Development and Validation of High Performance Liquid Chromatographic Method for the Determination of Nateglinide in Human Plasma by HPLC-UV Detection

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Abstract- A rapid, specific and accurate high performance liquid chromatographic method for the determination of Nateglinide in human plasma using Repaglinide as internal standard was developed and validated by UV detection. The extraction process involved a liquid-liquid extraction using ethyl acetate. Both Nateglinide and the internal standard were eluted under isocratic mode using a 150 X 4.6 mm i.d, 5 µm Phenomenex ODS 2 C18 column. The mobile phase composed a mixture of 50:50 % v/v Methanol and 10 mM phosphate buffer at a flow rate of 1.0 mL/minute. The wavelength of detection is 225 nm. The injection volume is 10 µL. The runtime of the method is 5 minutes. The method showed good linearity in the range of 50.42 – 10079.52 ng/mL. The mean recovery of Nateglinide from all the quality control samples is 89.34 % with a % c6efficient of variation of of 2.2 % and recovery of internal standard was 93.3% at MQC level. Matrix effects were not observed. The method is validated as per ICH guidelines.

Keywords- Anti-diabetics, Ethyl acetate, HPLC,Methanol, Nateglinide,Phosphate buffer, Repaglinide

1 INTRODUCTION

Nateglinide[1] is N-[[trans-4-(1-Methylethyl) cyclohexyl] carbonyl]-D-phenylalanine [Figure-1A], is classified as a oral hypoglycemic agent used for the treatment of non-insulin dependent diabetes mellitus. Nateglinide is official in the European pharmacopoeia[2] and the United states pharmacopoeia[3]. Nateglinide acts by binding to the β cells of the pancreas to stimulate insulin release. Nateglinide is an amino acid derivative that induces and early insulin response to means decreasing the postprandial blood glucose levels. Estimation of Nateglinide either alone or in combination by UV-Visible spectrophotometry or by RP-HPLC with other drugs in pharmaceutical dosage forms has been reported[4-16]. The enantiomeric separation of Nateglinide by using chiral stationary phase by HPLC or capillary electrophoresis has been reported [17-19]. The related substances of Nateglinide in bulk formulation are reported by He et al[20].

Mao et al[21], reported the HPLC based bioanalytical estimation of Nateglinide by column switching RP-HPLC technique. A pre-column derivatization using a coumarin-type fluorescent reagent has been used for estimation of Nateglinide in human plasma[22].

Determination of Nateglinide in human plasma by micellar electrokinetic chromatography and online sweeping technique has also been reported23. The most sensitive methods by the usage of liquid chromatography coupled to mass spectrometric detector were also reported[24-25]. Nateglinide has also been estimated in preclinical subjects[26].

Only one method has been reported for the estimation of Nateglinide in human plasma followed by UV detection[27]. It can therefore be understood that the usage of Nateglinide is versatile and most of the earlier reported methods require a modification by pre-column derivatization or column switching lesser sensitivity, and high noise in the base line indicating a need to develop a more efficient, sensitive, simple and rapid method in human plasma. Other techniques such as LC-MS/MS although sensitive yet they are costly. We therefore focused on to achieve the optimum chromatographic conditions for the determination of Nateglinide using Repaglinide as internal standard. To access the reproducibility and wide applicability of the developed method, it was validated as per FDA guidelines [28].

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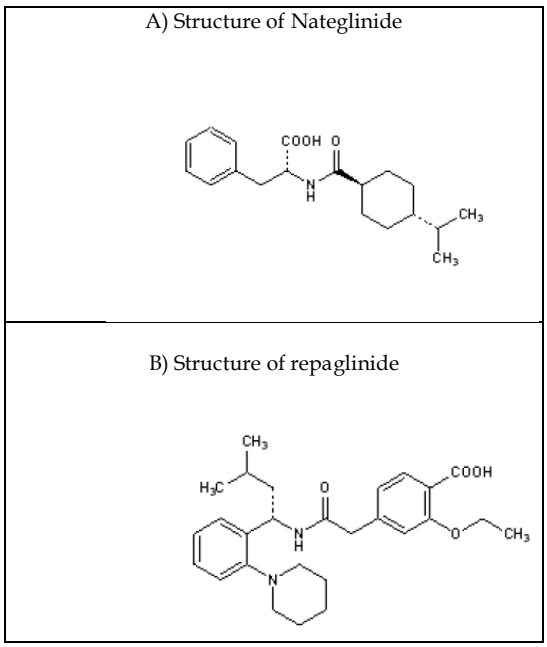
FIGURE 1: STRUCTURE OF NATEGLINIDE AND REPAGLINIDE



2 MATERIALS AND METHODS

2.1 Solvents and Chemicals

Nateglinide (purity 98.00 % w/w) was used as received from Lupin Laboratories Ltd. Repaglinide (used as Internal Standard, Purity 99.0 % w/w) is purchased Sigma Aldrich Inc. HPLC grade Methanol, potassium dihydrogen phosphate is purchased from S.D. Fine Chem, Mumbai. Ortho phosphoric acid was purchased from Merck Ltd (Mumbai, India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

2.2 Chromatographic System

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software. The detector is set at a wavelength of 225 nm. Chromatographic separations were accomplished using a Phenomenex prodigy ODS 2 C18, 5 µm, 150 mm×4.6 mm column. The mobile phase consists of a mixture of 50 parts of Methanol and 50 parts of 10 mM Potassium dihydrogen phosphate pH adjusted to 2.5 with orthophosphoric acid. The mixture was filtered through 0.22 µm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by flushing with nitrogen for 15 min. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min during analysis, at ambient temperature. The rinsing solution consists of a mixture of 50: 50 % v/v of methanol: HPLC Grade Water.

2.3 Preparation of Standard Solutions

A stock solution of Nateglinide is prepared in methanol such that the final concentration is approximately 1.0 mg/mL. Stock solution of Repaglinide (approx 2 mg/mL) is prepared in HPLC Grade methanol. The solutions were stored at 4°C and they were stable for at least two weeks. Aqueous stock dilution of Nateglinide is prepared in diluent solution (mixture of 50: 50 % v/v of methanol: HPLC Grade water).

2.3.1Sample Preparation

Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution is transferred into a 10 mL volumetric flask. The final volume is made up with screened drug-free K2EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0.0 (Blank; no Nateglinide added), 50.40, 100.80, 503.98, 2015.90, 4031.81, 6047.71, 8063.62 and 10079.52 ng/ml. Each of these standard solutions was distributed in disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.3 ml and stored at -70°C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 52.78, 155.22, 5039.76 and 7055.66 ng/ml respectively and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

2.3.2 Extraction procedure

The extraction of the plasma samples involved Liquid-Liquid Extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 200 µL is then transferred to pre-labeled 2.0 mL polypropylene centrifuge tubes. 50 µL of internal standard dilution (50 µg/mL) is then added and mixed. 1.5 mL of ethyl acetate is then added to extract the drug and internal standard. The samples are then kept on a reciprocating shaker and allowed to mix for 20 minutes. Samples are then centrifuged at 5000 rpm for 5 minutes at 4 oC. The samples are then subjected to flash-freezing using a mixture of dry-ice and acetone mixture. The supernatant is then transferred into prelabelled polypropylene tubes and allowed to evaporate to dryness under nitrogen at constant temperature of 40 oC. The dried residue is then dissolved in 500 µL of mobile phase and transferred into shell vials and containing vial inserts for analysis. 10 µL of the samples is then injected into the system for analysis. The autosampler temperature is maintained at 4 oC throughout the analysis. The column temperature oven is maintained at ambient temperature.

3 Validation of quantitative HPLC method

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, accuracy and

precision, limit of detection (LOD), limit of quantitation, % recovery, short term, long term, freeze–thaw, and auto sampler stability. The initial assay was fully validated for Nateglinide analysis in human plasma according to FDA guidelines.

3.1 Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free K2EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

3.2 Calibration curve

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of Nateglinide in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. Calibration curves for Nateglinide were represented by the plots of the peak-area ratio (Nateglinide / repaglinide) versus the nominal concentration of the Nateglinide in calibration standards. The regression line was generated using 1/concentration ^{2} factor as the mathematical model of best fit. Nateglinide concentrations in QC samples, recovery, and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

3.3 Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and the accuracy were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) along with three separate standard curves done in duplicates.

The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

Accuracy (%) = mean observed concentration/nominal concentration × 100

The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:

CV (%) =standard deviation/mean observed concentration× 100

3.4 Stability Studies

Autosampler, and freeze–thaw stability of Nateglinide was determined at low, medium and high QC concentrations. Bench top, long term stability of the plasma matrix is evaluated. To determine the impact of freeze–thaw cycles on Nateglinide concentration, samples were allowed to undergo 3 freeze (−70°C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the Nateglinide concentrations were analyzed in triplicates and compared to the control sample that had been stored at −70°C. Autosampler stability of extracted samples was determined by comparing Nateglinide concentration in freshly prepared samples and samples kept in autosampler at 4°C for 36 hrs. Aqueous solutions of Nateglinide and internal standard were also evaluated for bench top stability (approx 7 hrs) and refrigerated stability for 8 weeks.

3.5 Recovery

Recovery was determined by comparing the area under the curve (AUC) of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of Nateglinide as in the QC samples. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 50 µg/mL.

3.6 Data analysis

HPLC data acquisition and processing was performed by Shimadzu LC Solutions Ver 1.23 SP 1 software. Standard curves for quantitation of Nateglinide were constructed using a 1/concentration 2 weighted linear regression of the peak area ratio versus Nateglinide concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

4 RESULTS AND DISCUSSION

4.1 Method Development

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 we employed the usage of liquid-liquid extraction process. Initial experiments were performed by using non-polar solvents like t-butyl methyl ether, dichloromethane and diethyl ether. A mixture of 70 parts of t-butyl methyl ether and 30 parts of dichloromethane gave a comparable recovery to that of ethyl acetate. To avoid the preparation errors, we continued the usage of ethyl acetate as the extraction solvent. To get a better response the pH of the mobile phase is set to the acidic side. During our observation, a pH value around 2.5 resulted in better peak shape for the internal standard while that of the drug is not acceptable. Also, higher pH values of the mobile phase resulted in blunt peaks and also very late elution. Therefore experiments were performed using Potassium Dihydrogen phosphate in a limited pH range of 2.5 to pH 5.5. The response was checked at the detector using a connector (without the column). A pH value of 2.5 ± 0.1 gave maximum response for the analyte at 225 nm. Therefore the final mobile phase consisted of 50: 50 % v/v methanol and 10 mM potassium dihydrogen phosphate pH adjusted to 2.5 with orthophosphoric acid. The run time of analysis is higher when a longer reverse phase column (250 X 4.6 mm id) is used. The resolution between the peaks was decreased and peaks were not acceptable peak shape when the

experiment is performed using a shorter column (50 X 4.6 mm id). However better resolution, less tailing and high theoretical plates are obtained with a Phenomenex column C18 150 X 4.6 cm 5 µm column.

The flow rate of the method is 1.0 ml/min. The column temperature is maintained at ambient. At the reported flow rate, peak shape was acceptable, however increasing or decreasing the flow rate increased the tailing factor and resulting in poor peak shape and decreased resolution between the drug and internal standard. 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 10 mM potassium dihydrogen phosphate pH adjusted to 2.5 with orthophosphoric acid leading to better resolution of the drug and internal standard. Increasing the organic portion of the mobile phase caused Repaglinide to elute early. A mobile phase containing aqueous portion greater than 50 % led to very late elution and very poor peak shape for Nateglinide. The peaks were also broad with unacceptable asymmetry factor.

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.

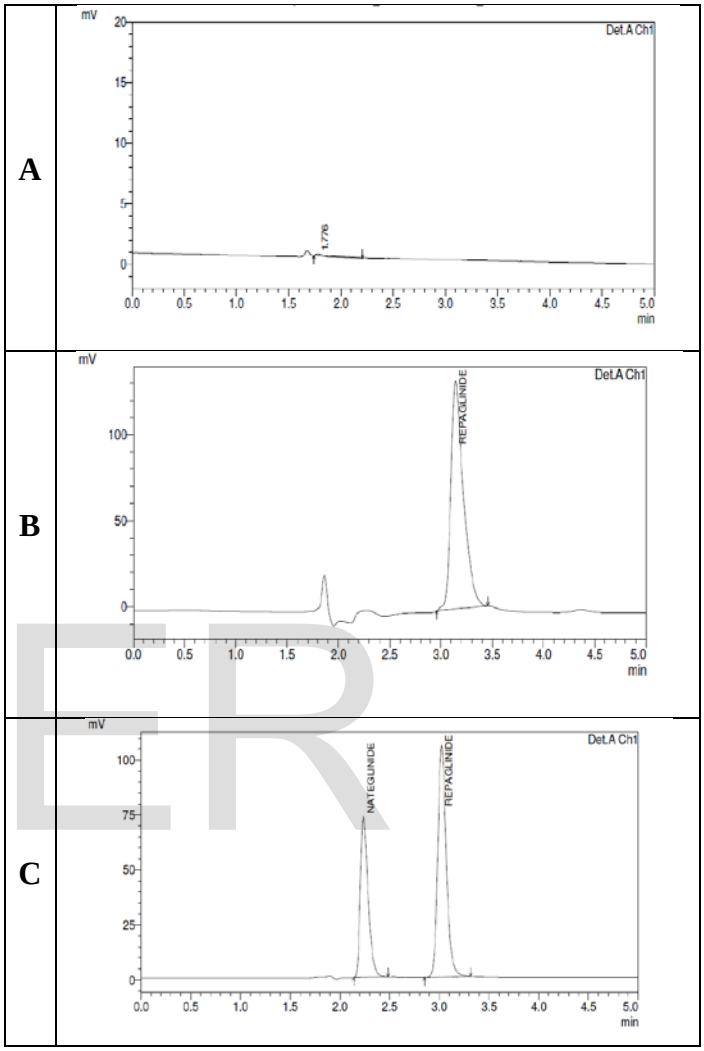
Extraction methods were initially attempted using Protein precipitation technique. Precipitation technique was adopted using Acetonitrile and or Methanol. Initial experiments of protein precipitation were done using 1: 3 ratio of plasma : Organic solvents. The recovery of the Nateglinide is moderate 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, we have done the final analysis using liquid-liquid extraction (LLE). SPE methods although 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.

4.2 Detection and chromatography

Figure 3 shows the typical chromatograms of a blank human plasma sample (A), a zero blank sample with Repaglinide (B), and (C) with a sample containing ULOQ sample extracted using Repaglinide as internal standard indicating the specificity of the method. The retention times for Nateglinide and Repaglinide were 2.23 and 3.02 min, respectively.

FIGURE 2: CHROMATOGRAMS OF (A) EXTRACTED BLANK SAMPLE (B) ZERO BLANK CONTAINING REPAGLINIDE AS INTERNAL STANDARD (C) NATEGLINIDE CONTAINING REPAGLINIDE AS INTERNAL STANDARD AT ULOQ LEVEL.



4.3 Method validation

4.3.1 Selectivity

The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for Nateglinide in any of the six independent blank plasma extracts evaluated (Figure 3-A).

4.3.2 Calibration curves

A system suitability exercise is performed before the initiation of the validation. A system is assumed to be suitable for analysis if and only if the % CV for the retention times of Nateglinide and internal standards is less than 2 %. For preparation of pooled plasma for the validation, Six different lots of blank plasma were screened for specificity. All the lots were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard.

The same human EDTA plasma lots free of interfering substances were used to prepare the calibration curve standards and the quality control samples for the validation study. The results of specificity were demonstrated in Table 1.

Calibration curves for Nateglinide in human plasma were fitted by weighted 1/concentration2 quadratic regression, with the r2 values of >0.99 for all curves generated during the validation. The calibration curve accuracy for plasma is presented in **Table 2** demonstrating that measured concentration is within ± 15% of the actual concentration point (20% for the lowest point on the standard curve, the LLOQ). Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and r2 value is depicted in Figure – 2.

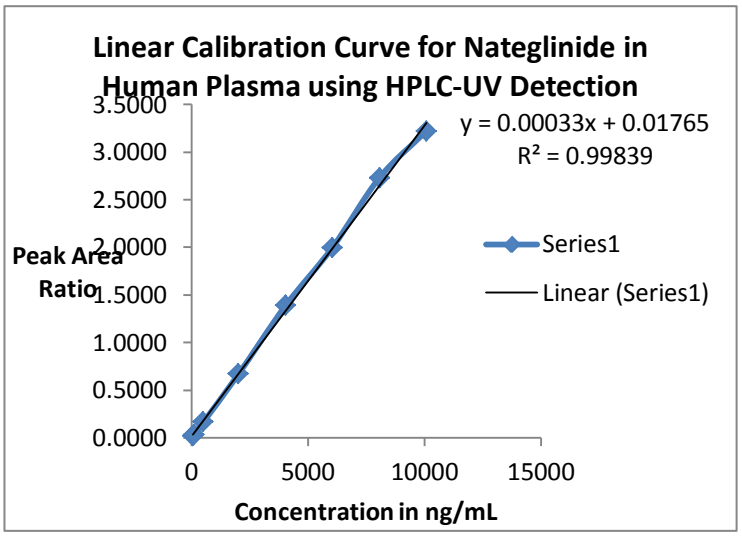
TABLE 1: SPECIFICITY EXERCISE: PERCENT INTERFERENCES AT THE RETENTION TIMES OF THE DRUG AND THE INTERNAL STANDARDS

Results for Nateglinide						
Human Plasma ID	Nateglinide			Repaglinide		
	Response in Blank	Response in LLOQ	% Interference	Response in Blank	Response in LLOQ	% Interference
1	155	4619	3.36	355.00	248810	0.14
2	142	5174	2.74	412.00	252001	0.16
3	161	4817	3.34	448.00	251456	0.18
4	175	5079	3.45	561.00	253346	0.22
5	121	4939	2.45	176.00	253065	0.07
6	96	5030	1.91	127.00	252435	0.05
Average	141.67	4943.0	2.87	346.50	251852.17	0.14
Total No. of Matrices	6	Number of Matrices Meeting the Requirements				6
Percentage of Matrices Meeting Selectivity Criteria				100.0%		

TABLE 2: RESULTS OF REGRESSION ANALYSIS OF THE LINEARITY DATA

Linearity parameters	Mean (n = 3)
Slope	0.000323
Intercept	0.019013
Correlation coefficient (r^2)	0.998333

FIGURE 2: CALIBRATION CURVE OF NATEGLINIDE



4.3.3 Accuracy and precision

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation is presented in Table 3. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines [28].

TABLE 3: INTRA AND INTER DAY ACCURACY AND PRECISION OF HPLC ASSAY

	Nominal Concentration (µg/mL)			
	LLOQ QC	LQC	MQC	HQC
	52.78	155.22	5039.76	7055.66
Precision and Accuracy Batch – 1				
Mean	53.017	154.528	4985.990	7030.587
S.D.	1.4464	4.1435	19.4156	55.1948
% CV	2.73	2.68	0.39	0.79
Precision and Accuracy Batch – 2				
Mean	52.540	152.210	5090.696	7150.107
S.D.	1.4334	4.0814	19.8234	56.1332
% CV	2.73	2.68	0.39	0.79
Precision and Accuracy Batch – 3				
Mean	52.440	166.472	5248.507	7328.859
S.D.	1.4307	4.1957	20.4379	57.5365
% CV	2.73	2.68	0.39	0.79

Each mean value is the result of triplicate analysis

4.3.4 Limit of detection and limit of quantification

LOD is defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 25 ng/mL. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (52.78 ng/mL) met the criteria for the LLOQ QC (Table 3). The method was found to be sensitive for the determination of Nateglinide in human plasma samples. The ULOQ has been accepted as the highest points on the standard curve with a relative standard deviation of less than 15%.

4.3.5 Carryover test

A critical issue with the analysis of many drugs is their tendency to get adsorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

4.3.6 Stability studies

The results of bench top, long term, autosampler and freeze–thaw stability are presented in **Table 4.** Determination of Nateglinide stability following three freeze–thaw cycles showed that for all QC samples there was a no significant change in the Nateglinide concentration. The stability of the aqueous solutions of Nateglinide and the internal standard were also represented in Table-5a and Table 5b respectively. Under refrigerated conditions there is no significant change in the concentration of the samples.

TABLE 4: SHORT TERM, LONG TERM AND FREEZE THAW STABILITY OF NATEGLINIDE (N=6)

	Nominal Concentration (µg/mL)	
	155.22 (LQC)	7055.66 (HQC)
Bench top stability (9 Hours)	99.17	98.,60
Long-term stability (12 Days)	98.23	98.59
Freeze – Thaw stability (3 Cycles)	99.66	99.42
Autosampler stability (3 Cycles)	99.69	98.60

TABLE 5A: BENCH TOP AND REFRIGERATED STABILITY OF NATEGLINIDE AND REPAGLINIDE

Sr. No.	Nateglinide Peak Area		Repaglinide (IS) Peak Area	
	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**	

TABLE 5B: LONG-TERM STABILITY OF NATEGLINIDE AND REPAGLINIDE IN STOCK SOLUTION

Sr. No.	Nateglinide Peak Area		Repaglinide (IS) Peak Area	
	Comparison (0.0 hour)	Stability (8.5 Days)	Comparison (0.0 hour)	Stability (8.5 Days)
1	324329	331431	333548	338769
2	321164	310986	340129	329678
3	320065	324538	329786	340132
4	318799	329356	327890	367590
5	320213	320067	337652	340002
6	315648	317865	329007	331104
Mean	**320036**	**322374**	**333002**	**341213**
±SD	**2847**	**7626**	**5003**	**13708**
%CV	**0.9**	**2.4**	**1.5**	**4.0**
% Stability	**100.7**		**102.5**	

4.3.7 Recovery

Percentage recovery of Nateglinide was measured by dividing the peak area values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of Nateglinide at LQC, MQC and HQC levels was 87.6 %, 89.00 % and 91.45 % respectively. The overall recovery is 89.34 % with a % Coefficient of variation of 2.2 %, respectively. The recovery of internal standard is 93.3 % at MQC level concentration of Nateglinide

5.CONCLUSION

A HPLC method was developed and validated for the determination of Nateglinide in human plasma. The extraction process was a single-step liquid–liquid extraction procedure employing the use of ethyl acetate. LLE method is usually devoid of polar interferences thus rendering the sample clean

for final analysis. The noise is usually absent or at minimum as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (200 µL). There is no carryover effect. Due to the LLE method of extraction, baseline noise is minimal. Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of approximately 52 ng/mL, acceptable recovery, stability, specificity and excellent efficiency with a total running time of 5.0 min per sample, which is important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of Nateglinide in human subjects.

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