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ORIGINAL RESEARCH

Development and validation of an HPLC method for determination of nateglinide in drug substances

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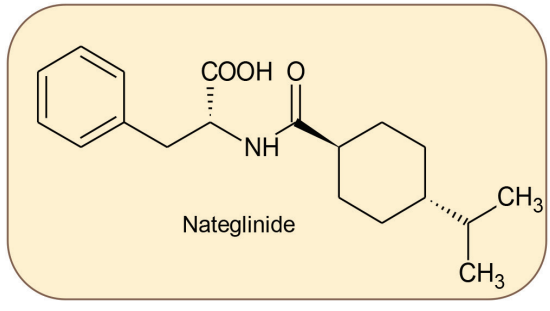
ABSTRACT

The scope of this study was the development and validation of a reliable, rapid, simple, sensitive, precise reversed-phase high performance liquid chromatography (RP-HPLC) method for the determination of nateglinide in drug substances. The method was developed on ACE C_{18} analytical column (150 x 4.6 mm i.d., particle size 5.0 µm) using a mobile phase of acetonitrile and 0.05% trifluoroacetic acid (25:25, v/v). The eluent was monitored with UV detection at 210 nm at a flow rate of 1.5 mL/min. Calibration curve was linear between the concentration range of 0.2846-1.0125 mg/mL. The retention time of Nateglinide was 7.07 min and the correlation coefficient (r) of the regression equation for the Nateglinide was greater than 0.99 in all cases.

Keywords: Nateglinide, HPLC assay, method validation

INTRODUCTION

Nateglinide, [*N*-(*trans*-4-isopropylcyclohexylcarbonyl)-D-phenylalanine]**,** is an oral antihyperglycemic agent used for the treatment of non-insulin-dependent diabetes mellitus (NIDDM) (1).



It belongs to the meglitinide class of short-acting insulin secretagogues, which act by binding to β cells of the

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pancreas to stimulate insulin release. Nateglinide is an amino acid derivative that induces an early insulin response to meals decreasing postprandial blood glucose levels. Activity of nateglinide is dependent on the presence functioning β cells and glucose.

In contrast to sulfonylurea insulin secretatogogues, nateglinide has no effect on insulin release in the absence of glucose. Rather, it potentiates the effect of extracellular glucose on ATP-sensitive potassium channel and has little effect on insulin levels between meals and overnight (2). As such, nateglinide is more effective at reducing postprandial blood glucose levels than fasting blood glucose levels and requires a longer duration of therapy (approximately one month) before decreases in fasting blood glucose are observed.

The insulinotropic effect of nateglinide is highest at intermediate glucose levels (3 to 10 mmol/L) and it does not increase insulin release already stimulated by high glucose concentrations (greater than 15 mmol/L) (3,4).

Nateglinide appears to be selective for pancreatic β cells and does not appear to affect skeletal or cardiac muscle or thyroid tissue. After oral administration, nateglinide is rapidly absorbed and peak plasma concentrations are reached after 0.5/1.0 h. The elimination of the substance is fast, with a half-life of approximately 1.4 h. Nateglinide is extensively metabolized in the liver and excreted in urine (83%) and feces (10%). The major metabolites possess less activity than the parent compound. One minor metabolite, the isoprene, has the same potency as its parent compound (5,6).

Several analytical methods have been developed for the separation and quantification of nateglinide from different matrices (7). For the determination of nateglinide in human plasma, high performance liquid chromatographic analysis with UV detection (8,9), LC-MS (10), coumarin-type fluorescent detection (6), and HPTLC/TLC methods (11) were reported. Spectrophotometric methods also have been reported for determination of nateglinide in bulk drug and in tablets (12).

Up to now, only a few methods have been developed that focus on the identification and determination of nateglinide in bulk drug and dosage forms and also in biological matrices. The aim of our present work was to develop and validate a new simple, rapid, selective, cost effective HPLC method for determination of nateglinide drug substances.

EXPERIMENTAL

Chemicals and Reagents

Trifluoroacetic acid was analytical grade and Acetonitrile was HPLC gradient grade and they were supplied from Merck (Germany). High purity water was prepared using Sartorius arium® RO 613L purification system.

Apparatus and Chromatographic Condition

The liquid chromatographic system, used in the present study, consisted of an Agilent Technologies (Waldbronn, Germany) 1200 series instrument equipped with a quaternary solvent system, an Agilent series UV detector and Agilent 1200 model online degasser. An Agilent ALS autosampler with a 100 µL sample loop was used for the injection of analytes. Chromatographic data were collected and processed using Agilent Chemstation software, The separation was conducted at ambient temperature, on a reversed phase ACE C_{18} column (150 x 4,6 mm; 5 µm particle size). All experiments were employed in the isocratic mode. The mobile phase was prepared by mixing acetonitrile and 0.05% trifluoroacetic acid (25:25, v/v) at a flow rate of 1.5 mL/min. The mobile phase was filtered through Millipore 0.45 µm membrane filter and degassed by sonication. Injection volume was set to 10 µL for the assay method. UV detection of the analytes was carried out at 210 nm.

Preparation of standard and test solutions

For the preparation of the standard solutions, 7.71 mg, 8.68 mg, 11.51 mg, 14.51 mg, 17.25 mg and 20.31 mg nateglinide was transferred into a 100.0 mL volumetric flasks. Nateglinide was dissolved by using (mixture of acetonitrile and water (11:9 , v/v)) and sonicated for 10 min. They were diluted to their volumes with diluent and they were mixed. They were filtered through Millipore 0.45 µm PVDF filter and injected to the HPLC system. For the preparation of the test solutions, 14,50 mg of sample was accurately weighed into a 20.0 mL volumetric flask. It was dissolved with diluent and sonicated for 10 min. It was diluted to its volume with diluent, mixed and filtered through Millipore 0.45 µm PVDF filter.

RESULTS AND DISCUSSION

Development of the chromatographic method

For the development of the chromatographic method, the relevant assay for nateglinid tablets existing in USP Pharmacopoeia was used. However, by using the column reported in the pharmacopoeial method, retention time for nateglinide peak was reported as 10 minutes. By changing the brand of column (Waters to ACE) and mobile phase composition (acetonitrile and 0.05% trifluoroacetic acid ratio (23:27) to (25:25), (v/v)) of the method improved the retention time the peak was obtained at 7 minutes. Analysis was carried out in a shorter analysis time.

Validation of the proposed method

The aim of method validation was to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2(R1) (13). The decribed method has been extensively validated in terms of system suitability, specificity, linearity, accuracy, precision (system precision, method precision and intermediate precision), robustness and solution stability.

Specificity

The specificity of an analytical method is its ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

To investigate the specificity of the analytical method, the blank has been injected into the chromatographic system. In the obtained chromatogram, no peaks has been observed in the same retention times as of nateglinide as presented in the chromatogram below (**Figure 1**).

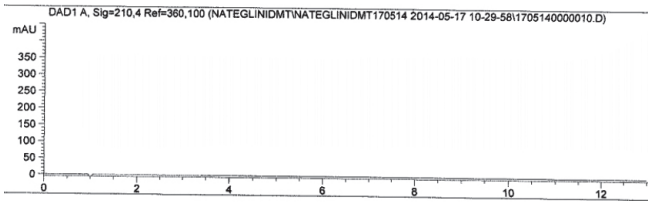

Figure 1. Blank chromatogram (Diluent: mixture of acetonitrile and water (11:9 , v/v))

Simultaneously, the test solutions that were prepared by using drug substance and have been injected into the chromatographic system. The retetion time was found 7.07 min for nateglinide and chromatogram was shown in **Figure 2**.

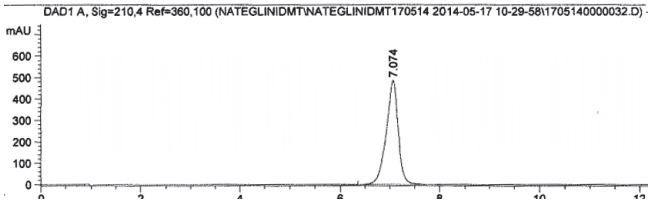

Figure 2. Sample chromatogram (14.58 mg of sample to be examined, accurately weighed, into a 20.0 mL volumetric flask. Dissolve with diluent and sonicate for 10 min. Dilute to volume with diluent, mix and filtered through Millipore 0.45 µm PVDF filter.)

Linearity (Calibration curve)

The linearity of an analytical method is its ability to elicit test results that are directly or a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range.

Linearity of the method has been investigated by injecting nateglinide standard solutions at six different concentrations in the defined range for the determination of assay .

Linear calibration plot for the nateglinide assay method was obtained over the calibration range of 40-140% of nominal concentration, of 0.2846-1.0125 mg/mL. During the study, the solutions corresponding to 100% of test concentration for assay determination have been injected six times while the remaining samples have been injected three times.

The regression equation and the correlation coefficient to that of have been calculated by using mean peak areas obtained by standard solution injections and exact concentrations as mg/mL. The correlation coefficient equals to 0.9999, indicating a strong linear relationship between the variables. Characterization of nateglinide calibration plot values was shown in **Table 1**.

Table 1. Characterization of Nateglinide calibration plot.

Parameter	Actual Result
Linearity Range	0.2846-1.0125 mg/mL
Correlation coefficient	0.9999
Slope	11048.1418
Intercept	-17.0397

Furthermore, for the statistical evaluation of linearity, regression analysis has been performed and the actual intercept and slope values have been found within the 95% confidence interval. Also 95% confidence interval of intercept has been found covering “0”.

It is verified that the analytical method is linear and therefore is capable to produce test results that are directly proportional to the nateglinide concentration in the sample.

The chromatogram of the standard solution corresponding to 100% of test concentration for assay determination has been given in **Figure 3**.

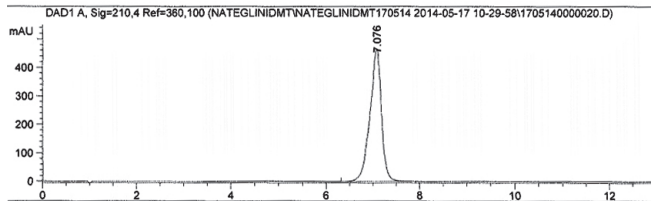

Figure 3. A typical chromatogram obtained from a standard solution of Nateglinide (0.7233 mg/mL).

Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value and is defined as recovery. Recovery is also defined as the ratio of the experimental concentration to the actual concentration.

In this validation study, accuracy of the method has been investigated by calculating the recovery values obtained by analyzing the solutions prepared with nateglinide standard corresponding to 80%, 100% and 120% of the test concentration by using the chromatographic parameters defined in the test procedure. The obtained results are presented in **Table 2**.

Table 2. Nateglinide accuracy study results.

Solution	Recovery%
80%	101.76
	101.36
	101.09
100%	101.25
	101.10
	100.91
120%	100.71
	101.01
	101.02
Mean of recovery	101.10
SD	2.44
RSD%	0.41%

Since, each individual recovery value obtained during the accuracy studies for the drug substances has been found in the range of 97.0% - 103.0% which is the acceptance criteria, hence it is determined that the analytical method is accurate and therefore is capable to produce test results that are close enough to nateglinide concentration of the sample.

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.

In this validation study, precision has been evaluated as: System precision, method precision and intermediate precision.

System Precision

System precision or repeatability of chromatographic system is a measure of chromatographic system performance.

In order to evaluate the system precision parameter, nateglinide standard solution corresponding to 100% of test concentration has been injected six times and the relative standard deviation of the peak areas has been calculated. The obtained result are presented in **Table 3**.

Table 3. System precision test results.

Injection	Nateglinide [Area]
1	7912.51660
2	7973.67725
3	7920.51221
4	7926.14307
5	7970.94971
6	7935.71436
Mean	7939.91887
SD	26.22
RSD%	0.33

Since the relative standard deviation has been found as less than the acceptance criteria, which is 1.0%, it is verified that repeatable results are obtained by the analysis system for the determination of assay.

Method precision

Method precision or repeatability of an analytical method is a measure of an analytical method performance.

In order to evaluate the method precision parameter, six different test solutions prepared by using either drug substance sample have been analyzed and the relative standard deviation of the experimental results have been calculated. The obtained results are presented in the **Table 4**.

Table 4. Method precision study results.

Test Solution	Nateglinide [%]
1	100.94
2	100.80
3	100.60
4	100.95
5	100.71
6	101.01
Mean	100.84
SD	0.16
RSD%	0.16

Since, the relative standard deviation of the experimental results obtained from the analysis system used to determine the assay has been found as less than the acceptance criteria which is 2.0%, it is verified that the analytical method is capable to produce test results that are unaffected by the small variations within the laboratory or sample handling and preparations.

Intermediate precision

Intermediate precision is a measure of repeatability within laboratory variation.

In order to evaluate the intermediate precision parameter, six different test solutions prepared by different analysts using either the same drug substance sample have been analyzed by using different chromatographs and the difference of the experimental results obtained by two analysts has been calculated. The obtained results are presented in **Table 5**.

Table 5. Intermediate precision study results.

Test Solution	Nateglinide [%] Analyst 1	Nateglinide [%] Analyst 2
1	100.94	101.56
2	100.80	101.48
3	100.60	101.46
4	100.95	101.04
5	100.71	101.34
6	101.01	101.17
Mean	100.84	101.34
SD	0.16	0.20
RSD%	0.16	0.20

Since, the difference between the results obtained by different analysts by using different analysis systems, is less than the acceptance criteria which is 2.0, it is verified that the analytical method used to determine the assay of drug substance, nateglinide is capable to produce test results that are unaffected by the small variations within the laboratory.

Robustness

The robustness of an analytical method 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.

In this validation study, robustness has been evaluated as: Changing of column temperature and flow rate.

In order to evaluate the robustness parameter, three different test solutions which have been prepared by using the same drug substance sample have been analyzed by repeating the analysis sequence at different column temperatures (20ºC, 25ºC, 30ºC) except all the other analytical parameters remaining the same and the difference of the experimental results obtained at different temperatures has been calculated. The obtained results are presented in **Table 6**.

Table 6. Effect of temperature variation on assay results.

Temperature	Assay (%)
20ºC	101.57%
25ºC	100.84%
30ºC	101.50%

Difference between the results obtained at 20ºC and 25ºC was found 0.72% and difference between the results obtained at 25ºC and 30ºC was found 0.66%, with in the acceptance criteria which is not more than 2.0%

In order to evaluate the robustness parameter, three different test solutions which have been prepared by using either the same drug substance sample or the same drug product sample have been analyzed by repeating the analysis sequence at different flow rates (1.3 mL/min, 1.5 mL/min, 1.7 mL/min) except all the other analytical parameters remaining the same and the difference of the experimental results obtained at different temperatures has been calculated. The obtained results are presented in **Table 7**.

Table 7. Effect of flow rate variation on assay results.

Flow rate (mL/min)	Assay (%)
1.3 mL/min	100.92 %
1.5 mL/min	100.84 %
1.7 mL/min	101.01 %

Difference between the results obtained at 1.3 mL/min and 1.5 mL/min was found 0.08% and difference between the results obtained 1.5 mL/min and 1.7 mL/min was found 0.17%, with in the acceptance criteria which is not more than 2.0%

Since, the difference calculated from the test results obtained at different temperatures and flow rates for the same lot of material is less than the acceptance criteria which is 2.0, it is verified that the analytical method used to determine the assay of drug product is capable capable of test results that are not being affected by the small variations in the method parameters.

Solution Stability

In order to evaluate the solution stability, test and standard solutions stored separately at room temperature have been injected to the same chromatographic system and the

percent difference of the peak areas comparing to initial have been calculated. For the test solution area difference was 0.16% and for the standard solution area difference was 1.07% in room temperature .

Since, the percents of differences between the peak areas obtained from the standard solution for the determination of assay at 67th hour have been found smaller than the acceptance criteria which is 2.0%, it is concluded that the standard solutions for assay determination are stable up to 67 hours when stored at room temperature.

CONCLUSION

A novel simple and sensitive reversed-phase HPLC isocratic method has been developed and validated for the determination of nateglinide drug substances by using UV detector. The method was found to be linear (r^2: 0.999) within the analytical range of 0.2846-1.0125 mg/mL. The obtained results proved that the method was accurate and reproducible. Therefore, the developed chromatographic method can be used for estimation of nateglinide drug substances.

Nateglinid Etken Maddesinin Miktar Tayini için HPLC Yönteminin Geliştirilmesi ve Validasyonu

ÖZET

Bu çalışmanın konusu, Nateglinid etken maddesinin miktar tayini için güvenilir, hızlı, basit, duyarlı, kesin zıt faz HPLC yönteminin geliştirilmesi ve validasyonunu içermektedir. Yöntem, ACE C18 analitik kolonda (150 x 4.6 mm, 5.0 µm) asetonitril ve % 0.05 trifloroasetik asit (25:25, h/h) mobil faz kullanılarak geliştirilmiştir. Nateglinid, 1.5 mL/dak akış hızında 210 nm dalga boyunda UV detektör yardımıyla gözlenmiştir. Kalibrasyon eğrisi 0.2846-1.0125 mg/mL derişim aralığında doğrusaldır. Nateglinidin alıkonma zamanı 7.07 dak. ve regresyon eşitliklerinin korelasyon katsayıları (r) her durumda 0.99'dan yüksek bulunmuştur.

Anahtar Kelimeler: Nateglinid, HPLC miktar tayini yöntemi, yöntem validasyonu

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