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Research Article

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Understanding the pharmaceutical stability of Nateglinide N-[(trans-4-Isopropylcyclohexyl)carbonyl]-D-phenylalanine

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Introduction

Nateglinide is an amino-acid derivative used for the treatment of non-insulin-dependent diabetes mellitus (NIDDM). It is an oral antihyperglycemic agent belongs to the meglitinide class of short-acting insulin secretagogues. It is used alone or in combination with other medications to treat type 2 diabetes (condition in which the body does not use insulin normally and therefore cannot control the amount of sugar in the blood) in people whose diabetes cannot be controlled by diet and exercise alone. It decreases the amount of glucose by binding on β cells of the pancreas to stimulate insulin release.

The mechanism of action is dependent upon functioning of beta-cells in the pancreatic islets. It interacts with the ATP-sensitive potassium (K+ATP) channel on pancreatic beta-cells. The subsequent depolarization of the beta cell opens the calcium channel, producing calcium influx and insulin secretion. The extent of insulin release is glucose dependent and diminishes at low glucose levels. It is highly tissue selective with low affinity for heart and skeletal muscle. It appears to be selective for pancreatic β cells and does not appear to affect skeletal or cardiac muscle or thyroid tissue.Side effects with the drug include hypoglycemia - sweating, trembling, dizziness, increased appetite, palpitations, nausea, fatigue, and weakness. Gastrointestinal symptoms, especially diarrhea and nausea are also may occur [1-9].

Materials and Methods

Stock solution of the standard drug

About 5mg of Nateglinide was weighed accurately and transferred into a 5mL volumetric flask containing 2mL of methanol. The contents were sonicated for 5min and then the volume made up with a further quantity of methanol to get a concentration of 1mg/ml.Then it was filtered through 0.45µm membrane filter paper. This solution was stored in a refrigerator below 10°C.

Stock solution of the internal standard

About 20mg of Repaglinide was weighed accurately and transferred into a 10mL volumetric flask containing 5mL of methanol. The solution was sonicated for 3min and then the volume made up with a further quantity of the methanol to get a concentration of 2mg/ml.Then it was filtered through 0.45µm membrane filter paper. This solution was stored in a refrigerator below 10°C.

The calibration curve solutions were prepared from Nateglinide stock solution as per the table 4.6 in the concentration range of 1.01-201.59µg/ml using diluent. These solutions (CC spiking solutions) were subsequently used for spiking the screened blank plasma.

Optimization of Chromatographic Conditions

The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of Nateglinide in Human Plasma.

Since both Nateglinide and internal standard are highly non-polar, liquid-liquid extraction process was employed. Initial experiments were performed by using non-polar solvents like t-butyl methyl ether, dichloromethane and diethyl ether in different ratio. A mixture of 70 parts of t-butyl methyl ether and 30 parts of dichloromethane gave a comparable recovery to that of ethyl acetate. However, ethylacetate was used as the extraction solvent to avoid preparation errors. Initially the pH of the mobile phase was adjusted to acidic to check the response. Better peak shape for the internal standard while that of the drug peak was not acceptable at low acidic pH and blunt peaks with high R_t values were observed at higher acidic pH. Therefore experiments were performed using Potassium dihydrogen phosphate in a limited pH range of 2.5 to pH 5.5. A pH value of 2.5 ± 0.1 gave maximum response for the analyte at 225 nm. Acetate buffer and formate buffers were initially used for the experiment but since they are classified as volatile buffers, the pH of the mobile phase is relatively unstable as compared to the non-volatile phosphate buffers. The buffer concentration is optimized at 10 mM. Higher buffer concentrations resulted in higher noise thus leading to the suppressing of the signal at lower values of quantification.

Therefore the final mobile phase consisted of 50: 50 % v/v methanoland 10mM potassium dihydrogen phosphate, pH adjusted to 2.5 with orthophosphoric acid, was selected. The resolution between the peaks was decreased and peaks were not acceptable when the experiment is performed using a shorter column (50 X 4.6 mm id). However better resolution, less tailing and high theoretical plates were obtained with a Phenomenex column C18 (150 X 4.6 mmi.d., 5 µm).

The flow rate of the method was set at 1.0ml/min and the column temperature was maintained at ambient. At the selected flow rate, peak shape and resolution was acceptable. There was no interference in the drug and internal standard, from the extracted blank. The peak symmetry were found to be good when the mobile phase composition of 50:50 v/v methanol and 10mM potassium dihydrogen phosphate, pH adjusted to 2.5 with orthophosphoric acid leading to better resolution of the drug and internal standard.

Extraction methods were initially attempted using Protein precipitation technique. Precipitation technique was adopted using Acetonitrile and / or Methanol. Initial experiments of protein precipitation were done using 1: 3 ratio of plasma: Organic solvents. The recovery of the Nateglinideis moderate while that of the internal standard is relatively unchanged as compared with liquid-liquid extraction.

Since the noise effects in solid phase extraction (SPE) method are similar to that of liquid-liquid extraction, final analysis was done using liquid-liquid extraction (LLE). SPE methods render a neat sample for final analysis; polar interferences do enter into the final sample during reconstitution. SPE is further expensive as compared to LLE technique. The recovery of Nateglinide and internal standard was high when Ethyl acetate was used individually.

Data acquisition and processing

The chromatograms were obtained, and data were processed by the peak area ratio method using the LC solution software. The concentrations of the unknown samples were calculated from the following equation of the regression analysis of the spiked plasma calibration graph using $1/X^2$ as weighting factor.

$$Y = m X + C$$

X = Concentration of Analyte/ Concentration of Internal standard
Y = Area of Analyte / area of Internal standard (area ratio)
m = Slope of the calibration curve
C = Y- intercept value

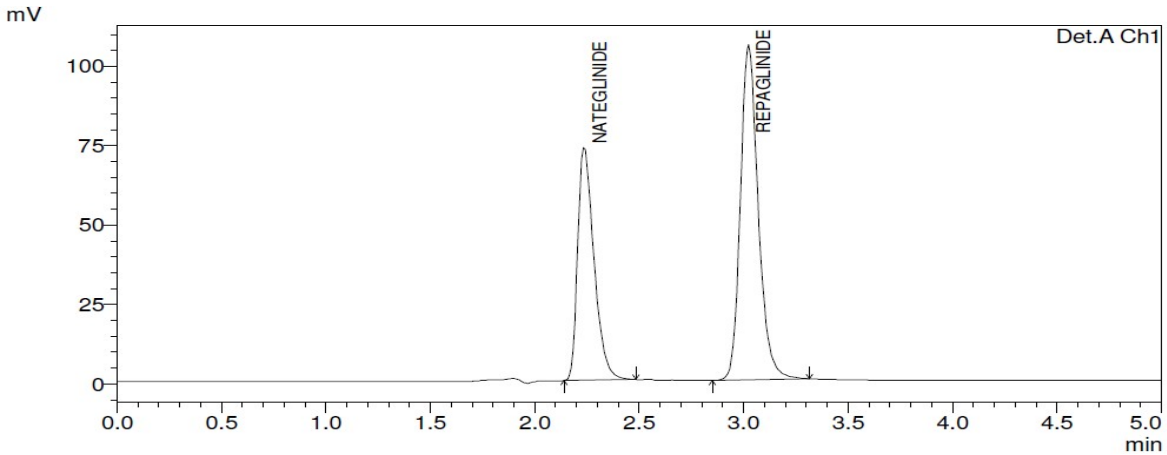

Figure 1: A chromatogram of Nateglinide (drug) and Repaglinide (IS) spiked blank plasma sample

Table 2: Precision and Accuracy of QC samples for Nateglinide

P & A ID	Observed Concentration (µg/ml)				
	QC ID	HQC	MQC	LQC	LLOQ QC
PA-01	1	7135.42	4987.43	154.11	51.21
	2	6999.48	4991.32	150.01	51.76
	3	6988.41	4987.63	149.31	55.32
	4	6995.23	4948.32	156.37	53.38
	5	7043.41	4999.11	158.49	53.44
	6	7021.57	5002.13	158.88	52.99
N		**6**	**6**	**6**	**6**
Average		**7030.587**	**4985.990**	**154.528**	**53.017**
Standard Deviation		**55.1948**	**19.4156**	**4.1435**	**1.4464**
CV (Precision %)		**0.79**	**0.39**	**2.68**	**2.73**
Nominal Concentration		**7055.66**	**5039.76**	**155.22**	**52.78**
Accuracy (%)		**99.64**	**98.93**	**99.55**	**100.45**
PA02	7	7256.72	5092.17	151.80	50.75
	8	7118.47	5096.14	147.76	51.29
	9	7107.21	5092.37	147.07	54.82
	10	7114.15	5052.23	154.02	52.90
	11	7163.15	5104.09	156.11	52.96
	12	7140.94	5107.17	156.50	52.51
N		**6**	**6**	**6**	**6**
Average		**7150.107**	**5090.696**	**152.210**	**52.540**
Standard Deviation		**56.1332**	**19.8234**	**4.0814**	**1.4334**
CV (Precision %)		**0.79**	**0.39**	**2.68**	**2.73**
Nominal Concentration		**7055.66**	**5039.76**	**155.22**	**52.78**
Accuracy (%)		**101.34**	**101.01**	**98.06**	**99.54**
PA 03	13	7438.14	5250.02	156.05	50.65
	14	7296.43	5254.12	151.90	51.20
	15	7284.89	5250.23	151.19	54.72
	16	7292.00	5208.85	158.34	52.80
	17	7342.23	5262.32	160.48	52.86
	18	7319.46	5265.50	160.88	52.41
N		**6**	**6**	**6**	**6**
Average		**7328.859**	**5248.507**	**156.472**	**52.440**
Standard Deviation		**57.5365**	**20.4379**	**4.1957**	**1.4307**
CV (Precision %)		**0.79**	**0.39**	**2.68**	**2.73**
Nominal Concentration		**7055.66**	**5039.76**	**155.22**	**52.78**
Accuracy (%)		**103.87**	**104.14**	**100.81**	**99.36**

Table 2: Short-term stability of Nateglinide and Repaglinide

Replicate No.	Nateglinide		Repaglinide (IS)	
	Comparison (0.0 hour)	Stability (7.25 hours)	Comparison (0.0 hour)	Stability (7.25 hours)
1	330046	317453	347313	340932
2	322319	318675	339283	350917
3	323249	320056	340262	345886
4	325762	327864	342908	354658
5	321804	331287	338741	349580
6	319783	321627	340151	349595
Mean	**323827**	**322827**	**341443**	**348595**
±SD	**3619**	**5517**	**3214**	**4694**
%CV	**1.1**	**1.7**	**0.9**	**1.3**
% Stability	**99.7**		**102.1**	

The objective of this work was to develop and validate a simple, rapid and sensitive assay method for the quantification of Nateglinide which is suitable to determine the drug in plasma samples. To achieve the objective, different options was evaluated to optimize sample extraction, detection parameters and chromatography during method development. The standard solution of Nateglinide was analyzed by Shimadzu HPLC equipment with SIL 10AD vp Auto sampler and separation was achieved on Phenomenex C_{18} column (150 X 4.6 mm id, 5 µm). A suitable maximum absorbing wavelength of 225nm was used as detector wavelength. The mobile phase flow rate was optimized in order to get well resolved symmetric peaks with accepted system suitable criteria. Finally at a mobile phase composition of 10mM mixed phosphate buffer (pH 2.5)and Methanol in the ratio of 50: 50 (v/v) with a flow rate of 1.0ml/min was found to be suitable for the analysis of Nateglinide. In this condition, retention time of Nateglinide was found to be 2.23min and retention time of Repaglinide was found to be 3.02min. In the optimized conditions, system suitable test was carriedout at a standard concentration.Number of theoretical plates were found to be 5400 and tailing factor was found to be 1.16 for standard drug. The resolution factor was found to be 7.42. The developed method was validated as per ICH guidelines. Peak area ratios of Nateglinide to the internal standard Repaglinide were measured. A representative calibration graph of Nateglinide was constructed with different concentration of Nateglinide. Good linear relation was observed in the concentration range of 50.40 to 10079.52ng/ml resulted in the regression equation ranged from 0.9983 to 0.9984 for Nateglinide.

The accuracy of an analytical method is the closeness of results obtained by that method to the true value for the sample. It is expressed as recovery (%), which is determined by the standard addition method. Samples were spiked with three different percentages of the standard and then analyzed. The experiment was performed in triplicate. Recovery (%) and RSD (%) were calculated for each concentration. Recovery for Nateglinidewas found to be 87.6% to 91.4% (Mean Recovery: 89.34%), whereas the mean recovery obtained for Repaglinide was 93.3%.Stability evaluations performed in EDTA human plasma, stock solutions and stock dilutions met the acceptance criteria, demonstrating insignificant degradation of Nateglinide over the specified storage duration and conditions. Stability of the samples was analyzed by Short-term stability and long-term stability. The percent stabilities were found to be 99.7 and 102.1 for Nateglinide and Repaglinide respectively in short term stability at 7.25 hrs sample kept at room temperature of ~ 25^0C. The percent long term stabilities obtained were 100.7 and 102.5 for Nateglinide and Repaglinide respectively at keeping the sample for 8.5 days in a refrigerator below 10^0C. Stability of the drug in biological matrix was determined by Freeze-thaw stability (96.83 % and 104.28 % at low and high concentrations respectively), Bench top stability (98.60 % and 99.17 % at low and high concentrations respectively), Auto-sampler stability (98.60% and 98.69% at low and high concentrations respectively), Long-term stability in plasma matrix (98.23% and 98.59 % at low and high concentrations respectively) and Dilution Integrity (100.67 % for 25 percent dilution and 99.88 % for 50 percent dilution). The results of the stability studies indicate that the proposed method was stable and can be applied for the estimation of drug.

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