Development and validation of analytical method for the estimation of nateglinide in rabbit plasma

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Abstract Nateglinide has been widely used in the treatment of type-2 diabetics as an insulin secretogoga. A reliable, rapid, simple and sensitive reversed-phase high performance liquid chromatography (RP-HPLC) method was developed and validated for determination of nateglinide in rabbit plasma. The method was developed on Hypersil BDSC-18 column (250 mm $\times$ 4.6 mm, 5 mm) using a mobile phase of 10 mM phosphate buffer (pH 2.5) and acetonitrile (35:65, v/v). The elute was monitored with the UV–vis detector at 210 nm with a flow rate of 1 mL/min. Calibration curve was linear over the concentration range of 25–2000 ng/mL. The retention times of nateglinide and internal standard (gliclazide) were 9.608 min and 11.821 min respectively. The developed RP-HPLC method can be successfully applied to the quantitative pharmacokinetic parameters determination of nateglinide in rabbit model.

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1. Introduction

Nateglinide (NTG) \([N(\text{trans-4-isopropylcyclohexylcarbonyl})-d-\text{phenylalanine}],\) a d-phenylalanine derivative lacking either a sulfonylurea or benzamil moiety, is a novel oral mealtime glucose regulator, and approved for the treatment of type-2 diabetes mellitus recently [1,2]. This meglitinide derivative (Fig. 1) works by stimulating the $\beta$-cell membrane of pancreas to release insulin by closing the ATP-dependent potassium channels, which leads to an opening of the calcium channels. The resulting influx of calcium induces insulin secretion. It is rapidly and completely absorbed from the gastrointestinal tract and peak plasma concentration reaches at 0.5–1.0 h. It is metabolized by cytochrome P-450 system to inactive metabolite and eliminated with half-life of 1.4 h [3]. Several analytical methods have been developed for the separation and quantification of nateglinide from different matrices. Ono et al.
illustrated the estimation of NTG and its main metabolites [4,5] with column switching HPLC after solid phase sample preparation. Ho et al. have detected 10 antidiabetic drugs including nateglinide from equine plasma and urine by liquid chromatography–tandem mass spectroscopy [6]. Nateglinide has been successfully determined in animal plasma by Yan et al. using micellar electrokinetic chromatography and on-line sweeping technique [7]. Yin et al. have studied chiral separation of nateglinide and its l-enantiomer on monolithic molecularly imprinted polymers [8]. Detection of metformin and nateglinide from human plasma by cation exchanging with normal-phase LC/MS has been reported [9]. All these methods employed a specific type of specialization and sophistication of the instruments and/or method of separations that requires high analytical cost.

In this work we developed and validated a rapid, simple and sensitive reverse phase high-performance liquid chromatographic method for the determination of NTG in rabbit plasma.

2. Materials and methods

2.1. Reagents and chemicals

Nateglinide and gliclazide were the generous gifts from Glenmark Pharmaceuticals Ltd., Nashik and Macleods Pharmaceuticals Ltd., Mumbai respectively. HPLC grade acetonitrile, methanol and dichloromethane were procured from Merck, Mumbai. Potassium di-hydrogen phosphate (KH2PO4) and ortho-phosphoric acid (H3PO4) were purchased from S.D. Fine Chem Ltd. (Mumbai, India). All other chemicals and solvents used were of analytical grade. Water used in the HPLC analysis was prepared by the water purifier (Arium® 611UF, Sartorius, Germany). The mobile phase and all the solutions were filtered through a 0.45 μm Ultipor® N66® membrane filter ( Pall Life Sciences, USA) prior to use.

2.2. Instruments

HPLC system (Waters, USA) consisting of quaternary pump (Water™ 600), 7725i rhodyne manual injector and UV–vis detector of module 2998 and empower-II software were used for analysis. The plasma samples were processed by using Micropipettes (Ependruff, USA), Spinix Vortexer (M37610-33, Barnstead International, USA), Biofuge Fresco Centrifuge (Heraeus, Germany), Ultra-Sonicator (Loba Chem, Mumbai), Nitrogen gas evaporator and Multi-Pulse Vortexer (Glas-COL, USA).

2.3. Experimental animals

Albino rabbits weighing 1.5–2.5 kg (housed in a temperature (22 ± 1°C) and relative humidity (55 ± 10%) controlled room) were used in the experiments. The rabbits were maintained in accordance with the principles declared by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, Government of India). The rabbits were orally administered the 60 mg nateglinide containing tablets.

2.4. Chromatographic conditions

HPLC analysis was performed using a Hypersil ODS C18 (average particle size 5 μm) column (250 mm, 4.6 mm). The mobile phase consisted of 10 mM phosphate buffer (pH 2.5) and acetonitrile (35:65, v/v). The eluent was monitored with the UV detector at 210 nm with a flow rate of 1 mL/min and sample size of 20 μL was carried out at column oven temperature 30 ± 2°C all over the study.

2.5. Preparation of standard solution

Stock solutions of nateglinide (665.965 μg/mL) and gliclazide (internal standard; 250 μg/mL) were prepared in methanol. Further dilution was carried out in mobile phase solvent for the preparation of working stock solution. Calibration standards were prepared freshly by spiking working nateglinide stock solution into the control blank plasma to give the concentration of 100.74, 239.85, 489.49, 699.26, 998.95, 1664.91 and 3329.83 ng/mL.

2.6. Quality control standards

Lowest quality control standards, median quality control standards and highest quality control standards were prepared by spiking drug free plasma with nateglinide to give solutions containing 239.85 ng/mL, 489.49 ng/mL and 998.95 ng/mL respectively. They were stored at −20°C till analysis.

2.7. Sample preparation

An aliquot quantity of 180 μL of rat plasma spiked with 10 μL of calibration standard drug was taken in a 2 mL stopper centrifuge tube and mixed for 20 s. To this 10 μL of internal standard (IS) solution (250 μg/mL) was added and mixed for 20 s. The drug was extracted by vortexing with 1.5 mL of selected extracting solvents containing acetonitrile, methanol and di-chloromethane in a spinix vortexer for 10 min followed by centrifugation at 10,000 rpm for 5 min at 4°C. The organic phase was withdrawn and dried using nitrogen evaporator. The residue was reconstituted with 100 μL of mobile phase and 20 μL was injected into the column.
2.8. Validation of method

The validation of an analytical method confirms the characteristics of the method to satisfy the requirements of the application domain [10]. The method was validated following the ICH Guidelines [11,12] for specificity, recovery, linearity, precision and stability. Under the validation program the following parameters were studied.

2.8.1. Specificity

The specificity criterion demonstrates that the result of the method is not affected by the presence of interferences, i.e. whether the compound of interest elutes without interfering with other compounds and components of plasma. The specificity of the method was determined by comparing the chromatograms obtained from the aqueous samples of NTG and IS with those obtained from blank plasma. Blank plasma samples from each of five rabbits were processed in the presence of IS and another set of five samples processed with NTG and IS to evaluate presence of interference around the peak of NTG. The solutions each containing 489.49 ng/mL were injected into the column under the optimized chromatographic conditions to obtain the chromatographic peaks of NTG in plasma in the presence of IS so as to differentiate them from the interfering peaks of plasma components.

2.8.2. Selection of solvent for recovery of drug

The recovery of an analyte is the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Different organic extraction solvents (acetonitrile, methanol and di-chloromethane) were tried in the experiment to recover NTG from plasma samples. Quality control samples were prepared in triplicate at three levels of 239.85 ng/mL, 489.49 ng/mL and 998.95 ng/mL of NTG and determined by HPLC method as described above. The extraction efficiency of NTG was determined by comparing the peak areas obtained from extracted quality control samples with the peak area of aqueous working solution containing same concentration of NTG at three levels.

2.8.3. Preparation of calibration curve

Quantitative analytical results are highly influenced by the quality of the calibration curve [13,14]. Nine different concentrations of NTG with fixed concentration of IS in blank plasma were processed and calibration curve was constructed in the specified concentration range (3329.83, 1664.91, 998.95, 699.264, 489.485, 342.639, 239.847, 167.893 and 100.736 ng/mL). The calibration curve was plotted between the ratio of NTG to IS and concentration of nateglinide by replicate analysis (n=6) at all concentration levels and the linear relationship was evaluated using the least square method using Microsoft Excel® program.

2.8.4. Precision and accuracy

Both repeatability (within a day precision) and reproducibility (between days precision) were determined as follows. Three quality control samples were subjected to the study. Five injections of each of the specified quality control samples at three levels were injected for analysis within the same day for repeatability, and over a period of 5 day for reproducibility. Mean and relative standard deviation were calculated and used to predict the accuracy and precision of the method. Accuracy was calculated as the percent of NTG found in the intra-day and inter-day samples to that of the actual.

2.8.5. Stability studies

The quality control standards containing 239.85, 489.49 and 998.95 ng/mL (n=6) of NTG were subjected to the detection of stability of the drug in plasma. The initial assay of the samples was conducted. One set of five samples each was kept in poly propylene tubes and subjected to three freeze-thaw cycles each at −20 ºC for 24 h and room temperature for 24 h. The second set of five samples each was kept at room temperature for 24 h and the third set of five samples each was kept at room temperature for 1 month. All the samples were analyzed by standard chromatographic conditions to determine their peak areas. Samples were considered to be stable, when the final assay values of samples were found similar to that of the initial assay value of the drug.

2.9. Pharmacokinetic study in rabbits

The method described above was applied to quantify the plasma concentration of nateglinide in a single-dose pharmacokinetic study conducted on three white male albino rabbits. The protocol was approved by the Institutional Ethical Committee at the Gayatri College of Pharmacy, Odisha, India. The experiments were conducted as per CPCSEA guidelines. The rabbits weighing 1.5–2.5 kg were housed with free access to food and water, except for the final 12 h before experimentation. After a single oral administration of 60 mg of nateglinide (Starlix®), 2.5 mL of blood samples were collected from the marginal ear vein at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 9, 12 and 24 h time points into heparinized collection tubes. The blood was immediately centrifuged (1000 x g) for 10 min at cooling temperature. The supernatant plasma layer was separated and stored at −20 ºC until analyzed. The plasma samples were analyzed for nateglinide concentrations as described above. The total area under the observed plasma concentration–time curve (AUC) was calculated using the linear trapezoidal rule. The first order elimination rate constant (K) was estimated by the least square regression of the points describing the terminal log-linear decaying phase. t1/2 was derived from K (t1/2=ln 2/K). The absorption rate constant (K) was determined by residual method. The maximum observed nateglinide concentration (cmax) and the time at which cmax was observed (tmax) were reported directly from the profile.

3. Results and discussion

3.1. Selection and development of chromatographic method

Normal phase chromatography can be used for the separation of non-ionic and non-polar substances, while chromatography (C8 and C18 column) can be used for the separation of non-ionic as well as ionic non-polar to semi polar substances. Thus, nateglinide (a ionizable semi polar weak acid) can be satisfactorily separated by reversed-phase chromatography. Octylsilane (C8) columns are similar to octadecysilane (C18). However, octylsilane columns are less retentive as compared...
to octadecylsilane. Majority of the ionizable pharmaceutical compounds can be very well separated on octadecylsilane reversed-phase columns [10]. Hence, octadecylsilane column was selected for NTG. A mixture of acetonitrile and phosphate buffer (pH 2.5–4.0) at flow rate 1 mL/min was used as mobile phase for the analysis of nateglinide [9,10]. The optimum ratio of acetonitrile to phosphate buffer (pH 2.5) used in the current investigation was 65:35 which was selected on the basis of resolution and absence of interferences. The best resolution and sensitivity of the method were obtained at 210 nm and 1 mL/min flow rate of mobile phase.

3.2. Validation of methods

3.2.1. Specificity
Typical chromatogram (Fig. 2) of mixture of NTG and IS revealed that they are well separated under HPLC conditions applied. A chromatogram of blank plasma sample is shown in Fig. 3. Retention time was 9.608 min for NTG and 11.821 min for IS. Fig. 4, which is the overlapping of the chromatogram of NTG and IS (Fig. 2) with the chromatogram of blank plasma (Fig. 3), shows the absence of interference of plasma components around the zone of retention time of NTG and IS. The chromatogram of medium level quality control sample i.e. 489.49 ng/mL of NTG (Fig. 5) showed a good resolution peaks for NTG and IS which are well differentiated from the peaks of plasma components.

3.2.2. Selection of extracting solvent for recovery of drug
The solvent extracted maximum amount of the drug from plasma sample was selected for processing of the samples. The recovery of the drug was found to be 87.36±0.70%, 72.41±1.70% and 55.27±1.70% in acetonitrile, methanol and di-chloromethane, respectively at all three concentration levels, which confirm the extraction efficiency of the solvents. Among the three solvents, acetonitrile showed maximum amount of the drug recovered, hence acetonitrile was selected as the extracting solvent. The recovery of IS was also found to be maximum in acetonitrile, which was 93.23±2.40%.

3.2.3. Linearity range
The ratio of peak areas of nateglinide to IS at various concentrations of nateglinide in plasma is shown in Table 1. A calibration curve was plotted between peak area ratio of NTG to

![Figure 2](image2.png)  
**Figure 2** Chromatogram of the mixture of nateglinide and Internal Standard in mobile phase. (1) Nateglinide-9.261; (2) internal standard-11.663.

![Figure 3](image3.png)  
**Figure 3** Chromatogram of blank plasma of rabbit.
IS versus plasma NTG concentrations. The chromatographic responses (ratio of peak areas of NTG to IS versus nateglinide concentration) were found to be linear over an analytical range of 100.74–3329.83 ng/mL with regression coefficient ($r^2$) value 0.999, which showed reproducibility of the method. The linear regression equation obtained was $Y=0.0015X-0.0084$.

Table 1  Peak area of the chromatogram of NTG and IS at different concentrations of NTG.

| Concentration of nateglinide (ng/mL) | Peak area of the chromatogram | Ratio of peak area |
|-------------------------------------|-----------------------------|-------------------|
|                                     | Nateglinide                 | IS (Gliclazide)   |                  |
| 100.74                              | 13,175                      | 118,875           | 0.11             |
| 167.89                              | 24,406                      | 104,728           | 0.23             |
| 239.85                              | 36,044                      | 104,448           | 0.35             |
| 342.64                              | 47,183                      | 94,784            | 0.50             |
| 489.48                              | 72,401                      | 111,815           | 0.65             |
| 699.26                              | 104,507                     | 100,611           | 1.04             |
| 998.95                              | 160,217                     | 103,005           | 1.56             |
| 1664.91                             | 275,030                     | 117,616           | 2.34             |
| 3329.83                             | 550,296                     | 113,730           | 4.84             |

Figure 4  Overlapping of chromatograms of NTG-IS mixture with blank plasma.

Figure 5  Chromatogram of medium level quality control sample of nateglinide (489.48 ng/mL). (1) Nateglinide-9.261; (2) internal standard-11.663.
3.2.4. Precision and accuracy

The accuracy of the measurements was determined by using three quality control samples and the results are reported in Table 2. The relative standard deviation (RSD) of intra-day assay of the drug was ranged from 4.95% to 10.58% and for the inter-day assay was from 7.91% to 10.71%. Accuracy data ranged from 98.19% to 99.83% for both the conditions indicated that there was no interference from endogenous plasma components. Inter-day as well as intra-day replicates of NTG resulted in a RSD value less than 10.72%, which revealed that the precision degree of the proposed method is very high.

3.2.5. Stability

The result of the stability validation is presented in Table 3. The result revealed that the final concentration of the drug each quality control sample undergoing three freeze–thaw cycles and storing at room temperature for 24 h and 1 month was similar with the initial concentration. The RSD value \((n=5)\) of final concentration of the drug after three freeze–thaw cycles, 24 h and 1 month was <13.78, 11.56 and <8.9 respectively. The accuracy of stored samples was found to be equivalent to 100%. Hence, it can be inferred that NTG was stable in rabbit plasma.

![Figure 6](image)

**Figure 6** Mean plasma drug concentration versus time profile after single oral administration of nateglinide (60 mg) in rabbits.

### Table 2

Precision and accuracy of the method for the estimation of nateglinide in rabbit plasma.

| Concentration (ng/mL) | Intra-day precision | Inter-day precision |
|-----------------------|---------------------|---------------------|
|                       | Mean (ng/mL) | RSD (%) | Accuracy (%) | Mean (ng/mL) | RSD (%) | Accuracy (%) |
| 239.85                | 236.81    | 4.95 | 98.73 | 235.51 | 10.17 | 98.19 |
| 489.48                | 485.32    | 10.58 | 99.15 | 486.05 | 10.72 | 99.30 |
| 998.95                | 996.22    | 7.56 | 99.73 | 997.24 | 7.91 | 99.83 |

### Table 3

Stability of nateglinide in rabbit plasma.

| Stability         | Concentration of NTG (ng/mL) | Initial concentration (mean; ng/mL) | Final concentration (mean; ng/mL) | Deviation (%) | RSD (%) | Accuracy (%) |
|-------------------|-----------------------------|-------------------------------------|----------------------------------|----------------|---------|---------------|
| Freeze–thaw       | 239.85                      | 236.67                              | 236.32                           | −0.15          | 8.72    | 98.53         |
|                   | 489.48                      | 486.60                              | 493.80                           | 1.48           | 13.78   | 100.88        |
|                   | 998.95                      | 999.83                              | 1003.00                          | 0.32           | 6.32    | 100.41        |
| 24 h stability    | 239.85                      | 243.73                              | 236.04                           | 2.90           | 3.81    | 98.67         |
|                   | 489.48                      | 484.92                              | 493.16                           | −0.57          | 11.56   | 99.63         |
|                   | 998.95                      | 996.55                              | 997.31                           | 0.92           | 8.71    | 98.84         |
| 1 month stability | 239.85                      | 236.04                              | 239.08                           | −1.29          | 7.29    | 99.68         |
|                   | 489.48                      | 493.16                              | 482.68                           | 2.13           | 2.42    | 98.61         |
|                   | 998.95                      | 997.31                              | 1007.42                          | −1.01          | 8.90    | 100.85        |
3.3. Pharmacokinetic study in rabbits

The developed method was applied to quantify nateglinide concentration in pharmacokinetic study carried out on rabbits. The mean plasma concentration versus time profile following a single oral administration of nateglinide to three rabbits is presented in Fig. 6. Various other pharmacokinetic parameters are summarized in Table 4.

4. Conclusion

A novel simple and sensitive reversed-phase HPLC isocratic method has been developed and validated for the estimation of NTG in rabbit plasma using UV detector. A good resolution was obtained between NTG and IS with retention time 9.608 min and 11.821 min respectively. There were no interference peaks observed around the retention time of NTG and IS. The method was found to be linear ($r^2=0.999$) within the analytical range of 100.74–3329.83 ng/mL. A maximum recovery of the drug from plasma was resulted using acetonitrile as extracting solvent in comparison to methanol and di-chloromethane. The results obtained proved that the method was accurate and reproducible, and the drug was stable in rabbit plasma. Therefore, the developed chromatographic method can be used for estimation of NTG in rabbit plasma with good resolution to evaluate the pharmacokinetic parameters of NTG.

Table 4 Pharmacokinetic parameters of single dose administration of 60 mg nateglinide to rabbits (n=3).

| Pharmacokinetic parameters | Observed value |
|----------------------------|----------------|
| Maximum plasma concentration, $c_{max}$ (µg/mL) | 13.407±0.476 |
| Time required to reach maximum plasma concentration, $t_{max}$ (h) | 1.167±0.373 |
| Area under the curve, AUC$_{0–∞}$ (h µg/mL) | 45.353±1.219 |
| Area under momentum curve, AUMC$_{0–∞}$ (h$^2$ µg/mL) | 115.939±6.618 |
| Volume of distribution, $V_d$ (L) | 2.714±0.116 |
| Elimination half life, $t_{1/2}$ (h) | 0.317±0.068 |
| Elimination rate constant, $K_e$ (h$^{-1}$) | 0.489±0.023 |
| Absorption rate constant, $K_a$ (h$^{-1}$) | 2.225±0.038 |
| Mean residence time, MRT (h) | 2.556±0.146 |
| Clearance, Cl (L/h) | 1.326±0.003 |

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