AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

A novel liquid chromatography–tandem mass spectrometric assay for nateglinide in human plasma and its pharmacokinetic application

Vijaya Kumari Karra[1], Nageswara Rao Pilli[1,2], Rambabu Maddela[1, 2], Jaswanth Kumar Inamadugu[2], Ramakrishna Gajula[2], J.V.L.N. Seshagiri Rao[3, *]

1.University College of Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad 500 085, India;

2.PCR Laboratories, Ramanthapur, Hyderabad–500 013, India;

3.Srinivasa College of Pharmacy, Madhurawada, Visakhapatnam 530 041, India

ABSTRACT

The authors proposed a simple, rapid and sensitive liquid chromatography / tandem mass spectrometry assay method for the determination of nateglinide in human plasma using carbamazepine as internal standard (IS). Analyte and the IS were extracted from the human plasma *via* liquid-liquid extraction using ethyl acetate. The chromatographic separation was achieved on a C_{18} column by using a mixture of 0.1% formic acid buffer –acetonitrile buffer (20:80, v/v) as the mobile phase at a flow rate of 0.8 mL/min. The calibration curve obtained was linear ($r^2 \geq 0.99$) over the concentration range of 10.0–10005 ng/mL. Method validation was performed as per FDA guidelines and the results met the acceptance criteria. The proposed method was found to be applicable to pharmacokinetic studies.

Keywords: Nateglinide; Liquid-liquid extraction (LLE); LC-MS/MS; Method validation; Pharmacokinetics

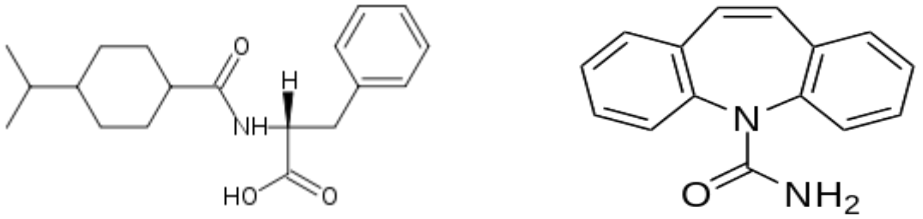
*Corresponding Author Email: vijayaanalysis@yahoo.co.in

Received 22 August 2014, Accepted 28 August 2014

Please cite this article in press as: Vijaya K *et al*., A novel liquid chromatography–tandem mass spectrometric assay for nateglinide in human plasma and its pharmacokinetic application American Journal of PharmTech Research 2014.

INTRODUCTION

Nateglinide is an oral, short-acting insulinotropic agent approved for the treatment of type-2 diabetes mellitus [1, 2]. It increases the short-lived insulin release from pancreatic β-cells through inhibition of ATP-dependent potassium channels and is dependent on the concentration of glucose [3]. Nateglinide has a rapid onset and short duration of insulinotropic action that results in reduction of mealtime glucose rise and lowers the post absorptive potential for hypoglycemia [4]. The drug is rapidly and completely absorbed from the gastrointestinal tract and peak plasma concentration reaches at 0.5–1.0 h [5].As per the literature, few liquid chromatography-mass spectrometry (LC-MS) methods have been reported for the determination of nateglinide in a variety of biological samples like human plasma [6, 7], rat plasma [8], equine plasma [9], monkey plasma [10] and in rabbit serum [11]. The method proposed by Han *et al.*, [6] described a single–quadrupole mass spectrometry (LC–MS) with selected–ion monitoring (SRM) mode to detect the precursor ion. This method is not sensitive enough for the determination of nateglinide concentrations for pharmacokinetic/bioequivalence studies because of its higher LLOQ (50 ng/mL). Moreover, the method employs protein precipitation (PP) for the samples preparation. PP is most likely to cause ion suppression, since this method fails to sufficiently remove the interference form the endogenous compounds. Hess *et al.*,[7] reported a LC–MS/MS method for the identification and quantification of 11 oral hypoglycaemic drugs including nateglinide in plasma. The rest of the LC-MS methods [8-11] reported so far were only applicable to animal models and these methods are not suitable for pharmacokinetic application in humans.The present paper describes a simple, selective and sensitive liquid chromatography–electrospray ionization tandem mass spectrometry (LC–MS/MS) assay method for the determination of nateglinide in human plasma using carbamazepine, as an internal standard (IS). The method employs a simple liquid-liquid extraction (LLE) technique for sample preparation using a smaller plasma volume (200 µL). The method ensured the estimation of nateglinide in real time samples collected from healthy male subjects with desired accuracy and precision to support a pharmacokinetic study in healthy volunteers.



Nateglinide Carbamazepine (IS)

Figure 1.Chemical structures of nateglinide and carbamazepine (IS).

MATERIALS AND METHOD

Standards and chemicals

The reference sample of nateglinide (99.48%) was obtained from Clearsynth Labs Limited (Mumbai, India), while carbamazepine (99.20%) was from Vivan Life Sciences Ltd., (Mumbai, India). Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, USA). Analytical grade formic acid and HPLC grade ethyl acetate were purchased from Merck Ltd (Mumbai, India). The control K_2 human plasma sample was procured from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) equipped with a Ace 5 C_{18} column (50 mm × 4.6 mm, 5 µm) (Make: Ace HPLC columns), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) was used for the study. Aliquot of 15 µL of the processed samples were injected into the column, which was kept at ambient (25±2°C) temperature. An isocratic mobile phase composed of a mixture of 0.1% formic acid and acetonitrile (20:80, v/v) was used to separate the analyte from the endogenous components and pumped at a flow rate of 0.80 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode for the analyte and the IS using an AB Sciex API–4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboionspray ™ interface at 500 °C. The ion spray voltage was set at 5500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 30, 40, 20, and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 80, 18, 10, 10 V for nateglinide and 80, 30, 10, 11 V for the IS. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 318.3 precursor ion to the *m/z* 166.0 for nateglinide and *m/z* 237.2 precursor ion to the *m/z* 194.1 product ion for the IS. Quadrupoles (Q1 and Q3) were set on unit resolution. Dwell time was set at 200 ms. The analysis data obtained were processed by Analyst software™ (version 1.4.2).

Preparation of calibration curve standards and quality control samples in human plasma

Two standard stock solutions of nateglinide were prepared separately in HPLC grade methanol (1 mg/mL) for the preparation of calibration curve standards and quality control samples,

respectively. Further dilutions of analyte were prepared in a mixture of acetonitrile and water (50:50, v/v; diluent). A 1 mg/mL of carbamazepine stock solution was prepared by dissolving the compound in HPLC grade methanol. The working concentration of carbamazepine (250 ng/mL) was prepared from the above stock solution using the diluent. Calibration samples were prepared by spiking 950 µL of control K_2 EDTA human plasma with the 50 µL working standard solution of the analyte as a bulk, to obtain riluzole concentration levels of 10.0, 20.0, 50.0, 200, 500, 2001, 4002, 8004 and 10005 ng/mL as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 10.0 (lower limit of quantitation quality control, LLOQ QC), 30.1 (low quality control, LQC), 1505 (medium quality control, MQC1), 5018 (MQC2) and 8505 ng/mL (high quality control, HQC) as a single batch at each concentration.

Sample processing

A 200 µL aliquot of human plasma sample was mixed with 20 µL of the internal standard working solution (250 ng/mL of carbamazepine). To this, 200 µL of 100mM ammonium formate buffer was added. After vortexing for 15 s, a 4 mL of ethyl acetate was added using Dispensette Organic (Brand GmbH, Wertheim, Germany). The sample was shaken for 10 min using a reciprocating shaker (Scigenics Biotech, Chennai, India) and then centrifuged for 4 min at 4000 rpm on Megafuse 3SR (Heraeus, Germany). The clear organic layer (3 mL) was transferred to a 5 mL glass test tube and evaporated at 45°C under a gentle stream of nitrogen. The dried extract was reconstituted with 500 mL of the mobile phase and a 15 µL aliquot of it was injected into the LC–MS/MS system.

Method validation parameters

The proposed method was validated as per recent US FDA guidelines [12]. The parameters determined were selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity and stability.

Pharmacokinetic study protocol design

A pharmacokinetic study was performed in healthy male subjects (n = 6). The Ethics Committee approved the protocol and the volunteers provided with written informed consent. All the volunteers were fasted for 12 h before the drug formulation administration. Blood samples were collected following oral administration of 120 mg nateglinide tablet at pre-dose and 0.33, 0.67, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.33, 3.67, 4, 5, 6, 8, 10, 12, 16 and 24 h and collected in K_2 EDTA vacutainer (5 mL) collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 ±

10 °C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. The pharmacokinetic parameters of nateglinide were calculated by non–compartmental model using WinNonlin Version 5.2.

RESULTS AND DISCUSSION

Method development

Mass parameters were optimized by infusing the nateglinide working solution (500 ng/mL) into the electrospray ionization chamber of the mass spectrometer operating in MRM mode. The signal responses obtained in positive mode were much higher than those in negative mode. Protonated form of analyte and the IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from m/z 318.3 to 166.0 for nateglinide and from m/z 237.2 to 194.1 for the IS. The dwell time for each transition was set at 200 ms. The method development includes mobile phase selection, column type, flow rate, and injection volume. Various combinations of methanol/acetonitrile with acidic buffers (ammonium acetate/ammonium formate–acetic acid/formic acid) in different ratios were tested. It was observed that 0.1% formic acid and acetonitrile (20:80, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Ace 5 C_{18} (50 mm × 4.6 mm, 5 µm) column gave good peak shape and response even at lowest concentration level for the analyte and the IS. The mobile phase was operated at a flow rate of 0.80 mL/min. The retention time of analyte and the IS were low enough (1.5 and 1.0 min) allowing a run time of 2.8 min.The earlier authors[6] have employed PP to extract nateglinide from human plasma samples. As a purpose to develop a simple and inexpensive extraction procedure LLE was tested. LLE was carried out using ethyl acetate, hexane, *tert* butyl methyl ether and dichloromethane alone or in combination as extraction solvents. Among the different solvents checked alone and in combination for their suitability ethyl acetate was found to be optimal, which can produce a clean chromatogram for a blank sample and yields the highest recovery for the analyte from the plasma. Addition 100mM ammonium formate buffer to the plasma samples as an extraction additive helped achieving reproducible and quantitative recoveries for the analyte and the IS. The use of stable labeled isotopes of the analyte as an internal standard is recommended for bioanalytical assays to increase assay precision and limit variable recovery between analyte and the IS. At the initial stages of this work, several compounds were investigated to find a suitable IS and finally carbamazepine was found to be best for the present purpose.

Selectivity and chromatography

The selectivity of the method was examined by analyzing blank human plasma extract obtained form six individual sources. As shown in Figure 2, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug–free human plasma at the retention time of the analyte and the IS. Figure 3 depicts a representative ion–chromatogram for the LLOQ sample (10.0 ng/mL).

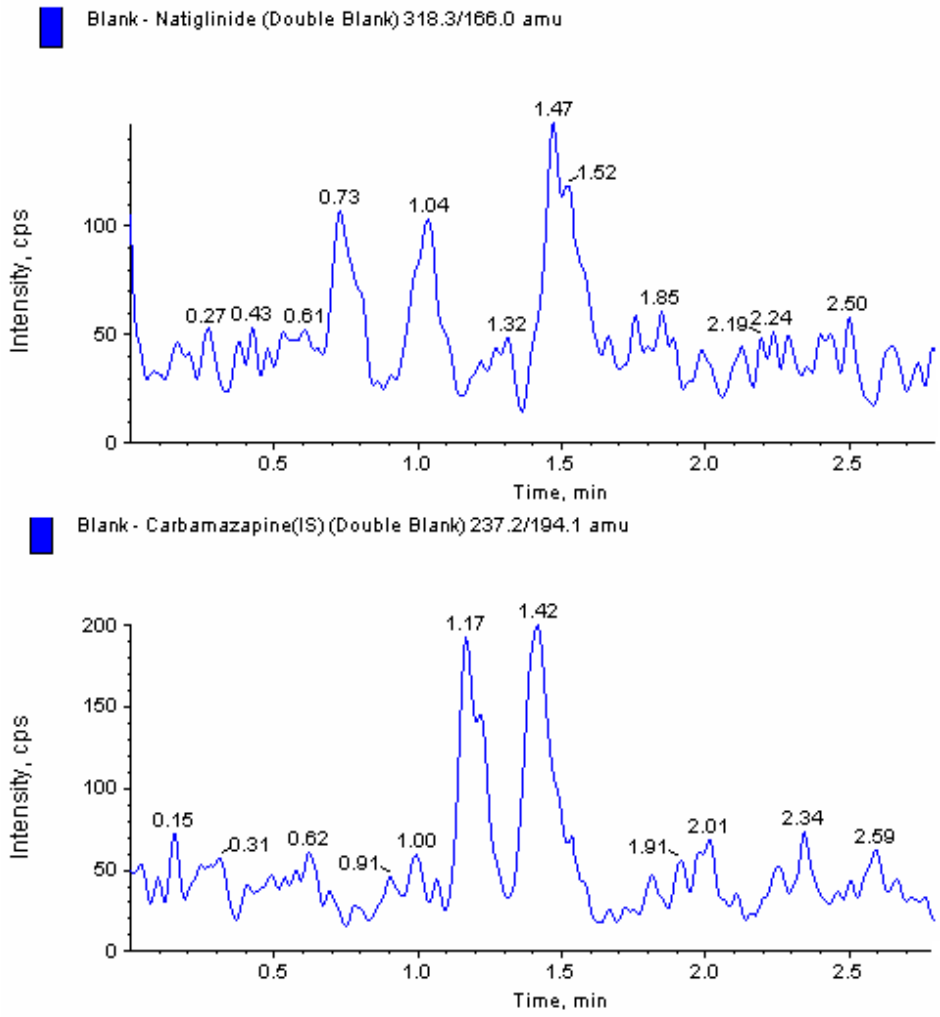


Figure 2. Typical MRM chromatogram of nateglinide and the IS in human blank plasma

Matrix effect

Matrix effect assessment was done with the aim to check the effect of different lots of plasma on the back calculated value of QC's nominal concentration. The precision and accuracy for nateglinide at LQC concentration were found to be 1.94% and 97.71%, and at HQC level they were 3.73% and 98.27%, respectively. Results revealed that no significant matrix effect was observed in all the six batches of human plasma for the analyte at low and high quality control concentrations.

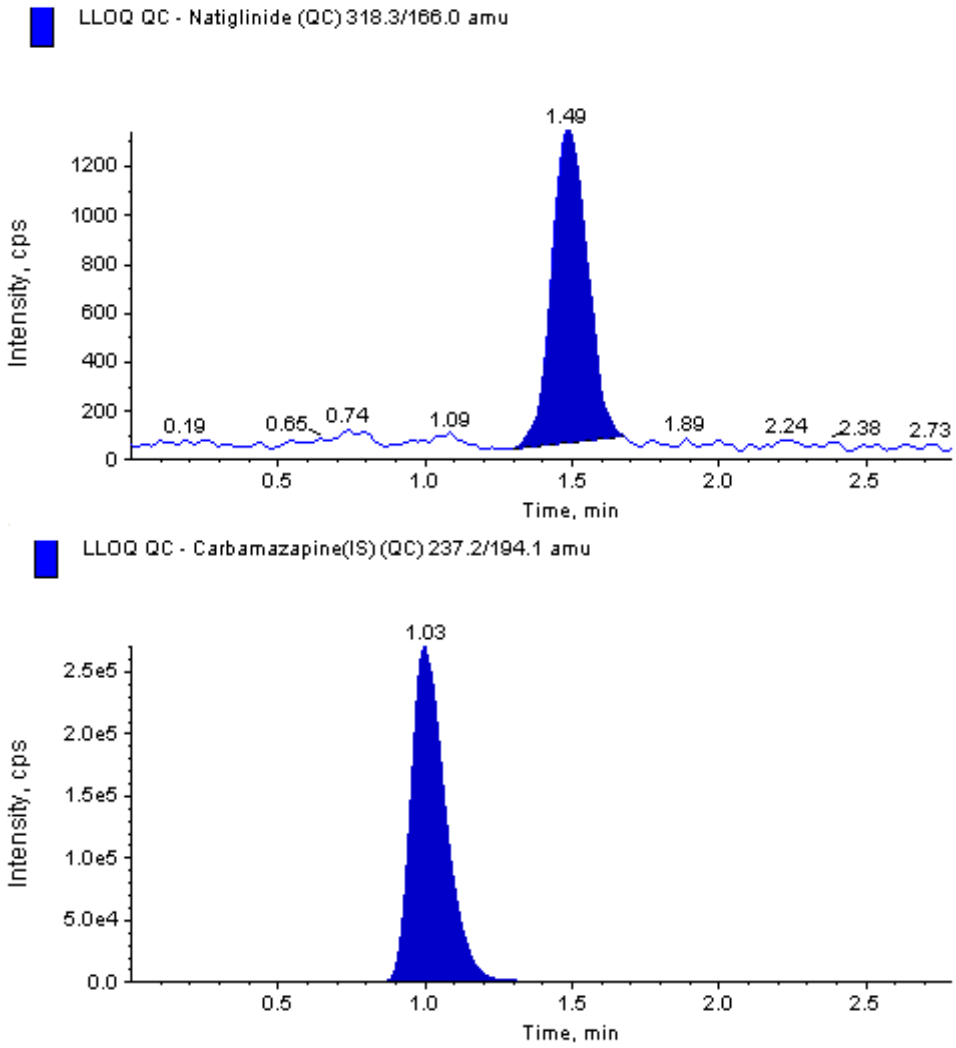


Figure 3. Typical MRM chromatogram of nateglinide and the IS in a LLOQ sample

Linearity, sensitivity, precision and accuracy

Five nateglinide calibration curves were linear over the concentration range of 10.0–10005 ng/mL with a correlation coefficient (r^2)≥0.9970. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship. The accuracy and %CV for the calibration standards ranged from 90.19% to 104.63% and 2.47% to 5.91%, respectively.The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ (10.0 ng/mL). The %CV and accuracy at LLOQ concentration were found to be 11.65% and 93.08%, respectively. The results for intra–day and inter–day %CV and accuracy in plasma quality control samples are summarized in Table 1. The intra–batch %CV ranged from 0.80 to 8.76% and the accuracy was within 100.56–107.83%. For inter–batch experiment, the %CV varied from 1.52 to 10.84% and the accuracy was within 98.00–106.27%.

Table 1:Precision and accuracy data for nateglinide.

Quality control	Run	Concentration found	% CV	Accuracy(%)
Intra–day variations (12 replicates at each concentration)				
LLOQ		10.7 ± 0.78	7.29	107.17
LQC		32.5 ± 2.84	8.76	107.83
MQC1		1620 ± 15.3	0.94	107.59
MQC2		5046 ± 40.3	0.80	100.56
HQC		8669 ± 91.8	1.06	101.93
Inter–day variations (30 replicates at each concentration)				
LLOQ		10.7 ± 1.16	10.84	106.27
LQC		31.1 ± 2.46	7.89	103.32
MQC1		1578 ± 69.2	4.39	104.80
MQC2		4918 ± 204	4.14	98.00
HQC		8673 ± 132	1.52	101.97

Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 10.0, 30.1, 1505, 5018, and 8505 ng/mL, respectively.

Recovery and dilution integrity

The mean overall recovery of nateglinide was 64.38±8.14% with the %CV range of 0.67–6.18% and the recovery of the IS was 51.37%. The recoveries of analyte and the IS were good and reproducible.The upper concentration limit of nateglinide can be extended to 16509 ng/mL by using two– and four–fold dilution with screened human blank plasma. The %CV for dilution integrity of half and quarter dilution was found to be 0.55% and 0.91%, while the accuracy results were found to be 96.25% and 99.24%, respectively.

Stability studies

In various stability experiments carried out namely bench top stability (11 h), autosampler stability (72 h), repeated freeze–thaw cycles (3 cycles), reinjection stability (33 h), wet extract stability (29 h at 2–8 °C) and long–term stability at –70 °C for 40 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2). Therefore, the results were found to be within the acceptable limits during the entire validation.

Table 2 Stability data for nateglinide in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Concentration found (ng/mL)	%CV	Accuracy/ Stability (%)
Process[a]	30.1	30.1 ± 1.05	3.49	100.06
	8505	9014 ± 50.6	0.56	105.99
Process[b]	30.1	30.9 ± 0.66	2.15	102.78
	8505	9023 ± 75.6	0.84	106.09
Bench top[c]	30.1	28.3 ± 0.97	3.43	94.09

	8505	8949 ± 52.2	0.58	105.22
FT[d]	30.1	30.0 ± 1.27	4.25	99.59
	8505	8996 ± 60.5	0.67	105.78
Re–injection[e]	30.1	30.5 ± 2.11	6.91	101.41
	8505	8548 ± 62.5	0.73	100.51
Long–term[f]	30.1	29.5 ± 1.29	4.37	97.95
	8505	8509 ± 149	1.75	100.05

[a] after 72 h in autosampler at 10°C; [b] after 29 h at 2-8 °C; [c] after 11 h at room temperature; [d] after 3 freeze and thaw cycles; [e] after 33 h of Reinjection; [f] at –70°C for 40 days

Pharmacokinetic study results

The proposed method was successfully used to quantify nateglinide plasma concentration for a pharmacokinetic study in healthy adult male subjects (*n*=6) under fasting condition. Fig. 4 depicts the mean plasma concentration *vs* time profile of nateglinide in health subjects. The maximum concentration (C_{max}) in plasma (3506±818 ng/mL) for nateglinide was attained at 1.45±0.27 h (t_{max}). The area under the plasma concentration–time curve from time zero to last measurable time point (AUC_{0-t}) and area under the plasma concentration time curve from time zero to infinity time point (AUC_{0-inf}) for nateglinide were 7105±1113 and 7220±1056 ng*h/mL, respectively. The terminal half–life ($t_{½}$) was found to be 7.00±4.68h.

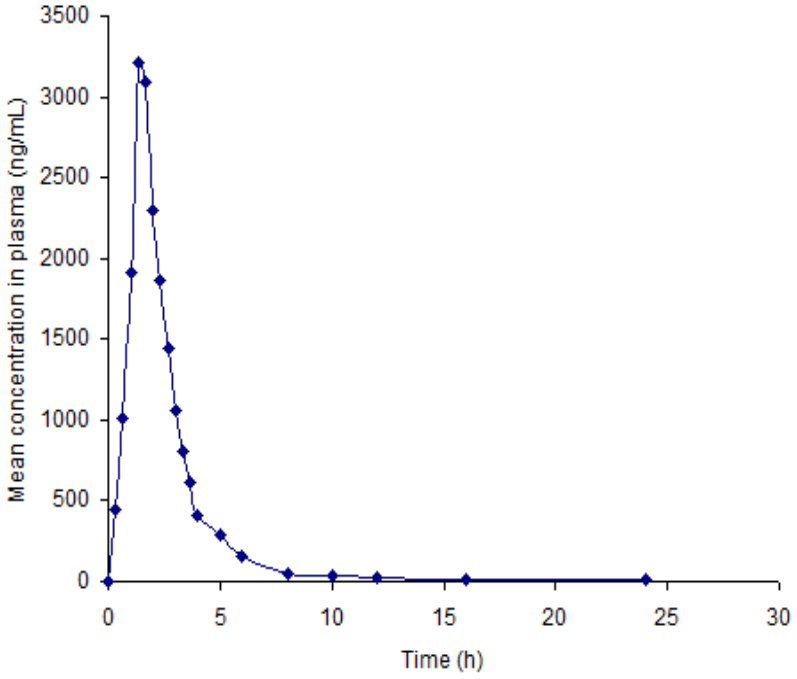


Figure 4.Mean plasma concentration–time profile of nateglinide in human plasma following oral administration of nateglinide (120 mg tablet) to healthy volunteers (*n*=6).

CONCLUSIONS

The proposed LC–MS/MS method is simple, rapid, specific and sensitive for determination of nateglinide in human plasma and is fully validated according to commonly acceptable FDA guidelines. The current method has shown acceptable precision and adequate sensitivity for the quantification of nateglinide in human plasma samples obtained for pharmacokinetic studies. The simple LLE with ethyl acetate method gave consistent and reproducible recoveries for the analyte and the IS from plasma. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge PCR Laboratories for providing necessary facilities to carry out this work.

REFERENCES

1. Fujitani S, Okazaki K, Yada T. The ability of a new hypoglycaemic agent, A-4166, compared to sulphonylureas, to increase cytosolic Ca2+ in pancreatic beta-cells under metabolic inhibition. Br J Pharmacol. 1997; 120: 1191–1198.
2. Karara AH, Dunning BE, McLeod JF. The effect of food on the oral bioavailability and the pharmacodynamic actions of the insulinotropic agent nateglinide in healthy subjects J Clin Pharmacol. 1999; 39: 172–179.
3. Fujitani S, Yada T. A novel D-phenylalanine-derivative hypoglycemic agent A-4166 increases cytosolic free Ca2+ in rat pancreatic beta-cells by stimulating Ca2+ influx. Endocrinology 1994; 134: 1395–1400.
4. Malli D, Gikas E, Vavagiannis A, Kazanis M, Daniilides K, Gennimata D, Panderi I. Determination of nateglinide in human plasma by high-performance liquid chromatography with pre-column derivatization using a coumarin-type fluorescent reagent. Anal Chim Acta. 2007; 599: 143-150.
5. Weaver ML, Orwig BA, Rodriguez LC, Graham ED, Chin JA, Shapiro MJ, McLeod JF, Mangold JB. Pharmacokinetics and metabolism of nateglinide in humans. Drug Metab Dispos. 2001; 29: 415–421.
6. Han D-E, Zheng Y, Li N, Zhao D, Zhang G, Yan H, Zhang L, Sun W, Wu Y-N, Lu Y, Chen X. Determination of nateglinide in human plasma by LC-ESI-MS and Its application to bioequivalence study. Chromatogr. 2010; 71: 299-304.

7. Hess C, Musshoff F, Madea B. Simultaneous identification and validated quantification of 11 oral hypoglycaemic drugs in plasma by electrospray ionisation liquid chromatography-mass spectrometry. Anal Bioanal Chem. 2011; 400: 33-41.
8. Varanasi KK, Sridhar V, Potharaju S, Shraddha R, Sivakumar SP, Sabapathi SK, Satheeshmanikandan TR, Kumar VV. Development and validation of a liquid chromatography/tandem mass spectrometry assay for the simultaneous determination of nateglinide, cilostazol and its active metabolite 3,4-dehydro-cilostazol in Wistar rat plasma and its application to pharmacokinetic study. J Chromatogr B Analyt Technol Biomed Life Sci. 2008; 865: 91-98.
9. Ho EN, Yiu KC, Wan TS, Stewart BD, Watkins KL. Detection of anti-diabetics in equine plasma and urine by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2004; 811(1): 65-73.
10. Wang M, Miksa IR. Multi-component plasma quantitation of anti-hyperglycemic pharmaceutical compounds using liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 856: 318-327.
11. He Z, Chen X, Zhong D, Zhao C, Liu X, Zhang R. Study on the bioavailability of nateglinide-hydroxypropyl-beta-cyclodextrin complex capsule in rabbits by liquid chromatographic-tandem mass spectrometry. Biomed Chromatogr. 2004; 18: 532-537.
12. US DHHS, FDA and CDER. Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Veterinary Medicine, 2001. Available at: http://www/fda.gov/cder/guidance/index.htm.