"DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR STRESS DEGRADATION STUDIES OF PREGABALIN"

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BY

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UNDER THE GUIDANCE OF

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CERTIFICATE

This is to certify that the dissertation work entitled "Development and Validation of RP-HPLC Method for Stress Degradation Studies of Pregabalin" submitted by Miss. Palak P. Thakkar with Reg No. (12MPH309) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University and Emcure Pharmaceuticals Limited under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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DECLARATION

I declare that the thesis "Development and Validation of RP-HPLC Method for Stress Degradation Studies of Pregabalin" has been prepared by me under the guidance of Mr. Nrupesh R. Patel, Assistant Professor, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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LIST OF ABBREVIATIONS

RP-HPLC	Reverse Phase High Performance Liquid Chromatography
USFDA	United States Food and Drug Administration
SIAM	Stability Indicating Assay Methods
IP	Indian Pharmacopoeia
USP	United states Pharmacopoeia
UV-Vis	Ultraviolet/Visible
FT-IR	Fourier Transformed Infrared
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of Detection
LOQ	Limit of Quantitation
NaOH	Sodium Hydroxide
HCl	Hydrochloric acid
ACN	Acetonitrile
H_2O_2	Hydrogen Peroxide
IPA	Isopropyl Alcohol
API	Active Pharmaceutical Ingredient
AR	Analytical Reagent
pKa	Partition coefficient
USP	United States Pharmacopoeia
TLC	Thin Layer Chromatography
HPTLC	High Performance Thin Layer Chromatography
LC/MS	Liquid Chromatography/Mass Spectroscopy
Rt	Retention Time
S.D.	Standard Deviation
R.S.D	Relative Standard Deviation
Ref.No.	Reference number
g	gram

mg	Milligram
µg	Microgram
Min	Minute(s)
mL	Mililiter
µl	Microliter
°C	Degree centigrade
±	Plus or Minus
λ	Lambda
%	Percentage
µL	Microliter
Vol.	Volume
v/v	Volume/Volume
w/v	Weight / Volume
r^2	Correlation coefficient
DP	Degradation Product

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Abstract

A simple and selective stability indicating Reverse-Phase High Performance Liquid Chromatographic (RP-HPLC) method has been developed and validated for estimation of pregabalin. The separation of all degradation peaks was achieved on 250 mm × 4.6 mm, 5µ, Alltima, C_{18} column, using gradient elution of Phosphate buffer : Acetonitrile (96:4)(Mobile phase A) and Acetonitrile (Mobile phase B) at flow rate of 1.0 mL/min and UV detection at 210 nm with the total run time of 75 min. Retention time of pregabalin was found to be 9.9 min. Forced degradation of pregabalin was carried out under acidic, basic, oxidative, thermal and humidity conditions. It was found that pregabalin degrades under basic and oxidative conditions. The method was validated for system suitability, specificity, linearity, accuracy, precision, LOD, LOQ and robustness as per ICH guidelines. Compatibility study of pregabalin was carried out with lactose. Pregabalin was reacted with lactose to generate lactose conjugate. It was found that there is decrease in peak area of pregabalin and lactum conjugate elutes at 46.02 min which very well coincides with the related literature. The method was found to be specific enough to separate degradation products from main analytes. Therefore the developed RP-HPLC method can be used for the estimation of pregabalin.

1.1 INTRODUCTION TO PREGABALIN

Pregabalin is an antiepileptic drug approved for adjunctive therapy of partial seizures. It has also been approved for the treatment of pain in diabetic neuropathy or post-herpetic neuralgia, spinal chord injury, in treatment of anxiety disorders and fibromyalgia. It is more potent successor to very well known anticonvulsant drug gabapentin.

Pregabalin (S-[+]-3-isobutylgaba) was designed as a lipophilic GABA (γ-amino butyric acid) analog. It was substituted at the 3′-position to facilitate diffusion across the blood-brain barrier.

Pregabalin exists in isomeric forms but S-(+)-3-isobutylgaba is the pharmacologically active enantiomer. Although pregabalin structurally resembles to GABA, it is not active at GABA receptors. Furthermore, pregabalin does not show affinity for receptor sites or modify responses associated with the action of several drugs for treating seizures or pain, suggesting that its mechanism of action is novel. [1]

Mechanism of Action

The mode of action of pregabalin has not been fully clarified, but it interacts with the same binding site as gabapentin. It is an α_2-δ ligand that has analgesic, anticonvulsant, anxiolytic, and sleep-modulating activities. Its main site of action appears to be on the α_2-δ subunit of presynaptic, voltage-dependent calcium channels which are distributed throughout the peripheral and central nervous system.

Pregabalin produces an inhibitory action on neuronal excitability , particularly in areas of the central nervous system deep in synaptic connections such as the neocortex, amygdala, and hippocampus. Ectopic activity is reduced. Like gabapentin, pregabalin is inactive at $GABA_A$ and $GABA_B$ receptors, also it is not converted into GABA or a GABA antagonist.

Pregabalin binds potently to the α_2-δ subunit and alters calcium influx at nerve terminals, and, thus reduces the synaptic release of several neurotransmitters, including glutamate, noradrenaline, serotonin, dopamine, and substance P.

As compared to other drugs acting on L type of calcium channels, pregabalin has no effect on arterial blood pressure or cardiac function.[1,2]

Clinical use in management of seizures

People suffering from epilepsy are prone to periods of uncontrolled electrical activity in the brain. These periods of uncontrolled electrical activity may lead to seizures. Pregabalin helps to control this electrical activity in the brain. This decreases the chances of having seizures. Pregabalin is taken as an add-on therapy with other drugs to treat epilepsy.[3]

Clinical use in management of diabetic neuropathic pain

People with diabetes have high blood sugar levels. High blood sugar over time or fluctuations in blood sugar damages nerves. This damage causes a different type of pain called diabetic nerve pain. Pregabalin works on these damaged nerves. It inhibits the release of neurotransmitters such as substance P and glutamate that are related with pain sensation.

Clinical studies of pregabalin with placebo showed that 3 times daily dosing resulted in a significant reduction in neuropathic pain. Generally central neuropathic pain is treated with antidepressants, anticonvulsants, opiods and cannabinoids but this medications may produce wide range of side effects. In 2011, the American Academy of Neurology (AAN) published a guideline for treating diabetic nerve pain. According to its evaluation, pregabalin was found to be more safer and effective in management of diabetic nerve pain.[4]

Clinical use in management of fibromyalgia

Fibromyalgia is a chronic disorder which leads to extensive musculoskeletal pain and tenderness. Fibromyalgia pain is originated by nerve-related changes, which cause nerve cells to fire off too many signals. This leaves a person sensitive to stimulus that are normally not painful.

Research shows that pregabalin decreases the number of nerve signals, and thus calms down over sensitive nerve cells. Studies shows that pregabalin improves symptoms such as disturbed sleep and fatigue.

Pregabalin has also been approved for generalized anxiety disorder.[5]

1.2 INTRODUCTION TO DISEASE

Epilepsy

Epilepsies are a group of disorders of central nervous system , characterized by paroxysmal cerebral dysrythmia, appearing as brief episodes (seizures) of loss or disturbance of consciousness with or without characteristic body movements (convulsions). Epilepsy has a focal origin in the brain, phenomenon depend on the site of the focus and regions into which the discharges spread.

Two major types of epilepsies are :

I. Generalised seizures

1. Generalised tonic-clonic seizures (grand mal epilepsy)

It is most common type and it lasts for about 1-2 min. The usual cycle is aura cry unconsciousness- clonic- spasm of all body muscles-conic jerking followed by prolonged sleep and depression of all CNS functions.

2. Absence seizures (minor epilepsy, petit mal)

These seizures are most prevelant in children. It lasts for about 1/2 min. There occurs momentary loss of consciousness. Patient apparently stares in one direction, no muscular movement or little bilateral jerking occurs.

3. Atonic seizures (Akinetic epilepsy)

It is characterized by unconsciousness with relaxation of all muscles due to excessive inhibitory discharges. Patient may fall.

4. Myoclonic seizures

There occurs shock-like momentary contraction of muscles of a limb or the whole body.

5. Infantile spasms (Hypsarrhythmia)

These are mostly seen in infants. Probably not a form of epilepsy, characterized by intermittent muscle spasm and progressive mental deterioration.

II. Partial seizures

1. Simple partial seizures

It lasts for 1/2-1 min. It is often secondary. Convulsions are restricted to a group of muscles or localized sensory disturbance depending on the area of cortex involved in the seizure, without loss of consciousness.

2. Complex partial seizures

Characterised by attacks of bizarre and confused behavior, emotional changes lasting 1-2 min along with loss of consciousness.

3. Simple partial or complex partial seizures

The partial seizure occurs first and evolves into generalized tonic-clonic seizures with loss of consciousness. Most of the cases are primary (idiopathic), some may be secondary to trauma/ surgery on head, intracranial tumour, tuberculoma, cysticercosis, cerebral ischaemia, etc.

Treatment is symptomatic and the same whether epilepsy is primary or secondary. [6]

1.3 BRIEF CLASSIFICATION OF ANTIEPILEPTIC DRUGS[6]

1**. Barbiturate** - Phenobarbitone

2. **Deoxybarbiturate** - Primidone

3. **Hydantoin** - Phenytoin, Fosphenytoin

4. **lminostilbene** - Carbamazepine, Oxcarbamazepin

5. **Succinimide** - Ethosuximide

6. **Aliphatic carboxylic acid** - Valproic acid (sodium valproate), Divalproex

7. **Benzodiazepines** – Clonazepam, Diazepam, Lorazepam

8. **Phenyltriazine** - Lamotrigine

9. **Cyclic GABA analogue** - Gabapentin

10. **Newer drugs** - Vigabatrin, Topiramate. Tiagabine. Zonisamide, Levetiracetam

1.4 DRUG PROFILE

1. Drug class[6] : Antiepileptic, Antianalgesic

2. Category[7] : Anticonvulsant

3. CAS number[8] : 148553-50-8

4. Official status[7] : Drug is official in IP 2010.

5. Chemical name[7] : (S)-4-amino-3-(2-methylpropyl)butyric acid

6. Molecular formula[7] : $C_8H_{17}NO_2$

7. Molecular weight[7] : 159.2 g/mol

8. Structural formula :

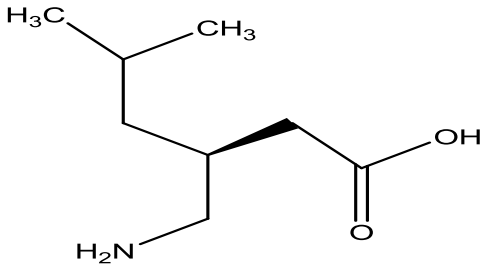

Figure 1.1 Structure of pregabalin

9. Physicochemical properties[7] :

a) Description : It is a white to off white powder.

b) Solubility : It is freely soluble in water and both basic and acidic aqueous solutions.

c) Melting point: 186 - 188°C

d) Optical rotation : +10.52°

e) Dissociation constant (pka) : 4.2,10.6

f) Partition coefficient (log p) : - 1.35 in pH 7.4

10. FDA approval[9] : 30th December 2004

11. Pharmacological action :

Pregabalin chemically resembles to gabapentin and both has same mechanism of action. Pregabalin binds with high affinity to the alpha 2 delta (α_2-δ) site which is a subunit of voltage-gated calcium channels in tissues of central nervous system. Pregabalin by binding to (α_2-δ) subunit , alters the function of calcium channels. This results in reduction in the release of several neurotransmitters, including glutamate, noradrenaline, serotonin, dopamine, and substance P. Research studies also suggest that the noradrenergic and serotonergic pathways originating from the brainstem may be involved with the mechanism of pregabalin. Although pregabalin is derived from inhibitory neurotransmitter gamma-aminobutyric acid (GABA), it does not bind directly to GABA or benzodiazepine receptors. It does not inhibit dopamine, serotonin, or noradrenaline reuptake. It also reduces the release of glutamate.[1,10]

12 . Metabolism[11] :

Pregabalin undergoes negligible metabolism in humans.

13. Dose[8] :

Adult: 150- 600 mg/day
Maximum dose : 600 mg/day

14. Pharmacokinetics[12]:

Pregabalin is absorbed rapidly after oral administration and peak plasma concentration is achieved within 1.5 hours. Oral bioavailability of pregabalin is about 90%. The rate but not the extent of absorption is reduced if given with food but this is not clinically significant. Steady state is achieved after 1 to 2 days. Pregabalin is not bound to plasma proteins and undergoes negligible metabolism. About 98% of dose is excreted in urine as unchanged drug.

15. Bioavailability[12] : Oral bioavailability is 90%

16. Half life[9] : 6.3 hrs

17. Volume of distribution[9] : 0.5 L/kg

18. Clearance[9] : 67.0 - 80.9 mL/min.

19. Protein binding[9] : Pregabalin does not bind to plasma protein.

20. Indication[12] : Pregabalin is used in treatment of

1) Partial Seizures
2) Diabetic nephropathy
3) Fibromyalgia
4) Spinal chord injury, Anxiety

21. Side effects[12] :

Dizziness, drowsiness, visual disturbance, ataxia, tremor, lethargy, euphoria, memory impairment, weight gain, dryness of mouth, constipation, depression, tachycardia, sweating, muscle cramp, flushing, hypotension.

22. Adverse effects[12] **:**

Congestive heart failure, reversible renal failure, rhabdomylosis, stevens- Johnson syndrome, haemangiosarcoma, hypersensitivity reactions including symptoms such as rash, blisters, urticaria.

23. Drug interaction[12]:

No major drug interactions are reported for pregabalin. When pregabalin is administered along with oxycodone, lorazepam, or ethanol, additive effects on cognitive and gross motor functioning were seen.

24. Storage[7] : Store protected from light and moisture.

25. Formulation[13] :

1) LYRICA – Pfizer Pharmaceuticals.
Capsules :75 mg, 150 mg

2) PREGASTAR – Lupin Pharmaceuticals, Inc
Capsules :75 mg, 150 mg

3) PREBEL – Johnlee Pharmaceuticals, Pvt. Ltd,
Capsules: 75 mg

4) PREGABA – Unichem laboratories Ltd.
Capsules : 50,75,150 mg

1.5 INTRODUCTION TO STABILITY INDICATING ASSAY METHODS (SIAMs)

Stability Indicating Assay Methods

The stability-indicating assay is a method that is used for the analysis of stability samples in pharmaceutical industry. With the advent of International Conference on Harmonization (ICH) guidelines, the establishment of a stability indicating method require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. Stability-indicating methods according to US-FDA stability guideline of 1987 were defined as the 'Quantitative analytical methods that are based on the structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient can be accurately measured.[14]

1.5.1 Types of stability indicating assay method

a) Specific Stability Indicating Assay Method

It can be defined as ‘A method that is able to measure unequivocally the drug in the presence of all degradation products, in the presence of excipients and additives expected to be present in the formulation.’

b) Selective Stability Indicating Assay Method

It can be defined as ‘A method that is able to measure unequivocally the drug and all degradation products in the presence of excipients and additives expected to be present in the formulation’.[15]

1.5.2 Development of validated stability indicating assay method

I Critical study of drug structure to assess the likely decomposition route(s)

There are defined functional group categories, like amides, esters, lactones, etc. that undergo hydrolysis, others like thiols, thioethers, etc. undergo oxidation, and compounds like olefins, aryl halo derivatives and those with aromatic nitro groups, N-oxides undergo photo decomposition.

II Collection of information on physicochemical parameters

It is important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug. The knowledge of pKa is important as most of the pH related changes occur at pH of the buffer to be used in the mobile phase. The knowledge of log P for the drug and the identified degradation products gives good information of the separation behavior likely to be obtained on a particular stationary phase.

III Preliminary separation studies on stressed samples

The stress samples obtained are subjected to primary analysis to study the number and types of degradation products formed during various conditions.

IV Final method development and optimization

To separate co-eluting peaks, the method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type

V Identification and characterization of degradation products and preparation of standards

Before proceeding to the validation of a SIAM, it is essential to identify the drug degradation products and arrange for their standards. These are required to determine specificity/selectivity of the method. Peak purity of the active substance is checked to confirm that the method is selective, and a single component peak is quantified.

Step VI: Validation of Stability Indicating Assay Methods

Validation of analytical methods, in general, has been widely covered in the ICH guidelines Q2A and Q2B, in the FDA guidance and by USP. [16-20]

Table 1.1: General protocol for stress degradation studies (API/DP)[14]

	Drug Substance		Drug Product	
Conditions	**Solid**	**Solution/ Suspension**	**Solid(Tablets, Capsules,Blends)**	**Solution (IV,Oral Suspension)**
Acid/Base	-	✓	-	×
Oxidative	×	✓	✓	✓
Photostability	✓	×	✓	✓
Thermal	✓	-	✓	✓
Thermal/ Humidity	✓	-	✓	-
✓ =Recommended; ×=Optional,suggested for some compounds				

1.5.3 Various regulatory guidelines available to carry out SIAM

Although FDA guidelines and ICH guidelines provide useful definitions of forced degradation studies, their directions regarding the extent, timing and best practice is very general and lacking in details.

Table1.2: ICH guidelines[21]

Guidelines	Reference Title
Q1A (R2)	Stability testing of new DS and DP
Q1B	Photo stability of new DS and DP
Q1E	Evaluation of stability data
Q2 (R1)	Validation of analytical procedures
Q3A (R2)	Impurities in new DS
Q3B (R2)	Impurities in new DP
Q5C	Stability testing of biotech/biological products
Q6A	New DP/DS (to determine content of DS/DP)

Table 1.3: Selection of FDA and EMEA guidelines and pharmacopeia chapter referencing SIAM [21]

Guidelines	Reference Title
USP <1086>	Impurities in official articles.
USP <11>	USP reference standard.
USP <1150>	Pharmaceutical stability.
USP <1191>	Stability consideration in dispensing practice.
USP <797>	Pharmaceutical compounding of sterile products, storage and beyond use dating.
EMEA March 2001	Note for guidance on in use stability testing of human medicinal products.
EMEA Dec 2004	Guidance on chemistry of new API.
FDA	Guidance for industry analytical procedures and method validation.

1.6 ANALYTICAL METHOD VALIDATION

Validation is a process of establishing documented evidence, providing a high degree of assurance that a specified activity will consistently produce a desired result or product meeting its predetermined specifications and quality attributes.

Method validation is the process of demonstrating that analytical procedures are appropriate for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of ascertainig that an analytical method is suitable for its intended purpose.

1.6.1 Objective of validation

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Validation is documented evidence, which provide a high degree of assurance for specific method. Any developed method may be influenced by variables like different assay times, different days, reagents. instruments, equipments, environmental conditions like temperature, etc. Therefore it is expected that after the method has been developed and before it is transferred from one lab to the other, it is properly validated.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (US FDA), American Association of Official Analytical Chemists (AOAC) United States Pharmacopoeia (USP), and International Union of Pure and Applied Chemists (IUPAC) provide a framework for performing such validations in a more efficient manner.

Method validation is required when

1. A new method is being developed.
2. There is need to revise an established method.
3. When established methods are used in different laboratories and with different analysts.
4. There is need to compare two methods.
5. When quality control requires changes in the method.[22]

The different parameters of analytical method development are discussed below as per ICH guidelines.[19,23,24]

1) Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Method

- When the impurities are available: Spiking of pure substance (drug substance or drug product) with appropriate levels of impurities/excipients demonstrate the result is unaffected.
- When the impurities are not available: Comparing the test results of sample containing impurities or degradation product to second well-characterized procedure. These comparisons should include sample under relevant stress condition.
- In chromatographic method: Peak purity test to be done by diode array and mass spectrometry.

Expression/calculation

- Proof of discrimination of analyte in the presence of impurities. E.g. chromatogram should be submitted.
- Peak purity test helps in demonstrating that the peak is not attributable to more than one component.
- For assay two results should be compared and for impurity tests two profiles should be compared.

Acceptance criteria: No interference peaks from blank or placebo at the R.T. of the drug should be observed

2) Linearity

The linearity of an analytical method is its ability (within given range) to obtain test results, which are directly proportional to the concentration of analyte in the sample.

Method

Drug with different dilutions or separately weighed synthetic mixture is to be taken. Response and plot response vs. concentration of analyte is to be measured. Demonstration of linearity should be done by,

• Visual inspection of plot

• Appropriate statistical methods

Recommendation

• Minimum of 5 concentrations are recommended.

Expression/calculation

• Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

Acceptance criteria: Correlation coefficient should not be less than 0.999.

3) Range

The range of analytical procedure is the interval between the uppermost and lowermost concentration of analyte in the sample (including these concentrations) for which it has been confirmed that the analytical procedure has a suitable level of precision, accuracy and linearity.

Method

Drug with different dilutions or separately weighed synthetic mixture is to be taken. Response and plot response vs. concentration of analyte is to be measured. Demonstration of linearity is done by,

• Visual inspection of plot

• Appropriate statistical methods

Recommendation

• Assay of drug/finished product: 80 – 120% of test concentration.

• For content uniformity: 70 – 130% of test concentration.

• For dissolution testing: ± 20% over specified range.

• For impurity: from reporting level to 120% of specification.

Expression/calculation

• Correlation coefficient, y-intercept, slope of regression, residual sum of squares.

4) Accuracy

The accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Method

• A sample of known concentration is analyzed and the measured value is to be compared with the 'true' value. However, a well characterized sample (e.g., reference standard) must be used.

• Spiked-placebo recovery method - In the spiked-placebo recovery method, a known amount of pure active constituent is to be added to formulation blank [sample that contains all other ingredients except the active], the resulting mixture is assayed, and the results obtained are to be compared with the expected result.

• Standard addition method - In the standard addition method, a sample is assayed, a known amount of pure active constituent is added, and the sample is again assayed. The difference between the results of the two assays is to be compared with the expected results.

• In both methods (spiked-placebo recovery and standard addition method), recovery is measured as the ratio of the observed result to the expected result expressed as a percentage.

Recommendation

Minimum of nine determinations

• Low concentration of range × 3 replicates

• Medium concentration of range × 3 replicates

• High concentration of range × 3 replicates

Expression/calculation

• Percent recovery by the assay of known added amount of analyte.

• Mean – Accepted true value with confidence interval.

Acceptance criteria: % Recovery should be in the range of 98-102%.

5) Precision

The precision of an analytical procedure expresses the closeness of agreement between the series of measurements obtained from multiple sampling of the same homogeneous sample under the stated conditions.

Method

• % Relative standard deviation (RSD) of response of multiple aliquots is to be determined.

Recommendation

a) Repeatability (Same operating condition over short interval of time)

Minimum of nine determinations

• Low concentration of range × 3 replicates

• Medium concentration of range × 3 replicates

• High concentration of range × 3 replicates

Or At target concentration × 6 determinations

b) Intermediate precision (within laboratory variation)

• Different Days

• Different Analysts

• Different Equipment etc.

Expression/calculation

• Standard deviation % RSD and confidence interval.

Acceptance criteria: % Relative standard deviation should be less than 2.

6) Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under finalised experimental conditions.

Method

1. By visual evaluation.

2. Based on S/N ratio

• Applicable to procedure, which exhibit baseline noise.

• Actual lowest concentration of analyte detected is to be compared with blank response.

3. Based on S.D. of response and slope.

LOD = 3.3 σ/s

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

• Standard deviation of blank response.

• Residual standard deviation of the regression line.

• Standard deviation of the y-intercept of the regression line.

Expression/calculation

• If based on visual examination or S/N ratio – relevant chromatogram is to be presented.

• If by calculation/extrapolation – estimate is validated by analysis of suitable no. of samples known to be near or prepared at detection limit.

Acceptance criteria

• S/N ratio > 3 or 2:1; not specified in other cases.

7) Quantitation limit

The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Method

1. By visual evaluation

2. Based on S/N ratio

• Applicable to procedure, which exhibit baseline noise.

• Actual lowest concentration of analyte detected is to be compared with blank response.

3. Based on S.D. of response and slope.

LOQ = 10 σ/s

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

• Standard deviation of blank response.

• Residual standard deviation of the regression line.

• Standard deviation of the y-intercept of the regression line.

Recommendation

• Limit should be validated by analysis of suitable no. of samples known to be near or prepared at quantitation limit.

Expression/calculation

• Limits of quantitation and method used for determining should be presented.

Acceptance criteria

• S/N ratio > 10:1; not specified in other cases.

8) 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.

Method

It should show the reliability of an analysis with respect to deliberate variations in method parameters. In case of liquid chromatography, examples of typical variations are

• Influence of variations of pH in a mobile phase.

• Influence of variations in mobile phase composition.

Different columns (different lots or suppliers).

• Temperature.

• Flow rate.

Recommendation

• Robustness should be considered early in the development of a method.

- If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement should be included in the method documentation.

Expression/calculation

- Effect of changed parameters on system suitability parameters.

Acceptance criteria

- The method must be robust enough to withstand slight changes and should allow routine analysis of sample.

9) Solution stability

Standard and sample are to be prepared as per test procedure and initial assay value is determined. Standard and sample preparation are stored up to 48 hours at room temperature. Determine the assay of sample preparation at an interval of 24 hours and 48 hours storage against freshly prepared standard and determine % response of standard preparation against initial standard response. The assay value of sample and % response of standard calculated after 24 hours and 48 hours should be matched against initial value and recorded.

If the stability of solution fails at 24 hour interval at room temperature, repeat the experiment allowing to stand solutions for 2, 4, 8, 12, and 18 hours at room temperature. If the stability of solution is found to be less than 24 hours at room temperature, then solution stability is to be established at 5°C±3°C as per the above procedure.

Calculation: Calculate results as follows:

Standard preparation stability: Calculate the % response of the standard preparation after specified period using the formula :

% Response =TA / SA × 100

Where,

TA = The peak area of standard preparation after standing for specified period.

SA = The initial peak area of standard preparation subjected for solution stability.

Sample preparation stability:

Calculate the % assay of the sample preparation after specified period as per the test procedure against freshly prepared standard. Calculate the difference of the result obtained after each interval against initial result.

Acceptance criteria

- The difference in the response of standard preparation should not be more than ± 2.0% from the initial value at any time interval.
- The absolute difference in the assay value of sample should not be more than ± 2.0% from the initial value at each time point.

11) System suitability testing

System suitability testing is an integral part of many analytical procedures. The tests are based on the theory that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.

Parameters such as plate count, tailing factors, resolution and reproducibility are determined and compared against the specifications set for the method.

Table 1.4: System suitability parameters and their recommended limits

PARAMETER	RECOMMENDATION
Capacity factor(k′)	The peak should be well-resolved from other peaks and the void volume, generally k′ > 2
Repeatability	RSD ≤ 2% (N ≥ 5 is desirable)
Relative retention	Not essential as the resolution is stated.
Resolution(Rs)	Rs of > 2 between the peak of interest and the closest eluting potential interfrent (impurity, excipients, degradation product, internal standard, etc.)
Tailing factor(T)	T ≤ 2
Theoretical plates(n)	generally should be > 2500

2.1. TITRIMETRIC AND COLORIMETRIC METHODS :

Sr No.	Matrix	Solvent/ Reagent	Method	Description	Ref
1	Capsule	Sanger's reagent	Complex formation	λmax - 365 nm Linearity – 2-18 µg/mL	[25]
2	Capsule Urine	2,4-dinitrofluoro-benzene,2,3,5,6-tetrachloro-1,4-benzoquinone	Colored complex formation	Linearity - 1-7 µg/mL	[26]
3	Bulk Capsule	Ninhydrin in phosphate buffer pH 7.4	Blue violet colored complex formation	Linearity - 50-1000 µg/mL	[27]
4	Bulk Capsule	-	Reaction with 7-chloro-4 nitrobenzofurazon	λmax - 460 nm Linearity - 0.5–7.0 µg/ mL	[28]
5	Bulk Capsule Urine	Distilled water	Single wavelength	λmax - 210 nm Linearity - 0.5–5.0 µg/mL	[29]
6	Bulk Capsule Urine	Distilled water	Reaction of drug with the mixture of potassium iodate and potassium iodide.	λmax - 353 nm Linearity - 0.5–3.5 µg/mL	[30]
7	Bulk Capsule	2,3 dichloro-5, 6-dicyano-1,4 benzquinone	Red colour charge–transfer complex	λmax - 465 nm	[31]

8	Bulk Capsule	1,2 napthaquinone-4- sulfonic acid sodium 2,4-dinitrophenyl-hydrazine	Orange colored Product forms Colored complex forms	λmax - 485 nm Linearity - 5- 45 µg/mL λmax - 461 nm Linearity - 50-450 µg/mL	[32]
9	Bulk Capsule	Benzoyl chloride	Zero order	λmax - 223 nm Linearity – 2.5 - 12.5 µg/mL	[33]
10	Bulk Capsule	2,6 dichloroquinone chlorimide 3-methyl-2-benzthiazolinone hydrazone (MBTH)	Colored oxidative coupling product Green colored product	λmax - 400 nm Linearity - 50-350 µg/mL λmax - 668 nm Linearity - 50-350 µg/mL	[34]
11	Bulk Capsule	-	Single wavelength	λmax - 210 nm Linearity - 6-14 µg/mL	[35]
12	Bulk Capsule	*p*-dimethylamino-benzaldehyde (*p*DMAB) in acid medium.	Single wavelength	λmax - 420 nm Linearity - 40-120 µg /mL	[36]

2.2. HPLC METHODS :

Sr No.	Other analyte	Matrix	Column	Mobile Phase	Description	Ref
1	Methyl-cobalamine	Capsule	C_{18} (250mm x 4.6mm, 5 µm)	Ammonium dihydrogen-o-phosphate (buffer 6.0): Acetonitrile: Methanol (75:15:10,v/v/v)	λmax - 210 nm Linearity- 3200-4800 µg/mL	[37]
2	Methyl-cobalamine	Capsule	Phenomenex C_{18} (250mm × 4.6mm, 5 µm)	Water: Methanol (60:40 v/v) adjusted to pH 6.5 with triethylamine (1%v/v) Flow rate - 1 mL/min	λmax - 218 nm Linearity- 50-300 µg/mL	[38]
3	-	Bulk Capsule	Kromasil C_{18} (100mm × 4.6mm, 5 µm)	Phosphate buffer pH 6.9: Acetonitrile (90:10,v/v) Flow rate - 1 mL/min	-	[39]
4	Methyl-cobalamine, Alpha lipoic acid	Capsule	C_{18} (250mm × 4.6mm, 5 µm)	Potassium dihydrogen orthophosphate buffer (20mM), pH-6: Methanol:	λmax - 210 nm Rt- 2.6 min Linearity- 187.5 - 750	[40]

				Acetonitrile (75:10:15,v/v/v) Flow rate - 1.2 mL/min	µg/mL	
5	-	Bulk Capsule	Spherisorb ODS (24.6mm x 250mm, 5µm)	Acetonitrile: Buffer (30:70,v/v) Flow rate - 1.0 mL/min	λmax - 210 Linearity- 200-800 µg/mL	[41]
6	Methyl-cobalamine	Bulk Capsule	C_{18} (250mm × 4.6mm, 5 µm)	Acetonitrile: Methanol: Ammonium acetate buffer (30:60:10,v/v/v), pH 4.5 Flow rate - 1.0 mL/min	λmax - 234 Rt- 2.1 Linearity- 5 - 50 µg /mL	[42]
7	-	Tablet	Phenomenex C_{18} ODS 2 (150mm × 4.6mm, 5 µm)	Methanol: 10mM Ammonium Acetate pH 3.0 (50:50,v/v) Flow rate - 0.7 mL/min	λmax - 210 Rt - 3.39 ± 0.10 min	[43]
8	Gabapentin Vigabatrin	Human serum	Alltima 3 C_{18}	-	Derivatising agent- *o* pthaldehyde 3-mercaptopropionic acid	[44]

9	-	Capsule	Hypersil BDS, C_8, (150mm x 4.6mm, 5 µm)	Phosphate buffer pH 6.9: Acetonitrile (95:05,v/v) Flow rate - 1 mL/min	λmax – 200 Linearity- 0.5 - 1.5 mg/mL	[45]
10	-	Bulk Capsule	Inertsil ODS-3V C_{18} (250mm x 4.6mm, 5 µm)	Buffer, Acetonitrile and Methanol in gradient elution Flow rate- 0.8 mL/min	λmax – 210 Rt - 6.5 min Linearity- 18.75 - 150 µg/mL	[46]
11	-	Bulk	Phenomenex C_{18} (250mm x 4.6 mm, 5 µm)	Acetonitrile: Phosphate buffer pH 3.5 (60:40,v/v) Flow rate - 0.5 mL/min	λmax -210 Rt - 2.5 min	[47]
12	-	Bulk	Inertsil ODS (250mm x 4.6 mm, 2.5 µm)	Phosphoric acid buffer: Acetonitrile (55:45,v/v) Flow rate - 1 mL/min	λmax - 340 nm Derivatising agent - Na-5-fluoro-2,4 dinitro-phenyl-5-L-alanine amide	[48]
13	-	Bulk Urine	C_{18} ODS hypersil (250mm x	Methanol: Acetonitrile: 0.02 M	λmax – 210 Linearity- 0.75 - 6.00	[49]

			4.6mm, 5 µm)	di-potassium hydrogen orthophosphate (K_2HPO_4),pH 7 (3: 1: 16,v/v/v)	µg/mL	

2.3. TLC AND HPTLC METHODS :

Sr No.	Other Analyte	Matrix	TLC Plate and Solvent System	Quantitative Analysis	Ref
1	Gabapentin	Capsule	Silica gel G60 F_{254} aluminum sheet (10 x 10 cm) Solvent - Ethyl Acetate: Methanol: Ammonia (6.0:4.0: 0.1,v/v/v)	Linearity - 2-12 ng / mL LOD – 0.4609	[50]
2	Methyl-cobalamine	Capsule	Silica gel G60 F_{254} Mobile Phase - Methanol: Toluene: Ammonia(30%) (8:2:0.4 ,v/v/v)	λmax - 497 Retention factor - 0.60 Linearity - 1500 - 7500 ng/band	[51]

2.4. LC- MS METHODS :

Sr No.	Matrix	Column and Solvent System	Quantitative Measurement	Ref
1	Human plasma	Column - C_{18} (50mm x 4.6mm, 5 µm) Mobile phase- Buffer: Methanol (20:80,v/v)	Linearity - 250 - 20000 ng/mL	[52]
2	Human plasma	Column - Kromasil 100 C_{18} (3.5 µm) Mobile Phase - Acetonitrile-0.5% formic acid (80:20)	Linearity - 50 - 8003.55 ng/mL	[53]

2.5. MISCELLANEOUS METHODS :

Sr No.	Method	Other Analyte	Matrix	Description	Ref
1	Ultra Performance Liquid Chromatography	Gabapentin Vigabatrin	Tablets Plasma	Column - Kinetex RP_{18} Mobile phase - 2 mM ammonium acetate supplemented with 0.1% formic acid in water and acetonitrile, LOD - 1.4×10^{-8} M Linearity - 0.03–25 mg/L	[54]
2	Gas Chromatography	Atorvastatin	Tablet	Capillary - Rtx 5 capillary with a dimension of 30 m x 0.25 mm Linearity 2-10 µg /mL	[55]

				Detector - FID Carrier gas pressure - 83.7 Kpa for 3.5 min Derivatising Agent -Ethyl Chloroformate	

2.6. OFFICIAL HPLC METHOD :

Sr No	Matrix	Column	Mobile phase	Description	Ref
1	Pregabalin API	Stainless steel column (25 ×4.6 mm, 5 µm)	Water : ACN (95:5)	λmax - 205 nm Flow rate - 1ml/min Detector - UV detector, Injection volume : 20µl	7
2	Pregabalin Capsule	Stainless steel column (25 ×4.6 mm, 5 µm)	Phosphate buffer(pH6.0): methanol: acetonitrile (92:05:03 %v/v/v)	λmax - 205 nm Flow rate - 1ml/min Detector - UV detector Injection volume : 20µl	7

3.1 RATIONALE FOR SELECTION OF DRUG

Pregabalin and gabapentin has similar mechanism of action. Both inhibits calcium influx and consequent release of excitatory neurotransmitters. But they differ in their pharmacokinetic and pharmacodynamic properties.

After oral administration, Gabapentin is absorbed very slowly with maximum plasma concentrations attained within 3–4 hours. Oral gabapentin shows saturable absorption which makes its pharmacokinetic profile less predictable. Absorption of gabapentin is restricted by saturable, dose-dependent transport in the gastrointestinal tract.

Orally administered pregabalin is absorbed more rapidly, with maximum plasma concentration attained within 1 hour. Its absorption is linear - first order which means its plasma concentrations increases proportionately with increasing dose. Pregabalin has an average bioavailability of more than 90% and is independent of dose.

For neuropathic pain, lower dose of pregabalin appears to reduce pain compared to the predicted maximum effect of gabapentin. Pregabalin is more effective antiepileptic than gabapentin as it reduces seizure frequency with high magnitude. Binding affinity of for the α_2-δ subunit and potency of pregabalin is six times more than that of gabapentin. Pregabalin possess some distinct pharmacokinetic advantages over gabapentin that eventually result into an enhanced pharmacodynamic effect.

Advantages of using pregabalin

- Pregabalin has been proved to be very effective and well-tolerated adjunctive therapy in the treatment of patients with partial seizures.
- Research studies shows that it can be used as first line treatment for management of diabetic neuropathic pain and fibromyalgia.
- Pregabalin has a favorable pharmacokinetic profile with predictable oral absorption.
- Pregabalin does not undergo hepatic metabolism and does not induce or inhibit liver enzymes such as the cytochrome P450 system .

- Pregabalin does not bind to plasma proteins therefore it is unlikely to cause pharmacokinetic drug–drug interactions.
- Pregabalin shows ease of dosing and favorable side effect profile. Its most common side effects are dizziness and somnolence. [56-59]

3.2 AIM OF PRESENT WORK

Pregabalin is official in Indian pharmacopoeia. The assay method and related substances method is reported by HPLC.

The literature review for pregabalin shows that number of analytical methods such as spectrophotometry, HPLC, TLC, HPTLC, LC/MS and titremetric methods for the individual/simultaneous estimation of pregabalin have been reported.
A stability indicating HPLC method has also been reported. It is stated that the drug is sensitive to oxidative conditions and stable under acidic and basic conditions. The amount of degradation obtained was not reported.

Emcure Pharmaceuticals, Ltd. wishes to launch a new formulation of pregabalin. Hence it is required to carry out stability study of the drug and its compatibility study with the excipients

So, the aim of the present work is to develop a complete degradation profile for the drug pregabalin by HPLC. Further, method is to be validated in terms of linearity, specificity, precision, accuracy, LOD, LOQ, robustness, solution stability and system suitability as per ICH guidelines.

Pregabalin capsules contains lactose as one of the major excipient. As pregabalin contains amine moiety, it reacts with lactose and undergoes maillard's reaction. This results in the formation of lactum conjugates.
So, another objective is to evaluate the lactum conjugates obtained by degradation reaction of pregabalin with lactose and to separate these conjugates using the developed method.

4. IDENTIFICATION OF PREGABALIN

Identification of pregabalin was carried out by melting point, UV-Visible spectroscopy and FT-IR spectroscopy.

Instrumentation

Melting point apparatus: T603160, (EIE Instruments, Pvt. Ltd.)

UV-Visible spectrophotometer: UV/Vis-1800, (Shimadzu Inc., Columbia, MD)

FT IR spectrophotometer: MB-3000 (ABB, Switzerland)

4.1. IDENTIFICATION BY MELTING POINT

Melting point of pregabalin has been determined using melting point apparatus. Melting point was taken by capillary method.

Table 4.1 Melting point of pregabalin

Drug	Reported Melting Point (^{o}C)[8]	Observed Melting Point (^{o}C)
Pregabalin	186ºC-188ºC	184ºC-186ºC

4.2. IDENTIFICATION BY UV VISIBLE SPECTROSCOPY

500 µg/mL solution of pregabalin was prepared in water and scanned in UV-Visible spectrophotometer in range of 190-400 nm to determine the absorption maxima of drug.

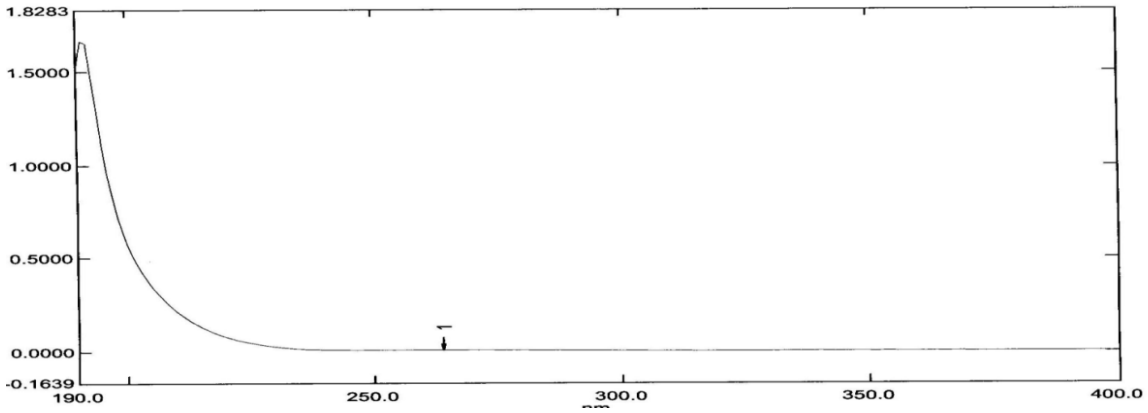

Figure 4.1 : UV spectra of pregabalin(500 µg/mL)

As λmax was not clearly visible, standard solutions of pregabalin at concentration range of 2500-7500 µg/mL were prepared in water and scanned in UV Visible spectrophotometer in range of 190-400 nm.

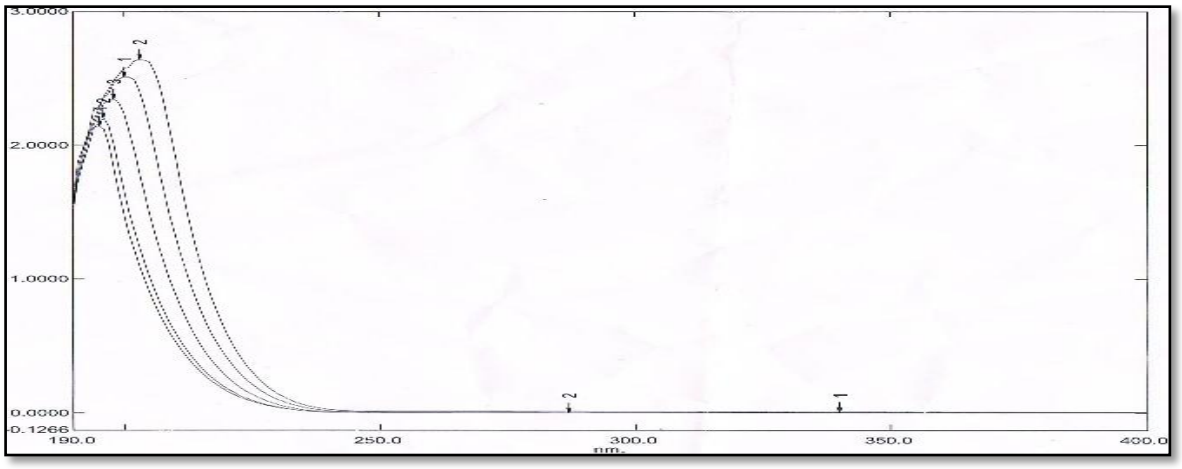

Figure 4.2 : UV overlay spectra of pregabalin(2500-7500 µg/mL)

From the above overlay spectra it can be seen that as we move towards higher concentration λmax is shifting towards 210 nm. Hence 210 nm was selected as detection wavelength.

4.3. IDENTIFICATION BY FT-IR SPECTROSCOPY

FT-IR spectra of pregabalin was taken using FT-IR spectrophotometer in attenuated total reflectance mode. Principle IR peaks were observed for the drug and are shown in Table 4.2

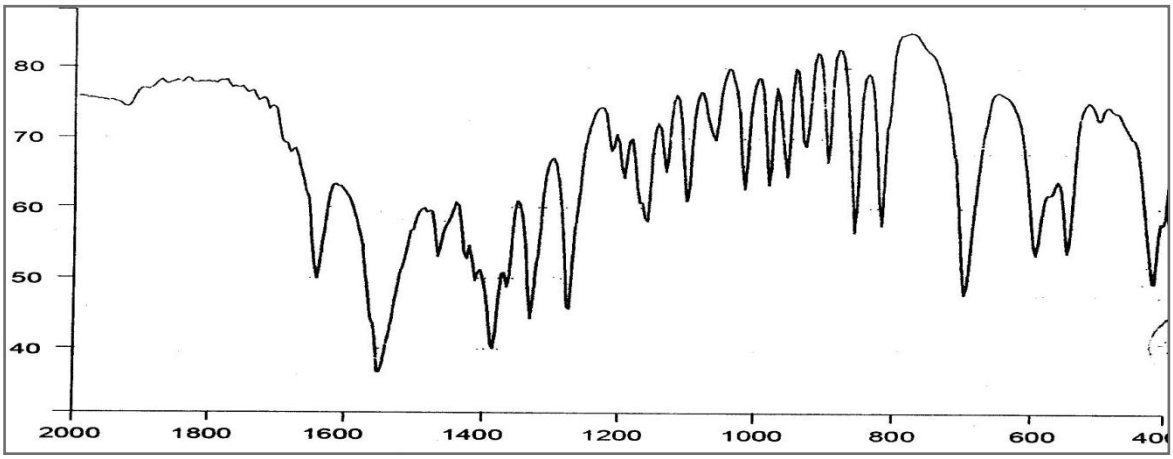

Figure 4.3 : Reported FT-IR spectra of pregabalin[7]

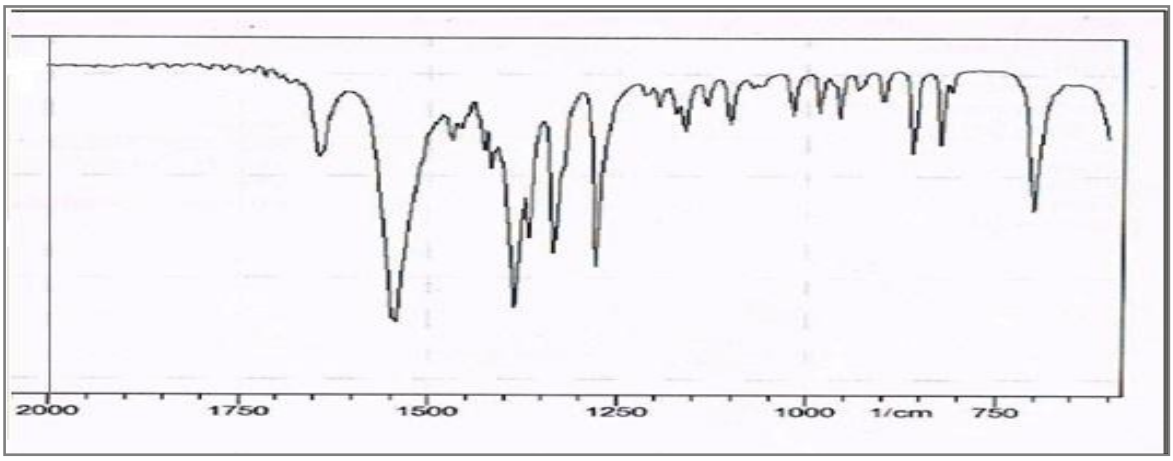

Figure 4.4 : Recorded FT-IR spectra of pregabalin

Table 4.2 - Interpretation of FT-IR spectra of pregabalin[60]

Wavenumber(cm^{-1})	Band assignment
1465.2	C-H (Bending)
3450.01	N-H (Primary amine) (Streching)
1742	C=O (carboxylic) (Streching)
1086	C-N (Bending)
3309.0	O-H (Alcohol) (Streching)

Conclusion:

The observed λmax, FT-IR frequencies and observed melting point meets the criteria and confirms that the solid white sample is of pregabalin.

5.1 INSTRUMENTATION

• HPLC system :

Waters Alliance HPLC system ,USA., Model - e2695, having 100 µL loop injector, 2489 UV detector , Software- Chromeleon 7 , version : 7.2.0.3972

• UV-Visible spectrophotometer :

Shimadzu Inc., Japan , Model: UV1800, Software: UV Probe 2.43

• FTIR spectrophotometer :

ABB, Switzerland, Model : MB 3000, Software: Horizon

• Analytical balance :

Metler Toledo, Switzerland, Model : XP205, weighing capacity : 2 to 220gm

• pH meter :

Metrohm India Ltd., Model: 780.

• Melting point apparatus :

EIE Instruments Pvt. Ltd., Model: T060316

• Sonicator :

Leelasonic Power

• Digital water bath , Vaccum pump, Hot plate, Hot air oven :

Patel Scientific Instruments

• Humidity chamber :

Mack, Model : MK2

• Water system :

Elix - Merk Millipore, Model : Elix®15

MilliQ - Merk Millipore, Model : Milli-Q®Reference

5.2 MATERIALS

1. Reagents

- HPLC grade water was obtained from Merck Millipore, India ,Ltd.
- HPLC grade acetonitrile was procured from Merck Specialities Pvt. Ltd, Worli, Mumbai.
- AR grade KH_2PO_4 , 30 % w/v H_2O_2, KOH pellets, NaOH pellets and concentrated HCL were procured from Merck Specialities Pvt. Ltd, Worli, Mumbai.

2. Apparatus and filters

- Glasswares like Volumetric flasks, Beakers, Pipettes and petridish used were of Borosilicate.
- Membrane filter (0.45 µ) and syringe filiter (0.45 µ) used were of Merk Millipores, India Ltd.

Marketed Formulation

Capsules containing 300 mg of pregabalin were supplied by Emcure Pharmaceuticals Ltd.

5.3 PREPARATION OF MOBILE PHASE

Phosphate Buffer (pH 6.5)

1.43 gm of potassium dihydrogen orthophosphate was accurately weighed and transferred to 1000 mL of water (10mM). pH of this solution was adjusted to 6.5 with 5N Potassium hydroxide solution. It was filtered through 0.45 µ filter.

Mobile phase A - Buffer : Acetonitrile (96:04 %v/v)

960 ml of phosphate buffer (pH 6.5) was mixed with 40 ml of acetonitrile to make (96:04) ratio. The solution was mixed and degassed.

Mobile phase(B) – Acetonitrile

Diluent : Mobile phase A -Buffer : ACN (96:4 %v/v)

5.4 PREPARATION OF SOLUTIONS

5.4.1 Preparation of standard solution of pregabalin

250 mg of pregabalin standard API was weighed and transferred to 50 mL volumetric flask. 15 mL of diluent was added and sonicated to dissolve. Volume was adjusted up to the mark with diluent and mixed (5000 µg/mL).

5.4.2 Preparation of diluted standard solution (specification level concentration 10 µg/mL)

From the standard stock solution of pregabalin (5000 µg/mL), 2.5 mL of aliquot was taken and transferred into 25 mL volumetric flask. Volume was adjusted upto the mark with diluent to produce diluted solution of 100 µg/mL of pregabalin. 1 mL of above solution was transferred in 10 mL of volumetric flask. Volume was adjusted to the mark with diluent to produce diluted solution of 10 µg/mL of pregabalin.

5.4.3 Preparation of sample solution of pregabalin capsule

20 capsules were weighed individually, content of the each capsule was emptied and reweighed. Capsule powder equivalent to 250 mg of pregabalin was accurately weighed and transferred to a 50 mL volumetric flask. 15 mL of diluent was added and sonicated to dissolve. Volume was adjusted up to the mark with diluent and mixed. Final solution was filtered through 0.45µ PVDF filter.

5.4.4 Preparation of pregabalin placebo solution

91.25 mg of placebo was accurately weighed and transferred to 50 mL volumetric flask. 15 mL of diluent was added and sonicated to dissolve. Volume was adjusted up to the mark with diluent and mixed. Resultant solution was filtered using 0.45µ PVDF filter.

5.5 OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Stationary phase : Alltima C_{18} column (250mm X 4.6mm, 5 µm).

Mobile phase :

Mobile phase(A) : Phosphate buffer pH 6.5 : ACN (96:04 v/v)

Mobile phase(B) : Acetonitrile

Time(min)	%Mobile Phase A	%Mobile phase B
0	98	02
15	98	02
55	60	40
65	60	40
66	98	02
75	98	02

Diluent : Mobile phase A

Flow rate : 1 mL/min

Injection volume : 100 µL

Wavelength : 210 nm

Run time : 75 min

5.6 METHOD VALIDATION

5.6.1 System suitability

I. System suitability at diluted standard level

The diluted standard solution of pregabalin (10 µg/mL) was prepared as given in section 5.4.2 and was injected 6 times into the HPLC system according to the chromatographic conditions mentioned in section 5.5. The theoretical plates, asymmetry and peak area were observed.

II. System suitability at test concentration level

Pregabalin standard solution (5000 µg/mL) was prepared as given in section 5.4.1 and was injected six times into the HPLC system with optimized chromatographic conditions (section 5.5). Peak area with asymmetry and number of theoretical plates were recorded.

5.6.2 Specificity :

For specificity study, Standard pregabalin solution (5000 µg/mL) (5.4.1), Pregabalin capsule solution (5000 µg/mL)(5.4.3) and pregabalin placebo solution (5.4.4) were prepared.

100µL of each solution was injected and analyzed as per the chromatographic conditions (section 5.5). Chromatograms were recorded and comparision is made within chromatogram of pregabalin standard, pregabalin capsule and placebo.

5.6.3 Linearity

I. Linearity study at diluted standard level

Preparation of linearity stock solution I

25 mg of pregabalin standard API was accurately weighed and transfered to 100mL volumetric flask. 15mL of diluent was added and sonicated to dissolve. Volume was adjusted upto the mark with diluent and mixed to get 250 µg/mL solution.

The linearity was determined at 6 levels from LOQ (2.5 µg/mL) to 150 % of the specification level concentration (10 µg/mL)

From the prepared linearity stock solution ,aliquots were taken to produce 2.5,5,7.5,10,12.5 and 15 µg/mL solution. 100 µL of each solution was injected and analyzed as per the optimized chromatographic conditions (section 5.5) at each level in triplicate.

A graph of mean area versus concentration in µg/mL was plotted and regression equation was determined.

II. Linearity at test concentration level

Preparation of linearity stock solution II

250 mg of pregabalin standard API was accurately weighed and transfered to 25 mL volumetric flask. 15 ml of diluent was added and sonicated to dissolve. Volume was adjusted upto the mark with diluent and mixed to get 10,000 µg/mL solution.

From the above prepared stock solution, aliquots were taken to produce 2500,3750,5000,6250 and 7500 µg/mL solutions. 100 µL of each solution was injected and analyzed as per the optimized chromatographic conditions (section 5.5) at each level in triplicate.

A graph of mean area versus concentration in µg/mL was plotted and regression equation was determined.

5.6.4 Precision

5.6.4.1 Repeatability study

I. Repeatability at diluted standard level

Pregabalin standard solution (10 µg/mL) was prepared as per section 5.4.2. and was injected six times into the HPLC system. Peak area was recorded and reported in terms of %RSD.

II. Repeatability at test concentration level

Repeatability was also checked by injecting pregabalin standard solution (5000µg/mL) (prepared as per 5.4.1) for six times. Peak area was recorded and reported in terms of %RSD.

5.6.4.2 Intraday precision

Intraday precision was checked by injecting three concentrations of pregabalin (2500, 5000 and 7500 µg/mL) into the HPLC system and were analyzed for three times on the same day. Results are reported in terms of %RSD.

5.6.4.3 Interday precision

Interday precision was checked by injecting three concentrations of pregabalin (2500, 5000 and 7500 µg/mL) into the HPLC system and were analyzed on three different days. Results are reported in terms of % RSD

5.6.5 LOD

From the linearity stock solution I, aliquot was taken to produce 1.5 µg/mL solution. It was injected into the HPLC system in triplicate and chromatograms were recorded. Peak area was recorded and reported in terms of %RSD.

5.6.6 LOQ

From the linearity stock solution I, aliquot was taken to produce 2.5 µg/mL solution. It was injected into the HPLC system in triplicate and chromatograms were recorded. Peak area was recorded and reported in terms of %RSD.

5.6.7 Accuracy

Accuracy of the method was determined by standard addition method at three different levels. To a fixed amount of placebo, 50% 100% and 150% of diluted standard concentration of pregabalin (10 µg/mL) (5.4.2) was added.

Level I

91.25 mg of placebo was taken in 50 mL of volumetric flask. It was spiked with 1 mL of standard solution of pregabalin (250 µg/mL) .Volume was made up to mark with diluent to obtain final concentration of 5 µg/mL.

Level II

91.25 mg of placebo was taken in 50 mL of volumetric flask. It was spiked with 2 mL of standard solution of pregabalin (250 µg/mL) .Volume was made up to mark with diluent to obtain final concentration of 10 µg/mL.

Level III

91.25 mg of placebo was taken in 50 mL of volumetric flask. It was spiked with 3 mL of standard solution of pregabalin (250 µg/mL) .Volume was made up to mark with diluent to obtain final concentration of 15 µg/mL.

5.6.8 Robustness

Standard solution of pregabalin (5000 µg/mL) was prepared as per section 5.4.1 and was injected into six replicates with following changes in optimized chromatographic conditions like:

1. Flow rate of mobile phase (±0.25mL/min) changed to 0.75 mL/min and 1.25 mL/min.
2. pH of mobile phase (±0.2 absolute) changed to 6.3 and 6.7.
3. Organic solvent composition (± organic strength) changed to 97:03 and 95:05 (Buffer : ACN)

Results were recorded in terms of %RSD.

5.6.9 Solution stability

Standard solution of pregabalin (5000 µg/mL) prepared as per section 5.4.1. was stored at room temperature for 48 hrs. The samples were injected into the system at interval of 12hrs, 24hrs and 48hrs. The results were recorded and analyzed according to chromatographic conditions in section 5.5. Peak area at each time point was compared with a freshly prepared standard solution and reported in terms of %RSD.

5.7 ANALYSIS OF CAPSULE FORMULATION OF PREGABALIN

The capsule sample was prepared as per section 5.4.3. The sample was then analyzed according to the chromatographic condition in section 5.5. Concentration was measured and the % assay of pregabalin in the capsule formulation was calculated.

5.8 FORCED DEGRADATION STUDY OF PREGABALIN

5.8.1 Acid degradation

250 mg of pregabalin was accurately weighed and transferred to 50 mL volumetric flask.15 mL of diluent was added to it and sonicated to dissolve the drug. 5 mL of 5N HCl solution was added to it and mixed. Volumetric flask was refluxed at 70°C for 1 hour on water bath. Mixture was cooled and neutralized with 5 mL of 5N NaOH. Volume was adjusted up to the

mark with diluent and it was injected into the HPLC system according to the chromatographic conditions in section 5.5.

5.8.2 Base degradation

250 mg of pregabalin was accurately weighed and transferred to 50 mL volumetric flask.15 mL of diluent was added to it and sonicated to dissolve the drug. 5 mL of 5N NaOH solution was added to it and mixed. Volumetric flask was refluxed at 70°C for 1 hour on water bath. Mixture was cooled and neutralized with 5 mL of 5N HCl. Volume was adjusted up to the mark with diluent and it was injected into the HPLC system according to the chromatographic conditions in section 5.5.

5.8.3 Peroxide degradation

250 mg of pregabalin was accurately weighed and transferred to 50 mL volumetric flask.15 mL of diluent was added to it and sonicated to dissolve the drug. 1 mL of 5% H_2O_2 solution was added to it and mixed. Volumetric flask was refluxed at 70°C for 5 minutes on water bath. Mixture was cooled .Volume was adjusted up to the mark with diluent and it was injected into the HPLC system according to the chromatographic conditions in section 5.5.

5.8.4 Thermal degradation

1 g of pregabalin standard API was transferred to the petridish. Petridish was kept in the hot air oven for 3 days at 60°C. Sample was cooled. From it 250 mg of the sample was taken and transferred to 25 mL volumetric flask. 15 mL of diluent was added and sonicated to dissolve the drug. Volume was adjusted up to the mark with diluent and it was injected into the HPLC system according to the chromatographic conditions in section 5.5.

5.8.5 Stability study in Humidity chamber

1 g of pregabalin standard API was transferred to the petridish. Petridish was kept in stability chamber for 3 days at 40°C/75% RH. Sample was cooled. From it 250 mg of the sample was taken and transferred to 25 mL volumetric flask. 15 mL of diluent was added and sonicated

to dissolve the drug. Volume was adjusted up to the mark with diluent and it was injected into the HPLC system according to the chromatographic conditions in section 5.5.

5.9 DEGRADATION STUDY OF PREGABALIN WITH LACTOSE

5.9.1 Preparation of degradation solution of lactose

250 mg pregabalin standard API was accurately weighed and transferred into 50 mL volumetric flask. 250 mg lactose was added into it. 15 mL diluent was added and sonicated to dissolve. Volume was adjusted upto the mark with diluent and mixed to get final concentration of 5000 µg/mL pregabalin and lactose each. Solution was heated for 4 hours and cooled. Resulting solution was filtered with 0.45µ PVDF filter. 100 µL of this solution was injected into HPLC system.

5.9.2 Formation of lactum conjugates[61]

0.8 gm of pregabalin standard API and 3.8 gm of lactose were dissolved in 5 mL of water with stirring and heat. The solution was then heated overnight at 90˚C in an open beaker on hot plate. The resulting solid was then redissolved in 20 mL of IPA by sonicating and heating. 250 mg residue was weighed and transferred to 50 mL volumetric flask, 15 mL of diluent was added and sonicated.

Volume was adjusted upto the mark with diluents. Solution was filtered with 0.45 PVDF filter. 100 µL of this solution was injected into the HPLC system. Chromatogram obtained was compared with the chromatogram of pregabalin lactose degradation solution.

Conclusion

A stability indicating HPLC method was developed and validated for the determination of pregabalin in presence of its degradation products. The column used was Alltima C_{18} column (250 x 4.6 mm, 5 µm). Mobile phase was optimized using various ratios of phosphate buffer and acetonitrile. The optimized mobile phase was phosphate buffer pH 6.5 : acetonitrile (96:4) as mobile phase A and acetonitrile as mobile phase B in gradient flow. The optimized flow rate was 1 mL/min with detection at 210 nm. The peak of pregabalin was observed at Rt of 9.9 min.

The method was validated as per ICH Guideline Q2 (R1) for specificity, linearity, accuracy, precision, LOD, LOQ, robustness, system suitability and solution stability. The linear regression analysis data showed good linear relationship at both diluted standard level (2.5 - 15 µg/mL) and test concentration level (2500-7500 µg/mL) with correlation coefficient of 0.997 and 0.999 respectively. LOD and LOQ of the method were found to be 1.5 µg/mL and 2.5 µg/mL respectively.

The % assay for the marketed formulation was 102.34% and there was no peak obtained at the Rt of degradation products. Thus the developed method can also be applicable to the routine analysis of pregabalin in marketed formulations.

Forced degradation was carried out according to ICH Guidelines Q1A (R2) and Q1B. Pregabalin was subjected to acid, base, thermal, oxidative and photo degradation. The degraded products obtained were well separated from the main peak using the developed method. The drug was found to be stable in acidic, thermal and humidity conditions. Degradation was observed in basic and oxidative conditions. Under basic condition two DP were obtained at Rt of 8.3 min and 46.03 min. When the drug was subjected to oxidative condition major DP was obtained at Rt of 22.98 min.

Degradation study of pregabalin was carried out with its major excipient lactose. It was found that pregabalin is degraded in the presence of lactose. Lactum impurity was generated at Rt of 46.02 min which was confirmed from related literature. This lactum impurity could be quantified with the help of developed method.

It was observed that, when pregabalin was reacted with lactose for 4 hours, % of lactum conjugate formed was found to be 0.11%. While when it was reacted with lactose for 24 hours, pregabalin was converted into lactum conjugate and % of lactum conjugate formed was found to be 94.54 %.

Future Scope

- Newer and simpler methods like Raman spectroscopy and Near IR spectroscopy can be developed for the quantification of pregabalin.

- Degradation products obtained during various stress conditions can be characterized by using hyphenated techniques such as LC/MS.

- Lactum impurities generated by reaction of pregabalin with lactose can be isolated from pregabalin using various isolation techniques such as preparative chromatography. Other impurities obtained along with lactum impurity can be characterized by various hyphenated techniques.

- Excipient compatibility study of pregabalin can be carried out with other excipients to confirm its stability. Studies can also be carried out to check the compatibility of pregabalin with other sugars.

REFERENCES

1. Gajraj, N. M.,Pregabalin: Its Pharmacology and Use in Pain Management. *Anesthesia & Analgesia:* **2007,** *105* (6), 1805-1815.
2. Taylor C. P., Angelotti T., Fauman E., Pharmacology and Mechanism of Action of Pregabalin: The Calcium Channel α2–δ (alpha2–delta) subunit as a Target for Antiepileptic Drug Discovery. *Epilepsy research* **2007,** *72* (2), 137-150.
3. http://www.nhs.uk/medicine/guides/pages/medicineoverview.aspx?condition =Anxiety& medicine=pregabalin
4. http://www.lyrica.com/DPN/relieving-diabetic-nerve-pain-with-lyrica.
5. http://www.webmd.com/fibromyalgia/guide/lyrica-for-fibromyalgia-treatment.
6. Tripathi, K. D., Essentials Of Medical Pharmacology ,6th Edition, Jaypee Brothers Medical Publishers. Ltd,New Delhi ,**2006**, 401-402.
7. Indian Pharmacopoeia, Government of India, Ministry of Helath and Family Welfare,The Indian Pharmacopoeial Commission, Vol III, **2010,** 1960.
8. The Merck Index. An Encyclopedia of Chemicals, Drugs and Biological; Merck Research Laboratories; USA; 14th edition, **2006**, 7843.
9. http://www.drugbank.ca/drugs/DB00230.
10. Satoskar R. S., Bhandarkar S. D., Rege N. N., Pharmacology and Pharmacotherapeutics, Popular Prakashan, 21 st edition, **2009** ,131.
11. http://www.accessdata.fda.gov/drugsatfda_docs/label/2005/019787s038lbl.pdf.
12. Sweetman, S. C., Martindale, The Complete Drug Reference, 33rd Edition, **2002**, 456.
13. CIMS ,Updated Prescribers Handbook, Published by UBM Medica India Pvt. Ltd.,Oct **2013**-Jan **2014**,176.
14. ICH, Stability Testing of New Drug Substances and Products (Q1AR2) , International Conference on Harmonization, IFPMA, Geneva. **2003**.
15. Singh, S. S., Bakshi, M., Development of Validated Stability Indicating Assay Methods: Critical Review. *Journal of Pharmaceutical and Biomedical Analysis.* **2002**, 28, 1011 – 1040.
16. Lund J. Pharmaceutical Codex: Principle and Practical of Pharmaceutics, 12th edition, Published by CBS ,**1994**, 696.

17. Jeffery G.H., Bassett J. Vogel's textbook of Quantitative Chemical Analysis, 5th edition, **1991**, 217-235.
18. Skoog D.A., Holler F.J., West D.M., Nierman T.A, Analytical Chemistry : An Introduction , 5th edition, Published by Cole, **1994**, 725-760.
19. FDA, Guidance for Industry: Analytical Procedures and Methods Validation (Draft guidance); Food and Drug Administration, Rockville, MD, **2000**.
20. The United States Pharmacopeia; 24th Revision; Asian Edition; United States, Pharmacopeial Convention, Inc., Rockville, MD, **2000**, 2149–2152.
21. Carstensen T.T., Rhodes C.T., Drug Stability: Principles and Practices; Marcel Dekker; New York, **2000**, 329–384.
22. Green J. M., A Practical Guide to Analytical Method Validation. Anal. Chem., **1996**, 44, 305-309.
23. US FDA. Technical Review Guide: Validation of Chromatographic Methods. **1993**.
24. ICH, Q2 (R1). Validation of Analytical Procedures: Text and Methodology: **2005.**
25. Singh R., Manirul S. K., Kumar P., A Sensitive Spectrophotometric Method for the Determination of Pregabalin in Bulk, Pharmaceutical Formulations and in Human Urine Samples. *International Journal of Biomedical Science.* **2009**, 5(4), 421–427.
26. Rasha M. , Spectrofluorimetric and Spectrophotometric Determination of Pregabalin in Capsules and Urine Samples. *International Journal of Biomedical Science*. **2010**, (3), 260–267.
27. Bali A., Gaur P., A Novel Method for Spectrophotometric Determination of Pregabalin in Pure Form and in Capsules. *Chemistry Central Journal.* **2011**,5(11).
28. Önal A, Sagirli O., Spectrophotometric and Spectrofluorimetric Methods for the Determination of Pregabalin in Bulk and Pharmaceutical Preparation. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. **2009**, 1(72),34-38.
29. Gujral R.S., Haque S. K., Shanker P., Development and Validation of Pregabalin in Bulk, Pharmaceutical Formulations and in Human Urine Samples by UV Spectrophotometry. *International Journal of Biomedical Science.* **2009**, 2(5),78-8.

30. Gujral R.S., Haque S. K, Shanker P., A Sensitive Spectrophotometric Method for the Determination of Pregabalin in Bulk, Pharmaceutical Formulations and in Human Urine Samples. *International Journal of Biomedical Science.* **2009**, 4(5),21-26.

31. Reddy A. J., New Spectrophotometric Determination of Pregabalin in Bulk and Pharmaceutical Dosage Form. *Journal of Drug Discovery and Therapeutics.* **2013**, 1(6),56- 58.

32. Sowjanya K., Thejaswini J. C., Gurupadayya B. M., Indupriya M., Spectrophotometric Determination of Pregabalin using 1, 2-Napthaquinone-4- sulfonic acid Sodium and 2, 4 Dinitrophenyl hydrazine in Pharmaceutical Dosage Form. *Der Pharmacia Lettre*. **2011**, 2(3),67-73.

33. Kaur N., Mittal K., Nagar R., Nepali K., Thakkar A., A Sensitive Spectrophotometric Method for the Determination of Pregabalin in Bulk, Pharmaceutical Formulations through Benzoylation. *International Research Journal of Pharmacy*. **2011**, 1,78-84.

34. Sowjanya K., Thejaswini J. C., Gurupadayya B. M., Indupriya M., Spectrophotometric Determination of Pregabalin Using Gibb's and MBTH reagent in Pharmaceutical Dosage Form. *Der. Pharma. Chemica.* **2011**, 3,25-28.

35. Shep S. G., Lahoti S. R., Development and Validation of UV Spectrophotometric Method of Pregabaln In Bulk And Pharmaceutical Formulation. *International Journal of Pharm Tech Research.* **2012**, 2(5),23-26.

36. Najam R., Shah M., Muzaffar S., Kinetic Spectrophotometric Determination of an Important Pharmaceutical Compound, Pregabalin. *Journal of Analytical Science and technology*. **2013,** 4(22).

37. Kannapan N., Nayak P., Venkatachalam T., Prabhakaran V., Analytical RP-HPLC Method for Development and Validation of Pregabalin and Methylcobalamine in Combined Capsule Formulation. *Journal of Applied Chemical Research.* **2010,** 1(13), 85-89

38. Bhatt K. K., Emanual M., Aswin M., Simultaneous Estimation of Pregabalin and Methylcobalamine in Pharmaceutical Formulation by RP-HPLC Method. *Journal of Analytical and Bioanalytical Techniques.* **2013**, 4, 254-265.

39. Sharma T., Gavaskar R., Development and Validation of RP-HPLC Method for the Assay of Pregabalin in Capsules. *Journal of Pharmacy and Pharmaceutical Sci*ence **2013,** (3), 521-535.

40. Singh V., Kumar P., Gupta R., Development and Validation of RP-HPLC Method for the Assay of Pregabalin in Capsules. *Journal of Pharmacy and Pharmaceutical Sci*ence. **2013**, (3), 703-711.

41. Sung P., Jui P., RP-HPLC Method for Estimation of Pregabalin in Bulk. *International Journal of Pharmaceutical Science.* **2010**, 18, 116-119.

42. Kavitha M., Rajasekhar P., A Validated HPLC Method for the Analysis of Pregabalin and Methylcobalamin in Bulk and Pharmaceutical Formulation. *Pharmacie Globale.* **2013**, 4,174 180.

43. Mohan A., Rajkumar B., Bhavya T., Ashok K., RP-HPLC Method Development And Validation For The Simultaneous Quantitative Estimation Of Pregabalin, Mecobalamin And Alpha Lipoic Acid In Capsules. *International Journal of Pharmacy and Pharmaceutical Sci*ence. **2014**, 6, 142-155.

44. Vermeij T., Edelbroek P. M., Simultaneous High-Performance Liquid Chromatographic Analysis of Pregabalin, Gabapentin and Vigabatrin in Human Serum by Precolumn Derivatization with O- phtaldialdehyde and Fluorescence Detection. *Journal of Chromatography.* **2004**, 87 96.

45. Faraqui M., Kasawar G. B., Development and Validation of HPLC Method for the Determination of Pregabalin in Capsules. *Indian Journal of Pharmaceutical Science.* **2010**, 72(4),517-519.

46. Reddy N. C., Chandra Sekhar K. B., RP-HPLC Determination of Related substances of Pregabalin in Bulk and Pharmaceutical Dosage Form. *International Journal of Chemistry and Pharmaceutical Science*. **2012**, 2(3),44-49.

47. Mishra S. K., Gurupadhyya B. M., Ver S., Stability Indicating RP-HPLC Method for Determination of Pregabalin using ICH Guidelines. *International Journal of Natural Products Science*. **2012,** 1(130),23-28.

48. Xiaohui C., Daolin Z., Jie D., Xiaotai F., Determination of Optical Impurity of Pregabalin by HPLC with Pre-Column Chiral Derivatization. *Journal of Chromatographic Science.* **2006**, 46.

49. Gujral R. S., Manirul H., Kumar S., A Novel Method for the Determination of Pregabalin in Bulk, Pharmaceutical Formulations and Human Urine Samples. *African Journal of Pharmacy and Pharmacology*. **2009**, 36,142 -151.

50. Prasad M., Chaitanya K., Sagar G., Sudhakar P., Simultaneous HPTLC Method for Estimation of Gabapentin and Pregabalin. *International Journal of Pharmacy and Pharmaceutical Science*. **2013,** 4(5).

51. Shah D. A., Patelia E. A., Mori A., Simultaneous Estimation of Pregabalin and Methylcobalamine in Pharmaceutical Formulation by HPTLC-Densitometry Method. *Journal of Chromatographic Seperation Techniques.* **2013**, 4(169),12-16.

52. Chhabra G. S., Bhalodiya H. K., Banerjee S. K., LC-MS-MS Method Validation of Pregabalin in Human Plasma. *Bulletin of Pharmaceutical Research*. **2012**, 2(2),90-94.

53. Uma G., Manimala M., Vasudevan M., Karpagam S., LC-MS-MS Method for the Determination of Pregabalin in Human Plasma. *International Journal of Pharmacy and Pharmaceutical Science*. **2012**, 4, 389 399.

54. Chahboun G., Burger D., Jeroen C., René M., Abraham J., Eleanora L., Rapid Quantification of Gabapentin, Pregabalin, and Vigabatrin in Human Serum by Ultraperformance Liquid Chromatography With Mass-Spectrometric Detection. *Therapeutic Drug Monitoring*. **2013**, 1(35).

55. Thejaswini J. C., Gurupadayya B. M., Raja P., Gas Chromatographic Determination of Pregabalin in Human Plasma using Ethyl Chloroformate Derivatizing Reagent. *Journal of Pharmaceutical Research.* **2012**, 6(5),56-63.

56. Wesche D., Bockbrader H., A Pharmacokinetic Comparison of Pregabalin and Gabapentin. *Journal of Pain American Pain Society*. **2005**, 6(3),29.

57. Frank B., Cousins M. J., Pregabalin in the Treatment of Neuropathic Pain Associated with Diabetic Peripheral Neuropathy. *Future of Neurology* **2008,** 3(6),631-639.

58. Bockbrader H. N., Wesche D., Miller R., Chapel S., Janiczek N., Burger P., A Comparison of the Pharmacokinetics and Pharmacodynamics of Pregabalin and Gabapentin. *Clinical Pharmacokinetics.* **2010**, 49(10), 661-669.

59. Thomas J. L., Walker R. R., Pregabalin (Lyrica) for the Management of Pain Associated with Diabetic Neuropathy. *American Family Physician*. **2006** , 74(12),2093-2094.

60. Pavia D., Lampman M., Kriz G., Introduction to Spectroscopy, Brooks Cole ,4th edition,**2008**,54.

61. Lovdahl M. J., Hurley T. R., Tobias B., Priebe S. R., Synthesis and Characterisation of Pregabalin Lactose Conjugate Degradation Products. *Journal of Pharmaceutical and Biomedical Analysis.* **2002**, 28,917 924.

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