A validated reverse phase chiral liquid chromatographic method for the enantiomeric purity determination of nateglinide in bulk drug samples and pharmaceutical dosage forms

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ABSTRACT

A simple isocratic reverse phase chiral HPLC method was developed for the enantiomeric purity determination of nateglinide in bulk drugs and dosage forms with a short run time of about 20 min. Chromatographic separation of nateglinide and its enantiomer was achieved on a bonded macro cyclic glycopeptides (Chirobiotic-T column (250×4.6) mm with 5μm particle size) stationary phase. Bonded macrocyclic glycopeptides stationary phase found to be enantioselective for L and D enantiomers of nateglinide with a resolution (R_s) of greater than 3.4. The mobile phase used was a mixture of buffer and acetonitrile in the ratio of 70: 30 (v/v). Buffer consists of 5 mM of tetra-n-butyl ammonium hydrogen sulfate, pH adjusted to 3.5 using diluted ammonia solutions (1 in 10). The test concentration is 1.0 mg mL⁻¹ in diluent (6:4 (v/v) acetonitrile and water). This method is capable of detecting the L-nateglinide up to 0.06μg wrt test concentration of 1000μg mL⁻¹ for a 10μL injection volume. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal degradation. There is no interference of degradants with D-nateglinide and L-nateglinide. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness. The percentage recovery of L-nateglinide of nateglinide in bulk drug samples and in dosage forms ranged from 93.0 to 102.0%. The test solution was found to be stable in the diluent for 48h after the preparation.

INTRODUCTION

The chemical name of nateglinide is (3R, 4S)-1-(4-flourophenyl)-3-[(3S)-3- (4-flourophenyl)-3-hydroxy propyl]-4-(4-hydroxyphenyl)-2-azetidinone (Figure 1) is non-sulfonylurea oral antidiabetic drug for the treatment of type II diabetes mellitus. nateglinide can reportedly stimulate a rapid, transient secretion of insulin from the pancreatic -cell, which is dependent on ambient glucose concentrations. An oral dose of 1.6 mg kg⁻¹ D-nateglinide can induce a 20% decrease in blood glucose, whereas approximately 100 mg kg⁻¹ of

KEYWORDS

Chiral HPLC; Enantiomeric purity; Validation and quantification; Forced degradation; nateglinide.
L enantiomer is required for equal potency. Few analytical methods were available in literature for the separation of L-nateglinide from D-nateglinide. According to the work of Sinkai Etal., enantiomer of nateglinide can be separated on a sumichiral OA-3000 column but the samples need to be derivatized before injection. This method has some limitations, the derivatization agent is very expensive and the derivatisation reaction needs vigorous control.

Based on the work of Meling Qi, the enantiomers of nateglinide can be resolved on a chiralcel OD-R column. The retention times of enantiomers (L-nateglinide and D-nateglinide) are about 41.1 min and 43.3 min, resolution is about 1.5 and flow rate is 0.2 mL min⁻¹. The disadvantage of the method is flow rate is not consistent at the level of 0.2 mL min⁻¹ and it takes almost 1 hr to analyze nateglinide sample to determine the other enantiomer (L-nateglinide).

The work contributed by Yang G liiz Etal., resolves the enantiomers of nateglinide on a sumichiral OA-3000 column, the disadvantage of the method was the resolution between the enantiomers was greater than 3.0 at very low concentration levels only. The separation (R >1.0) between L-nateglinide (at a level of 0.15%) and D-nateglinide (higher level of 100%) was less and difficult to quantify the L-nateglinide in D-nateglinide at low levels of L-nateglinide.

Molecular imprinted polymer has been prepared by non-covalent imprinting for the separation of L-nateglinide from D-nateglinide. However, these columns have to be specifically synthesized as they are not commercially available.

Moreover, an enantioselective method for the separation of nateglinide enantiomers in rat intestinal sacs has been described, shows separation of the enantiomers at very low concentration levels to investigate the enantiomers in intestinal samples. No separation between L-nateglinide (at a level of 0.15%) and D-nateglinide (higher level of 100%).

Keeping all the disadvantages in view, a simple and rugged RP-LC method with a shorter runtime of 20 min was developed. This paper describes the enantio-meric purity determination of nateglinide for accurate quantification of L-nateglinide in nateglinide with a resolution (Rₚ) of 3.4 in bulk samples and in pharmaceutical dosage forms.

**EXPERIMENTAL**

**Chemicals**

Samples of nateglinide and L-nateglinide were obtained from Dr.Reddy’s Laboratories Ltd, Hyderabad, India. Commercially available 60mg nateglinide tablets were purchased. HPLC grade acetonitrile was purchased from Merck, Darmstadt, Germany. Analytical reagent grade 35% ammonia solution and tetra-n-butyl ammonium hydrogen sulfate were purchased from Merck, Darmstadt, Germany. High purity water was prepared by using Millipore Milli-Q plus water purification system.

**Equipment**

The HPLC system employed in the chiral method development and validation was Agilent 1100 series (Agilent Technologies, Hewlett-Packard Strasse 8, Waldbronn, BW76337 GERMANY) LC system with a dual wavelength detector. The output signal was monitored using empower software (Waters) on pentium computer (Digital Equipment Co., Houston, USA). For the ruggedness and specificity studies, waters (waters Corporation, 34 Maple Street, Milford MA 01757) 2695 Photo diode array detector (PDA) was used. The output signal was monitored using millenium software (Waters) on pentium computer (Digital Equipment Co., Houston, USA). The chirobiotic T column was purchased from Astec Advanced separation technologies, 37 Leslie Court, Post Office Box 297, Whippany, NJ 07981 USA.
Chromatographic conditions

The analysis was carried out on chirobiotic T column, (250.4 6) mm with 5 μm particle size. The mobile phase contains a mixture of buffer and acetonitrile in the ratio of 70: 30: (v/v). Buffer consists of 5 mM of tetra-n-butyl ammonium hydrogen sulfate, pH adjusted to 3.5 using diluted ammonia solution (1 in 10).

The flow rate of the mobile phase was 1.0 mL min⁻¹. The column temperature was maintained at 27°C and the detection was monitored at a wavelength of 210 nm. The injection volume was 10 μL. acetonitrile: water (6:4 v/v) was used as diluent.

Preparation of solutions

1. Preparation of standard solutions

Stock solutions of D-nateglinide and L-nateglinide (1000 μg mL⁻¹) were prepared individually by dissolving appropriate amount in the diluent. A stock solution of sample and impurity mixture was prepared at 500 μg mL⁻¹ in diluent.

2. Preparation of sample solution

Twenty tablets were individually weighed to get the average weight of the tablets and powered in mortar. A sample of the powered tablets, claimed to contain 100 mg of nateglinide was transferred to 100 mL volumetric flask. About 75 mL of diluent was added and kept on rotatory shaker for 10 min to disperse the material completely and sonicated for 10 min and diluted to 100 mL. The content was centrifuged for 10 min at 3,000 rpm. The supernatant was collected and filtered using 0.45 μm nylon 66-membrane filter.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Forced degradation studies provide an indication of the stability indicating property and specificity of the proposed method. The stress conditions employed for degradation study includes light (carried out as per ICH Q1B), heat (60°C), acid hydrolysis (0.1N HCl), base hydrolysis (0.1 N NaOH and 0.1N NaHCO₃), water hydrolysis and oxidation (3% H₂O₂). For heat and light studies, study period was 10 days whereas for acid, base, water hydrolysis and oxidation, it was 48 h. Peak purity of stressed samples of nateglinide was checked by using 2996 Photo diode array detector of Waters (PDA).

Method validation

As per the ICH guidelines the method was validated in terms of following parameters⁹,¹⁰.

1. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements from multiple sampling of the same homogeneous sample under prescribed conditions. The precision of the developed method was checked by injecting six individual preparations of (1000μg mL⁻¹) nateglinide spiked with 0.15% L-nateglinide. The %RSD of area for L-nateglinide was calculated.

The intermediate precision of the method was also evaluated on different lot of column, on a different instrument, by different analyst on the same instrument, in different laboratories and the %RSD for six individual spiked preparations was calculated.

2. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for L-nateglinide was estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. The precision study was also carried out at the LOQ level by injecting six individual preparations of L-nateglinide and calculated the % RSD for the area.

3. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte sample. Linearity solutions were prepared by diluting the impurity stock solution (1000 μg mL⁻¹) to the required concentrations. The solutions were prepared at nine concentration levels from LOQ to 200% of the permitted maximum level of impurity wrt to 1000 μg mL⁻¹ of test concentration. (LOQ, 0.03 %, 0.045 %, 0.06 %, 0.075 %, 0.1125 %, 0.15 %, 0.1875%, 0.225 % and 0.30 %). The correlation coefficient, slope and Y-intercept of the calibration curve was reported.

4. Accuracy

Standard addition and recovery experiments were
RESULTS AND DISCUSSION

Method development and optimization

The main target of this work is to evaluate the enantiomeric purity of nateglinide and the accurate quantification of the undesired L-nateglinide. The racemic mixture of D and L enantiomers were used during the method development. The preliminary trails were carried on normal phase chiral columns were not fruitful in the separation of these enantiomers. In chiral AGP Column separation was observed, but the tailing of D-nateglinide is more than 2.0 and the resolution between L-nateglinide and D-nateglinide is very less (<1.5). In chirobiotic-T column (250x4.6) mm with 5μm particles no separation was observed with buffer and ethanol combination. Buffer and acetonitrile combination resolved the D and L-nateglinide peaks. When water and acetonitrile (700:300 v/v) was used as mobile phase, the separation was observed and the resolution between D-nateglinide and L-nateglinide was poor (less than 1.5). Introduction of ion pair agent (0.005 M tetra-n-butyl ammonium hydrogen sulfate) played significant role in achieving the separation between L-nateglinide and D-nateglinide. The ion pair reagent (Tetra-n-butyl ammonium hydrogen sulfate) and the stationary phase are found to be crucial in getting the separation between L and D-nateglinide. When pH was adjusted to 7.2 the resolution between L-nateglinide and D-nateglinide is very less (less than 1.4), when pH increased towards acidic side resolution increases and satisfactory resolution observed at pH 3.5. When the quantity of the acetonitrile increases, resolution between L-nateglinide and D-nateglinide decreases. On the other hand by decreasing the quantity of acetonitrile the retention time increased 30-40 min and the resolution too. Satisfactory resolution was obtained with buffer and acetonitrile in the ratio 70:30 (v/v). Detailed method development results were given in TABLE 1.

Satisfactory chromatographic separation was achieved on on chirobiotic-T column (250x4.6) mm with 5μm particles column, by using the mobile phase contains buffer and acetonitrile in the ratio of 70:30 (v/v). Buffer consist of 0.005 M tetra-n-butyl ammonium hydrogen sulfate, pH adjusted to 3.5 using ammonia solution. The flow rate of the mobile phase was
1.0 mL min\(^{-1}\) at 27°C column temperature. The system suitability results were given in TABLE 2. Analysis was performed for different batches of bulk drug samples.

### TABLE 2: System suitability report

<table>
<thead>
<tr>
<th>Name</th>
<th>Retention time (t(_r)) in min</th>
<th>Resolution (R(_S)) by tangent method (USP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Nateglinide</td>
<td>7.3</td>
<td>3.46</td>
</tr>
<tr>
<td>D-Nateglinide</td>
<td>9.4</td>
<td>-</td>
</tr>
</tbody>
</table>

### TABLE 3: Results of accuracy study for drug substance

<table>
<thead>
<tr>
<th>Added ((\mu g/ n=6))</th>
<th>Recovered ((\mu g))</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375</td>
<td>0.362</td>
<td>96.5</td>
<td>0.8</td>
</tr>
<tr>
<td>0.75</td>
<td>0.741</td>
<td>98.8</td>
<td>0.7</td>
</tr>
<tr>
<td>1.125</td>
<td>1.098</td>
<td>97.6</td>
<td>0.7</td>
</tr>
<tr>
<td>1.5</td>
<td>1.503</td>
<td>100.2</td>
<td>0.4</td>
</tr>
<tr>
<td>1.875</td>
<td>1.864</td>
<td>99.4</td>
<td>0.6</td>
</tr>
<tr>
<td>2.25</td>
<td>2.286</td>
<td>101.6</td>
<td>1.2</td>
</tr>
</tbody>
</table>

n = 6, Number of determinations

### TABLE 4: Results of robustness study

<table>
<thead>
<tr>
<th>S.no</th>
<th>Parameter</th>
<th>Variation</th>
<th>Resolution between L-Nateglinide and D-Nateglinide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Temperature</td>
<td>(a) At 22°C (b) At 32°C</td>
<td>3.5 (3.3)</td>
</tr>
<tr>
<td>2</td>
<td>Flow rate</td>
<td>(a) At 0.8 mL/min (b) At 1.2 mL/min</td>
<td>3.5 (2.8)</td>
</tr>
<tr>
<td>3</td>
<td>Different buffer pH</td>
<td>(a) At 3.3 (b) At 3.7</td>
<td>3.5 (3.0)</td>
</tr>
<tr>
<td>4</td>
<td>Different organic ratio</td>
<td>(a) At 90% (b) At 110%</td>
<td>3.5 (3.0)</td>
</tr>
</tbody>
</table>

### TABLE 5: Results of robustness study

<table>
<thead>
<tr>
<th>S.no</th>
<th>Parameter</th>
<th>Variation</th>
<th>Resolution between L-Nateglinide and D-Nateglinide</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Temperature</td>
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<tr>
<td>2</td>
<td>Flow rate</td>
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<td>3.5 (2.8)</td>
</tr>
<tr>
<td>3</td>
<td>Different buffer pH</td>
<td>(a) At 3.3 (b) At 3.7</td>
<td>3.5 (3.0)</td>
</tr>
<tr>
<td>4</td>
<td>Different organic ratio</td>
<td>(a) At 90% (b) At 110%</td>
<td>3.5 (3.0)</td>
</tr>
</tbody>
</table>
(n=3) and for pharmaceutical dosage forms (n=3). Results were given in TABLE 8. Stability study results as per ICH Q1A (R2) for nateglinide were given in TABLE 9 and TABLE 10.

Method validation

1. Precision

The %RSD for the area of L-nateglinide under precision study was found to be within 1.5 % confirming the good precision of the method.

The %RSD for L-nateglinide in intermediate precision study was within 2.0 confirming the ruggedness of the method. (TABLE 7).

2. Limit of detection and limit of quantification

The limit of detection for L-nateglinide was 0.06µg

3. Linearity

Linear calibration plot for L-nateglinide was obtained over the calibration ranges tested, i.e. LOQ to 0.3 % The correlation coefficient obtained was greater than 0.999. Slope and Y-Intercept values are 171.42 and 165.34 respectively. Linearity was checked for the enantiomeric purity method over the same concentration range for 3 consecutive days. The percentage of RSD, values of the slope and Y-intercept of the calibration curves were 3.5 and 6, respectively. The results show that an excellent correlation existed between the peak area and concentration for L-nateglinide.

4. Accuracy

The percentage recovery of L-nateglinide in bulk drug samples ranged from 96.0 to 102.0 (TABLE 3)
496 Enantiomeric purity determination of nateglinide

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5. Robustness

In all the deliberate varied chromatographic conditions carried out (flow rate, pH, mobile phase composition and column temperature), the resolution between the L-nateglinide and D-nateglinide was greater than 3.0, illustrating the robustness of the method (TABLE 5).

Solution stability and mobile phase stability

No significant changes were observed in the content L-nateglinide during solution stability and mobile phase experiments when performed using the developed enantiomeric purity method. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during the study was stable up to 48 h.

Results of forced degradation studies

Degradation was not observed in nateglinide stressed samples that were subjected to light, heat study and water hydrolysis. The degradation of drug substance was observed under acid, base hydrolysis, and oxidative conditions. nateglinide is highly sensitive towards base and it was degraded completely with 0.1N NaOH treatment. So the degradation was carried out in 0.05N NaHCO₃. The purity angle is within the purity threshold limit obtained in all stressed samples demonstrates the analyte peak homogeneity. There is no interference of degradants with D-nateglinide and L-nateglinide (TABLE 6).

CONCLUSIONS

A simple linear and accurate reverse phase chiral HPLC method was described for the enantiomeric purity evaluation of nateglinide on bonded macrocyclic glycopeptides. Chirobiotic-T column was found to be specific for the D and L enantiomers of nateglinide. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is robust in the quantification of L-nateglinide of nateglinide up to 0.16μg mL⁻¹ and can be used for the routine analysis of production samples.

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