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Nateglinide quantification in rabbit plasma by HPLC: Optimization and application to pharmacokinetic study

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Abstract

A rapid, simple, and sensitive HPLC method with UV detection was developed and validated for the determination of nateglinide (NTG) from rabbit plasma. The retention behavior of NTG and gliclazide (GLZ, internal standard-IS) as a function of mobile phase pH, composition and flow rate was investigated. Separation was developed on a reverse-phase C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size), using a mixture of acetonitrile (ACN):10 mM phosphate buffer (PBS, pH 3.0) in the ratio of 70:30 (%v/v) at a flow rate of 1.0 ml/min with UV detection at 203 nm within 8 min, and quantified based on drug/IS peak area ratios. The plasma samples were prepared by a simple deproteinization with a mixture of methanol and acetonitrile, yielding more than 97.86% extraction efficiencies. The calibration curve was linear (correlation coefficient of 0.9984) in the concentration range of 10–2500 ng/ml. The limit of detection (LoD) and limit of quantitation (LoQ) were found to be 2.91 and 9.70 ng/ml, respectively. Both the intra-day and inter-day precisions at four tested concentrations were below 1.32% R.S.D. The present method was selective enough to analyze NTG in rabbit plasma without any tedious sample clean-up procedure and was successfully applied for estimating the pharmacokinetic parameters of NTG following oral administration of a single 15 mg NTG to white albino rabbits.

Keywords: Nateglinide; HPLC; Optimization; Pharmacokinetic; Plasma

1. Introduction

Nateglinide (NTG) [*N*(trans-4-isopropylcyclohexylcarbonyl)-D-phenylalanine], a D-phenylalanine derivative lacking either a sulfonylurea or benzamido moiety, is a novel oral mealtime glucose regulator, and was approved for the treatment of type II diabetes mellitus recently [1,2]. This meglitinide derivative (Fig. 1) works by stimulating the pancreas to release insulin by closing the ATP-dependent potassium channels in the β-cell membrane, 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 gastrointestional 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].

This work is a part of the sustained release matrix formulation development of NTG and its pharmacokinetic evaluation in rabbit. We developed an HPLC method for the quantitation of NTG from rabbit plasma for comparison of developed formulation with that of the marketed formulation. Several analytical methods have been developed for the separation and quantitation 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 different anti-diabetics including nateglinide from equine plasma and urine by liquid chromatography–tandem mass spectrometry [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/MS has been reported [9]. All these methods employed

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a specific type of specialization and sophistication of the instruments and/or method of separations that requires high analytical cost.

Estimation of NTG in human plasma using HPLC with UV detector was reported by Bauer et al. [10]. This method elutes NTG at 14 min, has a 0.1 μg/ml limit of quantitation (LoQ) and requires a column oven to be maintained at 50 °C for the entire analysis. The limit of quantitation and retention time for above mentioned analytical method is much higher than the method reported here and the present method also eliminates the requirement of column oven. In addition, no method is reported till date for determination of nateglinide by HPLC-UV from rabbit plasma.

In the present study, the effect of mobile phase pH (which influences the ionisation state of the compounds), the mobile phase composition (which influences the peaks separation), and the flow rate (which influences the peaks shape, retention time, and resolution, etc.) were studied on various chromatographic parameters such as resolution, plates, height equivalent to theoretical plates, symmetry factor, retention time, peak areas, etc.

2. Experimental

2.1. Chemicals and reagents

Working standard of NTG was a generous gift from Alembic Ltd. (Vadodara, India) with purity of 99.87%. Gliclazide (GLZ, purity of 99.69%, Fig. 1), used as an internal standard (IS), was received from Relax Pharmaceuticals (Vadodara, India). Market sample of Glinate™-60 (Glenmark Pharmaceuticals Ltd., Mumbai, India) was procured from the retail pharmacy. Potassium di-hydrogen phosphate (KH_2PO_4) and 85% *ortho*-phosphoric acid (H_3PO_4) of analytical-reagent grade were purchased from S.D. Fine Chem Ltd. (Mumbai, India) and were used without further purification. HPLC grade methanol, acetonitrile (ACN) and water were purchased from the same supplier. All other chemicals and solvents were of analytical reagents grade. All buffers and solutions were prepared with HPLC grade water. Unless otherwise specified, all solutions were filtered through a 0.2 μm Ultipor® N_{66}® Nylon 6, 6 membrane filter (Pall Life Sciences, USA) prior to use.

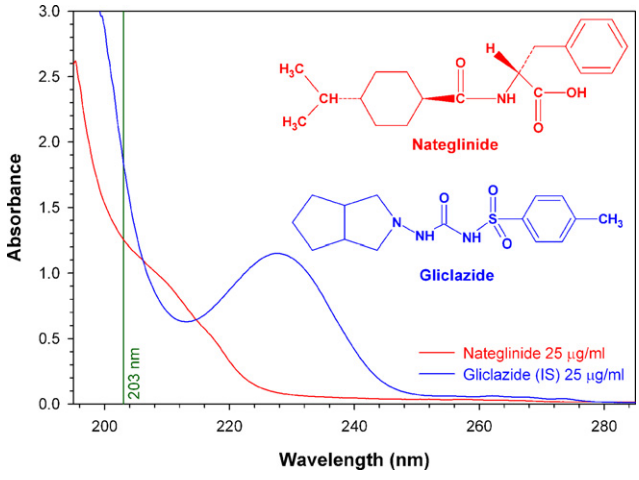

Fig. 1. Chemical structure of nateglinide (molecular weight 317.4, $pK_a = 3.1$) and gliclazide (molecular weight 323.4, $pK_a = 5.98$) along with their UV spectra in mobile phase.

2.2. Instrument and software

A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan) was composed of a LC-20AT Prominence solvent delivery module, a manual rheodyne injector with a 20-μl fixed loop and a SPD-20A Prominence UV–visible detector. Separation was performed on a Phenomenex C_{18} column (paricle size 5 μm; 250 mm × 4.6 mm i.d.; Phenomenex, Torrance, USA) preceded by an ODS guard column (10 μm, 10 mm × 5 mm i.d.) at an ambient temperature. Chromatographic data were recorded and processed using a Spinchrom Chromatographic Station® CFR Version 2.4.0.195 (Spinchrom Pvt. Ltd., Chennai, India).

2.3. Chromatographic conditions

Analysis was isocratic at 1.0 ml/min flow rate with ACN:10 mM KH_2PO_4 buffer solution (PBS) (adjusted to pH 3.0 with H_3PO_4) (70:30, v/v) as mobile phase. The mobile phase was prepared freshly everyday. The mobile phase was premixed, filtered through a 0.2 μm membrane filter to remove any particulate matter and degassed by sonication before use. The absorbance of NTG and IS were higher at 203 nm compared to generally preferred 210 nm (Fig. 1) and further it was free from any interference. Hence, the eluted peaks were detected at 203 nm. A previous UV (190–280 nm) scanning was done in order to select the optimal absorbance wavelength. The sensitivity of the detector was set at 0.01 AUFS. The substance was quantified using its peak area ratio of NTG to IS. Prior to injecting solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. Each solution was injected in triplicate, and the relative standard deviation (R.S.D.) was required to remain below 1.0% on NTG/IS peak area ratio basis.

2.4. Preparation of solutions

A stock solutions of NTG (500 μg/ml) and GLZ (500 μg/ml) were prepared in methanol and were stored at 2–8 °C until used. Aliquots of these solutions were diluted stepwise with the mobile phase to obtain 30 μg/ml of both NTG and GLZ. This solution was used for the optimization of the proposed method.

The spiking solutions of NTG (8 μg/ml) and GLZ (8 μg/ml) were prepared by diluting the suitable aliquots of stock solutions with HPLC grade water. Suitable aliquots of the spiking solutions were spiked to plasma in order to obtain the NTG concentrations in the analytical range of 10 to 2500 (10, 25, 100, 250, 500, 1000, and 2500) ng/ml and that of GLZ to be 2500 ng/ml for calibration curve.

2.5. Optimization of chromatographic conditions

Sometimes, the effects of different chromatographic conditions on the instrumental responses create a situation where one has to compromise between different experimental variables in order to achieve the best chromatographic separation. Chromatographic separations are significantly affected by the mobile phase conditions, such as the type and composition of the organic modifiers [11]. And therefore before selecting the conditions for the optimization, a number of preliminary trials were conducted with different combinations of different organic solvents and buffers at various pH, compositions, and flow rate to check the retention time, shape, resolution, and other chromatographic parameters of NTG and IS peaks individually. From those experiments the mobile phase combination of ACN and KH_2PO_4 buffer in the acidic pH range was found to be most suitable.

In order to achieve an optimum separation, following conditions were studied: (i) Mobile phase pH varied at 2, 3, and 4 keeping the composition of ACN:KH_2PO_4 buffer (10 mM) 70:30 and flow rate of 1.0 ml/min fixed. (ii) Mobile phase composition varied at 60:40, 70:30, and 80:20 with pH and flow rate kept constant at 3 and 1.0 ml/min, respectively. (iii) Flow rate was varied (0.8, 1.0, and 1.2 ml/min) with mobile phase composition and pH maintained at 70:30 and 3, respectively. Moreover, the effects of different level of all these three factors were systematically addressed on system suitability parameters such as resolution, theoretical plates, retention time, capacity factor, separation factor, asymmetry, and HETP etc.

All mobile phases used in optimization study were prepared by mixing the buffer system with the organic solvent in the desired proportions. The apparent pH of the mixtures was adjusted to desired value using H_3PO_4. Mobile phase was then filtered through 0.2 μm membrane filter and sonicated before being used for chromatography.

2.6. Estimation of NTG from rabbit plasma

In order to investigate the practical applicability of the method in biological analysis, the present method was applied to the estimation of NTG from rabbit plasma. Blank plasma of white albino rabbit was spiked with known concentrated aqueous standards of NTG and IS. In one 2-ml micro-centrifuge tube (Ependorff, USA), spiked plasma along with equal volume of methanol (1 ml) was added to precipitate plasma proteins followed by addition of 0.75 ml of ACN for complete extraction of drug and IS. The mixture was vortexed for 3 min and centrifuged (C-24.2/2002, Remi Cooling Centrifuge, Mumbai, India) at 3000 × *g* for 10 min. The supernatant transparent liquid was transferred into a glass centrifuge tube and evaporated to dryness at room temperature under a stream of nitrogen. The residue was reconstituted with 0.2 ml of mobile phase. Then, the samples were filtered through a 13 mm ø 0.2 μm membrane filter using syringe filter holder. An aliquot of 20 μl was injected into the rheodyne injector of an HPLC system for analysis.

2.7. Validation of the proposed method

Once the chromatographic method had been developed and optimized, it must be validated. The validation of an analytical method verifies that the characteristics of the method satisfy the requirements of the application domain [12]. The proposed method was validated in the light of ICH Guidelines [13–15] for linearity, precision, sensitivity, and recovery. Consequently, the following were performed.

2.7.1. Calibration curve (linearity)

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of an analyte in the sample [16,17]. Seven different concentrations of NTG with constant IS concentration were spiked to the blank plasma as described previously and calibration curve was constructed in the specified concentration range. The calibration plot (peak area ratio of NTG to IS versus NTG concentration) was generated by replicate analysis ($n = 6$) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel® program.

2.7.2. Accuracy and precision

Both repeatability (within a day precision) and reproducibility (between days precision) were determined as follows. Solutions containing lowest, intermediate, and highest concentrations of the calibration curve, i.e. 10, 500, 1000, and 2500 ng/ml were prepared. Six injections at each of the specified concentration levels were injected within the same day for repeatability, and over a period of 3 days (6 injections/day) for reproducibility. Mean and relative standard deviation were calculated and used to judge accuracy and precision of the method. Both intra-day and inter-day samples were calibrated with standard curves concurrently prepared on the day of analysis. Accuracy was calculated as the percent of ratio of NTG amount found to that of the actual.

2.7.3. Sensitivity

As per IUPAC [18] and ISO [19], the instrumental response sensitivity is the slope of the calibration line because a method with a large slope is better able to discriminate between small differences in analyte content. LoD and LoQ were determined according to following equation:

$$\text{LoD or LoQ} = \frac{ks_B}{S} \tag{8}$$

where k is a constant (3 for LoD and 10 for LoQ), s_B is the standard deviation of the analytical signal, and S is the slope of the concentration/response graph.

2.7.4. Specificity

The specificity criterion tries to demonstrate that the result of the method is not affected by the presence of interferences, i.e. whether the compound elutes without any other interfering compounds or not [12]. The specificity of the method was determined by comparing the chromatograms obtained from the

samples containing NTG and IS with those obtained from blank plasma. Five blank plasma samples from six lots of rabbit plasma were processed with and without the internal standard to evaluate presence of interfering peaks.

2.7.5. Stability

Blank plasma was spiked with the known amount of NTG to achieve the concentration of 50, 1000, and 2000 ng/ml ($n = 3$) and stored at −4 °C. The stability of these samples was checked for up to 1 month by comparing the results with fresh stock prepared on the day of analysis. Further, the freeze–thaw (−20 °C/room temperature) stability of the NTG spiked plasma samples were determined for three cycles. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision. No internal standard was added prior to the analysis.

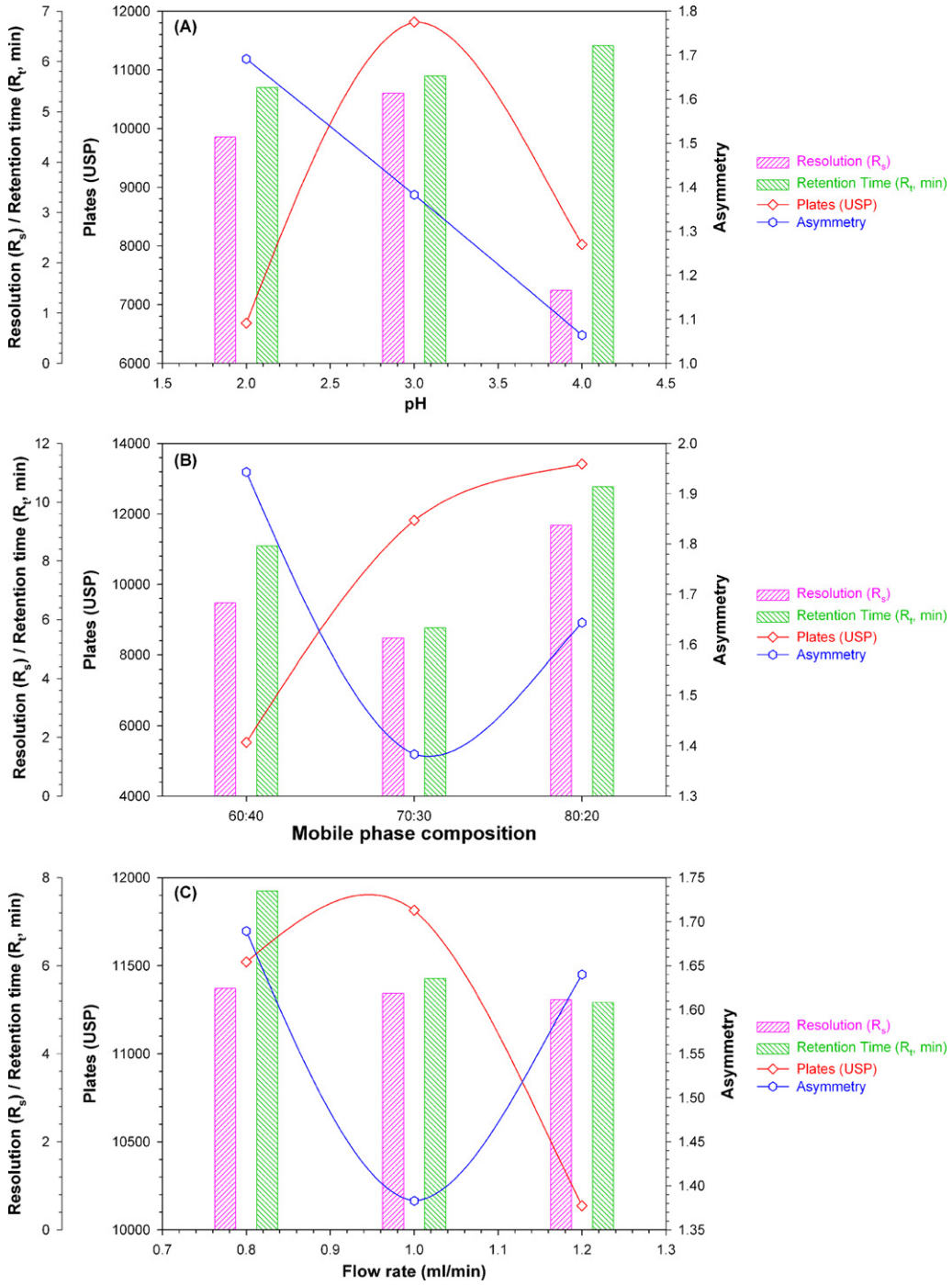

Fig. 2. Effect of (A) mobile phase pH (2, 3, 4) at mobile phase composition of 70:30 (ACN:PBS; v/v) and flow rate of 1.0 ml/min; (B) mobile phase composition (ACN:PBS, 60:40, 70:30, 80:20) at pH 3 and flow rate of 1.0 ml/min; and (C) mobile phase flow rate (0.8, 1.0, 1.2 ml/min) at mobile phase composition of 70:30 (ACN:PBS) and pH 3 on resolution, retention time, theoretical plates, and asymmetry.

2.7.6. Extraction efficiency

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 [20]. Different organic extraction solvents (ethyl acetate, dichloromethane, acetonitrile and chloroform) were tried in the experiment, and acetonitrile proved to be the most efficient in extracting NTG from rabbit plasma and had a small variation in extraction recoveries over the concentration range. Spiked plasma samples were prepared in triplicate at three concentrations 50, 1000, and 2000 ng/ml of NTG and 2500 ng/ml of IS, and assayed as described above. The extraction efficiency of NTG was determined by comparing the peak areas measured after analysis of spiked plasma samples with those found after direct injection of non-biological (unextracted) samples into the chromatographic system at the same concentration levels.

2.8. Application to study pharmacokinetics from rabbits

The method described above was applied to quantify the plasma concentration of NTG in a single-dose pharmacokinetic study conducted on three white albino rabbits. The protocol was approved by the Institutional ethical committee at the M.S. University of Baroda, India. The experiments were conducted as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals) 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 15 mg of NTG (Glinate^{TM}-60), 2.0 ml of blood samples were collected from the marginal ear vein at 0, 0.5, 1, 2, 3, 4, 5, 6, 9, and 12 h time-points into heparinized collection tubes. The blood was immediately centrifuged (1900 × g) for 10 min at an ambient temperature. The supernatant plasma layer was separated and stored at −20 °C until analyzed. The plasma samples were analyzed for NTG concentrations as described above. The total area under the observed plasma concentration–time curve (AUC) was calculated by using the linear trapezoidal rule. The first order elimination rate constant (k_{el}) was estimated by the least square regression of the points describing the terminal log-linear decaying phase. $T_{1/2}$ was derived from k_{el} ($T_{1/2} = \ln 2/k_{el}$). The absorption rate constant (k_a) was determined by residual method. The maximum observed NTG concentration (C_{max}) and the time at which C_{max} was observed (T_{max}) were reported directly from the profile.

3. Results and discussion

Normal phase chromatography can be used for the separation of non-ionic and/or non-polar substances, while reversed-phase chromatography (C_8, C_{18}) can be used for the separation of non-ionic as well as ion forming non-polar to medium polar substances. Thus, nateglinide (a ion-forming medium non-polar weak acid) can be satisfactorily separated by reversed phase chromatography. Octylsilane (C_8) columns are similar to octadecylsilane (C_{18}). 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 [21]. Hence, octadecylsilane was selected. Rapid, sensitive and novel HPLC method for determination of NTG in rabbit plasma was optimized and validated.

3.1. Optimization of chromatographic conditions

3.1.1. Effect of mobile phase pH

With the aim of the optimization of mobile phase pH (2, 3, and 4), the remaining two factors were kept constant, i.e. mobile phase composition (ACN: PBS; 70:30, v/v) and flow rate of 1 ml/min. Observed chromatographic responses were plotted against respective pH. As shown in the Fig. 2(A), retention time increases with the increase in pH while asymmetry decreases. The number of theoretical plates as well as resolution between NTG and IS was maximum at pH 3. Moreover, the changes in peak width, capacity factor, separation factor and HETP are enumerated in Table 1. Looking at the importance of the different chromatographic parameters, pH 3 was found to be optimum.

The dissociation constant (pK_a) of NTG is ∼3.1 at (21–24 °C). According to this value, ∼90%, ∼50%, and ∼10% of the drug will be unionized at pH 2, 3, and 4 respectively. As the pH increased, the retention time for NTG could have decreased with using octadecylsilane (ODS, C_{18}) column because of the less interaction between drug and C_{18} under ionized condition with increase in pH. However, the mobile phase contained 70% ACN which does not have much affinity for the ionized hydrophilic drug species. This could be the probable reason for the delay of retention time for NTG with increase in pH. The asymmetry value decreases with increase in pH. At lower pH the NTG will be carried out faster with mobile phase, however, due to higher unionized species, the drug has a tendency to stick/partition with stationary phase too. This result in tailing and hence increase in asymmetry value at lower pH. At higher pH value, the ionized hydrophilic species are not much portioned with stationary phase and hence gives symmetric peak. The resolution was poor at pH 4 using GLZ as an IS, but was highest at pH 3. Similarly, the plate number (highest for higher retention time and smallest peak width) was highest at pH 3. Thus, the best chromatographic separation was achieved at pH 3, and hence was considered to be optimum.

3.1.2. Effect of mobile phase composition

The effect of mobile phase composition (i.e. ratio of ACN:PBS was studied at 60:40, 70:30, and 80:20, v/v levels) at pH 3 and the flow rate of 1 ml/min is shown in Fig. 2(B). Minimum retention times of NTG and IS were obtained at 70:30, v/v level, which makes the method rapid, a one of the most desirable criteria. Though retention time was shorter, satisfactory resolution and asymmetry values were achieved. An adequate theoretical plates (∼12000) is indicative of a good column performance. As can be seen from Fig. 2(B), the asymmetry was >1.5 at 80:20, v/v and still higher at 60:40, v/v which indicates tailing of the peaks, but was <1.4 at 70:30, v/v. Other chromatographic parameters at different composition of mobile phase are listed in Table 1.

Table 1
Effect of mobile phase pH, composition, and flow rate on various chromatographic parameters

S. No.	Variable	Value	Unretained peak (t_0, min)	Retention time (R_t, min)	Width (W, min)	Width @ 5% ($W_{5\%}$, min)	Width @ 10% ($W_{10\%}$, min)	Width @ 50% ($W_{50\%}$, min)	Skewness	Capacity factor (k')	Separation factor (α)	HETP (h)
1	pH	2	0.9140	5.4858	0.2683	0.3857	0.3228	0.1565	1.5825	5.0020	0.7834	0.0374
		3	0.9490	5.7110	0.2102	0.2877	0.2427	0.1227	1.3141	5.0179	0.7874	0.0212
		4	0.9538	6.3130	0.2818	0.3894	0.3237	0.1562	0.9169	5.6188	0.9308	0.0311
2	Mobile phase composition (v/v)	60:40	0.9928	8.4978	0.4573	0.6847	0.5642	0.2637	1.7910	7.5595	0.6754	0.0453
		70:30	0.9490	5.7110	0.2102	0.2877	0.2427	0.1227	1.3141	5.0179	0.7874	0.0212
		80:20	1.0443	10.5257	0.3635	0.4378	0.3797	0.2123	1.5689	9.0789	0.6954	0.0186
3	Flow rate (ml/min)	0.8	1.0690	7.6972	0.2868	0.4133	0.3460	0.1678	1.5751	6.2003	0.7901	0.0217
		1	0.9490	5.7110	0.2102	0.2877	0.2427	0.1227	1.3141	5.0179	0.7874	0.0212
		1.2	0.9165	5.1685	0.2053	0.2985	0.2480	0.1202	1.5246	4.6394	0.7781	0.0247

Generally, increasing the organic solvent concentration in the mobile phase induces a decrease in the distance between the solute molecule and the terminal carbon atoms (C_{18}) in the ODS ligand, and it results in lower retention time [22]. Similar findings were observed in the present study up to 70:30, v/v composition, but further increase in ACN content resulted in increased retention time. This may be explained by following. The elution power of mobile phase decreased at 60:40, v/v because relative amount of ACN decreased and NTG was eluted at higher retention time. In contrast to this at 80:20, v/v composition, the drug affinity to stationary phase increased due to relatively lower buffer content in mobile phase and resulted in delayed elution. However, at 70:30, v/v composition, proper balance was attained between these two situations and resulted in least retention time. The least asymmetry at 70:30 compared to other two compositions can be explained on the same basis. Plate number increased with increase in ACN composition in mobile phase. However, the asymmetry value at 80:20 v/v was higher than that of at 70:30 v/v. These suggest that the increased plates at 80:20, v/v was due to higher retention time value (even though it had greater peak width due to tailing). Further, acceptable resolution (>2) was achieved at 70:30, v/v composition and so was considered to be optimum.

3.1.3. Effect of mobile phase flow rate

From Fig. 2(C), it can be observed that theoretical plates were highest at flow rate of 1 ml/min with asymmetry of less than 1.5. The change in flow rate had no significant effect on resolution while retention time decreased as the flow rate increased. The values of capacity factor, and separation factor (Table 1) also indicate optimum flow rate of 1 ml/min.

3.2. Proposed chromatographic method

Looking at the different chromatographic parameters during the method development, the finally recommended mobile phase consisted of ACN: 10 mM PBS of 70:30, v/v adjusted to pH 3. The best resolution and sensitivity of the method was obtained at 203 nm and mobile phase flow rate of 1 ml/min. Typical chromatogram at the optimized condition gave sharp and symmetric peak with retention time of 4.7 and 5.7 min for IS and NTG, respectively. Thus, within very short time the system became ready for the next sample injection without the need for additional wash time.

3.3. Validation of the proposed method

3.3.1. Calibration curve (linearity)

Calibration curve (peak area ratio of NTG to IS versus NTG concentration) in plasma was constructed by spiking seven different concentrations of NTG and fixed concentration of IS. The chromatographic responses were found to be linear over an analytical range of 10–2500 ng/ml and found to be quite satisfactory and reproducible with time. The linear regression equation was calculated by the least squares method using Microsoft Excel® program and summarized in Table 2. The correlation coefficient

Table 2
Spectral and statistical data for determination of nateglinide by proposed HPLC method

Parameters	Value
Absorption maxima, λ_{max} (nm)	203
Linearity range (ng ml^{-1})	10–2500
Coefficient of determination (r^2)	0.9969
Correlation coefficient (r)	0.9984
Regression equation (Y[a])	$Y = 0.0594 \cdot x + 0.0063$
Slope (b)	0.0594
t_{cal}[b]	0.0093
Confidence interval[c]	−1.7145 to 1.7271
Intercept (a)	0.0063
t_{cal}[b]	0.0056
Confidence interval[c]	−1.5313 to 1.6502
Limit of detection, LoD (ng ml^{-1})	2.91
Limit of quantitation, LoQ (ng ml^{-1})	9.70

[a] $Y = a + bx$, where x is the concentration (μg/ml).
[b] $t_{tab} = 2.57$ for 95% two sided confidence interval for 5 degrees of freedom.
[c] Confidence interval was calculated at 95% two sided t value for 5 degrees of freedom.

equals 0.9984, indicating a strong linear relationship between the variables.

The variance of response variable S^2_{Yx} calculated was 1.9634, indicates low variability between the estimated and calculated values. This further confirms negligible scattering of the experimental data points around the line of regression and good sensitivity of the proposed method. The variance of slope (S^2_b) and intercept (S^2_a) were obtained as 0.3761 and 0.4401, respectively. The calculated t-value for slope and intercept were reported in Table 2 and were less than tabulated t-values. This shows that the intercept is not significantly different from zero, indicating no interference in the estimations. Further the slope and intercept were within the confidence interval.

3.3.2. Accuracy and precision

Accuracy data in the present study ranged from 98.59 to 99.76% (Table 3) indicates that there was no interference from endogenous plasma components. Inter-day as well as intra-day replicates of NTG, gave an R.S.D. below 11.79 (should be less than 15 according to CDER guidance for Bio-analytical Method Validation [23]), revealed that the proposed method is highly precise. Accuracy of the method was evaluated by using t-test at four concentration levels including the lowest quantifiable level. The t-values obtained for 10, 500, 1000, and 2500 ng/ml were 0.66, 2.17, 1.39, and 0.96 for inter-day whereas 0.74, 1.17, 1.64, and 1.22 for intra-day, respectively. The t-value required for significance at 5% level at 5 degrees of freedom is 2.57, and the obtained values were well below this value. Thus no significant difference was observed between the amounts of drug added and recovered. Overall, the data summarized in Table 3, enables the conclusion that an excellent accuracy and high precision was obtained.

3.3.3. Sensitivity

The LoD and LoQ were found to be 2.91 and 9.70 ng/ml, respectively. When this method is applied to plasma samples, its sensitivity was found to be adequate for pharmacokinetic studies.

3.3.4. Specificity

Any potential interference (overlapping peaks) due to plasma endogenous components were within 2–4 min only (Fig. 3), later on there was no significant interference from blank plasma that affected the response of NTG and IS.

3.3.5. Stability

The spiked rabbit plasma samples stored at −4 °C, were injected over a period of 1 month did not suffer any appreciable changes in assay value and meet the criterion mentioned above. Hence, the samples were stable during 1 month. The NTG was found to be stable in rabbit plasma after three freeze–thaw cycles.

3.3.6. Extraction efficiency

Extraction efficiency was performed to verify the effectiveness of the extraction step and the accuracy of the proposed method. The extraction efficiency of NTG from rabbit plasma samples was satisfactorily ranged from 97.86 to 98.62% (R.S.D.

Table 3
Summary of inter-day ($n = 3$) and intra-day ($n = 6$) precision and accuracy of the method in rabbit plasma

Nominal concentration (ng/ml)	Mean concentration found[a] (ng/ml)	S.D.	Precision (R.S.D., %)	Mean accuracy[b] (%)	t_{cal}[c]	Confidence interval (CI)
Inter-day ($n = 3$)						
10	9.95	0.13	1.31	99.50	0.66	10 ± 0.19
500	492.93	5.57	1.13	98.59	2.17	500 ± 8.26
1000	993.23	8.40	0.85	99.32	1.39	1000 ± 12.47
2500	2493.68	11.38	0.46	99.75	0.96	2500 ± 16.88
Intra-day ($n = 6$)						
10	9.96	0.13	1.32	99.60	0.74	10 ± 0.14
500	497.19	5.85	1.18	99.44	1.17	500 ± 6.13
1000	995.21	7.12	0.72	99.52	1.64	1000 ± 7.47
2500	2494.11	11.79	0.47	99.76	1.22	2500 ± 12.37

[a] Average of three and six determinations at three concentration levels for inter-day and intra-day respectively.
[b] All the mean accuracies were calculated against their nominal concentrations.
[c] $t_{cal} = |100 - R|\sqrt{n}/\text{R.S.D.}$, where t_{cal} is the calculated t value, n is the number of replicates, and R is mean accuracy. Tabulated t-value for 95% two sided confidence interval for 5 degree of freedom was (t_{tab}=)2.57.

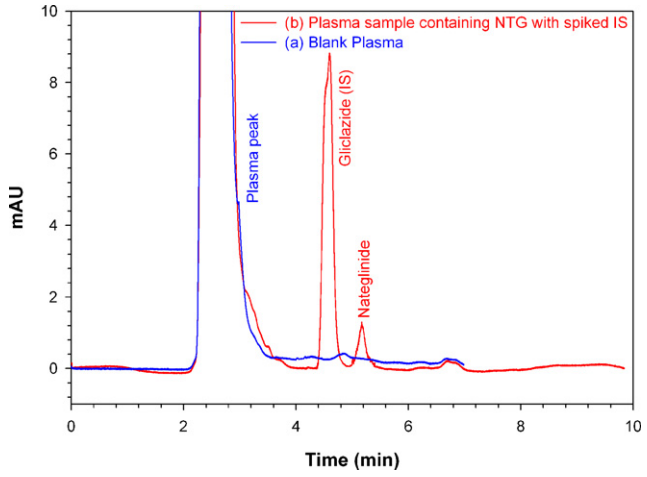

Fig. 3. Representative chromatograms of (a) blank plasma and (b) clinical plasma sample taken from a rabbit 30 min after a 15 mg oral dose of NTG (quantitated to be 26.42 ng/ml) spiked with IS.

was less than 2.05) at all three concentration levels, which confirm no interference effects due to plasma components. Recovery of IS was found to be 98.42% (R.S.D. = 1.47).

3.3.7. System suitability

System suitability tests, an integral part of a chromatographic analysis is used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis [24]. A system suitability test according to USP was performed on the chromatograms obtained from standard and test solutions to check different above mentioned parameters and the results obtained from six replicate injections of the standard solution are summarized in the Table 4.

3.4. Pharmacokinetics from rabbits

The developed method was applied to quantify NTG concentration in pharmacokinetic study carried out on rabbits. HPLC chromatogram of rabbit plasma is shown in Fig. 3, which shows (A) typical chromatograms of blank rabbit plasma and (B) NTG in plasma after 30 min of drug administration. Representative mean plasma concentrations versus time profiles following a single oral administration of NTG to three rabbits are presented in Fig. 4(A). Fig. 4(B) shows the natural log concentration versus time plot along with trendline for absorption and elimination rate constants. Various other pharmacokinetic parameters have been summarized in Table 5. The T_{max} and $T_{1/2}$ of NTG in the present study was similar, although the intake doses were different from those reported in literature [25].

Table 4
System suitability parameters

S. No.	Parameters	NTG[a]	GLZ[a]
1	Retention time, R_t (min)	5.19	4.52
2	Area (mAU·s)	10.38	66.90
4	Capacity factor (k')	7.504	4.834
5	Separation factor (α)	1.555	–
6	Theoretical plates (USP)	7928	7999
7	HETP (mm)	0.032	0.031
8	Resolution (R_s)	2.93	–
9	Asymmetry (A_s)	0.712	1.115
10	R.S.D. (%)		

[a] Average of six determination.

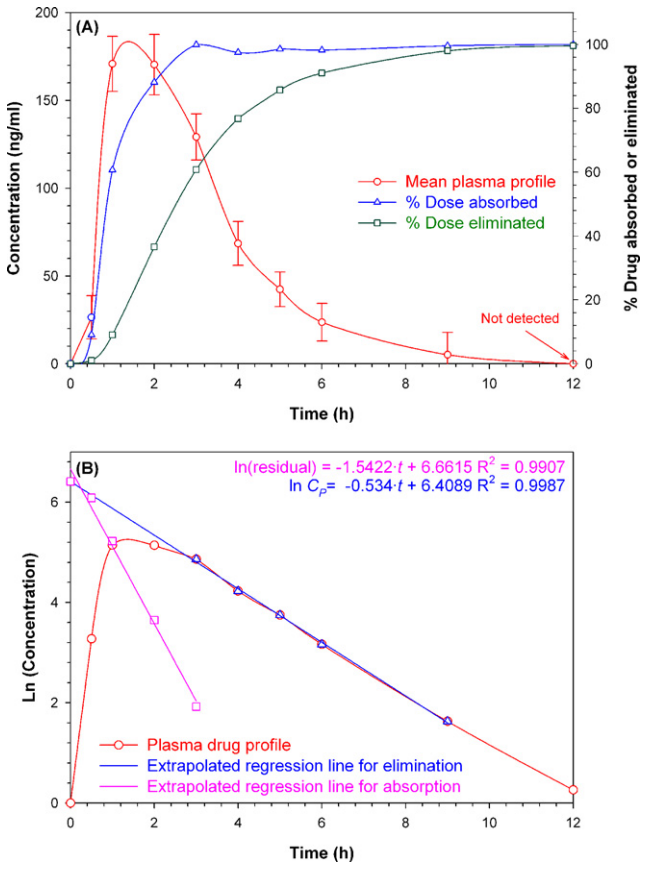

Fig. 4. (A) Representative mean plasma concentrations versus time profile following a single oral administration of NTG (15 mg) to three rabbits. (B) Natural log plasma concentrations versus time profile for determination of k_a and k_{el}.

Table 5
Pharmacokinetic parameters of NTG after a single oral dose of 15 mg NTG to three rabbits

S. No.	Pharmacokinetic parameters	Observed value
1	Absorption rate constant, k_a (h^{-1})	1.54
2	Elimination rate constant, k_{el} (h^{-1})	0.53
3	Time required for maximum plasma concentration, T_{max} (h)	1.38
4	Maximum plasma concentration, C_{max} (ng/ml)	183.5
5	Plasma half life, $T_{1/2}$ (h)	1.30
6	Area under curve at 12 h, $AUC_{(0\rightarrow12)}$ (ng h/ml)	616.29
7	Area under curve at infinite time, $AUC_{(0\rightarrow\infty)}$ (ng h/ml)	618.72
8	Area under momentum curve at 12 h, $AUMC_{(0\rightarrow12)}$ (ng·h^2/ml)	1793.81
9	Volume of distribution, V_d (ml)	40
10	Mean residence time, MRT (h)	2.91
11	Total clearance rate, TCR (l/h)	0.02

4. Conclusion

In the present work, a new rapid, simple and sensitive reversed phase HPLC method has been developed, optimized and validated for the estimation of NTG in rabbit plasma using UV detector and isocratic elution. Optimization showed that the mobile phase pH and composition are more crucial parameters to be controlled than flow rate for reproducible and quantitative estimation of the NTG. The short peak retention time of 5.7 min cuts down on overall time of sample analysis and thereby makes the method more cost effective. Method was found to be linear over an analytical range of 10–2500 ng/ml with LoD and LoQ of 2.91 and 9.70 ng/ml, respectively. The results of *t*-test applied to accuracy and precision data enabled the conclusion that an excellent accuracy and high precision was achieved. From the extraction efficiency data, the recovery of the active component was found to be quantitative. Selectivity of the method was demonstrated by the absence of any interfering peaks from other coexisting endogenous substances at the retention time of the drug as well as IS. Simple and reproducible sample extraction procedure along with reconstitution in minimum quantity of mobile phase offers the higher sensitivity (10 ng/ml) for animal studies and was successfully applied for determination of NTG from rabbits plasma. In summary, the optimized chromatographic estimation of NTG with good resolution in a short time can be used for evaluating the bioavailability and also applied to routine therapeutic monitoring of the NTG.

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