and accurate was shown in **Table 20, 21** and **22.**

Precision: Precision studies were carried out using analysis of drug by intra-day and inter-day variability. Results showed that the % RSD found less than 2. The precision study for ACL and PNT was carried out with inter-day, which is discussed in **Table 30, 31** and **32** and the intra-day study was shown in **Table 25, 26,** and **27.**

Limit of Detection (LOD): The limit of detection (LOD) for ACL and PNT was found to be 0.02667 µg/mL, 0.0153 µg/mL, and 0.0886µg/mL, respectively shown in **Table 29, 30** and **31.**

Limit of Quantitation (LOQ): The Limit of Quantitation (LOQ) for ACL and PNT was found to be 0.08084 µg/mL and 0.0464 µg/mL, respectively shown in **Table 29** and **30.**

Selectivity: The optimized parameter was found suitable as well as there was no observation of any peak of excipients or impurity other than the peak of ACL and PNT during experimental work shown in **Fig. 41, 44.** Hence this method was selected for the development.

Ruggedness: The ruggedness of the method was studied by special parameters like different laboratory conditions, different analysts, a different sources of reagents and solvents were used for the proposed method of estimation, as a result, there was no significant change in the optimized parameters of the proposed method was observed.

The analysis results of the tablet for ACL and PNT were found to be 99.73-99.48 and 99.17-99.76%, respectively shown in **Table 16.** The reagent from different sources such as Methanol from Loba Chem Pvt. Ltd., were utilized for ruggedness study and resulted obtained. The ruggedness study has shown that there is no variation in the results for different laboratory conditions, different analysts, a different sources of reagents and solutions. The % RSD for ruggedness analysis was found to be less than 2 as indicated in **Table 23.**

Robustness: The robustness of the analytical method is the measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The method must be robust enough to withstand slight changes and allow a routine analysis of the sample. The robustness of the method was determined by carrying out the analysis under conditions during which change in flow rate, change in the organic composition of the mobile phase, change in pH, and change in analytical wavelength was studied. Variation of organic composition in the mobile phase, pH, wavelength, and flow rate were seemed to have no significant impact on resolution, peak area, tailing factor, retention time, and theoretical plates. The observations of robustness are discussed in **Table 24, 26.**

Solution Stability: Stability in solution was evaluated by the standard solution and the test preparation. The solution was stored at 5 °C ambient temperature without protection from light and tested after 12, 24, 36, and 48 h. The stability study of the stored standard solution and test preparation were performed and solutions were found to be stable for up to 48 h. The assay values obtained after 36 h. were statistically identical with the initial value without measurable loss shown in **Table 28.**

TABLE 31: OPTICALREGRESSION CHARACTERISTICS AND VALIDATION PARAMETERS OF RP-HPLC METHOD FOR ANALYSIS OF ACL AND PNT

Parameters	ACL	PNT
⌐ max	276 nm	289 nm
Linear range	5-25 μg/mL	4-20 μg /mL
Regression coefficient (r^2)	0.997	0.999
Slope (s)	21879	22642
Accuracy	98.92±0.7071to 99.01±0.04695	99.05±0.7273to 98.38±0.0917
Precision (Interday)	109256±97.22 to 541898±315.61	104550±526.86 to 548315±235.24
Precision (Intraday)	109372±126.62 to 541929±97.505	104652±201.01to 548274±222.92
LOD	0.02667μg/mL	0.0153 μg/mL
LOQ	0.08084μg/mL	0.0464 μg/mL

CONCLUSION: In this dissertation a new UV spectroscopic and RP-HPLC method was described for simultaneous estimation of ACL and PNT. Simultaneous Equation method was applied for the spectrophotometric determination of ACL and PNT. Statistical calculations were done by use of 276 nm and 289 nm wave lengths.

In RP-HPLC method, the estimation of ACL and PNT carried out by ACN: Buffer (Phosphate acetate) (80:20 v/v) as mobile phase, pH 6.8 at a flow rate of 1.0 mL/min and YMC Triart C18 column (250 mm × 4.6 mm, 5μ), column. The detection of ACL and PNT was carried out at 295 nm. Retention time of ACL and PNT was found at 3.739 and 2.991 respectively.

The results of analysis in both the methods were validated as per ICH guidelines in terms of linearity, range, accuracy, precision, LOD, LOQ, ruggedness, robustness and solution stability from this study it was concluded that the developed method of RP-HPLC can be successfully used for the estimation of ACL and PNT in their combined tablet formulations. The developed RP-HPLC and UV spectroscopic methods were accurate, precise, sensitive, reliable, specific, reproducible, rapid and economical. No interference of additives or matrix was encountered in both the developed methods.

CONFLICTS OF INTEREST: No conflict of interest.

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