METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF PREGABALIN BY USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY IN API SUBSTANCE AND MARKETED PHARMACEUTICAL DOSAGE FORM

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Abstract

The objective of this study was to develop a simple, sensitive and reproducible method for estimation of Pregabalin by Ultra Performance Liquid Chromatography (UPLC). Pregabalin was separated on Endeversil C18 ODS (2.1 x 50mm, 3µm), using 0.1% Octane Sulphonic acid buffer with pH of 3.0: Acetonitrile (30:70) at the PDA detection of 226nm. Isocratic elution of buffer and acetonitrile was used as a mobile phase with different flow rates, eventually 30:70 v/v buffer and acetonitrile was being set with the flow rate of 0.2 ml per min. Pregabalin was eluted at a run time of 2 mins. The statistical validation parameters such as linearity, accuracy, precision, inter-day and intra-day variation were checked as suggested by ICH recommendations, further the limit of detection and limit of quantification of Pregabalin concentrations were found to be 2.98µg/mL and 9.97µg/mL. Recovery and assay studies of Pregabalin were within 99 to 102% indicating that the proposed method can be adoptable for quality control analysis of Pregabalin in bulk form and Marketed Pharmaceutical dosage form.

Key Words: Pregabalin, UPLC, Method Development, Validation, Accuracy, Precision.

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INTRODUCTION

Pregabalin[1] is a DEA Schedule V controlled substance. Substances in the DEA Schedule V have a low potential for abuse relative to substances listed in Schedule IV and consist primarily of preparations containing limited quantities of certain narcotics. Pregabalin is a 3-isobutyl derivative of gamma-amino butyric acid (GABA) with anti-convulsant, anti-epileptic, anxiolytic, and analgesic activities. Although the exact mechanism of action is unknown, Pregabalin selectively binds to alpha2delta (A2D) subunits of presynaptic voltage-dependent calcium channels (VDCCs) located in the central nervous system (CNS). Binding of Pregabalin[2] to VDCC A2D subunits prevents calcium influx and the subsequent calcium-dependent release of various neurotransmitters, including glutamate, norepinephrine, serotonin, dopamine, and substance P, from the presynaptic nerve terminals of hyper excited neurons; synaptic transmission is inhibited and neuronal excitability is diminished. Pregabalin does not bind directly to GABA-A or GABA-B receptors and does not alter GABA uptake or degradation. Pregabalin[3] is an inhibitor of neuronal activity used for therapy of painful neuropathy and as an anticonvulsant. Therapy with Pregabalin is not associated with serum aminotransferase elevations, and clinically apparent liver injury from Pregabalin has been reported but appears to be quite rare. The IUPAC Name of Pregabalin is (3S)-3-(amino methyl)-5-methylhexanoic acid. The Chemical Structure of Pregabalin is as follows

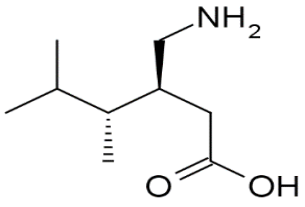

Fig-1: Chemical Structure of Pregabalin

MATERIALS AND METHODS

Table-1: List of Instruments Used

SL. No.	Instrument	Model
1	UPLC	WATERS, software: Empower, Acquity separation module, PDA detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil

Table-2: List of Chemicals Used

SL. No.	Chemical	Company Name
1	Pregabalin	Sun Pharma
2	KH_2PO_4	FINER chemical LTD
3	UPLC grade water	LICHROSOLV (MERCK)
4	UPLC grade Methanol	LICHROSOLV (MERCK)
5	UPLC grade Acetonitrile	MOLYCHEM
6	Ortho phosphoric Acid	MERCK
7	Octane Sulphonic acid	MERCK

PREPARATIONOF BUFFER AND MOBILE PHASE:

Preparation of 0.1% Octane Sulphonic Acid:

Accurately weighed 1 grams of Octane Sulphonic acid was taken in a 1000ml volumetric flask, dissolved and diluted to 1000ml with UPLC water and was adjusted to pH 3.0 with Orthophosphoric acid.

Preparation of Mobile Phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml of Acetonitrile UPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

Preparation of the Pregabalin Standard & Sample Solution:

Standard Solution Preparation:

Accurately weigh and transfer 10mg of Pregabalin working standard into a 50ml clean dry volumetric flask add about 30ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10mg Pregabalin (marketed formulation=240.4mg of tablet Powder) sample into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter. (Stock solution) Further pipette 1.5 ml of Pregabalin from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

Procedure:
Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for Pregabalin peaks and calculate the %Assay by using the formulae.

System Suitability:
Tailing factor for the peaks due to Pregabalin in Standard solution should not be more than 2.0
Theoretical plates for the Pregabalin peaks in Standard solution should not be less than 2000.

Calculation: (For Pregabalin)

$$\%\ Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

Where:
AT= average area counts of sample preparation.
AS= average area counts of standard preparation.
WS= Weight of working standard taken in mg.
P= Percentage purity of working standard
LC= Label Claim mg/ml.

Stability Studies
Stability studies should include testing of those attributes of the drug product that are susceptible to change during storage and are likely to influence quality, safety, and/or efficacy.

RESULTS AND DISCUSSION
UPLC Method Development
Mobile Phase Optimization:
Initially the mobile phase[4] tried was methanol: Ammonium acetate buffer and Methanol: phosphate buffer with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized with buffer (pH 3.0): Acetonitrile in proportion 30: 70 v/v respectively.

Wave length Selection:
UV spectrum of 10µg/ml Pregabalin in diluents (mobile phase composition) was recorded by scanning in the range of 200nm to 400nm. From the UV spectrum[5] wavelength selected as 226nm. At this wavelength both the drugs show good absorbance.

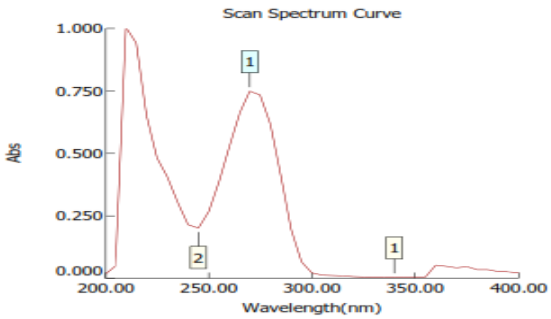

Fig-2: UV Spectrum of Pregabalin

Optimization of Column:
The method was performed with various columns like C18 column, Hypersil column, lichrosorb, and Inertsil ODS column. Endeversil ODS (2.1 x 50mm, 3µm) was found to be ideal as it gave good peak shape and resolution at 0.2 ml/min flow.

Optimized Chromatographic Conditions:
Instrument used: Waters UPLC with auto sampler and PAD or detector.

Temperature: Ambient
Column: Endeversil C18 ODS (2.1 x 50mm, 3µm)
Buffer: 0.1% Octa sulphonic acid
pH: 3.0
Mobile phase: 30% buffer 70% Acetonitrile
Flow rate: 0.2 ml per min
Wavelength: 226 nm
Injection volume: 2 µl
Run time: 2 min.

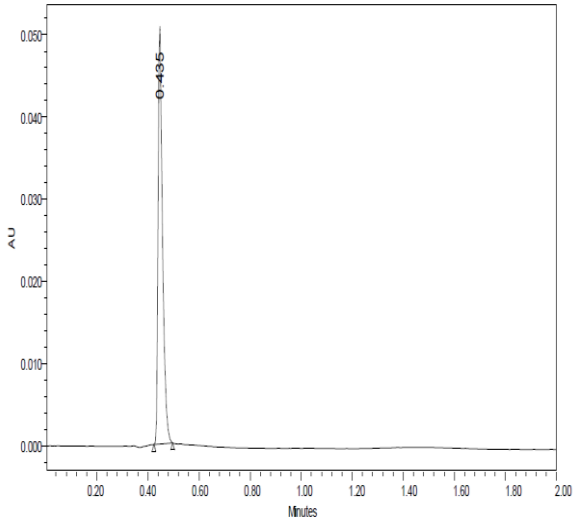

Fig-3: Optimized Chromatographic Condition

Validation of Method
Precision:
Preparation of stock solution:
Accurately weigh and transfer 10mg of Pregabalin working standard into a 50ml clean dry volumetric flask add about 30ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

Procedure: The standard solution[6] was injected for six times and measured the area for all six. Injections in UPLC. The %RSD for the area of six replicate injections was found to be within the specified limits. The results are summarized for Pregabalin

Table-3: Repeatability Results of Pregabalin

Injection	Area for Pregabalin
Injection-1	347358
Injection-2	345898
Injection-3	349624
Injection-4	351347
Injection-5	345567
Injection-6	349045
Average	**341839.8**
Standard Deviation	**2261.2**
%RSD	**0.6**

Acceptance Criteria: The % RSD[7] for the area of six standard injections results should not be more than 2%**.**

Intermediate Precision/Ruggedness:
To evaluate the intermediate precision[8] (also known as Ruggedness) of the method, Precision was performed on different day.

Preparation of stock solution:
Accurately weigh and transfer 10mg of Pregabalin working standard into a 50ml clean dry volumetric flask add about 30ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

Procedure:
The standard solutions prepared in the precision[9] were injected on the other day, for six times and measured the area for all six injections in UPLC. The %RSD for the area of six replicate injections was found to be within the specified limits. The results are summarized for Pregabalin

Table-4: Results of Intermediate Precision of Pregabalin

Injection	Area for Pregabalin
Injection-1	349537
Injection-2	342874
Injection-3	348593
Injection-4	345487
Injection-5	340784
Injection-6	345292
Average	**345427.8**
Standard Deviation	**3317.0**
%RSD	**1.0**

Acceptance Criteria: The % RSD for the area of six standard injections results should not be more

Specificity:
For Specificity[10] Blank and Standard are injected into system. There is no any interference of any peak in blank with the retention time of the analytical peaks.

Accuracy:

Preparation of Standard stock solution:
Accurately weigh and transfer 10mg of Pregabalin working standard into a 50ml clean dry volumetric flask add about 30ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 1.5 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration):
Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 5mg Pregabalin + 235.4mg of placebo mixture (marketed formulation=120.2mg of tablet Powder) into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter. (Stock solution)
Further pipette 1.5 ml of Pregabalin from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. (15ppm of Pregabalin)

For preparation of 100% solution (With respect to target Assay concentration):
Accurately weigh 10 tablets crush in mortar and pestle and transfer equivalent to 10mg Pregabalin + 230.4mg of placebo mixture (marketed formulation=240.4mg of tablet Powder) into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter. (Stock solution) Further pipette 1.5 ml of Pregabalin from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

For preparation of 150% solution (With respect to target Assay concentration):
Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 15mg Pregabalin + 345.6mg of placebo mixture (marketed formulation=360.6mg of tablet Powder) into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter. (Stock solution)
Further pipette 1.5 ml of Pregabalin from the above stock solution into a 10ml volumetric flask and

dilute up to the mark with diluent. (45ppm of Pregabalin)

Procedure:
Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Pregabalin and calculate the individual recovery[11] and mean recovery values. The accuracy[12] results for Pregabalin

Table-5: Accuracy Results of Pregabalin

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	175573	5	5.05	101.07	100.22
100%	347420	10	10.00	99.99	
150%	518990	15	14.94	99.58	

Acceptance Criteria:
The % Recovery[13] for each level should be between 98.0 to 102.0%

Linearity:
Preparation of stock solution:
Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10mg Pregabalin (marketed formulation=240.4mg of tablet Powder) sample into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered[14] through 0.44 micron Injection filter. (Stock solution)

Preparation of Level – I (10 ppm of Pregabalin):
0.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II (20 ppm of Pregabalin):
1 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III (30 ppm of Pregabalin):
1.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV (40 ppm of Pregabalin):
2.0 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – V (50ppm of Pregabalin)
2.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent[15].
Procedure:
Inject each level into the chromatographic system and measure the peak area.
Plot a graph of peak area versus concentration[16] (on X-axis concentration and on Y-axis Peak area) and calculate the correlation[17] coefficient.

Table-6: Results of Linearity Results: (for Pregabalin)

Linearity Level	Concentration	Peak Area
I	0	0
II	10	117116
III	20	234231
IV	30	351347
V	40	458463
VI	50	585578
Correlation Coefficient		**0.999**

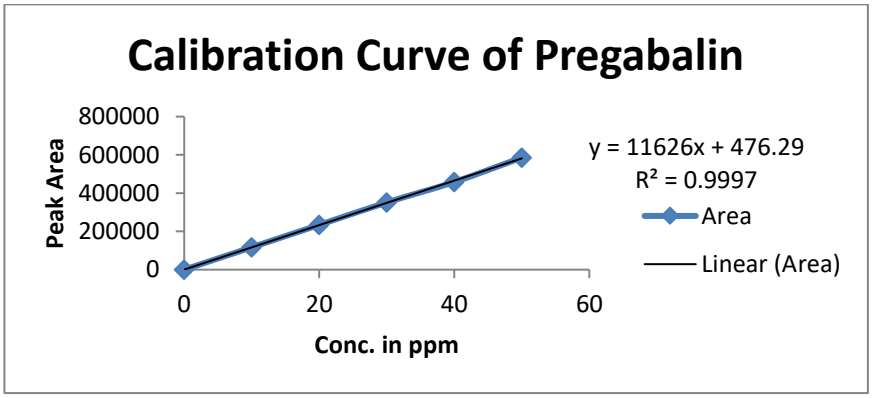

Fig-4: Calibration Curve of Pregabalin

Acceptance Criteria: Correlation coefficient should be not less than 0.99.

Limit of Detection: (For Pregabalin)
Preparation of 30µg/ml solution:
Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10mg Pregabalin (marketed formulation=240.4mg of tablet Powder) sample into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter. (Stock solution)
Further pipette 1.5 ml of Pregabalin from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

Preparation of 0.10 µg/ml solution:
Further pipette 1.0ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.
Further pipette 0.34ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Calculation of S/N Ratio:
Average Baseline Noise[18] obtained from Blank: 64 µV
Signal Obtained from LOD solution: 191 µV
S/N = 191/64 = 2.98

Acceptance Criteria:
S/N Ratio value shall be 3 for LOD[19] solution.

Limit of Quantification:
Preparation of 30µg/ml solution:
Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10mg Pregabalin (marketed formulation=240.4mg of tablet Powder) sample into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter[20]. (Stock solution)
Further pipette 1.5 ml of Pregabalin from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent. (30ppm of Pregabalin)

Preparation of 0.33 µg/ml solution:
Further pipette 1.0 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.
Further pipette 1.1 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Calculation of S/N Ratio:
Average Baseline Noise obtained from Blank: 64 µV
Signal Obtained from LOQ[21] solution: 638 µV
S/N = 638/64 = 9.97

Acceptance Criteria:
S/N Ratio value shall be 10 for LOQ solution.

Procedure for LOD and LOQ:
The LOD and LOQ solutions was prepared injected, for three times and measured the area for all three injections in UPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Robustness:
As part of the Robustness[22], deliberate change in the Flow rate, Mobile Phase composition, Temperature[23] Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.18 ml/min to 0.22 ml/min.
Standard solution 30ppm of Pregabalin was prepared and analysed using the varied flow rates along with method flow rate. On evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±10%.

Table-7: System suitability results for Pregabalin

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.18	3639.37	1.55
2	0.2	3248.37	1.53
3	0.22	3386.38	1.54

***** Results for actual flow (0.2 ml/min) have been considered from Assay standard.

B. The Organic composition in the Mobile phase was varied from 63% to 77%.
Standard solution 10µg/ml of Pregabalin was prepared and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method. On evaluation of the above results, it can be concluded that the variation in 10%. Organic composition[24] in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the Mobile phase ±10

Table-8: System Suitability results for Pregabalin

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		Plate Count USP	USP Tailing
1	10% less	3674.67	1.55
2	*Actual	3248.37	1.53
3	10% more	3465.33	1.53

***** Results for actual Mobile phase composition (30:70 Buffer (ph-3): Acetonitrile has been considered from Accuracy stand

Studies of Degradation:

The International Conference on Harmonization (ICH) guideline[25,26] entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Pregabalin using the proposed method.

Preparation of Stock Solution:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 10mg Pregabalin (marketed formulation=240.4mg of tablet Powder) sample into a 50ml clean dry volumetric flask add about 30 ml of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.44 micron Injection filter. (Stock solution)

Acidic Degradation: Pipette 1.5 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized[27] with 0.1 N NaOH and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Basic Degradation: Pipette 1.5ml of above solution into a 10ml volumetric flask into a 10ml volumetric flask and add 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then, the volumetric flask was kept at 60ºC for 6 hours and then neutralized with 0.1N HCl and make up to 10ml with diluent. Filter the solution with 0.22 microns syringe filters and place in vials.

Oxidative Degradation: Pipette 1.5ml above stock solution 2 into a 10ml volumetric flask solution into a 10ml volumetric flask 1 ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe[28] filters and place in vials.

Thermal Degradation: Pregabalin sample was taken in petridish and kept in Hot air oven at 110^0 C for 24 hours. Then the sample was taken and diluted with diluents and injected into UPLC[29] and analysed.

Photo Degradation: Pipette 1.5 ml above stock solution into a 10ml volumetric flask and expose to sunlight for 24hrs and the volume was made up to the mark with diluent[30]. Filter the solution with 0.45 microns syringe filters and place in vials.

Table-9: Results of Degradation Studies[31-35]

Sample Name	Pregabalin				
	Area	% Degraded	Purity Angle	Purity Threshold	Peak purity
Standard	346387	0.00	0.389	1.283	Passes
Acid	316528	8.62	0.339	1.250	Passes
Base	338212	2.36	0.208	1.252	Passes
Peroxide	324461	6.33	0.123	0.262	Passes
Thermal	340602	1.67	0.180	0.255	Passes
Photo	334402	3.46	0.168	0.253	Passes

CONCLUSION

A precise, linear, specific & suitable stability indicating UPLC method for analysis of Pregabalin, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over isocratic elution. In case of UPLC various columns are available, but here Endeversil C18 ODS (2.1 x 50mm, 3µm) column was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of

Pregabalin it is evident that most of the UPLC work can be accomplished in the wavelength range of 226nm conveniently. Further, a flow rate of 0.2ml/min & an injection volume of 2µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay which can help in the analysis of Pregabalin in different formulations.

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