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DEVELOPMENT, VALIDATION & STRESS DEGRADATION STUDIES OF PREGABALIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT: A simple, selective, rapid, precise and economical reverse phase high pressure liquid chromatographic method has been developed for the estimation of Pregabalin in pharmaceutical Tablet dosage form. The mobile phase consisted of 50:50 % (v/v) of Methanol & 10mM Ammonium Acetate (pH adjusted to 3.0 with glacial acetic acid) operated on isocratic mode. The flow rate is 0.7 ml/min. Chromatographic determination of Pregabalin was performed on Phenomenex C_{18} column (150 X 4.6 mm Id, ODS 2, 5µm). The wavelength of detection is 210 nm. The injection volume is 20µL. The retention time of Pregabalin is 3.39 ± 0.10 minutes. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantitation, solution stability, ruggedness, and robustness. The influence of Acid, Alkaline, Oxidative Stress, Photolytic stress conditions on Pregabalin was studied. Results indicated that pregabalin is stable under the experimental conditions with a high baseline noise observed in alkaline medium. The proposed method has been successfully used for the estimation in tablet dosage forms.

INTRODUCTION: Pregabalin (PGB), (S) - 3 - amino methyl hexanoic acid, is a structural analogues of γ - amino butyric acid (GABA) as shown in **(Figure 1)**. It is a white crystalline solid. It is soluble in water and in both basic and acidic aqueous solutions. It is a new anticonvulsant and analgesic medication that was recently approved for adjunctive treatment of partial seizures in adults in both the United States and Europe and for the treatment of neuropathic pain from post-therapeutic neuralgia and diabetic neuropathy.

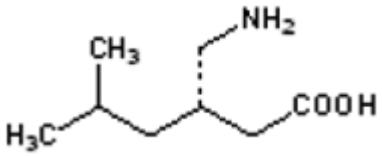

FIGURE 1: STRUCTURE OF PREGABALIN

It is both structurally and pharmacologically related to the anticonvulsant and analgesic medication gabapentin and both compounds were originally synthesized with the hope of modulating brain GABA receptors and GABA synthetic enzymes.

These compounds are inactive at $GABA_A$ and $GABA_B$ receptors [1]. The mechanism of action of pregabalin has been characterized only partially and in particular, the cellular and molecular details of its action to reduce neurotransmitter release are incompletely known.

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The primary high - affinity binding site for Pregabalin in forebrain tissues is the α2 – δ type 1 auxiliary subunit of voltage - gated calcium channels [2] and this interaction seems to the required for the pharmacological actions of the medications [3, 4].

The identification of the α2 – δ type 1 binding sites has led to the speculation that Pregabalin act pharmacologically specifically in neurons by modulating the action of synaptic calcium channels. This hypothesis is supported by several findings that Pregabalin reduce calcium influx into synaptosomes prepared from human brain [5,6] and it subtly reduce calcium dependent overflow of neurotransmitters from several different neuronal tissues and reduce synaptic responses.

PGB is thought to be useful for treating any other conditions, pain, physiological conditions associated with psychomotor stimulants, inflammation, gastrointestinal damage, alcoholism, insomnia, and various psychiatric disorders, including mania and bipolar disorder. There is no official method developed for the analysis of pregabalin till now and therapeutic importance of the drug has engendered development of assays for the quantification of PGB. A through literature search has revealed that only a few analytical methods are available for determination of Pregabalin in bulk drugs and pharmaceutical formulations [7].

Liquid chromatography - mass spectrophotometry (LC - MS), LC with fluorescence detection were used to determine pregabalin in human plasma and serum [8-12].

All of these methods are very expensive because these methods require long and tedious pretreatment of the samples, laborious clean up procedures (including extraction with solvent) and derivatization for the analysis of PGB. There is no HPLC method without derivatization for the analysis of PGB. So there is need for the development of a HPLC method for the analysis of PGB.

Hence, an attempt has been made to develop a simple, efficient and selective method for the analysis of PGB in pharmaceutical formulations. The method requires no derivatization steps. HPLC instrumentation with UV detection, which is readily available in most analytical and pharmaceutical laboratories, was used.

A total analysis run time of less than 5 min was achieved. The developed method was validated as per ICH Guidelines [12] and used successfully to evaluate 5 marketed PGB drug products.

EXPERIMENTAL:

Reagents and chemicals: Ammonium Acetate (AR Grade, SD Fine Chem ltd), Methanol (HPLC grade, Merck ltd), Milli-Q water, Pregabalin (reference standard is gifted by M/s Corpuscle Research Solutions), Acetic Acid (GR Grade, SD Fine Chem Ltd). All other chemicals are of the highest grade commercially available unless otherwise specified.

Apparatus and Chromatographic conditions: The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software. The mobile phase consisted of 50:50 % (v/v) of Methanol & 10mM Ammonium Acetate (pH adjusted to 3.0 with acetic acid) operated on isocratic mode. The flow rate is 0.7 ml/min. Chromatographic determination of Pregabalin was performed on Phenomenex C_{18} column (150 X 4.6 mm id, ODS 2, 5µm). The wavelength of detection is 210 nm. The injection volume is 20µL.

Preparation of standard solutions, Calibration Standards & Quality Control Samples: Stock solutions of Pregabalin (10 mg/mL) was prepared separately in a volumetric flask and labeled accordingly. Suitable dilutions of Pregabalin were prepared using 50:50 % (v/v) Methanol & Milli-Q water as diluent Solution. A Linear Calibration curve containing 8 non-zero standards were prepared using diluent solution in the concentration range of 249.25 – 7976.00 µg/mL. The linear standard calibration standard sample is then transferred into the auto sampler for analysis. Samples for Specificity (Sample with Drug; Blank Sample) were also prepared accordingly. For the preparation of quality control samples, a separate stock containing approximately the same concentration of the drug substance is prepared and labeled as quality control stock. From this stock, quality control samples were prepared at three concentration levels namely LQC (2492.50 µg/mL), MQC (4985.00 µg/mL), HQC

(7487.50 µg/mL) so as to obtain low, median and high concentration quality control samples. The performance of the linear calibration curve is then evaluated using quality control samples.

Assay: The assay of tablets containing Pregabalin is done using the procedure given in Indian Pharmacopoeia for tablets. Briefly, twenty tablets, each containing 75 mg of Pregabalin as labeled claim were weighed and finely powdered; a quantity of powder equivalent to 75.0 mg of Pregabalin was weighed and transferred to a 10 mL volumetric flask. To this 10 mL of methanol was initially added and vortexed thoroughly. The final volume is made up to volume with methanol. Suitable dilution is prepared using diluent solution so as to get a final concentration within the range of the calibration curve. This mixture is then carefully filtered using 0.45 µm membrane filter. The filtrate is then taken and suitably diluted and injected for analysis. The assay content was evaluated using the regression equation of linear calibration curve.

Method Validation:

1. **System Suitability:** The system suitability was assessed by six replicate analysis of the drug at a concentration of 4985.00µg/ml. The acceptance criterion is ± 2 % for the percent coefficient of the variation for the retention time for the drug.

2. **Detection and Quantization Limits (Sensitivity):** Limits of detection (LOD) and quantification (LOQ) (**Figure 2**) were estimated from both linearity calibration curve method and signal to noise ratio method. The detection limit was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantification limit was defined as the lowest concentration level that provided a peak area with signal to noise ratio higher than 5, with precision (%CV) and accuracy with (±) 20%.

3. **Linearity (Calibration Curve):** The calibration curve was constructed with eight concentrations ranging from 249.25 – 7976.00 µg/mL. The linearity was evaluated by linear regression analysis, which was calculated by least square method. It is depicted in (**Figure 3**).

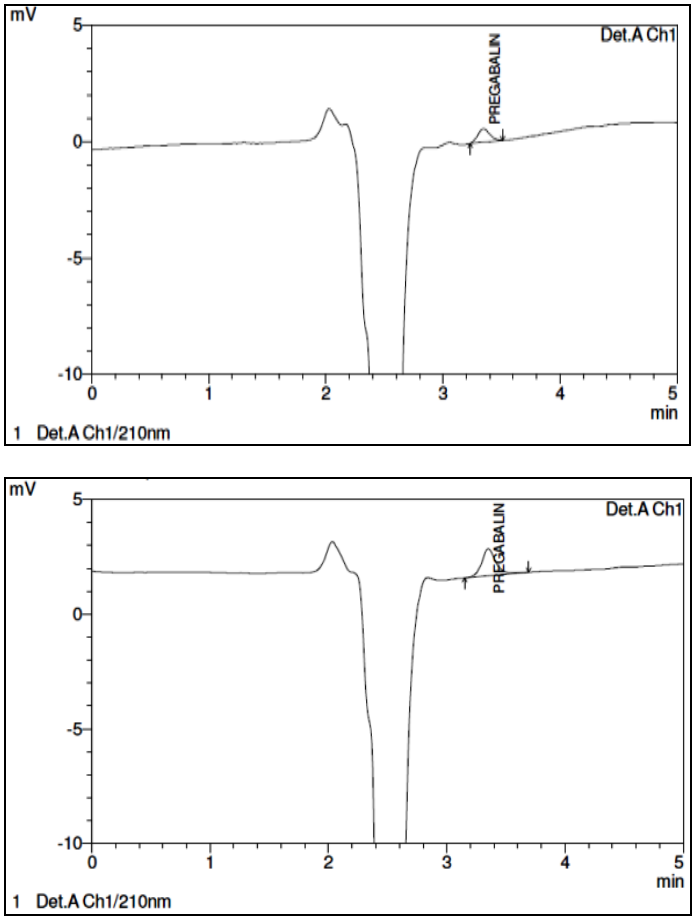

FIGURE 2: CHROMATOGRAMS SHOWN BELOW INDICATE LIMIT OF DETECTION (LOD) ABOVE AND LIMIT OF QUANTITATION (LOQ)

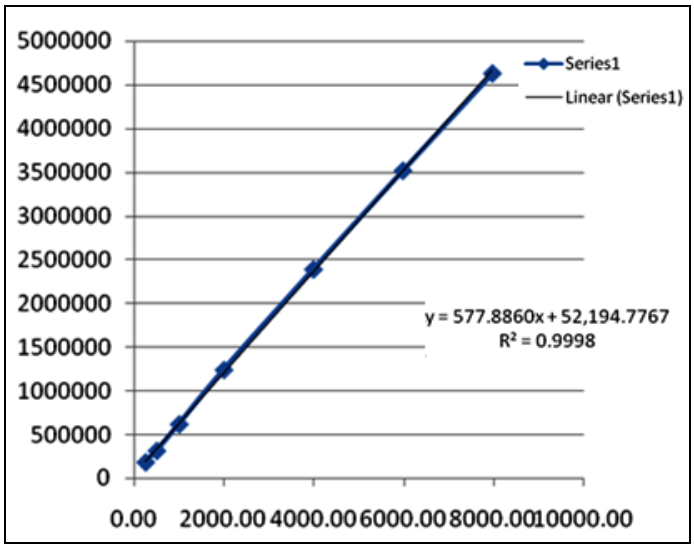

FIGURE 3: LINEAR CALIBRATION CURVE OF PREGABALIN

4. **Accuracy and Precision:** Accuracy of assay method was determined for both intra-day and inter-day variations using triplicate analysis of the QC samples. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day).

Repeatability refers to the use of the analytical procedure within the laboratory over the shorter period of the time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days).

5. **Specificity:** Specificity of the method was determined by comparing the Blank sample with that of the sample containing Pregabalin (**Figure 4**). A less than 20% interference of the peak area at the retention time of the drug in the blank sample is taken as acceptance criteria for the analyte. Sample Specificity is also observed in the degradation study of the drug. None of the degraded products must interfere with the quantification of the drug.

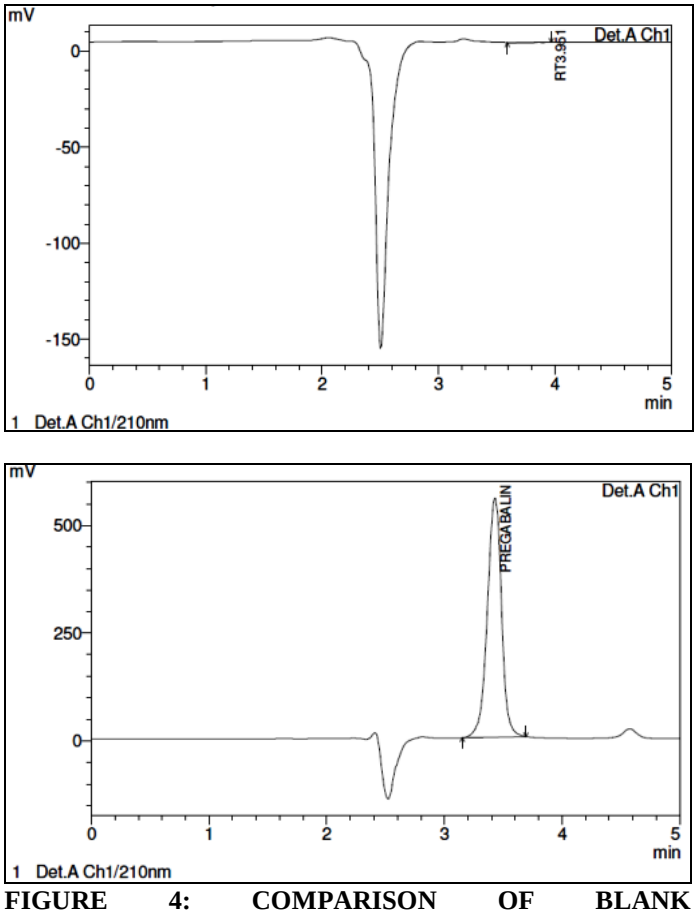

FIGURE 4: COMPARISON OF BLANK CHROMATOGRAM (ABOVE) TO THAT OF SAMPLE CONTAINING PREGABALIN (BELOW) STANDARD

6. **Stability:** The stability of the drug is determined by placing the MQC samples for the short term stability by keeping at room temperature up to 12 hours and then comparing the obtained peak area with that of the similarly prepared fresh sample. Further, auto-sampler stability for up to 24 hrs was studied and established.

7. **Stress Degradation Studies:** For Stress Degradation Analysis, 1 mL aliquots (in duplicate) of samples containing MQC level concentration are treated separately with 100 µL of 0.1N HCl (Acid stress), 0.1N NaOH (Alkaline stress), 5% v/v Hydrogen Peroxide (Oxidative Stress), for 24 Hrs. Samples for Photolytic stress are placed in a transparent glass vial & placed in a UV chamber for 24 Hrs. Samples are then injected for analysis. The results of analysis are then compared with similarly prepared fresh samples (**figure 5**).

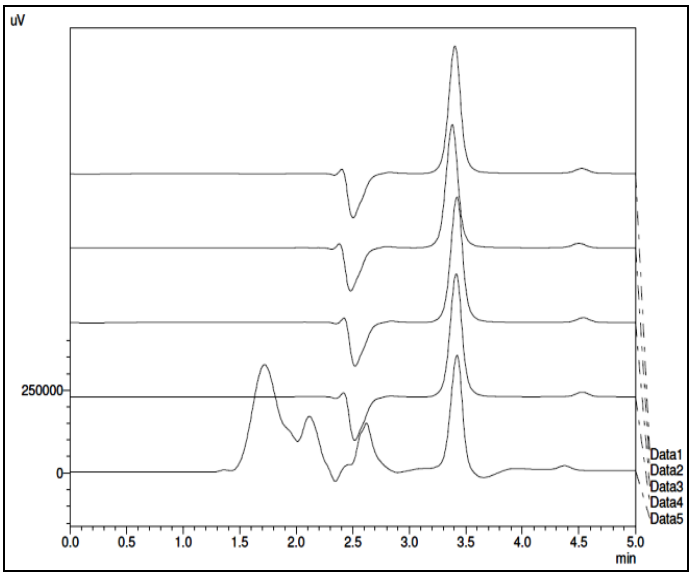

FIG. 5: OVERLAY CHROMATOGRAM SHOWING THE INFLUENCE OF VARIOUS STRESS CONDITIONS ON PREGABALIN; Data 1 – Freshly prepared Sample; Data 2 – Oxidative Stress; Data 3 – Photolytic Stress; Data 4 – Acid Stress; Data 5 – Alkaline Stress. Data 5 clearly indicates the spectral degradation of Pregabalin due to alkaline instability.

RESULTS AND DISCUSSION:

Method Development and Validation: The HPLC procedure was optimized with a view to develop a stability indicating assay method. Functional group analysis revealed the presence of Zwitter-Ionic character to the molecule due to the presence of both amino and carboxylic acid groups. The solubility of pregabalin in water is attributed to its highly ionic nature. Reverse phase HPLC separations usually require optimization of the mobile phase pH particularly for the highly ionic drugs.

Therefore, we evaluated the chromatographic behavior at different pH values ranging from pH 3.0 to pH 6.5 using various columns like Hypersil-BDS-C18, Symmetry C18, Ymc-Pack C18, Ymc-Pack

Pro, Spherisorb C18, Phenomenex C18 have been tried with different buffer salts such ammonium Formate, ortho phosphoric acid, di-potassium hydrogen orthophosphate, in combination with acetonitrile, methanol and tetrahydrofuran.

However less tailing and high theoretical plates are obtained with Phenomenex ODS 2 column C18 150 X 4.6 cm 5µm column. Mobile phase composition consisted of (50:50 v/v) of Methanol and 10mM Ammonium acetate (pH adjusted to 3.0 ± 0.1 with glacial acetic acid) on isocratic mode. The flow rate of the method is 0.7 ml/min. Calibration standards were prepared in diluents solution containing 50:50 % v/v of methanol and Milli-Q water. The wavelength of detection is 210nm. The column temperature is maintained at 25^{o}C. At the reported flow rate, peak shape was excellent; however increasing or decreasing the flow rate resulted in unacceptable tailing factor and poor peak shape. Hence, 0.7 ml/min was optimized flow rate decreasing the consumption of the mobile phase, which in turn proves to be cost effective for long term routine quality control analysis.

Method Validation:

1. **System Suitability:** The % RSD of the peak area and the retention time for both drug and internal standard are within the acceptable range (**Table 1**). The efficiency of the column was expressed as the number of theoretical plates for the six replicate injections was around 3975±32 and the USP tailing factor was 1.02 ± 0.01.

TABLE 1. SYSTEM SUITABILITY TEST FOR PREGABALIN

PREGABALIN				
SR NO	**Retention Time**	**Peak Area**	**Theoretical Plates**	**Tailing Factor**
1	3.39	3031271	4017	1.01
2	3.40	3044880	4004	1.02
3	3.40	3054691	3971	1.01
4	3.41	2895945	3979	1.03
5	3.41	3035137	3942	1.01
6	3.41	3005345	3938	1.02
MEAN	**3.403**	**3011211.500**	**3975.2**	**1.017**
ST DEV	**0.0082**	**58855.13**	**31.92**	**0.01**
% CV	**0.24**	**1.95**	**0.80**	**0.80**

2. **Determination and Quantification Limits (Sensitivity): Figure 2** represents the chromatogram of limit of detection and limit of quantification. The method is found to be sensitive which can be determined from the data obtained from **Table 2**.

TABLE 2: SENSITIVITY OF PREGABALIN BY HPLC

PREGABALIN			PREGABALIN		
LOD			**LOQ**		
Sr. No.	**Drug**		**Sr. No.**	**Drug**	
	Retention Time	**Peak Area**		**Retention Time**	**Peak Area**
1	3.34	4185	1	3.35	9840
2	3.33	4238	2	3.35	9814
3	3.35	4832	3	3.35	8338
MEAN	**3.340**	**4418.3**	**MEAN**	**3.350**	**9330.7**
ST DEV	**0.01**	**359.22**	**ST DEV**	**0.00**	**859.77**
% CV	**0.30**	**8.13**	**% CV**	**0.00**	**9.21**

3. **Linearity:** The linearity was demonstrated in triplicate. The results of the best fit line (y = mx + c) for the triplicate analysis is given in **Table 3**. The accuracy of the calibration standards was evaluated from the back calculated concentrations **Table 4**. All the standards were found to be within the range of 90 – 104 %.

4. **Accuracy and Precision:** Accuracy and precision calculated for the QC samples during the intra- and inter –day run are given in **Table 5**. The intra-day (day-1) and inter-day accuracy ranged from 98.00 to 102.00 %. The results obtained from intermediate precision (inter-day) also indicated a good method precision. All the data were within the acceptance criteria.

TABLE 3: RESULTS OF BEST-FIT LINE FOR TRIPLICATE ANALYSIS

Curve	Slope	Intercept	r^2
1	577.886	52194.77	0.9998
2	583.66486	52716.72	0.9999
3	592.33315	53499.64	0.9997
Mean	**584.63**	**52803.71**	**0.9998**

TABLE 4: LINEARITY AND RANGE FOR PREGABALIN DEMONSTRATING ACCURACY, CARRYOVER EFFECT AND SPECIFICITY OF THE METHOD (Data Represented for 1st Calibration Curve**)**

Sr. No.	Sample ID	Concentration (µg/mL)	Drug		Calculated Conc. (µg/mL)	Accuracy (%)
			Retention time	Peak area		
1	BLANK	0.000	0.00	NO PEAK	NA	NA
2	CC 01	249.25	3.36	183398	227.04	91.09
3	CC 02	498.50	3.37	314146	453.29	90.93
4	CC 03	997.00	3.36	618378	979.74	98.27
5	CC 04	1994.00	3.38	1239974	2055.37	103.08
6	CC 05	3988.00	3.39	2389838	4045.13	101.43
7	CC 06	5982.00	3.41	3519212	5999.44	100.29
8	CC 07	7976.00	3.43	4631730	7924.58	99.36
9	**Co Blank**	**0.000**	**0**	**No Peak**	**NAP**	**NA**

NA - Not applicable

5. **Specificity:** Specificity was determined by comparison of the Blank chromatogram with that of the Standard chromatogram (**Figure 4**).

6. **Room Temperature Stability:** Stability studies were done for short term stability up to 12 hrs on the bench top for the MQC levels conditions. Stability is calculated as the ratio of the mean peak area of the stability sample to the mean peak area of the fresh sample and expressed as the percentage (n=6). The room temperature stability was found to be 96.43 %. The results are tabulated in **Table 6**.

TABLE 5: RESULTS OF INTER AND INTRA-DAY ACCURACY & PRECISION FOR PREGABALIN BY HPLC

	Nominal Concentration (µg/mL)		
	2492.50	**4985.00**	**7477.50**
	DAY 1		
Mean Accuracy	99.99	101.69	101.15
SD	3.95	0.97	1.06
% CV	3.95	0.69	1.05
	DAY 2		
Mean Accuracy	101.21	100.69	102.34
SD	1.07	1.02	0.78
% CV	1.06	1.01	0.76
	DAY 3		
Mean Accuracy	100.28	101.07	102.69
SD	2.15	2.13	1.94
% CV	2.14	2.11	1.89

TABLE 6: ROOM TEMPERATURE STABILITY OF PREGABALIN (n = 6)

Fresh sample				Stability sample			
Sr. No	**Sample ID**	**Drug**		**Sr. No**	**Sample ID**	**Drug**	
		Retention time	**Peak area**			**Retention time**	**Peak area**
1	Fresh sample	3.4	2991777	1	Stability sample	3.40	2934788
2	Fresh sample	3.4	2967325	2	Stability sample	3.39	2937635
3	Fresh sample	3.39	3093143	3	Stability sample	3.39	3028658
4	Fresh sample	3.39	3071917	4	Stability sample	3.39	3025866
5	Fresh sample	3.39	3027765	5	Stability sample	3.39	2761565
6	Fresh sample	3.39	3003192	6	Stability sample	3.38	2935613
Mean			**3015478.50**	**Mean**			**2907681.33**
Std. Dev			**17375.73**	**Std. Dev**			**134346.15**
% CV			**0.58**	**% CV**			**4.62**

7. **Stress Degradation:** The stress studies involving acid, light (UV) and oxidation revealed that Pregabalin was stable under the stress conditions (**Figure 5**). However in alkaline conditions (0.1N NaOH), the baseline resulted in high noise without affecting the peak shape. For all stress conditions studied, the drug content was within 97 – 99 % indicating the stability and specificity of the analytical method to differentiate the degradation peaks.

8. **Robustness study:** Robustness is the measure of method capacity to remain unaffected by deliberate small changes in the chromatographic conditions. The experimental conditions were deliberately altered to evaluate the robustness of the method. The impact of flow-rate (0.7 ± 0.1 ml/min), and effect of mobile-phase composition (± 5%) on chromatographic parameters such as retention time, theoretical plates, and tailing factor, were studied**.** There was no significant variation due to the variation of mobile phase composition or flow rate variation.

Application of the method to Dosage forms: The HPLC method developed is sensitive and specific for the quantitative determination of Pregabalin. Also the method is validated for different parameters, hence has been applied for the estimation of drug in pharmaceutical dosage forms. Pregabalin tablets from two different manufacturers were evaluated. The amount of Pregabalin in tablet 1 is 99.05 ± 0.16 and tablet 2 is 99.35 ± 0.11. None of the tablets ingredients interfered with the analyte peak. The spectrum of Pregabalin is extracted from the tablets was matching with that of standard Pregabalin showing the purity of peak of Pregabalin in the tablets.

CONCLUSIONS: The method gave accurate and precise results in the concentration range of 249.25 to 7976.00 µg/mL. The mobile phase composition consists of (50:50 v/v) of Methanol and 10 mM Ammonium acetate (pH adjusted to 3.0 with glacial acetic acid), at the flow rate of 0.7 ml/min. The retention times of the drug are 3.39 minutes. The column is Phenomenex ODS 2 150 X 4.6mm, C18 column with the particle size of 5µm. A rapid sensitive and specific method for the determination of Pregabalin in the pharmaceutical formulations has been developed.

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ABBREVIATIONS:

PGB : Pregabalin
GABA: Gabapentine
LOD : Limit of Detection
LOQ : Limit of Quantification.
UV : Ultra violet
RSD : Relative Standard Deviation.

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