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Original Article

Development and validation of stability indicating RP-HPLC method for simultaneous estimation of pregabalin and aceclofenac in bulk and pharmaceutical dosage form

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ABSTRACT

A simple, precise and reproducible Reverse Phase High Performance Liquid Chromatography method was developed and validated for simultaneous estimation of Pregabalin and Aceclofenac in tablet dosage form.Chromatographic separation was achieved by Grace C_{18} (250 mm x 4.6 ID , Particle size- 5 micron) column and methanol : water pH3 (60:40v/v) as mobile phase, at a flow rate of 1 ml/min (millilitre per minute) using UV detection at 216nm. Forced degradation experiments were carried out by exposing Aceclofenac and Pregabalin standard and sample for thermal, photolytic, oxidative and acid-base hydrolytic stress conditions. The method has been validated for linearity, accuracy, precision, LOD, and LOQ. The retention time for Aceclofenac and Pregabalin were obtained as 4.296 min and 5.955 min respectively. Linearity of Aceclofenac and Pregabalin were found to be in range 20-100µg/ml and7.5-37.5µg/ml.(R^2=0.998) respectively. The accuracy of present method was evaluated at 50%,100%,150%. Recovery was found to be in a range from 99.80%-100.42% for both of the drugs. Intermediate precision studies were carried out and the RSD values were less than 2%. Lower values of LOD (0.35µg/ml for ACF and 0.18 µg/ml for PRE) and LOQ (1.08µg/ml for ACF and 0.56 µg/ml for PRE) indicated good sensitivity of the method.In this study, the optimization of mobile phase, flow rate, injection volume and wavelength were achieved.This demonstrate that the developed method is simple, precise,accurate and robust for simultaneous estimation of aceclofenac and pregabalin in tablet dosage form. The method was acceptable for degradation studies of heat, sunlight, acid, base, peroxide which meet the acceptance criteria for forced degradation studies.

Keywords: Aceclofenac, Pregabalin, RP-HPLC, Validation.

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INTRODUCTION

Aceclofenac (ACE) is chemically 2 [(2,6-dichlorophenyl) amino] phenyl acetoxy acetic acid. ACE is a non-steroidal anti-inflammatory drug which has analgesic and anti-inflammatory activity.It is used for the relief of pain and inflammation in rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. It is a cytokine inhibitor. It blocks the action of a substance in the body (cyclo-oxygenase), which may cause pain and inflammation. Pregabalin [(s) -3(amino methyl)-5-methyl hexanoic acid] is an anticonvulsant drug used for neuropathic pain and as an adjunct therapy for partial seizures with or without secondary generalization in adults. Pregabalin binds with high affinity to the alpha2-delta site (an auxiliary subunit of voltage-gated calcium channels) in central nervous system tissues.

The combination of the PRG (75 mg) and ACE (200 mg) is used for the relief of pain and inflammation in rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. This combination is also used as an adjunctive in the treatment of partial seizures, epilepsy, fibromyalgia and neuropathic pain. According to literature survey, there was not any developed analytical method which has been reported for simultaneous estimation of PRG and ACE in combined dosage form. So an attempt was being made to a developed simple, accurate, precise, economical and reproducible chromatographic method for simultaneous estimation of PRG and ACE in tablet dosage form. The developed method was validated in accordance with ICH guideline and successfully employed in the assay of PRG and ACE in combined tablet dosage form.

MATERIALS AND METHODS

Procurement of Drug Samples:

Table 1 : Drug sample suppliers and Manufacturer

Sr.No.	Name of Drugs	Drug supplies & Manufacturer
1	Pregabalin	Map laboratories pvt.ltd Mumbai, India.
2	Aceclofenac	Map laboratories pvt.ltd Mumbai, India.

Reagents and chemicals:

Following reagents and chemicals were used in whole experimental procedures.

Table.2 : Reagents and chemicals used.

Name	Supplied by	Grade
Distilled water	In House	Double distilled
Methanol	Merck	HPLC
O-Phosphoric acid	Lobachemie	AR
Potassium dihydrogen phosphate	Analab Fine Chemicals	AR
Sodium hydroxide	Molychem	LR
Hydrogen peroxide	Molychem	LR
Hydrochloric acid	Merck	LR
Potassium Di hydrogen phosphate	Merck	AR

Instrument Used:

Table. 3: List of apparatus/ instruments used

Sr. no.	Name	Model	Manufacturer/Supplier
1.	Weighing balance	PGB 100 Max : 100gm Min : 0.001gm	Wenser High Precision Balance
2.	Digital PH Meter	PICO+	Lab India pvt ltd.
3	Sonicator	WUC-4L Capacity -4 liter	Wenser Ultra Sonicator
4	Magnetic stirrer		Remi Equipment
5	HPLC	HPLC 3000 Series	Analytical Technologies Ltd.

Table. 4 : HPLC Instrument Information:

Parts of Instruments	Information
System	HPLC Binary Gradient System
Model no.	HPLC 3000 Series
Company	Analytical Technologies Ltd.
Pump	P-3000-M Reciprocating (40 MPa)
Column	Grace C18 (250mm×4.6ID, Partical size- 5 micron)
Detector	UV-3000-M
Software	HPLC Workstation

Preparation of Mixed Stock Solutions: Accurately weighed 100 mg of standard Aceclofenac and 37.5 mg of standard Pregabalin were transferred to a 100ml volumetric flask and dissolved in Methanol. The flask was sonicated for 15 minutes and volume was made up to the mark with Methanol to give a solution containing 1000 µg/ml Aceclofenac and 375 µg/ml Pregabalin. From these solutions, pipette out 1ml in to 10ml volumetric flask respectively, and dilute it with Methanol up to the mark to give a solution containing 100 µg/ml Aceclofenac and 37.5 µg/ml Pregabalin.

Preparation of Sample Solution: Twenty tablets were weighed accurately average weight was determined and ground to fine powder. A quantity of powder equivalent to 100 mg (Aceclofenac) and 37.5 mg (Pregabalin) was transferred into 100 ml volumetric flask containing 100 ml Methanol, sonicated for 30 min and diluted to mark with same solvent to obtain 500 µg/ml of Aceclofenac and 375 µg/ml of Pregabalin. The resulting solution was filtered using 0.45 µm filter (Mill filter, MA). From the above solution 1 ml was transferred into 10 ml volumetric flask and diluted to mark with same solvent. So, resultant solution was found to contain 50 µg/ml of Aceclofenac and 37.5 µg/ml.

Selection of wavelength: UV spectrum of 10µg/ml Aceclofenac and Pregabalin diluents (mobile phase composition- methanol : water (60:40v/v) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum wavelength selected as 216nm. At this wavelength, both the drugs show good absorbance.

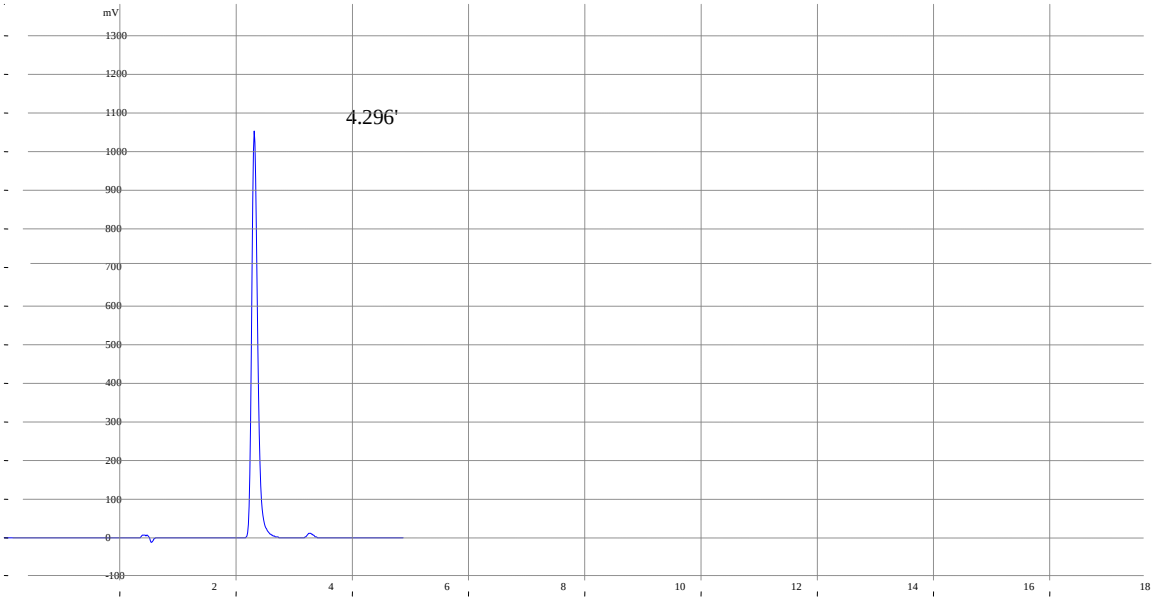
Selection of mobile phase: Based on sample solubility, stability, suitability and pKa various mobile phase compositions were tried to get a good resolution and sharp peaks. The standard solution containing mixture of two drugs, as well as individual drugs were run in various mobile phases containing different ratio of methanol, and water. The mobile phase containing Methanol: Water (60:40 v/v) proportion with detection. Wavelength 216 nm was selected.

RESULTS AND DISSCUSION

Optimized Chromatographic Conditions:

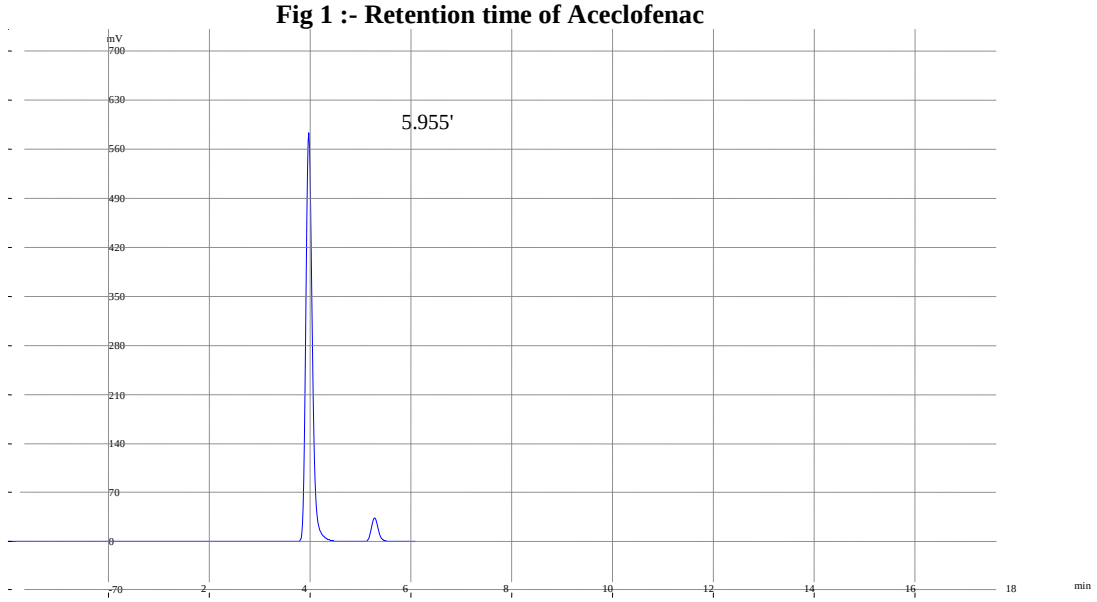
Table 5: Optimized Chromatographic Conditions

Parameters	Values
Column	Grace C18 (250mm x 4.6ID, Particle size: 5 micron)
Mobile Phase	Methanol :Water (60:40)
pH	3
Wavelength	216nm
Flow rate	1.0ml/min
Injection volume	20µl
Run time	7.78min
Retention time	4.296 min ACE and 5.955 min PRE



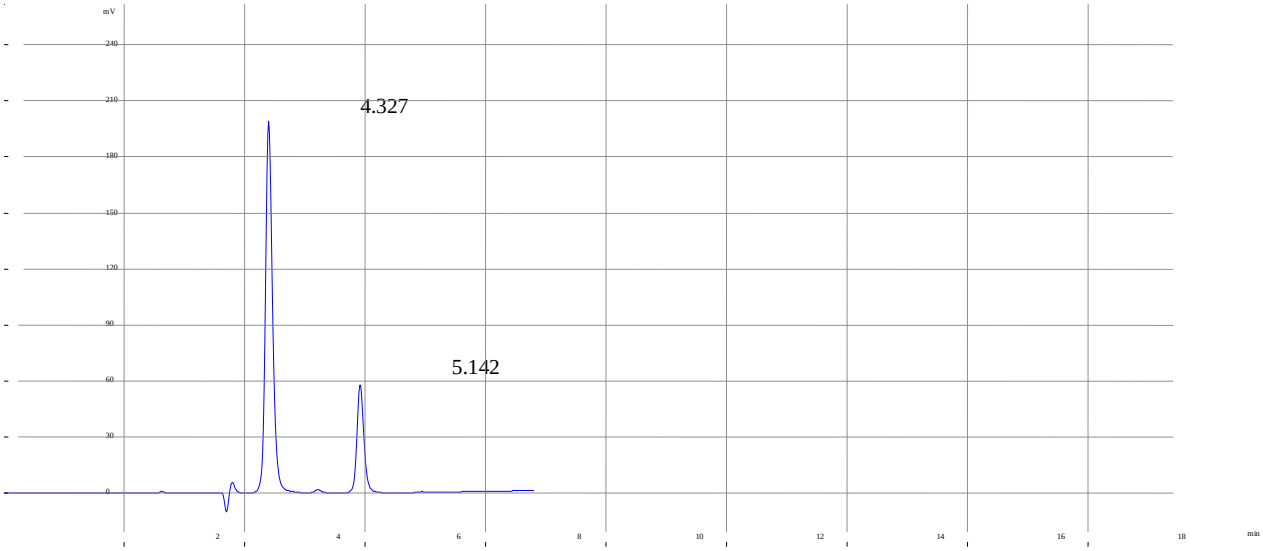
Time	Area	Resolution	T.Plate num	Asymmetry
4.296	8267591	0.00	7163	1.31

Fig 1 :- Retention time of Aceclofenac



Time	Area	Resolution	T.Plate num	Asymmetry
5.955	5205813	0.00	10428	1.25

Fig 2:-Retention time of Pregabalin



Time	Area	Resolution	T.Plate num	Asymmetry
4.327	710029	1.04	8433	1.21
5.142	361080	0.00	7333	1.07

Fig 3:- Chromatogram for standard solution of ACL and PRE

Method Validation

Linearity: The linearity of an analytical procedure is its ability within a given range to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. The linearity was tested for PRG and ACE in the concentration range value of 10-50 µg/mL.

Accuracy: To check the degree of accuracy of the method, recovery studies were performed in triplicate by the standard addition method at 25%, 75% and 125%. Known amounts of standard PRG and ACE were added to the pre-analyzed samples and were subjected to the proposed HPLC method.

Precision: The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). The repeatability was calculated by the relative standard deviation with three replications and three different concentrations during the same day. Intermediate precision was studied by comparing the assays on two different days.

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

$LOD = 3.3 \times N/S$

Where, N is the standard deviation of the peak area of the drug and S is the slope of the corresponding calibration curve.

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 for the determination of impurities and/or degradation products. Limit of quantification can be calculated using the following equation as per ICH guidelines.

$LOQ = 10 \times N/S$

Where, N is the standard deviation of the peak area of the drug and S is the slope of the corresponding calibration curve.

System Suitability: System suitability and chromatographic parameters were validated such as resolution, theoretical plates, and tailing factor was calculated.

Table 6: System suitability parameters for aceclofenac and pregabalin.

System suitability parameters	Aceclofenac	Pregabalin
Retention time	4.327min	5.142min
Theoretical plate no.	8433	7333
Tailing factor	1.21	1.07
Resolution	1.04	0.00

Linearity

The calibration curves exhibited linear relationship of peak area to concentration in the range 20-100 µg/mL for aceclofenac and 7.5-37.5 µg/mL pregabalin. The regression coefficients (r^2)for Aceclofenac were 0.998, and for Pregabalin were 0.998, maintaining good correlation close to unity. The graph of concentration Vs Average area was plotted which is showing straight line passing through all points. So as per ICH guidelines, the proposed HPLC method for the determination of aceclofenac and Pregabalin was found to be linear.

Fig4 –Calibration curve of aceclofenac

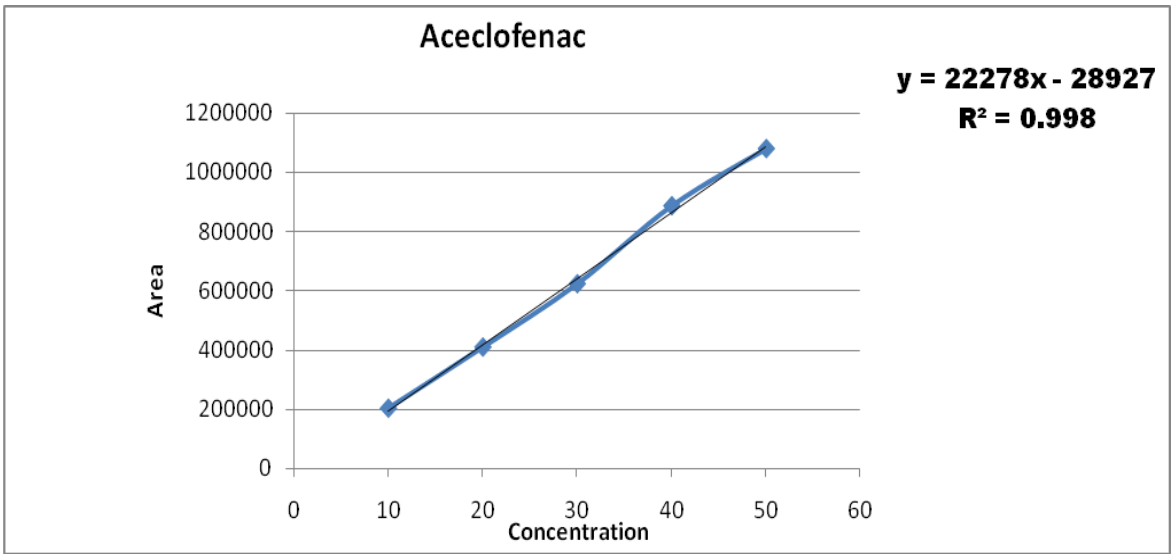
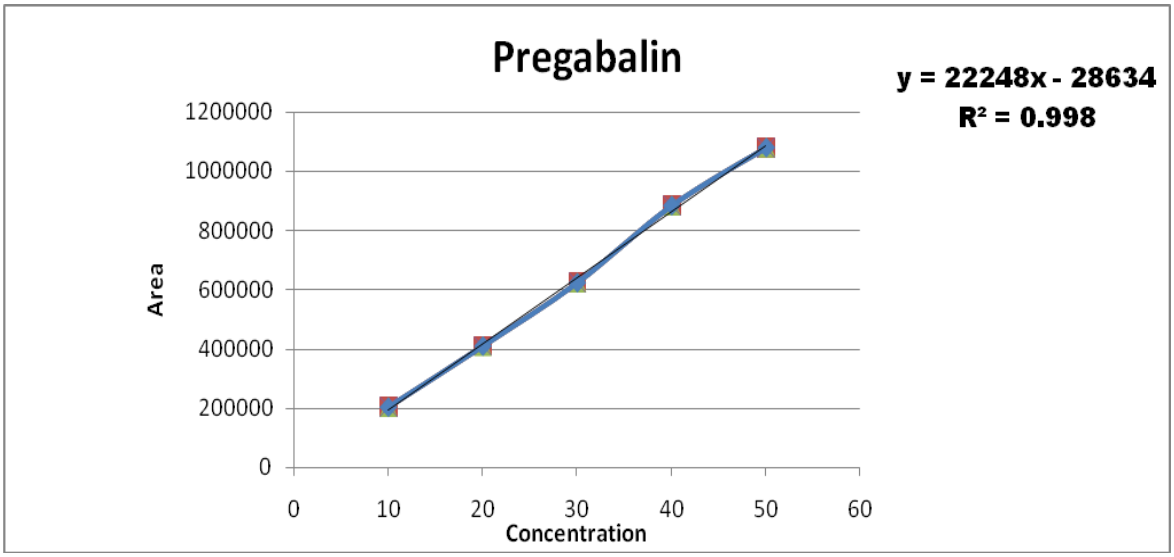


Fig5- Calibration curve of Pregabalin



Accuracy (% Recovery)

Accuracy was checked with standard drugs by placebo spiking method at three different concentration levels(multi –level recovery).Recovery of standard drugs added was found to be 100 % for ACF and 100.02% for PRE, indicating that the proposed method is accurate for the simultaneous estimation of aceclofenac and Pregabalin in presence of their degradation products and excipients.

Table7: Accuracy data for aceclofenac

Conc (%)	Sample amount (ppm)	Amount added (ppm)	Amount recovered(ppm)	% recovery	%mean recovery
50%	40	20	60.06	100.100	100.09
	40	20	59.95	99.93	
	40	20	60.14	100.24	
100%	40	40	79.84	99.80	99.9
	40	40	79.96	99.95	
	40	40	79.96	99.95	
150%	40	60	100.04	100.04	100.03
	40	60	99.95	99.95	
	40	60	100.10	100.10	

Table 8: Accuracy data for pregabalin

Conc (%)	Sample amount (ppm)	Amount added (ppm)	Amount recovered(ppm)	% recovery	%mean recovery
50%	15	7.5	22.46	99.85	100.08
	15	7.5	22.49	99.98	
	15	7.5	22.59	100.42	
100%	15	15	29.95	99.84	99.94
	15	15	29.99	99.98	
	15	15	30.00	100.01	
150%	15	22.5	37.56	100.17	100.05
	15	22.5	37.42	99.81	
	15	22.5	37.56	100.17	

Precision:

Repeatability: The RSDs for intra-day and inter-day precision were not more than 2.0% for Aceclofenac and Pregabalin. The low RSD values indicate the repeatability and reproducibility of the method. Therefore, as per the ICH guidelines, this HPLC method for the determination was precise.

Table 9: Repeatability data for Aceclofenac and Pregabalin.

Drug	Conc (ppm)	Area	Mean ±SD (n=3)	% RSD
Aceclofenac	20ppm	871908	1036.57	0.11
		873931		
		873312		
Pregabalin	7.5ppm	200230	1447.21	0.72
		199098		
		201971		

Table10: Interday Precision for Aceclofenac and Pregabalin.

Aceclofenac		**Pregabalin**	
Day 1	Area	Day1	Area
	1753841		622341
	1758216		624982
	1751451		624494
Day2	1752724	Day2	620908
	1757913		620207
	1750964		624556
Mean	1754185	Mean	622914
Standard deviation	3170	Standard Deviation	2056
%RSD	0.18%	%RSD	0.33%

Table 11: Intraday variability for Aceclofenac and Pregabalin

Aceclofenac		**Pregabalin**	
Morning	Area	Morning	Area
	1753841		622341
	1758216		624982
	1751451		624494
Evening	1756189	Evening	624975
	1754030		625251
	1755077		624738
Mean	1754801	Mean	624463.5
Standard deviation	2297	Standard Deviation	1070
%RSD	0.13%	%RSD	0.17%

Robustness:
Small deliberate changes in chromatographic conditions such as a change in mobile phase ratio (±2%), change in wavelength (±2units), and flow rate (±2units) were studied to determine the robustness of the method.

Table 12 :Data for Robustness (At Different Flow Rate)

Drug Sample	**Flow rate(ml/min)**	**Area**	**Mean**	**SD**	**%RSD**
Aceclofenac	**0.9**	1342872	1341293	1556.02	0.116
	1.0	1341247			
	1.1	1339761			
Pregabalin	**0.9**	409664	407097	1166.96	0.285
	1.0	408678			
	1.1	407339			

Table 13: Data for Robustness (At different wavelength)

Drug Sample	**Wavelength**	**Area**	**Mean**	**SD**	**%RSD**
Aceclofenac	218	1341247	1341729.6	1099.55	0.081
	216	1340954			
	214	1342988			
Pregabalin	218	408678	407097.3	1376.66	0.338
	216	406161			
	214	406453			

Limit of detection (LOD): The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. LOD data is Shown in table 14.

Limit of quantification (LOQ): 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 for the determination of impurities and/or degradation products. LOQ data is shown in table 14.

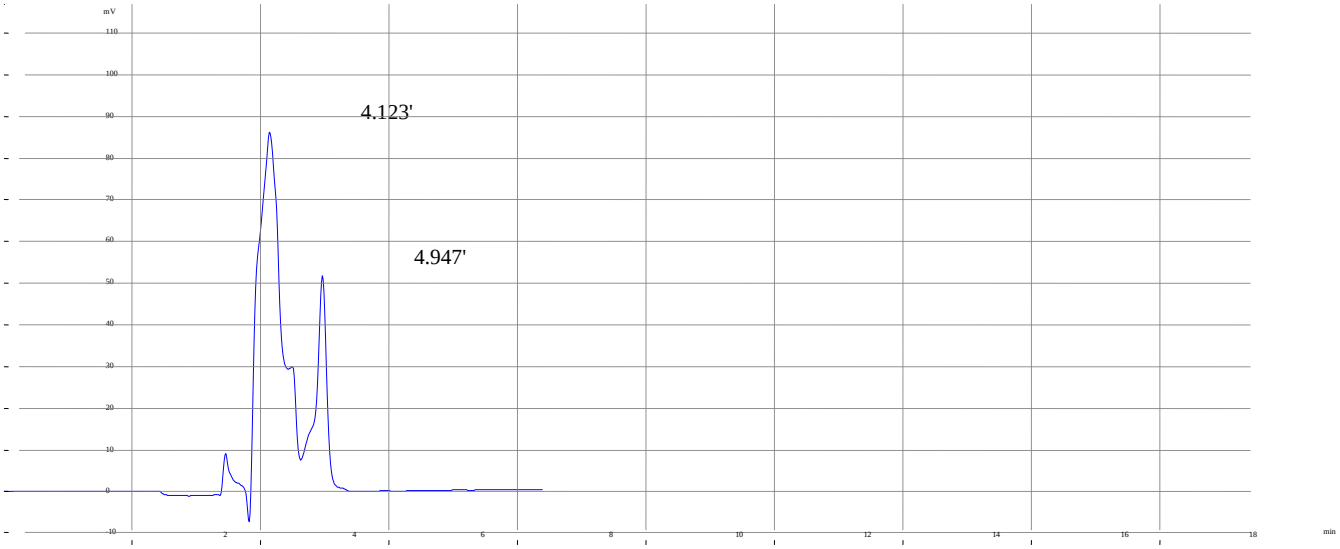
Table 14 : Data for LOD and LOQ

Sr.No.	**Drug**	**LOD**	**LOQ**
1	Aceclofenac	0.35	1.08
2	Pregabalin	0.18	0.56

FORCED DEGRADATION STUDIES

The study was intended to ensure the effective separation of PRE,ACE and its degradation peaks of formulation ingredients at the retention time of PRE,ACE. Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the methods.

Acid /Alkali hydrolysis: For Acid/Alkali hydrolysis, 2ml of 0.1M Hydrochloric acid (HCL) / 2ml of 0.1N Sodium hydroxide (NaOH) was added to solutions. These solutions were kept aside for 1hr at 60^0C. Resultant solutions were injected in to system after neutralization and chromatograms were recorded to access stability.



Time	Conc	Area	Resolution	T.Plate num	Asymmetry
4.123	100ppm	2209541	2.12	5709	0.94
4.947	37.5ppm	763736	0.00	6028	0.90

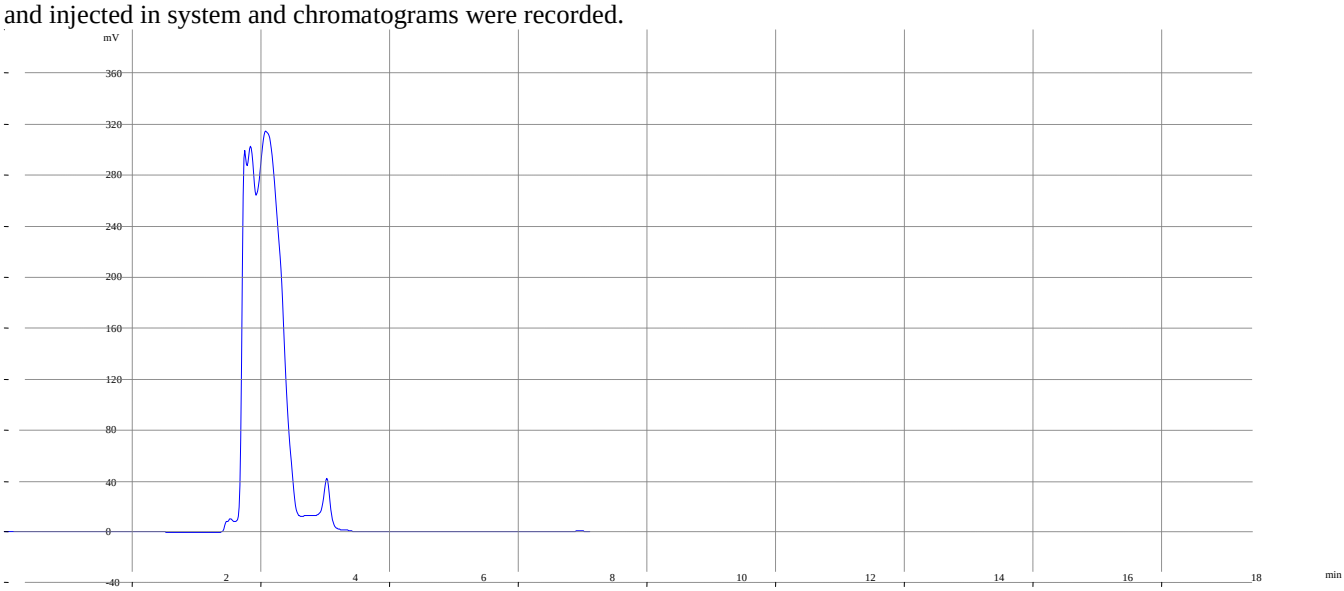
Fig 6: Chromatogram for acid degradation of aceclofenac and pregabalin 1hr at 60^0C.

Time	Conc	Area	Resolution	T.Plate num	Asymmetry
4.288	100ppm	1456071	2.23	8065	1.02
5.001	37.5ppm	719590	0.00	7197	0.93

Fig 7: Chromatogram for alkaline degradation of aceclofenac and pregabalin 1hr at 60^0C.

Oxidation Degradation:

For oxidation degradation, 3ml of 2% Hydrogen Peroxide(H_2O_2) was added and kept aside for 24hrs at 60˚C and injected in system and chromatograms were recorded.

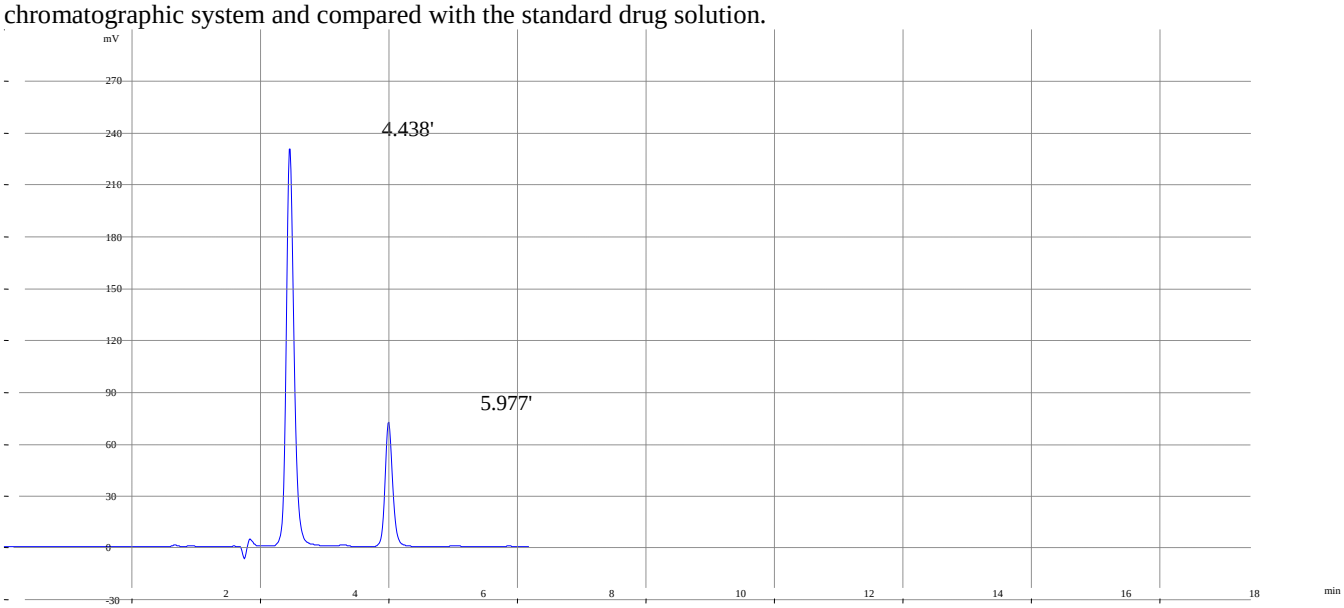


Time	Conc	Area	Resolution	T.Plate num	Asymmetry
3.821	100ppm	1971288	2.38	6970	1.09
4.056	37.5ppm	766875	0.00	5956	0.95

Fig 8: Chromatogram for oxidation degradation of aceclofenac and pregabalin 24hrs at 60˚C.

Photo Degradation:

For photo degradation solutions were exposed near UV light for 24hrs and resultant solutions were injected in chromatographic system and compared with the standard drug solution.



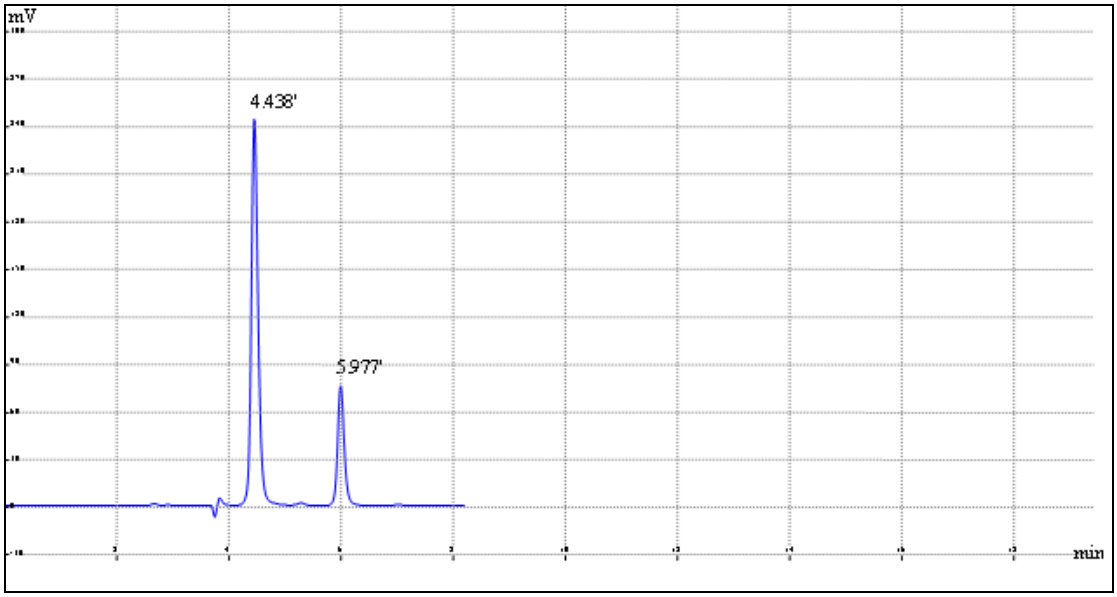
Time	Conc	Area	Resolution	T.Plate num	Asymmetry
4.438	100ppm	2543282	5.66	6364	1.20
5.977	37.5ppm	969604	0.00	8060	1.15

Fig 9: Chromatogram for photo degradation of aceclofenac and pregabalin at 24hr

Thermal degradation:

Aceclofenac and Pregabalin were transferred to petri plate separately and kept in a hot air oven at 70˚C for 12hrs. From the above stressed sample, 10mg was weighed accurately and transferred to 10ml

volumetric flask separately and volume was made up to the mark with the methanol to get the concentration of 1000µg/ml of both drug solution. 5ml of above solution transferred in 10 ml volumetric flask and volume was made with diluents.



Time	Conc	Area	Resolution	T.Plate num	Asymmetry
4.438	100ppm	2582716	5.70	6361	1.20
5.977	37.5ppm	900218	0.00	8292	1.15

Fig 10: Chromatogram for thermal degradation of ACF and PRE at 70˚C for 12hrs

Table 15: Summary of degradation data for Aceclofenac.

Stress Condition	Retention Time	Area of Peak	Degradation (%)	API after degradation %
Std. Drug	4.296	2604620	-	-
Acidic (0.1N HCL)	4.123	2209541	84.83160691	15.16839309
Alkaline (0.1 N NaOH)	4.288	1656071	63.58205804	36.41794196
Oxidation (3% H2O2)	4.056	1971288	75.68428408	24.31571592
Photolytic (UV)	4.438	2543282	97.64503075	2.354969247
Thermal	4.438	2582716	99.1590328	0.840967204

Table 16: Summary of degradation data for Pregabalin

Stress Condition	Retention Time	Area of Peak	Degradation (%)	API after degradation %
Std. Drug	5.955	1078099	-	-
Acidic (0.1N HCL)	4.947	763736	82.89925137	17.10074863
Alkaline (0.1 N NaOH)	5.001	719590	66.74618936	33.25381064
Oxidation (3% H2O2)	5.164	766875	71.1321502	28.8678498
Photolytic (uv)	5.977	969604	89.93645296	10.06354704
Thermal	5.977	900218	83.50049485	16.49950515

CONCLUSION:

Development and validation of RP-HPLC method was found to be linear, accurate, precise, specific and robust according to acceptance criteria and with high level of LOD and LOQ. The results show that the HPLC method presented here can be considered suitable for the analytical determination of PRG and ACE in bulk and tablet dosage form. The developed method was validated. The good % recovery in tablet forms suggests that the excipients present in the dosage forms have no interference in the determination. The %RSD was also less than 2% showing high degree of precision of the proposed method. The method was successfully applied to the available marketed formulation without any interference due to the excipients and can have an application in the industry.

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