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A Validated Stability Indicating LC Method for Nateglinide

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ABSTRACT A simple, isocratic, rapid and accurate reverse phase high-performance liquid chromatography (RP-HPLC) method was developed for the quantitative determination of Nateglinide. The developed method is also applicable for determination of related substance in bulk drugs. The chromatographic separation was achieved on a Hypersil C18 (250 × 4.6 mm 5 μm) column using aqueous mixture of 0.025 M potassium hydrogen phosphate and 0.1% triethyl amine, v/v (pH 3.0 with dilute phosphoric acid)–methanol (25:75, v/v) as a mobile phase. Solution concentrations were measured on a weight basis to avoid the use of an internal standard. The chromatographic resolutions between Nateglinide and its potential impurities A and B were found to be greater than four. Forced degradation studies were performed for Nateglinide using acid (0.5 N hydrochloric acid), base (0.5 N sodium hydroxide), oxidation (3% hydrogen peroxide) heat (60°C) and UV light (254 nm). The limit of detection and limit of quantification of Nateglinide, impurities A and B were found to be 0.05 and 0.15 μg /mL, respectively for 20 μL injection volume. The percentage recovery of Nateglinide was ranged from 98.4 to 100.9. The percentage recovery of impurities in Nateglinide sample was ranged from 96.8 to 103.5. The developed RP-HPLC method was validated with respect to linearity, accuracy, precision, robustness, and forced degradation studies prove the stability indicating power of the method.

KEYWORDS Nateglinide, Validation, Forced degradation, RP-LC, Solution and mobile phase stability

INTRODUCTION

Nateglinide [*N*-(*trans*-4-isopropylcyclohexylcarbonyl)-D-phenylalanine] (Fig. 1) is a novel, highly physiologic, mealtime glucose regulator approved for the treatment of type II diabetes mellitus. Nateglinide has a rapid onset and short duration of insulinotropic action that results in reduction of mealtime glucose rise and lowers the postabsorptive potential for hypoglycemia in humans and experimental animals (Weaver et al., 2001).

Nateglinide is an oral hypoglycemic agent approved for use alone or in combination with metformin as an adjunct to diet and exercise for the treatment of type 2 diabetes mellitus. Nateglinide, an amino acid derivative of D-phenylalanine, stimulates the secretion of insulin by binding to the ATP potassium channels in pancreatic beta cells. The result is an increase in beta-cell

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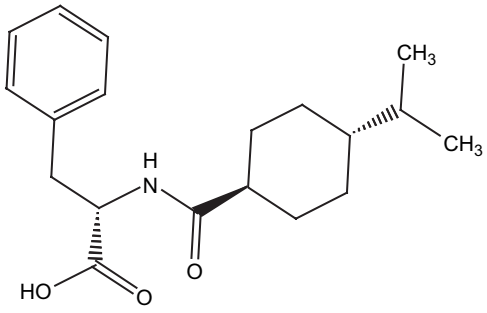
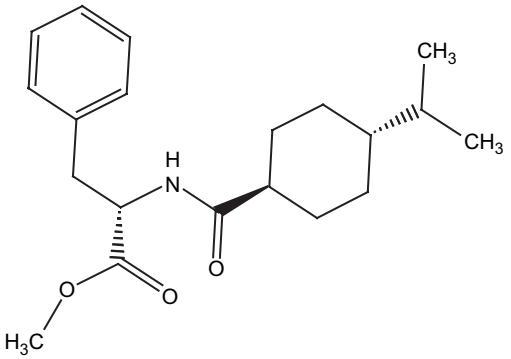
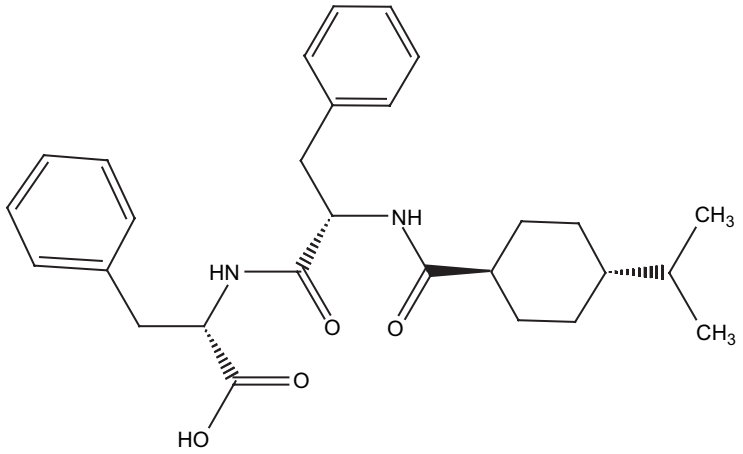


2-[(trans-4-Isopropyl-cyclohexanecarbonyl)-amino]
- 3-phenyl-propionic acid
Nateglinide



2-[(trans-4-Isopropyl-cyclohexanecarbonyl)-amino]
- 3-phenyl-propionic acid methyl ester
Impurity B



2-{2-[(trans-4-Isopropyl-cyclohexanecarbonyl)-anino]-3-phenyl-propionylamino}
-3-phenyl-propionic acid Impurity A

FIGURE 1 **Chemical Structures of Nateglinide, Impurities A and B.**

calcium influx, which leads to rapid, short-lived insulin release (Halas, 2001).

A literature survey revealed a few high-performance liquid chromatographic (HPLC) methods for determination of Nateglinide in human plasma (Ono et al., 1997; Bauer et al., 2003). The HPLC method for the determination of Nateglinide in tablet formulation (Meiling et al., 2002) and chiral reversed-phase liquid chromatographic method for the determination of the L-enantiomer of Nateglinide in a bulk drug substance (Qi et al., 2003). So far, to our present knowledge, no stability indicating analytical method for Nateglinide was available in literature. It was, therefore, felt necessary to develop a stability indicating LC method for the related substance determination and quantitative estimation of Nateglinide.

This paper deals with the forced degradation of Nateglinide under stress condition like acid hydrolysis, base hydrolysis, oxidation, heat, and UV light. This paper also deals with the assay and related substances method validation for accurate quantification of Nateglinide and its impurities.

EXPERIMENTAL

Chemicals

Samples of Nateglinide and its two potential impurities namely impurity-A impurity-B (Fig. 1) were received Veritec Paharma Ltd., Hyderabad, potassium hydrogen phosphate, orthophosphoric acid and HPLC grade acetonitrile were purchased from Merck (Darmstadt, Germany). High pure water was prepared by using Millipore Milli Q plus purification system.

Equipment

Quantitative analysis was performed on a high-performance liquid chromatography (HPLC) which consisted of a dual piston reciprocating two LC-10AT VP pumps from Shimadzu Crop. Japan (model HPLC class 10AT), photo-diode array detector from Shimadzu Crop. Japan (model SPD-10M VP) and auto sampler of SCL-10A series. The HPLC system was equipped with data acquisition and processing software "LC Solution" Shimadzu Crop. Japan.

Sample Preparation

A stock solution of Nateglinide (5000.0 µg/mL) was prepared by dissolving appropriate amount substance in Mobile phase. Working solutions of 500 and 50 µg/mL were prepared from above stock solution for related substances determination and assay determination, respectively. A stock solution of impurities (mixture of A and B) at 500 µg/mL was also prepared in mobile phase.

Chromatographic Conditions

The experiment was performed on a Hypersil C18 (250 mm × 4.6 mm 5 µm) column. The mobile phase was aqueous mixture of 0.025 M Potassium dihydrogen phosphate and 0.1% triethyl amine, v/v (pH 3.0 with dilute ortho phosphoric acid)–methanol (25:75, v/v).The mobile phase was filtered through a nylon membrane (pore size 0.45 µm) and degassed with a helium spurge for 10 min. The chromatography was performed at room temperature using a flow rate of 1 mL/min. The column temperature was maintained at 25°C and eluents were monitored at a wavelength of 215 nm. The volume of each injection was 20 µL.

VALIDATION OF THE METHOD

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities (ICH, 2000). Testing of the drug substance under stress conditions can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule.

The specificity of the developed LC method for Nateglinide was determined in the presence of its impurities, namely impurities A, B, and degradation products. Intentional degradation was attempted to stress condition of UV light (254 nm), heat (60°C), acid (0.5 N HCl), base (0.5 N NaOH), and oxidation (3.0% H_2O_2) to evaluate the ability of the proposed method to separate Nateglinide from its degradation product (ICH, 2000). For heat and light studies, study period was 10 days whereas for acid, base, and oxidation, it was 48 hr. Peak purity test was performed for Nateglinide peak by using PDA detector in stress samples. Assay studies were performed for stress samples against qualified Nateglinide reference standard. Assay also calculated for Nateglinide bulk sample by spiking both impurities (A and B) at the specification level (i.e., 0.15%).

Precision

Intra Assay Precision

Intra Assay precision was evaluated by performing six independent assays of the Nateglinide at three-concentration levels 80, 100 and 120% of assay analyte concentration i.e., 40, 50 and 60 µg/mL on the same day against qualified reference standard and calculating the % CV of assay.

The intra assay precision of the related substance method was checked by injecting six individual preparations of Nateglinide (500 µg/mL) spiked with 0.075, 0.15, and 0.225% each of impurity–A and B with respect to Nateglinide analyte concentration on the same day and calculating the % CV of impurities A and B.

Inter Assay Precision

Inter assay precision was evaluated by performing six independent assays of the Nateglinide at three-concentration levels 80, 100 and 120% of assay analyte concentration i.e., 40, 50, and 60 µg/mL for 6 days against qualified reference standard and calculating the % CV of assay.

The intra assay precision of the related substance method was checked by injecting six individual preparations of Nateglinide (500 µg/mL) spiked with each of impurity–A and B at 0.12, 0.15, and 0.18% with respect to Nateglinide analyte concentration for 6 days and calculating the % CV of impurities A and B.

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

The LOD and LOQ for Nateglinide, impurities A and B were estimated at a signal-to-noise ratio of 3:1 and

10:1, respectively, by injecting a series of dilute solutions with known concentration (ICH, 1995). Precision study was also performed at the LOQ level by injecting six individual preparations of Nateglinide, impurities A and B and by calculating the % CV of the area.

Accuracy

The accuracy of the assay method was evaluated in triplicate at three-concentration levels i.e., 25, 50, and 75 μg/mL in bulk drug sample. The percentage of recoveries was calculated from slope and Y-intercept of the calibration curve obtained. Accuracy/recovery experiments were performed in triplicate.

The bulk sample showed the presence of 0.02% of impurity A. Standard addition and recovery experiments were conducted to determine accuracy of the related substance method for the quantification of impurities A and B in Nateglinide samples.

The study was performed in triplicate at 0.075, 0.15, and 0.225% of the analyte concentration (500 μg/mL). The percentage of recoveries for impurities A and B were calculated from the slope and Y-intercept of the calibration curve obtained.

Linearity

Linearity test solutions for assay method were prepared from stock solution at six concentration levels from 50 to 150% of assay analyte concentration (25, 37.5, 50, 62.5, and 75 μg/mL). The peak area versus concentration data was performed by least-squares linear regression analysis.

Linearity test solutions for related substance method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 200% (1.5 μg/mL) of the specification level (LOQ, 0.075, 0.15, 0.187, 0.225, and 0.3%).

Linearity test was performed for 3 consecutive days in same concentration range for both assay and related substance method. The % R.S.D. value of the slope and Y-intercept of the calibration curve was calculated.

Robustness

To determine the robustness of the developed method, experimental conditions were purposely altered and the resolution between Nateglinide and impurity A was evaluated.

The flow rate of the mobile phase was 1.0 mL/min. To study the effect of flow rate on the resolution, it was changed by 0.2 units from 0.8 to 1.2 mL/min. The effect of the column temperature on resolution was studied at 20 and 30°C instead of 25°C. The effect of the percent organic strength on resolution was studied by varying acetonitrile by −3 to +3% while other mobile phase components were held constant.

Solution Stability and Mobile Phase Stability

The solution stability of Nateglinide in the assay method was performed by leaving both the test solutions of the sample and reference standard in tightly capped volumetric flasks at room temperature for 48 hr. The same sample solutions were analyzed for 6 hr interval up to the study period. Furthermore, mobile phase stability was also performed by analyzing the freshly prepared sample solutions against freshly prepared reference standard solution for 6 h interval up to 48h. Mobile phase prepared was kept constant during the study period. The % RSD for the assay of Nateglinide was calculated during mobile phase and solution stability experiments.

The solution stability of Nateglinide and its impurities in the related substance method was performed by leaving spiked sample solution in tightly capped volumetric flask at room temperature for 48 hr. Content of impurities A and B were determined for every 6 hr interval up to the study period. Mobile phase stability was also performed for 48 hr by injecting the freshly prepared sample solutions for every 6 hr interval. Content of impurities A and B were checked in the test solutions.

RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

The main target of the chromatographic method is to get the separation of critical closely eluting impurities, namely A and B. Impurities were coeluted by using different stationary phases like C18, C8, phenyl, and cyano and different mobile phases. The chromatographic separation was achieved on a Hypersil C18 (250 mm × 4.6 mm 5 μm) column using aqueous mixture 0.025 M Potassium hydrogen phosphate and 0.1% triethyl amine,

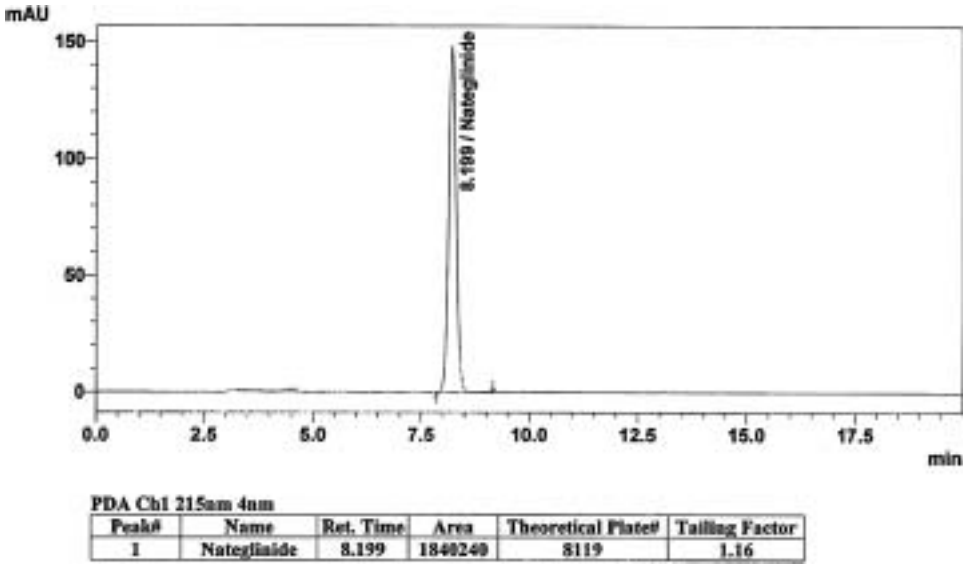


FIGURE 2 **Typical HPLC Chromatogram of Nateglinide (Unspiked) Sample.**

v/v (pH 3.0 with dilute phosphoric acid)–methanol (25:75, v/v) as a mobile phase. The flow rate of the mobile phase was 1.0 mL/min, at 25°C column temperature, the peak shape of Nateglinide was found symmetrical. In optimized chromatographic conditions Nateglinide, impurities A and B were well separated with resolution grater than 4, typical retention time were about 8.2, 11.0, and 13.2 min, respectively (Fig. 2). The developed LC method was found to be specific for Nateglinide and its two impurities.

Results of Forced Degradation Studies

Degradation was not observed in Nateglinide stressed samples that were subjected to light, heat, base and peroxide. Nateglinide was degraded during acid hydrolysis. Peak purity test results confirmed that the Nateglinide peak is homogenous and pure in all the analyzed stress samples. The assay of Nateglinide is unaffected in the presence of impurities A and B and its degradation products confirm the stability indicating power of the developed method. The summary of forced degradation studies is given in Table 1.

TABLE 1 **Summary Report of Forced Degradation Study**

Stress condition	Degradation time	% Assay of Nateglinide
Acid hydrolysis (0.5 N HCl)	48 hr	97.2
Base hydrolysis (0.5N NaOH)]	48 hr	99.6
Oxidation (3% H_2O_2)	48 hr	99.8
Thermal (60°C)	10 days	99.2
UV (254 nm)	10 days	99.4

TABLE 2 **Intra Assay Precision of Nateglinide**

Concentration (μg) (*n* = 6)	% Concentration	% Assay	% CV
40	80	79.9	1.0
50	100	98.8	1.4
60	100	119.8	1.7

n = Six determinations.

TABLE 3 **Inter Assay Precision of Nateglinide**

Concentration (μg) (*n* = 6)	% Concentration	% Assay	% CV
40	80	80.2	1.0
50	100	98.7	1.9
60	100	110.1	2.5

n = Six determinations.

Precision

The intra and interassay coefficient of variation for assay of Nateglinide during assay method precision study were within 1.7 and 2.6%, respectively. (Tables 2 and 3).

The intra assay and inter assay coefficient of variation for impurities A and B in related substance method precision study were within 3.4 and 3.8%, respectively, conforming good precision of the method.

Limit of Detection and Limit of Quantification

The limit of detection and limit of quantification of Nateglinide was 0.05 μg\mL and 0.15 μg\mL for 20 μL injection volume. The coefficient of variation of Nateglinide area at LOQ concentration was below 4.0%.

The limit of detection of Nateglinide, impurities A and B were 0.05 μg\mL for 20 μL injection volume. The limit of quantification of impurities A and B were 0.15 μg\mL for 20 μL injection volume. The coefficient of variation at LOQ concentration for impurities A and B were below 4.9%.

Accuracy

The percentage recovery of Nateglinide in bulk drug samples was ranged from 98.5 to 101.2 (Table 4).

TABLE 4 **Recovery Result of Nateglinide Sample**

Added (μg) (n = 3)	Recovered (μg)	% Recovery	% RSD
26	25.6	98.5	0.8
51	51.6	101.2	0.7
75	74.4	99.2	0.5

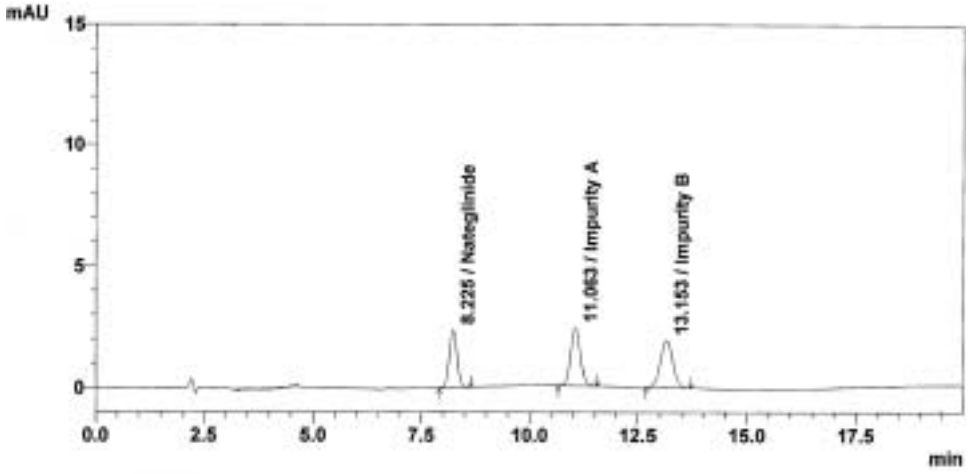
n = Number of determinations.

The percentage recovery of impurities A in Bulk sample was ranged from 97.8 to 102.4. The percentage recovery of impurities B in bulk samples ranged from 96.7 to 101.3. HPLC chromatogram of spiked with impurities A and B in Nateglinide bulk sample are shown in Fig. 3.

Linearity

Linearity calibration plot for assay method was obtained over the calibration ranges tested, i.e., 25–75 μg/mL (Table 5) and correlation coefficient obtained was greater than 0.999 (Fig. 4) Linearity was checked for assay method over the same concentration range for 3 consecutive days. The % RSD values of the slope and Y-intercept of the calibration curves were 1.6 and 2.4, respectively. The result shows that an excellent correlation existed between the peak area and concentration of the analyte.

Linear calibration plot for related substance method was obtained over the calibration ranges tested, i.e., 0.15 μg\mL (LOQ) to 1.5 μg\mL for impurities A and B. The correlation coefficient obtained was greater than 0.998. Linearity was checked for related substance method over the same concentration range for 3 consecutive days. The % RSD values of the slope and Y-intercept of the calibration curves were 3.1 and 2.6, respectively. The result shows that an excellent correlation existed between the peak area and concentration of impurities A and B.



PDA Ch1 215nm 4nm

Peak#	Name	Ret. Time	Area	Resolution	Theoretical Plate#	Tailing Factor
1	Nateglinide	8.225	29404	0.00	8121	1.14
2	Impurity A	11.063	36489	7.07	10213	1.10
3	Impurity B	13.153	38233	4.24	9229	1.09

FIGURE 3 **HPLC Chromatogram of Spiked Impurities A and B in Nateglinide Sample.**

TABLE 5 **Linearity Data of Nateglinide Sample**

S.No.	Concentration injected (μg\mL)	Average peak area (*n* = 3)	% RSD
1	25	445532	0.860
2	37.5	901038	0.790
3	50	1325233	0.680
4	62.5	1795429	0.533
5	75	2303142	0.626

n = Number of determinations.

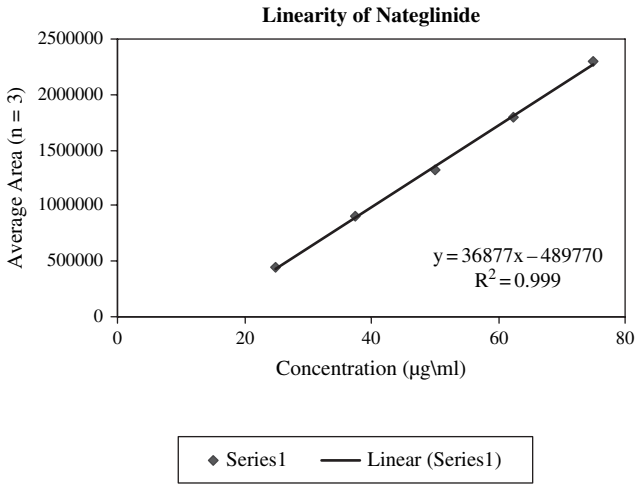


FIGURE 4 **Linearity Graph of Nateglinide Average Peak Areas Response Against Concentration Injected.**

Robustness

In all the deliberate varied chromatographic conditions performed (flow rate, column temperature, and organic solvent), the resolution between closely eluting impurity and Nateglinide was greater than 2.0, illustrating the robustness of the method.

Solution Stability and Mobile Phase Stability

The % RSD of assay of Nateglinide during solution stability experiments was within 1.5%. No significant changes were observed in the content of impurities A and B during solution stability and mobile phase stability experiments when performed using related substances method. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during assay and related substance determination were stable up to 48 hr.

CONCLUSION

The reverse phase high-performance liquid chromatography (RP-HPLC) method was developed for quantitative and related substance determination of Nateglinide is precise, accurate, rapid, and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method can be used for the routine analysis and also to check the stability of Nateglinide.

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