

## VALIDATION OF A DEVELOPED ANALYTICAL METHOD FOR DETERMINATION OF NATEGLINIDE AND METFORMIN HCL IN PURE AND PHARMACEUTICAL DOSAGE FORM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS DEGRADATION STUDIES

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### ABSTRACT

**Objective:** The objective of the study was to develop a versatile analytical method and validate according to International Council for Harmonization guidelines for simultaneous estimation of nateglinide and metformin HCl by reversed-phase high-performance liquid chromatography (RP-HPLC) in active pharmaceutical ingredient and in tablet dosage form.

**Methods:** Analytes, metformin and nateglinide, are separated and eluted from stationary phase luna phenyl hexyl column (150 mm × 4.6 mm, 3.5 μm) (micrometer) using polar mobile phase composed of acetonitrile:1% orthophosphoric acid 30:70 v/v, with flow rate of 1 ml/min for 8 min at ambient column temperature, at 221 nm (nanometer) detection. Acid, base, peroxide, thermal, and photolytic-induced degradation studies were performed on nateglinide and metformin.

**Results:** Through isocratic flow, both metformin and nateglinide are detected at retention times of 2.79 min and 5.13 min, respectively, at 221 nm. The linearity and range of analytical method for nateglinide and metformin were 0.61–9.15 μg/ml and 7.5–75.15 μg/ml, respectively. The R<sup>2</sup> value for nateglinide was 0.9998 and for metformin HCl was 0.9991. The limit of detection and limit of quantification for nateglinide were 0.21 μg/ml and 0.63 μg/ml and for metformin were 4.8 μg/ml and 14.6 μg/ml, respectively. The % relative standard deviation for method precision was found to be 0.22% and 0.64% for both nateglinide and metformin, respectively. The mean %recovery for nateglinide and metformin was 99.88% and 99.21%, respectively. The %thermal degradation was identified as 17.7% and 17.5% for nateglinide and metformin, respectively.

**Conclusion:** The developed chromatographic (RP-HPLC) method was selective, specific, economic, precise, and accurate. Hence, it can be one of the preferred analytical methods of choice for the estimation of nateglinide and metformin by RP-HPLC in pure and in tablet dosage form.

**Key words:** Nateglinide, Metformin, Reversed-phase high-performance liquid chromatography, Isocratic, Acetonitrile.

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### INTRODUCTION

Nateglinide is chemically 3-phenyl-2-[(4-propan-2-yl cyclohexane carbonyl) amino] propanoic acid (Fig. 1) with molecular formula C<sub>19</sub>H<sub>27</sub>NO<sub>3</sub>. It acts by blocking adenosine triphosphate sensitive potassium channels of beta cells of pancreas, causes membrane depolarization results in calcium influx and their by stimulation of insulin secretion. Metformin HCl is chemically N, N-Dimethyl imidodicarbonimidic diamide hydrochloride (Fig. 2) with molecular formula C<sub>4</sub>H<sub>11</sub>N<sub>5</sub>.HCl. The main mechanism of metformin HCl was lowering glucose intestinal absorption, inhibition of hepatic glucose production, and improving glucose uptake and utilization [1-6].

It was found that very few articles are available in detailed literature survey on simultaneous estimation of nateglinide and metformin HCl by reversed-phase high-performance liquid chromatography (RP-HPLC) in pure and dosage form [7-9]. The resting literature was found on analytical and bioanalytical methods by HPLC, LC-MS/MS, RP-LC, high-performance thin-layer chromatographic, and ultraviolet (UV) spectrophotometric estimations, in combination with glinides (nateglinide, repaglinide, and mitiglinide) and metformin HCl [10-21].

The comprehensive literature survey disclosed diverse analytical techniques of estimating nateglinide and metformin HCl in single and in combination with other drugs. The present study was taken up to

develop a sensitive, accurate, precise, and simple method of analysis for the estimation of both drugs in combined dosage forms.

### METHODS

#### Chemicals and reagents

The active pharmaceutical ingredients (APIs), nateglinide and metformin hydrochloride, were supplied as a gift sample by Care Labs, L.B Nagar, Hyderabad, and marketed formulation was purchased from the local market. HPLC grade orthophosphoric acid, acetonitrile, and water were of Merck grade. Waters autosampler RP-HPLC, e2695 pump, and 2998 photodiode array (PDA) detector with Empower2 software were employed in this method.

#### Selection and preparation of mobile phase and diluent

In RP-HPLC, pure API mixture containing nateglinide and metformin HCl at lower concentration levels were prepared, injected, and run with different solvent systems. Different combination of solvents using acetonitrile, triethylamine, and orthophosphoric acid at different compositions, flow rates, and ratios were tried to optimize the mobile phase. Finally from the trials, mobile phase and diluent (acetonitrile and 0.1% orthophosphoric acid in a ratio of 30:70 v/v) are selected since they were fulfilling the requirements and the results obtained were within the acceptable limits.

#### Preparation of standard stock solution

Powder analytes equivalent to 6 mg and 50 mg of nateglinide and metformin HCl, respectively, were accurately weighed and transferred

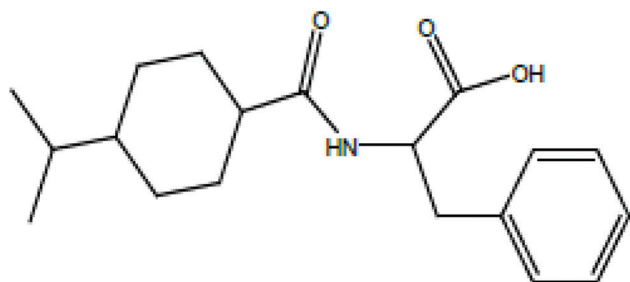


Fig. 1: Chemical structure of nateglinide

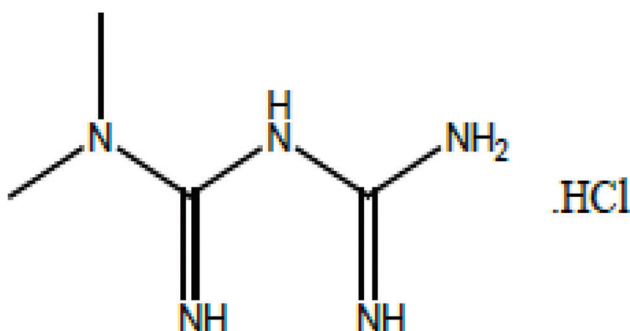


Fig. 2: Chemical structure of metformin HCl

to calibrated 100 ml volumetric flask, added 50 ml of diluent, sonicated for 10 min, and filled up to the mark with diluent.

#### Preparation of working standard solution

Five milliliters of standard stock solution were transferred into 50 ml calibrated volumetric flask and filled up to the mark with the diluent.

#### Selection of detection wavelength ( $\lambda_{max}$ ) (maximum absorbance wavelength)

Nateglinide (6  $\mu\text{g/ml}$ ) and metformin HCl (50  $\mu\text{g/ml}$ ) solutions were prepared separately using diluent and scanned separately in Shimadzu 1800 UV-visible spectrophotometer at a range of 200–400 nm.

#### Optimized chromatographic conditions

Many trials have been conducted to optimize all the chromatographic conditions required for simultaneous estimation of nateglinide and metformin HCl. Finally, reverse phase C18 column, luna phenyl hexyl column (150 mm $\times$ 4.6 mm, 3.5  $\mu$ ), mobile phase containing acetonitrile:0.1% orthophosphoric acid (30:70 v/v ratio), flow rate 1.0 ml/min, run time 8 min, injection volume – 10  $\mu\text{l}$ , and a detection wavelength – 221 nm using a PDA detector are the best possible optimized chromatographic conditions which gave the best resolution, s/n (signal to noise) ratio, peak tailing, and United States Pharmacopeia (USP) plate count for the present estimation.

#### Preparation of calibration curve solutions

Eight standard solutions were prepared to construct calibration curve. Different concentrations of nateglinide (0.61  $\mu\text{g/ml}$ –9.15  $\mu\text{g/ml}$ ) and metformin HCl (5.01  $\mu\text{g/ml}$ –75.15  $\mu\text{g/ml}$ ) were prepared using diluent and injected into stabilized RP-HPLC system which was in already mentioned optimized chromatographic conditions. The peak areas (on y-axis) obtained from respective concentrations (on x-axis) were taken into consideration to plot a calibration curve. The linearity, range, intercept, slope, and  $r^2$  were calculated from calibration curve.

#### Analysis of nateglinide and metformin HCl from marketed tablets

From 20 tablets weight, average weight of each tablet was calculated. Triturated the tablets into fine powder and equivalent to 6 mg of nateglinide and 50 mg of metformin HCl of tablet powder was weighed

and transferred to 100 ml volumetric flask mixed with 70 ml of diluent, sonicated for 30 min, and filled up to the mark with the same. Filtered the resulting solution through 0.45  $\mu\text{m}$  filter paper and from the filtrate 5 ml was transferred in 50 ml of volumetric flask mixed and adjusted the volume to the mark with diluent and sonicated the final solution for 5 min. From the prepared solution, 10  $\mu\text{l}$  replicates were injected into RP-HPLC chromatographic system. The peak areas obtained from chromatograms were tabulated for amount of nateglinide and metformin HCl present in each tablet.

#### System suitability

To ensure that the system was working perfectly and exploring the feasible results throughout the analysis, system suitability test was performed. To evaluate system suitability, six working standard solutions were injected and the parameters such as resolution (Rs), retention time (Rt), tailing factor (Tf), USP plate count (N), and %RSD of peak areas were calculated from the chromatograms of analytes of interest.

#### Validation of analytical method [22-25]

##### Selectivity and specificity

The developed method was said to be selective, when nateglinide and metformin HCl are completely separated from each other with fixed resolution and retention time at optimized chromatographic conditions. Selectivity of the recommended method was evaluated by repeated injections of working standard solutions.

The purpose of testing specificity was to identify the interference peaks from the impurities or from excipients of dosage form at the same retention times of compounds of interest at optimized chromatographic conditions. By injecting tablet extract, placebo blank, standard drug solutions, and mobile phase (blank) specificity of the method was evaluated.

#### Accuracy

According to the guidelines to estimate the accuracy of the method, triplicates of three different concentration levels of standard solutions (50%, 100%, and 150%) containing both nateglinide and metformin HCl were injected into RP-HPLC system. Before standard injections, a blank solution (mobile phase) was injected. From each chromatogram obtained, percentage of drug recovery, mean % of drug recovery, and %RSD are calculated.

#### Precision

##### System precision

System precision or chromatographic system performance was estimated by injecting six replicate freshly prepared working standard solutions containing 6  $\mu\text{g/ml}$  of nateglinide and 50  $\mu\text{g/ml}$  of metformin HCl (100% test concentration) into HPLC system. The percentage relative standard deviation (%RSD) was computed from the peak areas of nateglinide and metformin HCl chromatograms.

##### Method precision

To determine method precision of proposed analytical method, six replicates of working standard solutions and six replicates of sample solutions containing 6  $\mu\text{g/ml}$  of nateglinide and 50  $\mu\text{g/ml}$  of metformin HCl was injected into HPLC system without changing the optimized chromatographic conditions. From the peak areas obtained, calculated the presence of percentage of analytes in each injection. The %RSD for mean peak areas and assay was computed.

##### Intermediate precision

Six samples of same batch were analyzed to calculate the intermediate precision of the proposed analytical method by different analyst on different day on different instrument. The %RSD for mean peak areas and assay was computed.

##### Robustness

To ensure the robustness of the proposed analytical method, the flow rate was adjusted to 0.8 ml/min, 1.2 ml/min and organic composition of

mobile phase was changed to  $\pm 10\%$  and notified the changes occurred in the chromatograms after injecting the working standard solutions and sample solutions containing nateglinide and metformin HCl.

#### Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ are defined as the lowest concentrations to detect and to quantify the analyte(s) respectively. To estimate LOD and LOQ, a series of dilutions of both nateglinide and metformin HCl was injected and plotted a calibration graph between peak areas and concentration. Finally, LOD and LOQ values were finalized using regression analysis.

The preferable formulas to calculate LOD and LOQ are as follows:

$$\text{LOD} = \frac{3.3 \sigma}{S}$$

$$\text{LOQ} = \frac{10 \sigma}{S}$$

Where  $\sigma$  = Standard deviation of the response and S = Slope of calibration curve.

#### Forced degradation studies

Sample stock solution for forced degradation studies was prepared by mixing 62.1 mg of sample powder with 70 ml of diluent in 100 ml volumetric flask and sonicated for 30 min and diluted to the mark with the same diluent.

#### Acid-induced degradation

Five milliliters of sample stock solution and 1 ml of 1 N HCl were taken in a 50 ml volumetric flask, placed in water bath for 30 min at 60°C constant temperature. Further after cooling to room temperature, 1 ml of 1N NaOH was added and diluted to the mark with diluent and stored at room temperature. The resulting solution was injected after 24 h.

#### Alkali-induced degradation

Five milliliters of sample stock solution and 1 ml of 1 N NaOH were taken in a 50 ml volumetric flask, placed in water bath for 30 min at 60°C

constant temperature. Further after cooling to room temperature, 1 ml of 1 N HCl was added and diluted to the mark with diluent and stored at room temperature. The resulting solution was injected after 24 h.

#### Peroxide-induced degradation

Five milliliters of sample stock solution and 1 ml of 30% H<sub>2</sub>O<sub>2</sub> were taken in a 50 ml volumetric flask, placed in water bath for 30 min at 60°C constant temperature. Further after cooling to room temperature diluted to the mark with diluent and stored at room temperature. The resulting solution was injected after 24 h.

#### Thermal-induced degradation

The sample powder was exposed at 105°C for 72 h. 62.1 mg of this sample was weighed and transferred into 100 ml volumetric flask containing 70 ml of diluent and sonicated for 30 min and diluted to the mark with the same diluent. Further, 5 ml was diluted to 50 ml with diluent, stored at room temperature, and injected into RP-HPLC system after 24 h.

#### Photolytic degradation

The sample powder was exposed under UV light for 24 h. 62.1 mg of this sample was weighed and transferred into 100 ml volumetric flask containing 70 ml of diluent and sonicated for 30 min and diluted to the mark with the same diluent. Further, 5 ml was diluted to 50 ml with diluent, stored at room temperature, and injected into RP-HPLC system after 24 h.

## RESULTS AND DISCUSSION

#### Selection of detection wavelength ( $\lambda_{\text{max}}$ )

In the UV spectrum, nateglinide has shown  $\lambda_{\text{max}}$  at 212.3 nm and metformin HCl has shown  $\lambda_{\text{max}}$  at 258.3 nm. In the overlain spectra, both nateglinide and metformin HCl show absorbance at 221 nm as common middle wavelength (Fig. 3).

#### Construction of calibration curve

As shown in Figs. 4 and 5, straight lines are obtained on calibration plots with linearity and range from 0.61  $\mu\text{g/ml}$  to 9.15  $\mu\text{g/ml}$ ; whereas, the regression equation was  $y=197,824x + 458.32$  for nateglinide and for metformin HCl, the linearity and range from 7.5  $\mu\text{g/ml}$  to 75.15  $\mu\text{g/ml}$ ; whereas, the regression equation was  $y=50,772x + 47,812$ . The R<sup>2</sup> values for nateglinide and metformin HCl are 0.9998 and 0.9991, respectively (Table 1).

#### Analysis of nateglinide and metformin HCl from marketed tablets

One of the marketed formulations was analyzed with the proposed RP-HPLC method and the mean assay was identified as 100.6% and 99.18% against the label claim of nateglinide (60 mg) and metformin HCl (500 mg), respectively. The %RSD of mean assay of the formulation was within the acceptable limits  $\leq 2$  (Table 2).

#### System suitability

From the data obtained for six injections, it was observed that the resolution (Rs) was  $>2$ , tailing factor (Tf) was  $<2$ , USP theoretical plates

Table 1: Linearity results

Parameter	Nateglinide	Metformin HCl
Linearity and range ( $\mu\text{g/ml}$ )	0.61-9.15	7.5-75.15
Slope (a)	197,864	50,772
Intercept (b)	458.32	47,812
Coefficient correlation ( $R^2$ )	0.9998	0.9991
Regression equation	$y=197,864x+458.32$	$y=50,772x+47,812$
SD of intercept	12,583.41	74,355.68

SD: Standard deviation,  $\mu\text{g/ml}$ : Microgram per milliliter

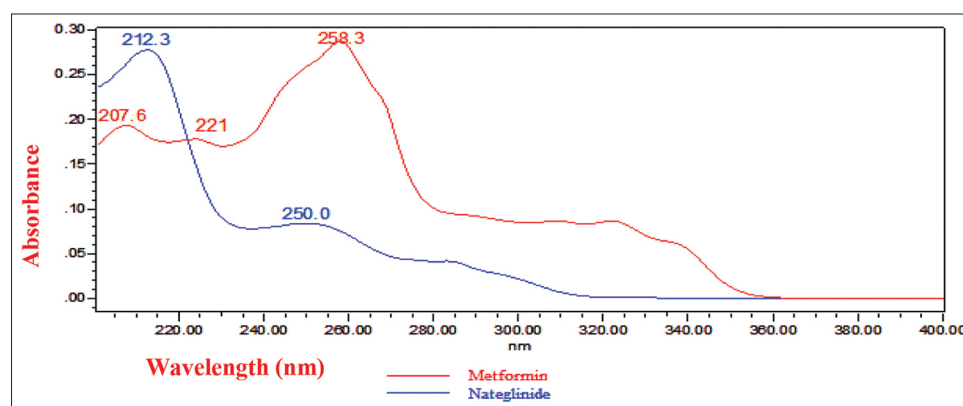


Fig. 3: Selection of isosbestic point for nateglinide and metformin HCl

count (N) was >2000, and the %RSD of peak areas of working standard solution was <2. The numerals are represented in Table 3.

### Validation of proposed analytical method

#### Selectivity and Specificity

From the chromatograms Fig. 6 on observation, it was found that the retention times and resolution of nateglinide and metformin HCl were

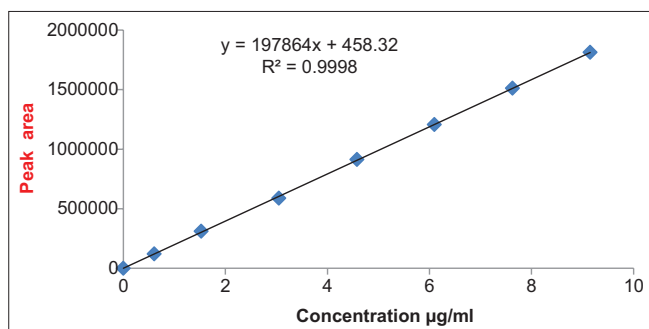


Fig. 4: Linearity curve of nateglinide

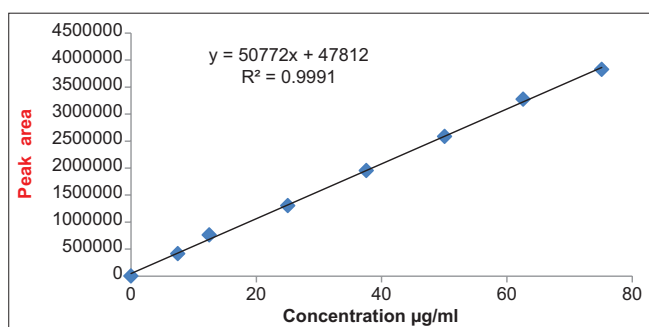


Fig. 5: Linearity curve of metformin HCl

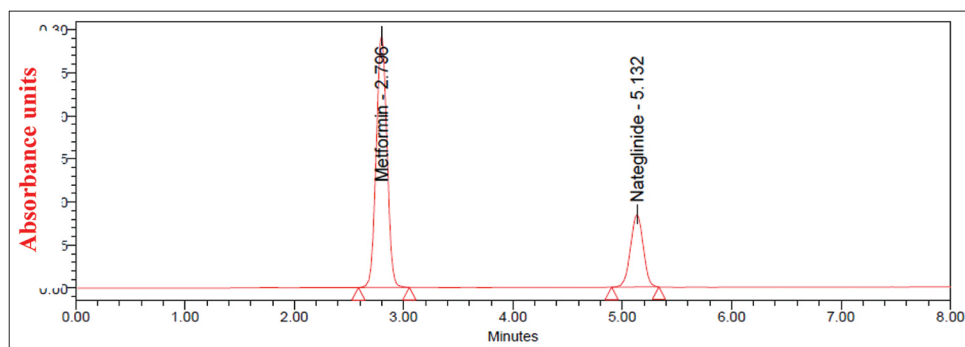


Fig. 6: Chromatogram of mixture of standard nateglinide and metformin HCl with retention time of 2.796 min and 5.132 min, respectively

Table 2: Analysis of nateglinide and metformin HCl from marketed tablets

Brand name	Drugs	Label claim	Assay*	Amount recovered	Amount recovered ±%RSD
GLINATE-MF	Nateglinide	60 mg	99.18%	59.51mg	59.51±0.61
	Metformin HCl	500 mg	100.60%	503 mg	503±0.82

\*Average of five determinations, RSD: Relative standard deviation

Table 3: System suitability results

Parameter	Observed results*		%RSD	
	Metformin HCl	Nateglinide	Metformin HCl	Nateglinide
Retention time (Rt)	2.79±0.00	5.11±0.00	0.093	0.068
Peak areas	25,532.67±92.44	12,587.5±151.88	0.362	1.207
Theoretical plates (N)	3557±34.28	8841.67±52.27	0.964	0.591
Tailing factor (Tf)	0.87±0.11	0.95±0.024	-	-
Resolution (Rs)	-	11.28±0.15	-	1.38

\*Average of six determinations, Rt: Retention time, Rs: Resolution, USP: United States Pharmacopeia, Tf: Tailing factor: RSD: Relative standard deviation

fixed same as such without any changes confirm the selectivity of the analytical method.

The perusal of Fig. 7 reveals that the mobile phase scan and placebo scan did not show any peaks at the retention time of analytes of interest. However, the tablet extracts scan and working standard solutions scan gave characteristic peaks of nateglinide and metformin HCl. These results convey specificity of the method.

#### Accuracy

The calculated mean % recoveries were 99.21% and 99.88% for metformin HCl and nateglinide, respectively. Hence, the recovery study proves the accuracy of the proposed RP-HPLC method (Table 4).

#### Precision

The %RSD of peak areas of six replicate injections of working standard solution was estimated as 0.14% and 0.41% for metformin HCl and nateglinide, respectively. This indicates that the system precision was within the limits of acceptability.

The mean assay of six replicate injections of sample solutions was estimated as 99.53% for metformin HCl and 99.05% for nateglinide. Hence, the method was reproducible.

Six replicate injections of sample solutions when analyzed on different day by different analysts with different column and the mean assay was calculated as 99.65% for metformin HCl and 99.17% for nateglinide. Hence, the method was reproducible on different instrument on different day (Table 5).

#### Robustness

To ensure the robustness of the proposed method, sample solutions were injected for 3 times after each change in optimized chromatographic parameters. From the procured data, mean peak area of sample, %RSD of peak area, and mean assay for sample were calculated. The %RSD of sample peak areas at all the deliberate conditions was identified as ≤2% (Table 6).

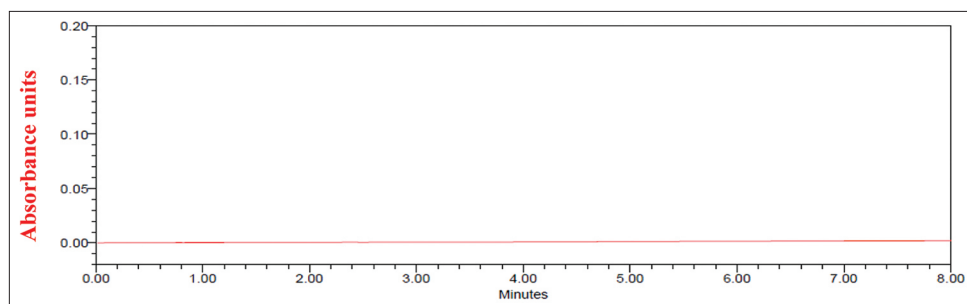


Fig. 7: Chromatogram of placebo

Table 4: Accuracy results

Drugs	Level %	Amount added ( $\mu\text{g/ml}$ )	Amount found ( $\mu\text{g/ml}$ )	%Recovery $\pm$ SD (n=3)	%RSD
Metformin HCl	50	25.1	24.78	98.73 $\pm$ 0.3	0.30
	100	50.1	49.99	99.73 $\pm$ 0.25	0.25
	150	75.1	74.50	99.16 $\pm$ 0.94	0.94
Nateglinide	50	3.1	3.13	100 $\pm$ 0.79	0.79
	100	6.2	6.17	99.53 $\pm$ 0.15	0.15
	150	9.2	9.24	100.1 $\pm$ 0.43	0.429

STD: Standard, SD: Standard deviation RSD: Relative standard deviation

Table 5: Precision results

Parameter	Drug	Mean peak area*	%RSD
System precision	Metformin HCl	2,517,052 $\pm$ 3624.25	0.14
	Nateglinide	1,199,129 $\pm$ 4929.91	0.41
Method precision	Metformin HCl	Mean % recovery* 99.53 $\pm$ 0.35	0.35
	Nateglinide	99.05 $\pm$ 0.63	0.63
Intermediate precision	Metformin HCl	Mean % recovery* 99.53 $\pm$ 0.35	0.35
	Analyst-1	99.65 $\pm$ 0.39	0.39
	Analyst 2	Mean % recovery* 99.05 $\pm$ 0.63	0.39
	Nateglinide	99.17 $\pm$ 0.4	0.41

\*Average of six determinations, SD: Standard deviation, %RSD: Percentage relative standard deviation

Table 6: Robustness results

Parameter	Metformin HCl			Nateglinide		
	Area*	Rt*	Tf*	Area*	Rt*	Tf*
Flow plus (1.2 ml/min)	2,266,340	2.31	1.04	1,046,318	4.23	1.01
Flow minus (0.8 ml/min)	2,853,697	3.44	1.06	1,569,444	6.35	1
Organic solvent ratio (+10%)	2,262,110	2.45	1.04	1,153,106	3.84	1.01
Organic solvent ratio (-10%)	2,753,249	3.25	1.05	1,348,965	7.36	0.98

\*Average of three determinations, Rt: Retention time, Tf: Tailing factor

Table 7: LOD and LOQ results

S. No.	Parameter	Nateglinide	Metformin HCl
1	LOD	0.21 $\mu\text{g/ml}$	4.8 $\mu\text{g/ml}$
2	LOQ	0.63 $\mu\text{g/ml}$	14.6 $\mu\text{g/ml}$

LOD: Limit of detection, LOQ: Limit of quantification

#### Flow rate 1.2 ml

The sample assay at 1.2 ml/min was calculated as 99.87% for nateglinide, and for metformin HCl, it was 99.83%.

#### Flow rate 0.8 ml

The sample assay at 0.8 ml/min was calculated as 99.53% for nateglinide, and for metformin HCl, it was 99.83%.

#### More organic mobile phase (+10%)

The mobile phase organic composition when changed to +10% the calculated assay was 98.8% for nateglinide, and for metformin HCl, it was 99.07%.

#### Less organic mobile phase (-10%)

The mobile phase organic composition when changed to -10% the calculated assay was 99.67% for nateglinide, and for metformin HCl, it was 99.73%.

#### LOD and LOQ

SD of intercept was calculated by regression analysis. Using slope of calibration plot and SD of intercept, LOQ and LOD are calculated.

The estimated LOD and LOQ values for nateglinide are 0.21  $\mu\text{g/ml}$  and 0.63  $\mu\text{g/ml}$ , respectively; for metformin HCl, the LOD and LOQ are 4.8  $\mu\text{g/ml}$  and 14.6  $\mu\text{g/ml}$ , respectively (Table 7).



**Table 8: Stability indicating method data for nateglinide and metformin HCl**

Degradation condition	Nateglinide		Metformin HCl	
	Peak area	% degradation	Peak area	% degradation
Control	1,202,125	-0.1	2,523,178	-0.1
1 N HCl (acid)	102,3478	14.8	2,151,437	14.8
1 N NaOH (alkaline)	994,732	17.2	2,139,878	15.2
30% H <sub>2</sub> O <sub>2</sub> (peroxide)	105,7824	12	2,128,941	15.6
Thermal (105°C)	994,712	17.2	2,091,462	17.1
Photolytic	988,793	17.7	2,082,987	17.5

N: Normality, HCl: Hydrochloric acid, NaOH: Sodium hydroxide, H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide

### Forced degradation studies

Estimated assay after forced degradation with 1 N HCl was 85.3% and 85.3% of nateglinide and metformin HCl, respectively, it was 82.9% and 84.3% of nateglinide and metformin HCl, respectively, with 1 N NaOH, and with peroxide, it was 88.1% and 84.5% of nateglinide and metformin HCl, respectively. When the sample was exposed at 105°C, the estimated assay was 82.9% and 83.0% of nateglinide and metformin HCl, respectively, and sample on exposure with UV radiation the assay was 82.4% and 82.6% of nateglinide and metformin HCl, respectively (Table 8).

### CONCLUSION

From the available literature, it was observed and notified that only few articles are reported the stability indicating simultaneous estimations of nateglinide and metformin HCl by RP-HPLC. A New stability indicating method was developed and fully validated with mobile phase (acetonitrile:0.1% orthophosphoric acid 30:70 v/v), column (luna phenyl hexyl 150 mm × 4.6 mm, 3.5 μ), detection wavelength at 221 nm, and at other optimized chromatographic conditions. From the validation report, it was found that the developed RP-HPLC method was suitable, simple, economic, specific, and precise for the estimation of nateglinide and metformin HCl in tablet dosage forms. Stability studies indicated that the nateglinide and metformin HCl could be evaluated simultaneously by RP-HPLC in the presence of their degradation products. Hence, this method can be applied and implemented to study stability samples in the industry.

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### AUTHORS' CONTRIBUTIONS

K Md Ismail: Concept and design of work, data collection, data analysis, drafting and revision of article, and final approval of the revision to be published.

Dr. A. Lakshmana Rao: Design of work, drafting and revision of article, and final approval of the revision to be published.

### CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

### AUTHORS' FUNDING

Nil.

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