METHOD DEVELOPMENT, VALIDATION AND STABILITY INDICATING ASSAY PROCEDURE OF PREGABALIN BY USING RP-HPLC

Jabeen*, Md. Akram, Abdul Sayeed
MESCO College of Pharmacy, Hyderabad (T.S)

Abstract:
A simple, rapid and precise Reverse phase liquid chromatographic method has been developed and subsequently validated for estimation of Pregabalin in tablet dosage form. The method is based on High Performance Liquid Chromatography (HPLC) on a reversed-phase column. The separation was carried out using a mobile phase consisting of Phosphate buffer of pH 6.2 and acetonitrile (70:30 v/v). The column used was reversed-phase, Develosil C18, (5µm, 150 x 4.6 mm i.d. column) with flow rate of 1.0 ml/min, column temperature at 40°C using UV detection at 225 nm. The drug was well resolved on the stationary phase and the retention times were around 2.33 minute. The method was validated and shown to be linear for Pregabalin. The correlation coefficient for Pregabalin is 0.998. The relative standard deviations for five replicate measurements in two sets of each drug in the tablets is always less than 2% and mean % error of active recovery not more than ±1.5%. Pregabalin was subjected to various degradation conditions like acidic, alkaline, thermal, oxidation, photo, UV degradation and neutral conditions for a period of 24 hrs and it was found to degrade to different extent in different conditions. Pregabalin was found to be highly degraded at oxidation and photo degradation and comparatively stable under neutral condition. The method was validated for precision and accuracy. The proposed method was successfully applied to the pharmaceutical dosage forms containing the above mentioned drug without any interference by the excipients.

Keywords: Pregabalin and RP-HPLC method.

Corresponding author:
Jabeen,
Assistant professor,
Mesco College of pharmacy,
Hyderabad.
Email : syed.jabeen2@gmail.com

Please cite this article in press Jabeen et al., Method Development, Validation and Stability Indicating Assay Procedure of Pregabalin by Using RP-HPLC, Indo Am. J. P. Sci, 2018; 05(03).
INTRODUCTION:
The term ‘Chromatography’ covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

Modes of Chromatography (1-16)
Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography).

Different modes of chromatography are as follows:
- Normal Phase Chromatography
- Reversed Phase Chromatography
- Reversed Phase – ion pair Chromatography
- Ion Chromatography
- Ion-Exchange Chromatography
- Affinity Chromatography
- Size Exclusion Chromatography

Reversed Phase Chromatography:
Since 1960’s chromatographers started modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed-phase chromatography.

A large number of chemically bonded stationary phases based on silica are available commercially. Silica based stationary phases are still most popular in reversed phase chromatography however other absorbents based on polymer (styrene-di-vinyl benzene copolymer) are slowly gaining ground.

The retention decreases in the following order: aliphatics > induced dipoles (i.e. CCl₄) > permanent dipoles (e.g.CHCl₃) > weak Lewis bases (ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases.

As a general rule the retention increases with increasing contact area between sample molecule and stationary phase i.e. with increasing number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

Adsorption Chromatography or Normal Phase Chromatography (17):
In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents. The silica structure is saturated with silanol groups at the end. These OH groups are statistically disturbed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase. This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present.
- Dipole-induced dipole
- Dipole-dipole
- Hydrogen bonding
- π-Complex bonding

Chromatographic methods can be classified most practically according to the stationary and mobile phases, as shown in the table:

<table>
<thead>
<tr>
<th>Stationary phase</th>
<th>Mobile phase</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid</td>
<td>Liquid</td>
<td>Adsorption column, thin-layer, ion exchange, High performance liquid chromatography.</td>
</tr>
<tr>
<td>Liquid</td>
<td>Liquid</td>
<td>Partition, column, thin-layer, HPLC, paper chromatography. Gas–Liquid Chromatography.</td>
</tr>
<tr>
<td>Liquid</td>
<td>Gas</td>
<td>Partition, column, thin-layer, HPLC, paper chromatography. Gas–Liquid Chromatography.</td>
</tr>
</tbody>
</table>

The modern form of column chromatography has been called high performance, high pressure, high-resolution and high-speed liquid chromatography.

High-Performance Liquid Chromatography (HPLC) is a special branch of column chromatography in which the mobile phase is forced through the column at high speed. As a result the analysis time is reduced by 1-2 orders of magnitude relative to classical column chromatography and the use of much smaller particles of the adsorbent or support becomes possible increasing the column efficiency substantially.

The systems used are often described as belonging to one of four mechanistic types, adsorption, partition, ion exchange and size-exclusion. Adsorption chromatography arises from interaction between solutes on the surface of the solid stationary phase.
Partition chromatography involves a liquid stationary phase, which is immiscible with the eluent and coated on an inert support. Adsorption and partition systems can be normal phase (stationary phase more polar than eluent) or reversed phase (stationary phase less polar than eluent). Ion-exchange chromatography involves a solid stationary phase with anionic or cationic groups on the surface to which solute molecules of opposite charge are attracted. Size-exclusion chromatography involves a solid stationary phase with controlled pore size. Solutes are separated according to their molecular size, the large molecules enable to enter the pores eluting first.

Method development by HPLC:
According to ICH guidelines this following procedure is applicable to the development of new analytical methods by HPLC.

Procedure:
Selection and optimization:
The primary objective in selection and optimization of mobile phase is to achieve optimum separation of all the individual impurities and degradants from active analyte peak. The selection of mobile phase is done always in combination with selection of Stationary phase (column).
The following are the parameters which shall be taken into consideration while selecting and optimizing the mobile phase:
1. Buffer and its strength (if any)
2. pH of the buffer or pH of the mobile phase
3. Mobile phase composition

Buffer and its strength (if any):
Buffer and its strength play an important role in deciding the peak symmetries and separations. The following are some of the most commonly used ones.
a. Phosphate buffer-KH₂PO₄, K₂HPO₄, NaH₂PO₄, Na₂HPO₄, NaH₂PO₄, H₃PO₄ etc.
b. Acetate buffer-Ammonium acetate, Sodium acetate etc.
c. Triethyl amine/Diethyl amine buffers
d. Buffers with various ion pair reagents like tetra butyl ammonium, Hydrogen sulphate, Butane sulphonic acid, Hexane sulphonic acid, Heptane sulphonic acid etc.,

It is important to use the buffers with suitable strength to cope up for the injection load on the columns; otherwise peak tailing may arise due to ionic changes during chromatography. The retentions times also depend on the molar strength of the buffer. Molar strength is inversely proportional to retention times.

Ideally the strength of the buffers shall be opted between 0.01M to 0.20M. The selection of buffer and its strength is done always in combination with selection of organic phase composition in mobile phase. The strength of the buffer can be increased if necessary to achieve the required separations. But it is to be ensured that the higher buffer strengths shall not result in precipitations or turbidities either in mobile phase or in standard and test solutions. Experiments shall be conducted using different buffers having different strengths to obtain the required separations. (9, 10)

pH of the buffer or pH of the Mobile Phase:
pH plays an important role in achieving the chromatographic separations as it controls the elution properties by controlling the ionization characteristics. Depending on the pKₐ of drug molecules retention time may vary.
Ex: Acids show an increase in retention time as the pH is reduced, while base show a decrease in retention time.

It is important to maintain the pH of the mobile phase in the range of 2.0 to 8.0 as most columns does not withstand to the pH which are outside this range. This is due to the fact that the siloxane linkages are cleaved below pH 2.0 while at pH values above 8.0 silica may dissolve. If a pH outside the range of 2.0 to 8.0 is found to be necessary packing materials (stationary phase) which can withstand to that ranges shall be chosen. E.g.; Waters Xterra, Merck Purospher.

pH of the buffer or pH of the mobile phase which gives separation of all individual impurities from each other and from active analyte peak shall be selected. Experiments shall be conducted using buffers or mobile phase having different pH to obtain the required separations.

Mobile Phase Composition:
In reverse phase chromatography, the separation is mainly controlled by hydrophobic interactions between drug molecules and the alkyl chains on the column packing material. Most chromatographic separations can be achieved by choosing the optimum mobile phase composition. This is due to the fact that a fairly large amount of selectivity can be achieved by choosing the qualitative and quantitative composition of aqueous and organic portions.

Most widely used solvents in Liquid chromatography are methanol and acetonitrile. Tetra hydrofuran is also used but to a lesser extent. Experiments shall be
conducted with mobile phases having buffers with different pH and different organic phases to check for the best separation of impurities and degradants from each other and from active analyte peak.

Method Validation:
The simple meaning for method validation is methods which give reliable results and checking the reliability of the results in all aspects. Other definitions include “Establishing documented evidence that a system does what it purports to do. FDA defines validation as “the documented program providing high degree of assurance that specific process or equipment will consistently produce product, meeting predetermined specification and quality attributes.

The steps involved in method validation are:
- Method validation protocol definition
- Laboratory method validation
- Validated test method generation
- Validation report

For an efficient validation process, it is of utmost importance to specify the right validation parameters & acceptance criteria. The more parameters, the more time it will take to validate. The more stringent the specifications or acceptance limits, the more often the equipment has to be re-calibrated & probably also re-qualified, to meet the higher specifications at any one time. It is not always essential to validate every analytical performance parameter, but it is necessary to validate the minimum important parameters required.

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Analytical methods need to be validated or revalidated before their introduction into routine use; Whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix); and whenever the method is changed and the change is outside the original scope of the method. USP has published specific guidelines for method validation for compound evaluation.

Plan of Work
Objective:
According to the literature survey it was found that few analytical methods such as (HPLC, UV-Visible analysis and LC-MS) were reported for the estimation of Pregabalin. The objective of the proposed method is to develop simple and accurate methods for the determination of Pregabalin by RP-HPLC method in pharmaceutical dosage forms & its stability indicative studies.

The Plan of the Proposed Work Includes the Following Steps:-
1. To undertake solubility and analytical studies of Pregabalin and to develop initial U.V. and chromatographic conditions.
2. Setting up of initial UV and chromatographic conditions for the method development in pure and pharmaceutical dosage forms.
3. Optimization of initial chromatographic and spectrophotometric conditions.
4. Analytical method validation of the developed RP-HPLC method.
5. Quantitative determination of Pregabalin in pharmaceutical dosage form using the method developed and validated.

EXPERIMENTAL:
MATERIALS AND METHODS:
The following are the list of instruments/equipments, chemicals/reagents and standards to perform the HPLC Analysis of the drug Pregabalin.
1. A suitable HPLC having isocratic system equipped with manual injector with UV detector.
2. Analytical Balance, capable of measuring the 0.01mg.
3. Sonicator
4. Usual laboratory glass war HPLC grade water e of class-A
Chemicals and Reagents: HPLC grade water, Dipotassium hydrogen orthophosphate, Potassium dihydrogen orthophosphate and Ortho phosphoric acid were procured from Sd fine-Chem ltd; Mumbai. Methanol and Acetonitrile were procured from Loba Chem,Mumbai.

Procedure:
HPLC Instrumentation & Conditions:
The HPLC system employed was HITACHI L2130 with D Elite 2000 Software with Isocratic with UV-Visible Detector (L-2400).

Standard & sample preparation for UV-spectrophotometer analysis:
25 mg of Pregabalin standard was transferred into 25 ml volumetric flask, dissolved in mobile phase & make up to volume with mobile phase. Further dilution was done by transferring 0.1 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase. The standard & sample stock solutions were prepared separately by dissolving standard & sample in a
solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It scanned in the UV spectrum in the range of 200 to 400 nm. This has been performed to know the maxima of Pregabalin, so that the same wave number can be utilized in HPLC UV detector for estimating the Pregabalin. While scanning the Pregabalin solution we observed the maxima at 225 nm. The UV spectrum has been recorded on ELICO SL-159 make UV–Vis spectrophotometer model UV-2450. The scanned UV spectrum is shown in the results.

**Preparation of Buffer and Mobile Phase:**

i) Preparation of Phosphate buffer:
 Accurately weighed 136.09 gms of Potassium dihydrogen ortho phosphate was taken into a 1000 ml volumetric flask, dissolved and diluted to 1000 ml with HPLC water and the volume was adjusted to pH 6.2 with NaOH.

ii) Preparation of mobile phase: Accurately measured 700 ml (70%) of above buffer and 300 ml of Acetonitrile HPLC (30%) were mixed and degassed in an ultrasonic water bath for 5 minutes and then filtered through 0.45 μ filter under vacuum filtration. The Mobile phase was used as Diluent.

- Optimized Chromatographic Conditions:
  - **Column**: C₁₈ Develosil ODS HG-5 RP 150mm x 4.6mm 5μm particle size
  - **Mobile Phase**: ACN: phosphate buffer (3:7)
  - **Flow Rate**: 1.0 ml/minute
  - **Wave length**: 225 nm
  - **Injection volume**: 20 μl
  - **Run time**: 10 minutes
  - **Column temperature**: Ambient
  - **Sampler cooler**: Ambient

- **Optimization of Column**: The method was performed with various columns like Develosil ODS HG-5 RP C₁₈, 5μm, 15 cm x 4.6 mm i.d. was found to be ideal as it gave a good peak shape and resolution at 1.0 ml/min flow.

**Preparation of Solutions:**

Preparation of Standard solution:
 Working concentration should be around 10 µg/ml. Accurately weighed around 25 mg of Pregabalin working standard, taken into a 25 ml volumetric flask, then dissolved in mobile phase and diluted to volume with the mobile phase to obtain a solution having a known concentration of about 1000 mcg/ml. Further dilutions has been made to get the final concentration of 10 µg/ml

Preparation of Test solution:
 Diluted quantitatively an accurately measured volume of label claim solution with diluents to obtain a solution containing about a linear range.

**Preparation of Buffer and Mobile Phase:**

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- The mobile phase used in this analysis consists of a mixture of Acetonitrile & Phosphate buffer (pH=6.2) in ratio 30:70

- **MOBILE PHASE OPTIMIZATION**: Initially the mobile phase tried was Phosphate buffer: Acetonitrile and Triethyl amine buffer: acetonitrile with various combinations of pH as well as varying proportions. Finally, the mobile phase was optimized with Acetonitrile and Phosphate buffer (pH 6.2), in proportion 30:70 respectively.

- **OPTIMIZATION OF COLUMN**: The method was performed with various columns like Develosil ODS HG-5 RP C₁₈, 5μm, 15 cm x 4.6 mm i.d. was found to be ideal as it gave a good peak shape and resolution at 1.0 ml/min flow.

**Assay Procedure:**

A solution of 20 µL standard, sample separately were injected into the chromatographic system and areas for pregabalin peak was measured and the percentage assay calculated by using the formulae. Recorded the chromatogram and measured the peak responses. Calculated the mean and percentage RSD for the same.

\[
\text{Assay} \% = \frac{\text{AT} \times \text{WS} \times \text{P} \times \text{Avg W}}{\text{AS} \times \text{DS} \times 100} \times \text{Label claim}
\]

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 of drug mg/ml

**Forced Degradation Studies:**

Following protocol was strictly adhered to for forced degradation of Pregabalin Active Pharmaceutical Ingredient (API).

The API (Pregabalin) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. This is one type of accelerated stability studies that helps us determining the fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing.

The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation and oxidative degradation.

1. **ACID HYDROLYSIS:**

An accurately weighed 25 mg. of pure drug was transferred to a clean & dry 25 ml volumetric flask.
To which 0.1 N Hydrochloric acid was added & make up to the mark & kept for 24 hours and from that 4 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl (after all optimized conditions).

2. BASIC HYDROLYSIS:
An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 10 ml volumetric flask. To which 0.1 N Sodium hydroxide was added & make up to the mark & kept for 24 hrs. from that 4 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

3. THERMAL DEGRADATION:
An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 100 ml volumetric flask, make up to the mark with mobile phase & was maintained at 50°C for 24 hrs and then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

4. PHOTOLYTIC DEGRADATION:
Approximately 10 mg. of pure drug was taken in a clean & dry Petri dish. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 1 mg. of the UV exposed drug was transferred to a clean & dry 10 ml volumetric flask. First the UV exposed drug was dissolved in methanol & make up to the mark. Then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

5. OXIDATIVE DEGRADATION:
Accurately weighed 10 mg. of pure drug was taken in a clean & dry 100 ml volumetric flask and 30 ml. Hydrogen peroxide (3%) and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml. using water to prepare 100 ppm solution. The above sample was injected into the HPLC system.

METHOD VALIDATION
Accuracy: Recovery study:
To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of PREGABALIN were taken and added to the pre-analyzed formulation of concentration 10μg/ml. From that percentage recovery values were calculated. The results were shown in table-5.

Preparation of sample pregabalin (formulation) stock solution:
Accurately weight and transfer 91.3mg of pregabalin drug formulation into 25 ml clean dry volumetric flask and add about 20ml of diluent and sonicate to dissolve it completely and make up to the mark with the same solvent(1000ppm solution).

Pipette out about 0.2ml of the above solution into 10ml clean dry volumetric flask & make up to the mark with the same diluent (20ppm solution).

Preparation of Standard stock solution:
Accurately weigh and transfer 10 mg of Pregabalin working standard into a 10mL clean dry volumetric flask add about 7ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation Sample solutions:
Preparation of 80% solution (With respect to target Assay concentration):
Accurately pipette out 0.16 ml of pregabalin working standard into a 10mL clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 1.0 ml of Pregabalin the above stock solution into a10ml volumetric flask and add 1ml of sample pregabalin (formulation) up to the mark with diluents.

Preparation of 100% solution (With respect to target Assay concentration):
Accurately pipette out 0.2 ml of pregabalin working standard into a 10mL clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1.0 ml of Pregabalin the above stock solution into a10ml volumetric flask and add 1ml of sample pregabalin (formulation) up to the mark with diluents.

Preparation of 120% solution (With respect to target Assay concentration):
Accurately pipette out 0.24 ml of pregabalin working standard into a 10mL clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 1.0 ml of Pregabalin the above stock solution into a10ml volumetric flask and add 1ml of sample pregabalin (formulation) up to the mark with diluents.

Precision:
Repeatability:
Procedure:
The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of five replicates of a fixed amount of drug. Pregabalin (API) the percent relative standard deviations were calculated for Pregabalin is presented in the table. Accurately weigh and transfer 10 mg of Pregabalin working standard into a 10mL clean dry volumetric flask add about 10ml of Diluent and sonicate to dissolve it completely and make volume up to the mark with the
same solvent. Further pipette 0.1ml of Pregabalin the above stock solution into a 10 ml volumetric flask and dilute up to the mark with diluent. The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was calculated. Calculated the mean and percentage RSD for the same. These results are shown in table -6.

**Intra-assay & inter-assay:**

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Pregabalin revealed that the proposed method is precise. The results were shown in the table 7.

**Preparation of stock solution:**

Accurately weighed amount of 10mg pregabalin were taken to a 10 ml cleaned and dried volumetric flask. This was then diluted with 7 ml of diluent and sonicate. The volume was made to10ml with the same solvent. This was marked and labelled as Stock solution.

**Preparation of Level – I solution:**

0.1ml of Pregabalin stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

**Preparation of Level – II solution:**

0.2ml of Pregabalin stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluents

**Preparation of Level – III solution:**

0.3ml of Pregabalin stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent.

**Preparation of Level – IV solution:**

0.4ml of Pregabalin stock solution has taken in 10ml of volumetric flask dilute up to the mark with diluent. Chromatogram was shown in fig. 20-24.

The calibration curve showed good linearity in the range of 0-100 µg/ml, for Pregabalin (API) with correlation coefficient ($r^2$) of 0.998. A typical calibration curve has the regression equation of $y = 69655x + 12056$ for Pregabalin.

**Method Robustness:**

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2°C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-8, % RSD < 2%) the developed RP-HPLC method for the analysis of Pregabalin (API).

**LOD & LOQ:**

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.01 & 0.03 µg/ml respectively.

**System Suitability Parameter:**

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. These results are shown in table -4.

**RESULTS AND DISCUSSION:**

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Pregabalin, different chromatographic conditions were applied & the results observed are presented below.

Method Development:

1) Ultraviolet-Visible spectrum of pregabalin:

Scanned UV-Visible spectra of pregabalin in method development.
2) Different trails for chromatographic conditions:

**TRAIL: 1**
- **Column**: Develosil ODS HG-5 RP C<sub>18</sub>, 5µm, 15cmx4.6mm i.d.
- **Mobile phase**: methanol: water (80:20)
- **Flow rate**: 0.5 ml per min
- **Wavelength**: 230 nm
- **Temperature**: ambient.
- **Run time**: 10 min

**Observation:**
From the above chromatogram it was observed that the Pregabalin showed low response peak.
**TRAIL: 2**

Column: Develosil ODS HG-5 RP C\textsubscript{18}, 5µm, 15cmx4.6mm i.d.
Mobile phase: methanol: water (40:60)
Flow rate: 0.5 ml per min
Wavelength: 230 nm
Temperature: ambient.
Run time: 10 min

**Observation:**
From the above chromatogram it was observed that the Pregabalin showed very low response peak.

**TRAIL: 3**

Column: Develosil ODS HG-5 RP C\textsubscript{18}, 5µm, 15cmx4.6mm i.d.
Mobile phase: acetonitrile: water (5:5)
Flow rate: 1.0 ml per min
Wavelength: 225 nm
Temperature: ambient.
Run time: 10 min

**Observation:**
From the above chromatogram it was observed that the Pregabalin showed tailing peak.
**Observation:**

From the above chromatogram it was observed that the Pregabalin showed broad peak.

**Observation:**

From the above chromatogram it was observed that the Pregabalin peak shape was good and sharp.
Observation:
From the above chromatogram it was observed that there are no interferences.

Table 2: Different Trials for Chromatographic Conditions

<table>
<thead>
<tr>
<th>Column Used</th>
<th>Mobile Phase</th>
<th>Flow Rate</th>
<th>Wavelength</th>
<th>Observation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Develosil ODS HG-5 RPC18,5μm,15cmx4.6mm i.d.</td>
<td>Methanol:Water(80: 20)</td>
<td>0.5 ml/min</td>
<td>230nm</td>
<td>Low response</td>
<td>Method rejected</td>
</tr>
<tr>
<td>Develosil ODS HG-5 RPC18,5μm,15cmx4.6mm i.d.</td>
<td>Methanol:Water(40 :60)</td>
<td>0.5 ml/min</td>
<td>230nm</td>
<td>Very low response</td>
<td>Method rejected</td>
</tr>
<tr>
<td>Develosil ODS HG-5 RPC18,5μm,15cmx4.6mm i.d.</td>
<td>ACN: water(5:5)</td>
<td>1.0 ml/min</td>
<td>225nm</td>
<td>Tailing peak</td>
<td>Method rejected</td>
</tr>
<tr>
<td>Develosil ODS HG-5 RPC18,5μm,15cmx4.6mm i.d.</td>
<td>ACN: Phosphate buffer (5:5)</td>
<td>1.0 ml/min</td>
<td>225nm</td>
<td>Broad Peak</td>
<td>Method rejected</td>
</tr>
<tr>
<td>Develosil ODS HG-5 RPC18,5μm,15cmx4.6mm i.d.</td>
<td>ACN: phosphate buffer (3:7)</td>
<td>1.0 ml/min</td>
<td>225nm</td>
<td>Good sharp peak</td>
<td>Method accepted</td>
</tr>
</tbody>
</table>

FORCED DEGRADATION STUDIES:
ACID HYDROLYSIS:

Fig. 07: chromatogram for blank injection

Fig. 08: Chromatogram showing degradation for pregabalin in 0.1 N HCl
BASIC HYDROLYSIS:

Fig. 09: Chromatogram showing degradation related impurity in 0.1 N NaOH

THERMAL DEGRADATION:

Fig. 10: Chromatogram showing thermal degradation studies

PHOTOLYTIC DEGRADATION:

Fig. 11: Chromatogram showing photolytic degradation.
OXIDATIVE DEGRADATION:

![Chromatogram showing oxidative degradation.](image)

Table 3: Results of force degradation studies of Pregabalin API.

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Time</th>
<th>Assay of active substance</th>
<th>Assay of degraded products</th>
<th>Mass Balance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Hydrolysis (0.1 M HCl)</td>
<td>24Hrs.</td>
<td>2.19</td>
<td>97.34</td>
<td>99.53</td>
</tr>
<tr>
<td>Basic Hydrolysis (0.1 M NaOH)</td>
<td>24Hrs.</td>
<td>1.59</td>
<td>95.02</td>
<td>96.61</td>
</tr>
<tr>
<td>Thermal Degradation (50 °C)</td>
<td>24Hrs.</td>
<td>99.13</td>
<td>----------</td>
<td>97.39</td>
</tr>
<tr>
<td>UV (254nm)</td>
<td>24Hrs.</td>
<td>66.94</td>
<td>43.76</td>
<td>100.7</td>
</tr>
<tr>
<td>3 % Hydrogen peroxide</td>
<td>24Hrs.</td>
<td>69.41</td>
<td>30.14</td>
<td>99.55</td>
</tr>
</tbody>
</table>

Results of degradation studies:
The results of the stress studies indicated the specificity of the method that has been developed.

METHOD VALIDATION
SYSTEM SUITABILITY:

![Chromatogram showing system suitability testing of standard solution of pregabalin.](image)

Acceptance criteria:

Table 4: Data of System Suitability Parameter

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameter</th>
<th>Limit</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Resolution</td>
<td>Rs &gt; 2</td>
<td>9.15</td>
</tr>
<tr>
<td>2</td>
<td>Asymmetry</td>
<td>T ≤ 2</td>
<td>Pregabalin=0.12</td>
</tr>
<tr>
<td>3</td>
<td>Theoretical plate</td>
<td>N &gt; 2000</td>
<td>Pregabalin=3246</td>
</tr>
</tbody>
</table>

Peak results:

<table>
<thead>
<tr>
<th>Sr no.</th>
<th>Name</th>
<th>RT</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pregabalin</td>
<td>2.39</td>
<td>4949627</td>
</tr>
</tbody>
</table>
ACCURACY:

Fig. 14: Chromatogram showing Accuracy 80% of pregabalin.

Fig. 15: Showing Chromatogram showing Accuracy 100% of pregabalin.

Fig. 16: Showing Chromatogram showing Accuracy 120% of pregabalin.

Table 5: Accuracy Readings

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Concentration (µg/ml)</th>
<th>% Recovery of</th>
<th>Statistical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pure drug</td>
<td>Formulation</td>
<td>Pure drug</td>
</tr>
<tr>
<td>S1: 80 %</td>
<td>8</td>
<td>10</td>
<td>101.3</td>
</tr>
<tr>
<td>S2: 80 %</td>
<td>8</td>
<td>10</td>
<td>99.25</td>
</tr>
<tr>
<td>S3: 80 %</td>
<td>8</td>
<td>10</td>
<td>100.27</td>
</tr>
<tr>
<td>S4: 100 %</td>
<td>10</td>
<td>10</td>
<td>99.14</td>
</tr>
<tr>
<td>S5: 100 %</td>
<td>10</td>
<td>10</td>
<td>99.29</td>
</tr>
<tr>
<td>S6: 100 %</td>
<td>10</td>
<td>10</td>
<td>99.11</td>
</tr>
<tr>
<td>S7: 120 %</td>
<td>12</td>
<td>10</td>
<td>99.21</td>
</tr>
<tr>
<td>S8: 120 %</td>
<td>12</td>
<td>10</td>
<td>99.54</td>
</tr>
<tr>
<td>S9: 120 %</td>
<td>12</td>
<td>10</td>
<td>99.63</td>
</tr>
</tbody>
</table>

Acceptance criteria:
The percentage Recovery for each level should be between 98.0 to 102.0%.
**PRECISION:**

Repeatability: 1

![Chromatogram showing precision of standard injection-1 for pregabalin.](image1)

Fig. 17: Chromatogram showing precision of standard injection-1 for pregabalin.

Repeatability: 2

![Chromatogram showing precision of standard injection-2 for pregabalin.](image2)

Fig. 18: Chromatogram showing precision of standard injection-2 for pregabalin.

Repeatability: 3

![Chromatogram showing precision of standard injection-3 for pregabalin.](image3)

Fig. 19: Chromatogram showing precision of standard injection-3 for pregabalin.

Repeatability: 4

![Chromatogram showing precision of standard injection-4 for pregabalin.](image4)

Fig. 20: Chromatogram showing precision of standard injection-4 for pregabalin.
Repeatability: 5

![Chromatogram showing precision of standard injection-5 for pregabalin.]

Repeatability 6:

![Chromatogram showing precision of standard injection-6 for pregabalin.]

**Table 6: Precision Study**

<table>
<thead>
<tr>
<th>HPLC Injection Replicates of Pregabalin</th>
<th>Retention Time</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate – 1</td>
<td>2.33</td>
<td>1414087</td>
</tr>
<tr>
<td>Replicate – 2</td>
<td>2.33</td>
<td>1467177</td>
</tr>
<tr>
<td>Replicate – 3</td>
<td>2.35</td>
<td>1460891</td>
</tr>
<tr>
<td>Replicate – 4</td>
<td>2.34</td>
<td>1471809</td>
</tr>
<tr>
<td>Replicate – 5</td>
<td>2.33</td>
<td>1482648</td>
</tr>
<tr>
<td>Replicate – 6</td>
<td>2.33</td>
<td>1489902</td>
</tr>
<tr>
<td>Average</td>
<td>2.335</td>
<td>1464419</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.0083666</td>
<td>26794.24</td>
</tr>
<tr>
<td>% RSD</td>
<td>0.35831245</td>
<td>1.829684</td>
</tr>
</tbody>
</table>

**Observation:**
The percentage RSD for the standard solution is below 2, which is within the limits hence method is precise.

**Intra assay and inter assay:**

**Table 7: Results of intra-assay & inter-assay**

<table>
<thead>
<tr>
<th>Conc. Of Pregabalin (API) (µg/ml)</th>
<th>Observed Conc. Of Pregabalin (µg/ml) by the proposed method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-Day</td>
</tr>
<tr>
<td></td>
<td>Mean (n=6)</td>
</tr>
<tr>
<td>10</td>
<td>10.03</td>
</tr>
<tr>
<td>30</td>
<td>30.49</td>
</tr>
<tr>
<td>100</td>
<td>99.14</td>
</tr>
</tbody>
</table>
Observation:
The percentage RSD for the standard solution is below 2 percentage, which is within the limits hence method, is precise.

LINEARITY:
The linearity range was found to lie from 10ppm to 1000ppm of Pregabalin chromatograms are shown below.

Linearity 1:

![Fig. 23: Chromatogram showing linearity level-1 of Pregabalin.](image1)

Linearity 2:

![Fig. 24: Chromatogram showing linearity level-2 of Pregabalin.](image2)

Linearity 3:

![Fig. 25: Chromatogram showing linearity level-3 of Pregabalin.](image3)

Linearity 4:

![Fig. 26: Chromatogram showing linearity level-4 of Pregabalin.](image4)
Linearity 5:

Fig. 28: Chromatogram showing linearity level-5 of Pregabalin.

METHOD ROBURSTNESS:

Table 08: Result of method robustness test

<table>
<thead>
<tr>
<th>Change in parameter</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow (1.1 ml/min)</td>
<td>0.04</td>
</tr>
<tr>
<td>Flow (0.9 ml/min)</td>
<td>0.09</td>
</tr>
<tr>
<td>Temperature (27°C)</td>
<td>0.01</td>
</tr>
<tr>
<td>Temperature (23°C)</td>
<td>0.13</td>
</tr>
<tr>
<td>Wavelength of Detection (227 nm)</td>
<td>0.07</td>
</tr>
<tr>
<td>Wavelength of detection (223 nm)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

ESTIMATION OF PREGABALIN IN CAPSULE DOSAGE FORM:

Table 09: Recovery Data for estimation Pregabalin in gabafit

<table>
<thead>
<tr>
<th>Brand name of Capsules</th>
<th>Labelled amount of Drug (mg)</th>
<th>Mean (±SD) amount (mg) found by the proposed method (n=6)</th>
<th>Assay + % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabafit</td>
<td>75</td>
<td>75.10 (±0.498)</td>
<td>100.13(0.494)</td>
</tr>
</tbody>
</table>

Result: The amount of drugs in gabafit capsule was found to be 99.10 (±0.498) mg/tab for Pregabalin and 100.13 (±0.343) mg/tab for Pregabalin.

DISCUSSION:
To develop a precise, linear, specific & suitable stability indicating RP-HPLC 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 gradient elution.

In case of RP-HPLC various columns are available, but here Develosil C18, 5µm, 150 x 4.6 mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good.

Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1N HCl).

The drug was found to be highly soluble in methanol and slightly soluble in acetonitrile. Drug was soluble in water. Using these solvents with appropriate composition newer methods can be developed and validated.

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 HPLC work can be accomplished in the wavelength range of 220-280 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20 µl were found to be the best analysis.

The result shows the developed method is yet another suitable method for assay and which can help in the analysis of Pregabalin in different formulations.

CONCLUSION:
A sensitive & selective RP-HPLC method has been developed & validated for the analysis of pregabalin API.

Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Pregabalin in different formulations. The results obtained on the validation parameters met ICH requirements. It inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision. Thus the purpose of the present investigation was successfully achieved.

REFERENCES:
17. www.drugbank.com