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# STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF PREGABALIN AND METHYLCOBALAMINE BY RP-HPLC IN ITS BULK FORM

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## *ABSTRACT*

*The present work is associated with the application of simple, accurate, economic and precise Stability indicating RP-HPLC method for the simultaneous estimation of Pregabalin andMethylcobalamine in its bulk form.Pregabalin and Methylcobalamine are primarily used for Neuropathic pain. The HPLC instrument used was Waters-Alliance 2690/95 with PDA detector. Inertsil ODS 3V C-18 column with dimensions of 250 mm length, particle size of 5 microns and internal diameter of 4.6 mm was used for separation. The mobile phase consisted of 70 volumes of methanol and 30 volumes of water at a flow rate of 1.0 ml/min. The sample was injected using a 20 µl fixed loop and the total run time was 8 min. The retention times of Pregabalin and Methylcobalamine were found to be 3.661 min and 6.453 min respectively. The detection was carried out at 353 nm. The developed method was validated according to ICH Q2 (R1) guidelines for linearity, system suitability, accuracy, precision, limit of detection, limit of quantification, and robustness, forced degradation studies were performed which included acid, alkali, oxidative and thermal degradation. Linearity for both the drugs was found to be in the range of 20-80 µg/mL. The LOD values for Pregabalin and Methylcobalamine were 0.23 and 0.201 respectively. The LOQ values for Pregabalin and Methylcobalamine were 0.72 and 0.610 respectively. Both the drugs were found to be stable in all the stress conditions. The proposed method can be used for routine laboratory analysis.*

***KEYWORDS:*** *Pregabalin, Methylcobalamine, Neuropathic pain, Stability indicating RP-HPLC.*

## INTRODUCTION

Pregabalin (PGB) is an anticonvulsant drug used in neuropathic pain and also in adjunct therapy for partial seizures with or without secondary generalization in adults. Pregabalin is S-enantiomer of 3-aminomethyl-5- methylhexanoicacid [8, 9]. Pregabalin is a GABAergic anticonvulsant and depressant of the central nervous system (CNS). It is classified as a GABA analogue and gabapentinoid. It is a close analogue of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) which does not bind directly to $GABA_A$, $GABA_B$, $GABA_\varrho$ or benzodiazepine receptors nor blocks sodium channels but binds with high affinity to the $\alpha_2\delta$ subunit-containing voltage-gated calcium channels (VDCC) and increases extracellular GABA concentrations in the brain by producing a dose-dependent increase in L-Glutamic acid decarboxylase (GAD). This enzyme is responsible for making GABA and decreasing the calcium influx and thereby reducing the release of calcium dependent neurotransmitters and thus postsynaptic excitability and pain decreases. Methylcobalamine (MC) (mecobalamin, MeCbl, or MeB12) is a cobalamin, a form of vitamin B12, used in the treatment of megaloplasticaneamia, diabetic neuropathy and peripheral neuropathy. The Chemical formula of MC is $C_{63}H_{91}CoN_{13}O_{14}$ and IUPAC name is cobalt (3+) mecobalamin. It is equivalent physiologically to vitamin B12 and can be used to prevent or treat pathology arising from a lack of vitamin B12, such as pernicious anemia, in the treatment of peripheral neuropathy, diabeticneuropathy, and as a preliminary treatment for amyotrophic lateral sclerosis. Unlike the most common form of vitamin B12, cyanocobalamin, methycobalamin is required to protect against neurological diseases and aging. The liver converts a small amount of cyanocobalamin into methylcobalamine but higher amounts are needed to actually correct neurological defects and prevent aging. High amounts of methylcobalamine are needed to regenerate neurons and myelin sheath that protects nerve axons and peripheral nerves [1-7, 10].

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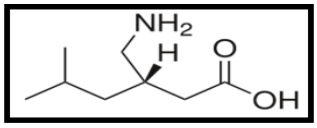


**Fig. 1: Structure of Pregabalin**

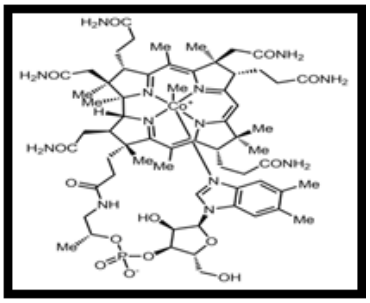

**Fig. 2: Structure of Methylcobalamine**

## EXPERIMENTAL

**Chemicals and materials:**

Pregabalin and Methylcobalamine were procured from Aurobindo Pharma Limited. Methanol, Acetonitrile and water used were HPLC grade and obtained from Standard Reagents Private Limited, Hyderabad.

**Instrument and Software:**

Waters-Alliance of model 2690/95 equipped with PDA detector and Empower Software version 2 was used in the current research.

**Chromatographic conditions:**

Inertsil- C18, ODS 3V column of dimensions (250 x 4.6 mm, 5 µ) was employed for separation. Mobile phase was Methanol: Water (70:30),flow rate was maintained at 1 ml/min and effluents were monitored at 353nm. The sample was injected using a 20 µL fixed loop and the total run time was 8 min.

**Preparation of Standard solution:**

Accurately 10 mg of Pregabalin and Methylcobalamine were weighed individually and transferred into 10 ml volumetric flask each and about 6-7 ml of methanol was added separately to each flask. Sonication was performed to dissolve completely and volume was made up to the mark with the same diluent.

**Preparation of Working Standard:**

Working standards were prepared by taking 0.4 ml of Pregabalin and 0.4 ml of Methylcobalamine from the stock solutions into a 10 ml volumetric flask and adjusted the volume with diluent.

**Method Development:**

Trials were performed using different mobile phases and changing their corresponding ratios. Various mobile phases like Acetonitrile, Methanol and water were considered. The chromatograms were studied for good peak shapes and resolution.

**Method Optimization:**

The mobile phase was optimized to Methanol: water in the ratio of 70:30 % v/v. The optimized method shows good peaks with resolution 6.6. The optimized chromatographic conditions are reported in table 1 and chromatogram in figure 3.

**Method Validation:**

Analytical method Validation is called as method validation. It is aregulatory requirement which assures that the selected method is capable of producing reproducible and reliable results adequate for intended purpose.According to ICH Q2 (R1) guidelines,the developed method was validated for System Suitability, linearity, accuracy, precision, Limit of detection, Limit of quantification and robustness.

***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 of analyte in the sample. Six concentrations were prepared by taking serial dilutions of 20, 30, 40, 50, 60, 70 and 80µg/ml from working standard and were analyzed for detector of linearity. The peak areas of chromatograms were plotted against concentrations of Pregabalin and Methylcobalamine to obtain caliberation curves. Regression analysis was conducted to calculate correlation coefficient and caliberation equation. They were shown in table 2 and linearity curves in figures 4 and 5.

***System Suitability:***

System suitability testing (SST) is an integral associated part of many of the analytical procedures. System suitability test provides the assurance that on a specified occasion the method is providing suitable accurate and precise results. System suitability tests are conducted every time a method is applied that may be either before or during analysis.In this scenario,the evaluated parameters include,Tailing factor, USP Plate count, Resolution, etc. For performing this procedure, five replicates of 40 ppm concentration each of Pregabalin and Methylcobalamine were injected and evaluated. The results obtained were within the limits and the method passes system suitability test. Results are given in table 3.

***Accuracy:***

Accuracy can be defined as the degree of closeness of agreement between the accepted true value or reference value and value obtained.It was tested by taking triplicates that are 50%, 100% and 150% concentrations and the average % recovery of Pregabalin and Methylcobalaminewere calculated. The results obtained were in the range of 98 % – 102 %which depicts that the test method is accurate.Results of accuracy are tabulated in table 4.

***Precision:***

Precision is a description of random errors, a measure of statistical variability it refers to the closeness of measurements to each other from multiple sampling of the same homogenous sample under prescribed conditions. The chromatogram was recorded and the results were given in Table 5, 6, 7

***a) System precision:***

In this, the solution of known concentration is injected to the system at the same conditions (optimized method). Five replicates (40 ppm of Pregabalin and Methylcobalamine each are injected and peak retention times, areas and % RSD is evaluated.

$$\% \text{ RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

***b) Method precision:***

Here, six replicates of the solution are injected and % RSD is calculated. Method precision is to check the reproducibility of results.

***c) Intermediate precision or Ruggedness:***

Precision under defined set of conditions is called as Intermediate Precision.

Peak area and % RSD for the areas were calculated for the six replicate injections. The test results obtained for precision were under the prescribed limits which suggest that method developed is precise.

***Robustness:***

The robustness of an analytical procedure is a 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. Various parameters like change in pH, variation in flow rate, change in mobile phase composition, change in temperature, change in column, etc. are assessed.In the proposed method, variation of flow rate (1 ml ± 0.2 ml) showed no significant effect thereby stating that the proposed method is robust and not affected by minor variations.Results are tabulated in table 8.

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

***LOD:*** The lowest amount of analyte in the sample which can be detected but not necessarily quantified as an exact value is defined as limit of detection.

$$\text{LOD} = \frac{3.3\,\sigma}{S}$$

Where,

σ = standard deviation of response

S = slope of calibration curve

***LOQ:*** Limit of Quantification is the lowest amount of sample which can be determined quantitatively with suitable amount of accuracy and precision.

$$\text{LOQ} = \frac{10\,\sigma}{S}$$

Where,

σ = standard deviation of response

S = slope of calibration curve

Results are given in table 9.

**Forced Degradation Studies:**

In forced degradation studies, the sample is subjected to different stress conditions like acid, alkali, thermal and oxidative degradation. There was no significant degradation products formed. Percent degradation was very low whereby we can conclude that the method is stable in all stress conditions applied.Results are given in table 10 and 11.

***1) Acid hydrolysis:***

Acid-induced, forced degradation was performed by adding an aliquot of stock solution (1 mg/ml) of Pregabalin and Methylcobalamine into 10 ml each of methanol and 0.1 M HCl and refluxing the mixture at 60°C for approximately six hours. The solution was then left to reach room temperature, neutralized to pH 7 by the addition of 0.1 M NaOH, and diluted to 100 ml with the mobile phase so as to get a final concentration of 10 µg/ml. Chromatogram is shown in figure 6.

***2) Alkaline hydrolysis:***

Here, forced degradation was performed by adding an aliquot of stock solution (1 mg/ml) of Pregabalin and Methylcobalamine to 10 ml each of methanol and 0.1 M NaOH, and refluxing the mixture at 60°C for approximately six hours. The solution was then cooled to room temperature, neutralized to pH 7 by addition of 0.1 M HCl, and diluted to 100 ml with the mobile phase, so as to get a final concentration of 10 µg/ml.Chromatogram is shown in figure 7.

***3) Oxidative degradation:***

To study the effect of oxidizing conditions, an aliquot of stock solution (1 mg/ml) of Pregabalin and Methylcobalamine was added to 10 ml of 30% $H_2O_2$ solution and the mixture was refluxed at 60°C for approximately six hours. The solution was left to reach room temperature and diluted to 100 ml with the mobile phase, so as to get a final concentration of 10 µg/ml.Chromatogram is shown in figure 8.

***4) Thermal degradation:***

To study the effect of temperature, approximately 50 mg Pregabalin and Methylcobalaminewas stored at 100°C in a hot air oven for 24 hours and then dissolved in 10 ml of methanol and the volume was adjusted to 50 ml with the mobile phase. The above solution was further diluted with the mobile phase, to give a solution of final concentration equivalent to 10µg/ml of Pregabalin and Methylcobalamine. Chromatogram is shown in figure 9.

$$\% \text{ Degradation} = \frac{Au - At}{Au} \times 100$$

Where:
Au=Area of Untreated Solution
At= Area of Treated Solution.

## RESULTS AND DISCUSSION

A simple, rapid, sensitive and reproducible RP-HPLC method was developed for simultaneous estimation of Pregabalin and Methylcobalamine in bulk. The optimised mobile phase Methanol: water in the ratio 70:30 v/v was found to be satisfactory and gave two symmetric and well resolved peaks for Pregabalin and Methylcobalamine. The wavelength of detection for Pregabalin and Methylcobalamine was measured at 353 nm. The retention time of Pregabalin and Methylcobalamine were found to be 3.661 min and 6.453 min respectively with run time of 8 minutes. The developed method was validated according to ICH Q2 (R1) guidelines. Thecaliberation curve forPregabalin and Methylcobalamine was found to be linearin the concentration range of 20-80 µg/mL(Regression equation of Pregabalin is y = 76817x - 3691.7 and Methylcobalamine is y = 5095.5x - 666.39; where y and x are peak area and concentration, respectively). Correlation coefficient was found to be 0.999.The detection of limits for Pregabalin and Methylcobalime were found to be 0.23µg/ml and 0.201µg/ml respectively, while quantification limits for Pregabalin and Methylcobalamine were found to be 0.72 µg/ml and 0.610 µg/ml respectively. The statistical data and recovery data reveal good accuracy and precision of the proposed method. System precision, method precision, intermediate precision was determined. The % RSD values obtained were found to be less than 2%. The accuracy of the method was determined by calculating recoveries of Pregabalin and Methylcobalamine by standard addition. Their % recoveries were found to be 100.23% for Pregabalin and 100.62 % for Methylcobalamine which indicates that the method is accurate. From stability studies results it was observed that % degradation was low and no degradation products were formed.

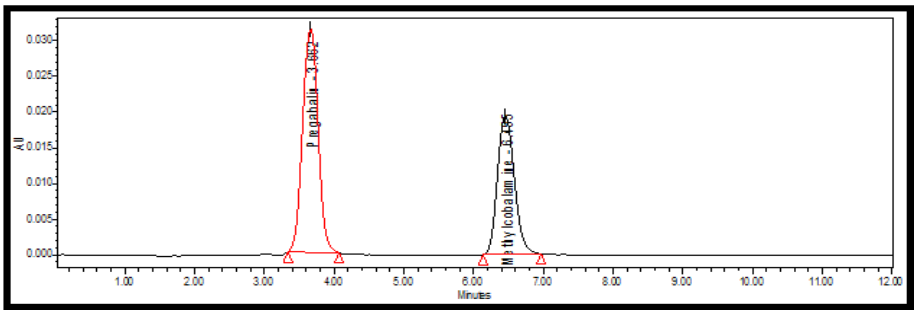


**Fig. 3: Chromatogram for Optimised method**

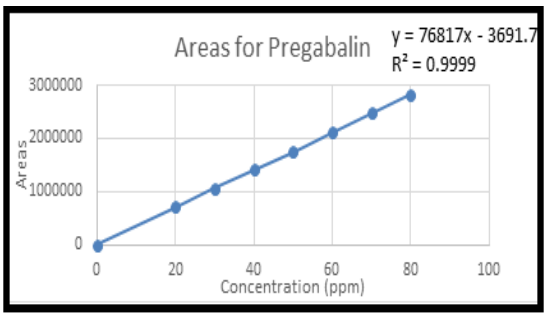


**Fig. 4: Linearity Plot (Concentration Vs Response) of Pregabalin**

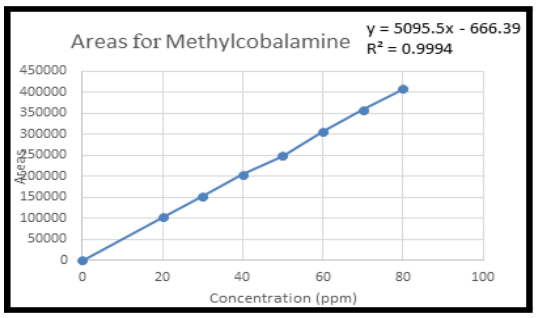


**Fig. 5: LinearityPlot (Concentration Vs Response) of Methylcobalamine**

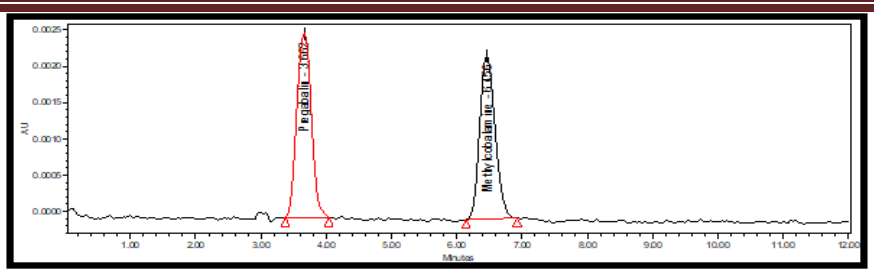


**Fig. 6: Chromatogram for Acid Degradation study**

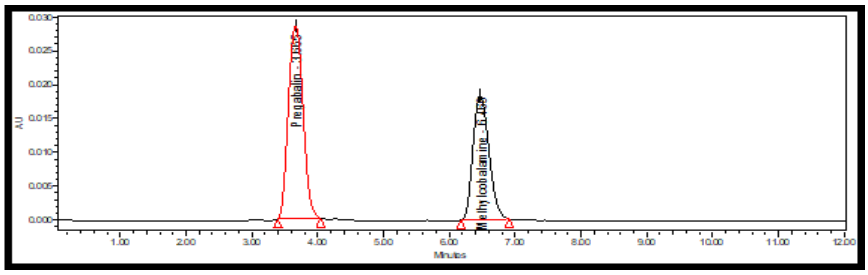


**Fig. 7: Chromatogram for Base Degradation study**

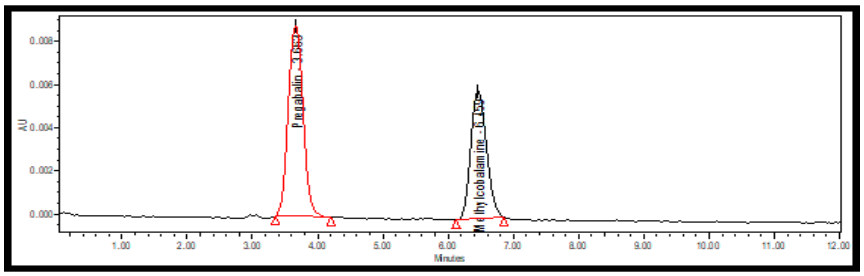


**Fig. 8: Chromatogram Oxidative Degradation study**

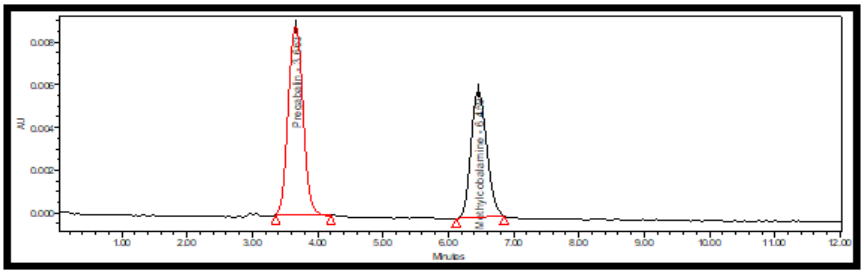


**Fig. 9: Chromatogram Thermal Degradation study**

**Table No. 1: Optimised Chromatographic conditions**

| Parameters | Method |
|---|---|
| **Stationary phase (column)** | Inertsil -ODS 3V $C_{18}$(250 x 4.6 mm, 5 µ) |
| **Mobile Phase** | Methanol : water (70:30 v/v) |
| **Flow rate** | 1.0 ml/min |
| **Run time (minutes)** | 8 min |
| **Column temperature (°C)** | Ambient |
| **Detector** | Photo Diode Array (PDA) |
| **Volume of injection loop** | 20 µl |
| **Detection wavelength (nm)** | 353 nm |
| **Drug Retention time(min)** | 3.661 min for Pregabalin and 6.453 for Methylcobalamine |

**Table No. 2: Linearity results of Pregabalin and Methylcobalamine**

| S.no | Concentration(ppm) | Areas for Pregabalin | Areas for Methylcobalamine |
|---|---|---|---|
| 1. | 0 | 0 | 0 |
| 2. | 20 | 706445 | 102056 |
| 3. | 30 | 1059486 | 153287 |
| 4. | 40 | 1412450 | 204256 |
| 5. | 50 | 1745687 | 248598 |
| 6. | 60 | 2119450 | 306482 |
| 7. | 70 | 2472451 | 357589 |
| 8. | 80 | 2825606 | 408513 |
| Correlation Coefficient | | 0.999 | 0.999 |

**Table No. 3: System Suitability results of Pregabalin and Methylcobalamine**

| S.no | Drug | Retention( | Mean area | USP Platecount | Tailing | Resolution |
|---|---|---|---|---|---|---|
| 1 | Pregabalin | 3.662 | 1412455 | 10023.845712 | 1.158 | 6.6 |
| 2 | Methylcobalamine | 6.455 | 204258 | 8325.875412 | 1.036 | |

**Table No. 4: Accuracy results of Pregabalin and Methylcobalamine**

| Drug | Spiked level (%) | % | % RSD |
|---|---|---|---|
| Pregabalin | 50 | 100.28 | 0.033 |
| | 100 | 100.23 | 0.083 |
| | 150 | 100.01 | 0.025 |
| Methylcobalamine | 50 | 100.88 | 0.043 |
| | 100 | 100.62 | 0.084 |
| | 150 | 100.46 | 0.050 |

**Table No. 5: System precision results**

| Concentration | Injection | Peak Areas of Pregabalin | Peak Areas of Methylcobalamine |
|---|---|---|---|
| 40ppm | 1 | 1413654 | 204511 |
| | 2 | 1415782 | 204687 |
| | 3 | 1412748 | 204015 |
| | 4 | 1412320 | 204194 |
| | 5 | 1414875 | 204906 |
| | 6 | 1415501 | 204735 |
| Statistical Analysis | Mean | 1414146 | 204508 |
| | SD | 1454.28 | 341.6513 |
| | % RSD | 0.102 | 0.167 |

**Table No. 6: Method precision results**

| Concentration | Injection | Peak Areas of Pregabalin | Peak Areas of Methylcobalamine |
|---|---|---|---|
| 40ppm | 1 | 1414856 | 204324 |
| | 2 | 1413894 | 204578 |
| | 3 | 1419876 | 204689 |
| | 4 | 1418700 | 204714 |
| | 5 | 1411845 | 204177 |
| | 6 | 1410780 | 204866 |
| Statistical Analysis | Mean | 1414991 | 204558 |
| | SD | 3646.13 | 259.491 |
| | % RSD | 0.257 | 0.126 |

**Table No. 7: Intermediate precision results**

| Concentration | Injection | Peak Areas of Pregabalin | Peak Areas of Methylcobalaine |
|---|---|---|---|
| 40ppm | 1 | 1410056 | 204148 |
| | 2 | 1405487 | 204025 |
| | 3 | 1418789 | 204298 |
| | 4 | 1414010 | 204151 |
| | 5 | 1416545 | 204286 |
| | 6 | 1415930 | 204568 |
| Statistical Analysis | Mean | 1413469 | 204246 |
| | SD | 4888.23 | 187.32 |
| | % RSD | 0.345 | 0.091 |

**Table No. 8: Robustness results**

| Parameter(flow rate) | Pregabalin (% RSD) | Methylcobalamine(% RSD) |
|---|---|---|
| 0.8 ml | 0.084 | 0.154 |
| 1.0ml | 0.186 | 0.163 |
| 1.2 ml | 0.139 | 0.013 |

**Table No. 9: LOD and LOQ results**

| DRUG | LOD(µg/ml) | LOQ(µg/ml) |
|---|---|---|
| Pregabalin | 0.23 | 0.72 |
| Methylcobalamine | 0.201 | 0.610 |

**Table No. 10: Forced degradation studies of Pregabalin**

| Mode of Degradation | Condition | Peak Area | % Degradation as compared with Control |
|---|---|---|---|
| Control sample | No treatment | 1402455 | - |
| Acid | 0.1 M HCl | 1361153 | 2.944 |
| Base | 0.1 M NaOH | 1401632 | 0.058 |
| Oxidative | 30% $H_2O_2$ | 1365421 | 2.640 |
| Thermal | 100°C | 218900 | 0.114 |

**Table No. 11: Forced degradation studies of Methylcobalamine**

| Mode of Degradation | Condition | Peak Area | % Degradationascompared with Control |
|---|---|---|---|
| Control sample | No treatment | 204258 | - |
| Acid | 0.1 M HCl | 196854 | **3.624** |
| Base | 0.1 M NaOH | 200564 | **1.808** |
| Oxidative | 30% $H_2O_2$ | 202153 | **1.031** |
| Thermal | 100°C | **201164** | **1.514** |

## CONCLUSION

Stability indicating RP-HPLC method was developed for simultaneous estimation of Pregabalin and Methylcobalamine in its bulk form. For routine analytical purpose, it is always necessary to establish methods capable of analyzing huge number of samples in a short period with accuracy and precision. The method was validated according to ICH guidelines. This developed and validated method for simultaneous analysis was found be very rapid, simple, accurate, precise and economical. Degradation studies were performed and no significant degradants were seen for oxidative, alkali, acid and thermal degradation. Finally, it can be concluded that this method can be employed for routine laboratory analysis.

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