PART-[A]

ENANTIOSELECTIVE METHOD
DEVELOPMENT AND VALIDATION
OF SOME PHARMACEUTICALS

SECTION-3

Enantioselective LCMS Method
Development and Validation of
Pregabalin
[1] Introduction

The importance of stereochemistry in drug action is gaining greater attention in medical practice and over the past fifteen to twenty years, the stereochemistry is gaining prime importance in pharmaceutical practice. As a result of advancement in chemical technologies associated with the synthesis, separation, identification and analysis of single enantiomer present in racemic compound, several chiral drugs are presented as a single enantiomer for approval to regulatory authorities. Rather to introduce a racemic compound, a single enantiomer always have better selectivity on receptor resulting in superior therapeutics action with less metabolic load and less side effects. The enzymes or amino acids or binding sites have been recognized to be stereoselective which is considered in chiral drug development. Each enantiomer interacts differently with the receptor, elicits the response differently and potency of enantiomer depends on the eudismic ratio or eudismic index or stereospecific index of the compound. Therefore Eudismic ratio is also an important tool in chiral drug designing.

The enantiomers of a chiral drug are best identified on the basis of their absolute configuration or their optical rotation. Other designations such as D and are used for sugars and amino acids but are specific to these molecules and are not generally applicable to other compounds. The terms $d$, or dextro, and $l$, or levo, are considered obsolete and should be avoided. Instead, the $R/S$ system for absolute configuration and the $+/-$ system for optical rotation should be used. The absolute configuration at a chiral center is designated as $R$ or $S$ to unambiguously describe the 3-dimensional structure of the molecule. $R$ is from the Latin rectus and means to the right or clockwise, and $S$ is from the Latin sinister for to the left or counterclockwise. There are precise rules based on atomic number and mass for determining whether a particular chiral center has an $R$ or $S$ configuration.

A chiral drug may have more than one chiral center, and in such cases it is necessary to assign an absolute configuration to each chiral center. Optical rotation is often used because it is easier to determine experimentally than absolute configuration, but it does not provide information about the absolute configuration of an enantiomer.
For a given enantiomer pair, one enantiomer can be designated (+) and the other as (−) on the basis of the direction they rotate polarized light. Optical rotations have also been described as dextrorotatory for (+) and levorotatory for (−). Racemates can be designated as (R, S) or (±).

1.1 Description

3-Isobutylgaba exists in isomeric forms and the S-(+)-3-isobutylgaba known as a Pregabalin (Lyrica™) is the pharmacologically active enantiomer (Fig. 1). The pregabalin is the potent anticonvulsant and it is sold under the trade name of “Lyrica™” [1]. Pregabalin has also been found to be useful for the treatment of neuropathic pain, epilepsy, in therapy of partial seizures in adults, pain from diabetic neuropathy and the treatment of anxiety disorders [2, 3].

Its molecular formula is C₈H₁₇NO₂ having molecular weight 159.23 g/mol. The CAS number of pregabalin is 148553-50-8.

![Figure 1: Pregabalin and (R)-enantiomer](image-url)
1.2 Indication

Pregabalin is an anticonvulsant drug used for neuropathic pain, as an adjunct therapy for partial seizures, and in generalized anxiety disorder.

1.3 Mechanism of action

Pregabalin binds with high affinity to the alpha2-delta site (an auxiliary subunit of voltage-gated calcium channels) in central nervous system tissues. Although the mechanism of action of pregabalin is unknown, results with genetically modified mice and with compounds structurally related to pregabalin (such as gabapentin) suggest that binding to the alpha2-delta subunit may be involved in pregabalin's antinociceptive and antiseizure effects in animal models. In vitro, pregabalin reduces the calcium-dependent release of several neurotransmitters, possibly by modulation of calcium channel function. Studies also suggest that the descending noradrenergic and serotonergic pathways originating from the brainstem may be involved with the mechanism of pregabalin. Interestingly, although pregabalin is a structural derivative of inhibitory neurotransmitter gamma-aminobutyric acid (GABA), it does not bind directly to GABA or benzodiazepine receptors. The sodium channels, opiate receptors, and cyclooxygenase enzymes are not involved with the mechanism of pregabalin. It is also inactive at serotonin and dopamine receptors and does not inhibit dopamine, serotonin, or noradrenaline reuptake.

1.4 Pharmacodynamics

Pregabalin is a new anticonvulsant drug indicated as an add on therapy for partial onset seizures and for certain types of neuropathic pain. It was designed as a more potent successor to a related drug, gabapentin. Pregabalin binds to the alpha2-delta subunit of the voltage-gated calcium channel in the central nervous system. While pregabalin is a structural derivative of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), it does not bind directly to GABAA, GABAB, or benzodiazepine receptors, does not augment GABAA responses in cultured neurons, does not alter rat brain GABA concentration or have acute effects on GABA uptake or degradation. However, in cultured neurons prolonged application of pregabalin increases the density of GABA transporter protein and increases the rate of functional GABA transport. Pregabalin does
not block sodium channels, is not active at opiate receptors, and does not alter
cyclooxygenase enzyme activity. It is inactive at serotonin and dopamine receptors and
does not inhibit dopamine, serotonin, or noradrenaline reuptake

1.5 Pharmacokinetics

Pregabalin oral bioavailability is approximately 90% and it is well absorbed after
oral administration. Peak plasma concentrations are reached within 1.5 h. Following
repeated administration, steady state is achieved within 24 to 48 h. Pregabalin does not
bind to plasma proteins. The apparent volume of distribution of pregabalin following oral
administration is approximately 0.5 L/kg. Pregabalin is a substrate for system L
transporter, which is responsible for the transport of large amino acids across the blood
brain barrier. Pregabalin has been shown to cross the blood brain barrier in experimental
animals.

Pregabalin undergoes negligible metabolism in humans. Approximately 90% of
the administered dose can be recovered in the urine as unchanged pregabalin. Pregabalin
is eliminated from the systemic circulation primarily by renal excretion as unchanged
drug with a mean elimination half-life of approximately 6 h in subjects with normal renal
function. Overall, pregabalin demonstrates highly predictable and linear
pharmacokinetics, which makes it easy to use in clinical practice.

1.6 Absorption

Pregabalin is well absorbed after oral administration. When an oral administration
of pregabalin under fasting conditions is given, the pharmacokinetic parameters are as
follows: Tmax = 1.5 h; Oral bioavailability = >90% (independent of dose); Time to
steady state = 24-48 h. It is also a substrate for the L-type transport system

1.7 Metabolism

Pregabalin undergoes negligible metabolism in humans. Pregabalin does not bind
to plasma proteins and is excreted virtually unchanged (<2% metabolism) by the kidneys.
It is not subject to hepatic metabolism and does not induce or inhibit liver enzymes such
as the cytochrome P450 system [4].
1.8 Adverse Reaction

Most common adverse reactions (≥5% and twice placebo) are dizziness, somnolence, dry mouth, edema, blurred vision, weight gain and thinking abnormal (primarily difficulty with concentration/attention). Several renal failure patients developed myoclonus while receiving pregabalin, apparently as a result of gradual accumulation of the drug. Acute over-dosage may be manifested by somnolence, tachycardia and hypertonicity. Plasma, serum or blood concentrations of pregabalin may be measured to monitor therapy or to confirm a diagnosis of poisoning in hospitalized patients [5-7].

1.9 Macrocyclic Glycopeptide Based Chiral Stationary Phase

Glycopeptide antibiotics present a large, cyclic heptapeptide scaffold that is rich in aromatic fragments, surrounded by polar and ionizable groups and carrying carbohydrate moieties at the macrocycle periphery. These sugar units are attached through glycosidic bonds to phenolic or secondary hydroxyl groups of the aglycone. They play important roles in delivering the antibiotic to its target by enhancing its water solubility. The cyclic peptide backbone has a conformationally rigid cup-shaped architecture, with the aromatic fragments rigidly interlocked in a well-defined stereochemical disposition [8]. It is noteworthy the presence of one cis peptide bond, which is essential to keep the structures in a rigid macrocyclic form. Glycopeptides are soluble in water, buffers, and acidic aqueous solutions and less soluble at neutral pH. They are moderately soluble in polar aprotic solvents such as DMF and DMSO and are relatively insoluble in most organic solvents [9]. The chemistry, biology, and medicine of the glycopeptide antibiotics are discussed in detail in an attractive comprehensive review [10]. It is now well understood that all glycopeptide antibiotics exert antibiotic activity against Gram-positive bacteria because they stereospecifically bind to the precursor peptidoglycan peptide terminus N-acyl-d-alanyl-d-alanine produced during bacterial cell wall biosynthesis, thereby inhibiting the action of bacterial enzymes that would otherwise use these termini to form new cross-links in peptidoglycan [11].
Figure 2: Chemical structure of Ristocetin A

Nocardia lurida produces glycopeptides ristocetin A and B, differing in the number of sugar moieties attached to the heptapeptide backbone [12]. Ristocetin A consists of an aglycone portion with four joined macrocyclic rings, which contains seven aromatic rings (lettered A through G), none of which are chlorosubstituted. There are also a primary amine group, one methyl ester and four ionizable phenolic moieties. The sugar units are d-mannose, the amino sugar l-ristosamine, and a heterotetrasaccharide made of d-arabinose, d-mannose, d-glucose, and l-rhamnose (Fig. 2), attached to the peptide nucleus via d-glucose. Ristocetin A (C$_{95}$H$_{110}$N$_8$O$_{44}$) has a molecular mass of 2066 and contains 38 stereogenic centers. Ristocetin B lacks arabinose and mannose of the tetrasaccharide fragment, while the mannose residue directly attached to the heptapeptide scaffold is still in place [13]. Ristocetin A was the third macrocyclic antibiotic evaluated as selector for the synthesis of HPLC CSPs [14].
[2] Literature Overview

A through literature review, it has been observed that Pregabalin has been determined and studied by several procedures. Pregabalin has nearly no UV absorption and it is polar in nature, therefore most of the reports describe the pre-column derivatization methods as an indirect approach for optical purity measurement [16-20].

Xiaohui Chen, Daolin Zhang, Jie Deng and Ziaotai Fu have reported a new method for determining the optical purity of Pregabalin is developed. The method is based on Pregabalin and its isomer can be derivatized with N-5-fluoro-2,4-dinitrophenyl-5-L-alanine amide. These derivated compounds can be separated by an ordinary C-18 chromatography column. The method shown the satisfactory results for sensitivity, accuracy, and repeatability is satisfied.

Another report describes the normal phase method using Pregabalin analysis by LC-MS technique, which yields the advantage of avoiding the derivatization process and helps in achieving the high throughput. There are two published reports which described the LC-MS/MS analysis of pregabalin for achiral and chiral bio-analytical applications [21, 22].

V.V. Vaidya, S.M. Yetal, Shikha M. N. Roy, N.A. Games, S.S. Joshi have reported quantification method for pregabalin in human plasma using electrospray tandem mass spectrometry (LC-MS/MS). The assay of pregabalin was linear calibration curve over the range of 10.000-10000.000 ng and lower limit of quantification in plasma sample was 10.000 ng/mL.

Yizhong Zhang, C. Hollimn, Daniel Tang, Douglas Fast, and S. Michael have reported direct chiral method separation of Pregabalin from its (R)-enantiomer using HPLC-MS/MS. This method described the quantitative estimation of Pregabalin in rat kidney samples using Chirobiotic T chiral column.
Aim of Present Study

Pregabalin (S-enantiomer) has approximately 10-times more affinity for alpha2-delta binding than the R-enantiomer [23]. Therefore, only (S)-enantiomer is applied for clinical use and (R)-enantiomer is considered as an optical impurity and its concentration needs to be monitored and control in the final product. In order to increase awareness towards biologically important isomers, the US Food and Drug Administration has issued certain guidelines for the marketing of racemic compounds [24]. This new trend resulted in the determination of chiral impurities at a concentration below 0.1 % and this place heavy demand on the chiral analytical methods [25, 26].

There are various reports for the direct estimation of pregabalin in biological samples using hyphenated LC-MS/MS. There are many laboratories which have only single quadrupole LC-MS and can not procure such expensive LC-MS/MS. There is no published report for direct chiral estimation of pregabalin in formulation sample using single quadrupole LC-MS.

The major objective of this present work was to develop and validate the direct LC-MS method for direct enantioselective estimation of pregabalin in pharmaceutical formulation to support routine analysis of quality control pharmaceutical laboratories. This will avoid the traditional derivatization process to detect the pregabalin. The present work deals with the systematic method development for the direct separation of Pregabalin and (R) -enantiomer using macrocyclic glycopeptide chiral stationary phase (CSP). The method was validated for the parameters; eg. specificity, linearity, accuracy, precision, limit of detection and quantification.
[4] Experimental

4.1 Chemicals and Drugs

HPLC grade water, isopropylalcohol, ethanol, methanol and acetic acid were purchased from Merck. (S)-3-(aminomethyl)-5-methylhexanoic acid (pregabalin), (R)-3-(aminomethyl)-5-methylhexanoic acid, racemic compound and capsule formulation of pregabalin were obtained from local market.

4.2 High Performance Liquid Chromatography

The chiral separation was performed on an Agilent 1200 HPLC system consist of a quaternary pump, column oven, photo diode array detector and an auto injector. CHIROBITIC chiral columns were used for method development to separate pregabalin enantiomers. The HPLC system was controlled and analytical data were processed using Agilent ChemStation software (Version B.04).

4.3 Mass Spectrometry Conditions

Detection of enantiomers was achieved using a G6110A single quadrupole mass spectrometer (Agilent Technologies, U.S.A.) equipped with electrospray ion source. The analysis performed in positive mode with parameters mentioned in parentheses: fragmentator voltage (70V); capillary voltage (3500V); gas temperature (250°C); drying gas flow (nitrogen, 8 L/min); nebulizer pressure (nitrogen, 30psi). The pregabalin detection was performed in single ion monitoring (SIM) mode using mass-to-charge of 160.2 (M+H). The LC-MS system was controlled and analytical data were processed using Agilent LC-MSD ChemStation software (Version B.04.03).

4.4 Chromatographic Conditions

The enantiomeric separation was at 25°C column oven temperature using Chirobiotic R (250 × 4.6 mm, 5 µm particle size, Astec®) chiral column. The mobile phase consisted of methanol and isopropylalcohol (50:50, v/v). Mobile phase was chosen as the diluent to achieve clean blank chromatogram without any interference. Flow rate was 0.4 mL/min and injection volume was 0.1 µl. The run time was set to 25 min.
4.5 Preparation of Stock Solutions

Stock solutions of (R)-enantiomer, (S)-enatniomer (pregabalin) and racemic compound by dissolving appropriate amount of standard samples in to 5mL of methanol and further dilutions were made in diluent. A stock solution concentration was fixed at 500 µg/mL.

4.6 Preparation of Sample Solutions

For formulation sample, 4 capsules (25 mg of pregabalin label claim) were opened to a 50 mL volumetric. This was equivalent to 100 mg of pregabalin which was extracted into 40 mL of methanol by ultrasonication. The final volume has made up with methanol and the resultant mixture was filtered through a 0.45 µm membrane filter. This solution corresponds to analyte concentration of 500 µg/mL, and further dilutions were prepared in diluent. To perform the recovery study, the placebo corresponding to the capsule formulation was used.

4.7 Method Validation

4.7.1 Selectivity

Selectivity of a method refers to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other components in the mixture. The term specific generally refers to a method that produces a response for a single analyte only and if the response is distinguished from all other responses, the method is said to be selective. Selectivity of this method was indicated by the absence of any endogenous interference at retention times of enantiomeric peaks. The absence of interfering peak was evaluated by injecting a blank consisting of diluent and placebo.

4.7.2 Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision of the method was checked by analyzing six replicate samples of pregabalin (at analyte
concentration, i.e. 250.00 µg/mL) spiked with 0.1% (0.25 µg/mL) of (R)-enantiomer on different days and RSD of area under the peaks was calculated.

### 4.7.3 Linearity

Linearity corresponds to the capacity of the method to supply results directly proportional to the concentration of the substance being determined within a certain interval of concentration. Detector response linearity was assessed by preparing 12 calibration sample solutions covering from 0.12 to 250 µg/mL (0.12, 0.24, 0.49, 0.98, 1.96, 3.91, 7.81, 15.63, 31.25, 62.50, 125.00, 250.00 µg/mL), Regression curve was obtained by plotting peak area versus concentration, using the least squares method.

### 4.7.4 Sensibility

Lower limit of detection (LLOD) defined as lowest concentration of analyte that can be clearly detected above the base line signal, and was estimated at a signal to noise ratio of 3:1. Lower limit of quantification (LLOQ) defined as lowest concentration of analyte that can be quantified with suitable precision and accuracy, and was estimated at a signal to noise ratio of 10:1. LLOD and LLOQ were achieved by analyzing six injections of a series of dilute solutions, prepared for linearity study.

### 4.7.5 Accuracy

The accuracy of an analytical method is defined as the degree to which the determined value of analyte in a sample corresponds to the true value. The standard addition and recovery experiments of (R)-enantiomer in formulation were conducted to determine accuracy of the present method.

The study was carried out in triplicate by spiking placebo with three concentrations (500, 625 and 750 ng/mL) of standard (R)-enantiomer and assaying for the chromatographic method. The recovery for (R)-enantiomer was calculated from the slope and Y-intercept of the calibration curve, drawn in the concentration range of 0.24-250 µg/mL.
4.7.6 Ruggedness

Ruggedness is measure of reproducibility test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst. The Ruggedness of an analytical method is degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as; different laboratories, analysts, instruments, reagents, temperature, time etc. To determine the ruggedness, the recovery experiments were carried out for (R)-enantiomer in another laboratory by using a different HPLC instrument.

4.7.7 Robustness

To evaluate the robustness of the developed method, the experimental conditions were deliberately altered and the resolution between enantiomeric peaks was evaluated. To study the effect of flow rate on the resolution, the flow rate changed by 10 %, i.e. 0.36 and 0.44 mL/min from the actual flow rate of 0.4 mL/min. The effect of column temperature on resolution was studied at 22 and 28°C instead of 25°C. The effect of the mobile phase composition was checked by varying the composition ± 5 % from actual value and other mobile phase components were held constant as stated in HPLC conditions. The change in chromatographic resolution between enantiomers was evaluated for the study.

4.7.8 Solution stability

The sample was analyzed for 24 h at room temperature, i.e., at 25°C. Resolution and composition of (R)- and (S)- enantiomers were observed during the study period.
[5] Result and Discussion

5.1 Method Development and Optimization

Pregabalin is (3S)-3-(aminomethyl)-5-methylhexanoic acid. The racemic sample solution of 100 µg/mL concentration was used for the method development and optimization. To determine the λmax, the racemic solution was scanned between 200 to 400 nm using the UV diode array detector. As Pregabalin has no strong chromophoric groups, we did not find any λmax with proper U.V. response (Fig.3).

![Figure 3: UV spectra of Pregabalin](image)

Pregabalin has two hydrogen bond acceptor and one hydrogen bond donor. It has two pKa values, 4.2 and 10.6 corresponding to the carboxylic acid and the amine group respectively. It has a logP value of -1.35 and freely soluble in water.

All these properties make possible to detect Pregabalin by electrospray ionization mass spectrometry technique (ESI-MS). Pregabalin sample solution of 250 µg/mL concentration was used for the optimization of ESI-MS parameters. Monoisotopic mass of Pregabalin is 159.2 and in positive ion polarity we got 160.2 (M+H) as a molecular ion.
peak. In order to get the maximum sensitivity and lower detection limit, single ion monitoring (SIM) mode had chosen for this study (Fig.4).

![SIM spectra of Pregabalin (M+H)]

**Figure 4:** SIM spectra of Pregabalin (M+H)

The fragmentator voltage is very important parameter in ESI-MS and need to be optimized with respect to the compound of interest. In order to optimize the fragmentator voltage, the racemic solution was injected without column to ESI-MS. The abundance of 160.2 mass was monitored at different fragmentator voltages between 50 and 100 V.

<table>
<thead>
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<th>SN</th>
<th>Fragmentator Voltage (V)</th>
<th>Abundance of 160.2 (M+H)</th>
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</tr>
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**Table 1:** Results of fragmentator voltage optimization
The maximum response corresponding to M+H was found at 70 fragmentator voltage (Table 1).

5.1.1. Development and Optimization of Chromatographic Conditions

Pregabalin is an amino acid. It has two charged groups (a negatively charged carboxylic acid and positively charged amine) at neutral pH. Because of the charged groups, pregabalin is readily soluble in water. The direct chiral separation of amino acid enantiomers requires three simultaneous points of interaction between the functionalities of the amino acid and the stationary phase [27]. The separation is more effective when the analyte functionalities are adjacent to the chiral center as they are for α- and β-amino acids. The direct separation of pregabalin from its enantiomer has proven difficult because pregabalin is a γ-amino acid with both the amine and carboxylic functionalities separated from the chiral center by a methylene group.

Macrocyclic glycopeptide-based stationary phases were introduced in 1994 and have been used to separate an array of enantiomeric compounds that includes amino acids [28–37]. The macrocyclic glycopeptide provides a number of chiral centers, binding pockets, sites of aromaticity, and hydrogen donors/acceptors enabling a variety of interactions such as hydrophobic, hydrogen bonding, dipole, π–π, steric repulsion, and ionic. The large diversity and combinations of binding site greatly increases the likelihood that three points of simultaneous interaction can be achieved. A broad number of specificities can be interrogated on a single macrocyclic phase through systematic investigation of different mobile phase types, such as polar ionic, polar organic, normal phase, and reversed phase modes.

The objective of this study was to separate two enantiomers of the title compound, using a macrocyclic glycopeptide based chiral column. The racemic mixture of 250 µg/mL concentration was used for the method development. In order to achieve the enantiomer separation different chiral columns namely Chirobiotic R, Chirobiotic V, Chirobiotic T and Chirobiotic TAG were employed. There was poor enantiomeric separation on Chirobiotic T (Teicoplanin) column using a mobile phase consisting of methanol (Fig. 5).
Figure 5: Column: Chirobiotic T, Mobile phase: methanol

Figure 6: Column: Chirobiotic R, Mobile phase: methanol

There was a published report which describes the enantioselective study of pregabalin in bio-analytical filed using Chirobiotic T CSP, so Chirobiotic R CSP was
selected for our method development work. Chirobiotic R uses the Ristocetin antibiotic and has the greatest number of stereogenic centers (i.e. 37) within four fused macrocyclic rings [38]. Over 230 chiral separations achieved on Chirobiotic R column in three different modes were reported in a single paper [39]. The enantiomeric separation was better with Chirobiotic R (Ristocetins) compare to Chirobiotic T column using methanol as a mobile phase (Fig. 6). The effect of acid modifier on enantiomeric separation was tried by adding 0.01 % TFA in methanol. After adding acid in to the mobile phase, the peak shape becomes sharp but we lost the resolution due to the early evolution of the enantiomers (Fig.7).

![Figure 7: Column: Chirobiotic R, Mobile phase: methanol containing 0.01 % (v/v) TFA](image)

In order to increase the enantiomeric separation, the less polar alcohol i.e. ethanol and isopropylalcohol were tried in combination with methanol as a mobile phase. The separation was improved with combination of methanol and ethanol (50:50, v/v) but there was no base to base separation (Fig. 8). The addition of isopropylalcohol improved the
separation and enantiomeric resolution of 1.34 could achieve with a mobile phase combination of methanol and isopropylalcohol of 70:30, v/v (Fig. 9).

**Figure 8:** Column: Chirobiotic R, Mobile phase: methanol and ethanol (50:50, v/v)

**Figure 9:** Column: Chirobiotic R, Mobile phase: methanol and isopropylalcohol (70:30, v/v)
Optimum enantiomeric separation between (R)- and (S)- enantiomers could achieve with a combination of methanol and isopropylalcohol in a ratio of 50:50 (v/v). In final method the enantiomeric separation achieved at 25°C column oven temperatures using Chirobiotic R (250 × 4.6 mm, 5 µm particle size). Mobile phase was chosen as the diluent to achieve clean blank chromatogram without any interference. The flow rate was 0.4 mL/min and injection volume was 0.1 µL. In final method the typical retention time of both enantiomers was about 17 and 19 min (Fig. 10). In the final method, the resolution corresponding to enantiomeric peaks was 1.7.

Figure 10: Mobile phase: methanol and isopropylalcohol (50:50, v/v)  
Column: Chirobiotic R, Flow : 0.4 mL/min Column Temperature: 25 °C  
To identify the (R)- and (S)- enantiomers, the pregabalin has analyzed in developed chiral method. The undesired (R)-enantiomer was eluted first than the desired (S)-enantiomer, which was avoided the possible interference of major (S)-enantiomer in the enantiomer purity determination of pregabalin (Fig. 11).
Figure 11: Typical chromatogram of Pregabalin sample in final method

Figure 12: Typical chiral chromatogram of Pregabalin sample spiked with (R)-enantiomer
In order to have further confirmation of the retention time of enantiomers in final developed method, pure (S)-enantiomer (pregabalin) sample has spiked with undesired enantiomer, i.e. (R)-enantiomer at low concentration. The typical chiral HPLC chromatogram of pregabalin formulation sample spiked with (R)-enantiomer at 5 % w/w concentration level has shown in Fig. 12.

5.2 Results of Method Validation

5.2.1 Results of System suitability

The system suitability results are summarized in Table 2.

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<th>N</th>
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<td>5509</td>
<td>1.71</td>
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<td>Pregabalin</td>
<td>1.59</td>
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</table>

(T: USP tailing factor, N: number of theoretical plates, Rs: USP resolution, α: enantioselectivity)

Table 2: System suitability results.

5.2.2 Results of Selectivity

To evaluate the selectivity, the chromatogram obtained by analyzing the blank run consisting of diluent and placebo was compared in order to check the absence of any peaks likely to interfere at RTs of (S)- and (R)-enantiomers. The retention times of pregabalin and (R)-enantiomer were 17 and 19 minutes respectively.

As can be seen in overlay of racemic (LLOQ level) and blank chromatogram (Fig. 13), blank chromatograms is free from any interference at RTs of (R)- and (S)-enantiomers. The blank run was acquired by injecting the the placebo solution, prepared using the diluent.
5.2.3 Results of Method Precision

Repeatability was checked by analyzing six replicate resumes samples. Relative standard deviation (%RSD) of retention time and area under the peaks were calculated for (S)- and (R)- enantiomers. As can be seen in Table 3, the precision study results were also satisfactory, illustrating the excellent precision of the method.

The intermediate precision was determined in another laboratory by performing six successive injections. In the intermediate precision study, results showed that %RSD values were in the same order of magnitude than those obtained for repeatability. The results for method precision and robustness are summarized in Table 4.
### Precision Data

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*Table 3: Results of precision study*

### Intermediate Precision Data

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<td>3</td>
<td>15.09</td>
<td>396765</td>
</tr>
<tr>
<td>4</td>
<td>15.03</td>
<td>397634</td>
</tr>
<tr>
<td>5</td>
<td>14.78</td>
<td>398895</td>
</tr>
<tr>
<td>6</td>
<td>15.24</td>
<td>382532</td>
</tr>
<tr>
<td>Average</td>
<td>15.02</td>
<td>395507</td>
</tr>
<tr>
<td>SD</td>
<td>0.16</td>
<td>6455.91</td>
</tr>
<tr>
<td>%RSD</td>
<td>1.08</td>
<td>1.63</td>
</tr>
</tbody>
</table>

*Table 4: Results of intermediate precision study*

#### 5.2.4 Results of Linearity

The results show that good correlation existed between the peak area and concentration for both enantiomers (Fig.14). The described method was linear over the
concentration range from 0.12 to 250 µg/mL. The regression was found to be linear all over the wide concentration range and correlation coefficient values were 0.9980 and 0.9984 for (R) - enantiomer and pregabalin respectively (Table 5).

**Table 5**: Linearity results

<table>
<thead>
<tr>
<th></th>
<th>(R)-enantiomer</th>
<th>Pregabalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration Range (µg/mL)</td>
<td>0.12-250.00</td>
<td>0.12-250.00</td>
</tr>
<tr>
<td>Calibration Points</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Intercept</td>
<td>26528.6</td>
<td>21972.8</td>
</tr>
<tr>
<td>Slope</td>
<td>13019.7</td>
<td>13442.8</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.9980</td>
<td>0.9984</td>
</tr>
</tbody>
</table>

**Figure 14**: Linearity Chart for (R)-enantiomer and Pregabalin
5.2.5 Results of Sensibility

The LLOD and LLOQ concentration were estimated to be 240 and 120 ng/mL for both enantiomers respectively. The results are summarized in Table 6.

<table>
<thead>
<tr>
<th>LOD (ng/mL)</th>
<th>(R)-enantiomer</th>
<th>Pregabalin</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>7.4</td>
<td>6.2</td>
</tr>
<tr>
<td>LOQ (ng/mL)</td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>13.4</td>
<td>11.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 6: Results of Sensibility

5.2.6 Results of (R)-enantiomer Recovery in Pregabalin Formulation

The standard addition and recovery experiments were conducted to determine the accuracy of the present method. The study was carried out in triplicate by spiking placebo with three concentrations (500, 625 and 750 ng/mL) of standard (R) -enantiomer and assaying for the chromatographic method. The recovery for (R)-enantiomer was calculated from the slope and Y-intercept of the calibration curve, drawn in the concentration range of 0.24-250 µg/mL.

<table>
<thead>
<tr>
<th>Laboratory A</th>
<th>% Level of test concentration</th>
<th>Added (ng)</th>
<th>Recovered (ng)</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>500</td>
<td>455.5</td>
<td>91.1</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>625</td>
<td>613.8</td>
<td>98.2</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>750</td>
<td>720.8</td>
<td>96.1</td>
<td>8.6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Laboratory B</th>
<th>% Level of test concentration</th>
<th>Added (ng)</th>
<th>Recovered (ng)</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>500</td>
<td>463.5</td>
<td>92.7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>625</td>
<td>608.1</td>
<td>97.3</td>
<td>9.2</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>750</td>
<td>686.3</td>
<td>91.5</td>
<td>8.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Recovery result of (R)-enantiomer in pregabalin
The same recovery experiments were also conducted using a different system in laboratory B at the same concentration levels as tested in laboratory A and results were well in agreement. This confirmed the ruggedness of the method and the results of recovery study are summarized in Table 7.

5.2.7 Results of Solution Stability

The sample was analyzed for 24 h at room temperature, i.e., at 25°C. Resolution and composition of (R)- and (S)- enantiomers were observed during the study period. No significant change was observed in resolution and peak area composition of enantiomers during the solution stability study.

<table>
<thead>
<tr>
<th>Time interval (h)</th>
<th>% area bias</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(R)-enantiomer</td>
<td>Pregabalin</td>
</tr>
<tr>
<td>Initial</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>12</td>
<td>0.01</td>
<td>-0.03</td>
</tr>
<tr>
<td>18</td>
<td>-0.18</td>
<td>-0.19</td>
</tr>
<tr>
<td>24</td>
<td>-0.22</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Table 8: Results of solution stability study

The data are presented in Table 8, It can be seen from the data that % bias of area for enantiomers was less than 1% hence sample solution and mobile phase are stable for 24h at room temperature, i.e., at 25°C.

5.2.8 Results of Robustness

In robustness study, the racemic pregabalin sample was analyzed with change of different experimental conditions as a part of robustness study. The resolution between (R)- and (S)- enantiomer peaks were remain more than 1.65 for all deliberately changed chromatographic conditions and this confirmed the robustness of the method. The results are summarized in Table 8.
Table 8: Results of robustness study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Resolution between two enantiomers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (mL/min)</td>
<td></td>
</tr>
<tr>
<td>0.36</td>
<td>1.73</td>
</tr>
<tr>
<td>0.4</td>
<td>1.71</td>
</tr>
<tr>
<td>0.44</td>
<td>1.66</td>
</tr>
<tr>
<td>Column temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>1.72</td>
</tr>
<tr>
<td>25</td>
<td>1.72</td>
</tr>
<tr>
<td>28</td>
<td>1.71</td>
</tr>
<tr>
<td>Methanol content (% v/v)</td>
<td></td>
</tr>
<tr>
<td>52.5</td>
<td>1.68</td>
</tr>
<tr>
<td>50</td>
<td>1.72</td>
</tr>
<tr>
<td>48.5</td>
<td>1.72</td>
</tr>
</tbody>
</table>

[6] Conclusion

This report describes the development and validation of chiral HPLC method for direct enantioselective analysis of pregabalin in pharmaceutical formulation. To achieve baseline separation various glycopeptides based chiral stationary phase and combination of various alcohols have been studied. The baseline separation was achieved on Chirobiotic R column using mobile phase consisted of methanol and isopropylalcohol (50:50, v/v).

The method was validated showing satisfactory data for all the tested validation parameters and the method was found to be sensitive and linear over the thousand fold concentration range. The accuracy data proved that the developed method can be used for the direct quantitative determination of undesired (R)-enantiomer in pregabalin formulations (Fig. 16). This method can be used for routine pharmaceutical analysis in quality control laboratories.
Figure 16: Typical chiral chromatogram of Pregabalin capsule sample
[7] References


