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DEVELOPMENT AND VALIDATION OF LC-MS/MS METHOD FOR SIMULTANEOUS QUANTIFICATION OF METFORMIN AND NATEGLINIDE IN HUMAN PLASMA AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

A selective, sensitive and rapid LC-MS/MS method has been developed and validated for simultaneous quantification of metformin and nateglinide in human plasma using repaglinide as internal standard (IS). The analytes and IS were extracted from 100 µL of plasma by liquid-liquid extraction technique using methyl tert - butyl ether which offers high sensitivity, wide linearity without interferences form endogenous matrix components. Chromatographic separation was achieved in 1.50 min run time on Hypersil gold C_{18} column using a 5 mM ammonium formate/acetonitrile mobile phase in gradient mode. The quantification of target compounds was performed in a positive electrospray ionization mode and multiple reaction monitoring (MRM). The proposed method was validated over the concentration ranges of 1-20000 ng/mL for metformin and 0.01-200 ng/mL for nateglinide. The intra- and inter-day precision and accuracy results were acceptable as per FDA guidelines. Stability of compounds were established in a battery of stability studies, i.e. bench top, auto sampler, dry extract and long term storage stability as well as freeze-thaw cycles. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic studies.

KEYWORDS: Metformin; Nateglinide; LC-MS/MS.

INTRODUCTION

Type 2 diabetes mellitus is the 7th leading cause of death in world wide. Approximately 1.5 million deaths occurred in 2014 are directly due to type 2 diabetes mellitus. More than 80% of diabetes deaths occurring in developing countries. Unhealthy diet, lack of physical exercise, stress and more usage of tobacco increasing the risk of developing type 2 diabetes mellitus.[1]

Type 2 diabetes mellitus is a chronic metabolic disease characterized by impaired insulin secretion and insulin resistance in insulin-targeting tissues (liver, skeletal muscle and adipocytes).[2] Insulin resistance in these tissues is accompanied by decreased glucose utilization by muscle and fat cells, and increased hepatic glucose output, leads to hyperglycemia. Insulin resistance is compensated by excessive insulin secretion. The over secretion of insulin gradually decreases the islet beta-cell reserve and its function.[3] The complexity in treatment of type 2 diabetes mellitus is welcoming new combinations for specific cure.

Metformin is chemically 3-(diaminomethylidene)-1, 1-dimethylguanidine. Metformin is an effective oral hypoglycemic drug used in the treatment of type 2 diabetes mellitus. It lowers blood glucose levels predominantly by decreasing hepatic glucose production and release and also by increasing glucose uptake into skeletal muscle.[4] Mechanism of action involved in this is metformin increases AMP-activated protein kinase enzyme activity results in the stimulation of glucose disposal into skeletal muscle, fatty acid oxidation in muscle and liver, and the inhibition of liver gluconeogenesis, cholesterol and triglyceride synthesis and lipogenesis. In this way it acts as effective anti-hyperglycemic agent and improves lipid profiles and decreases cardiovascular risk. Like other hypoglycemic agents, it does not cause a gain in body weight and hypoglycemia. The most common side effects associated with metformin are mild, transient, gastrointestinal symptoms, which are usually self-limiting.[5]

Nateglinide is chemically (2R)-3-phenyl-2-{[4-(propan-2-yl) cyclohexyl] formamido} propanoic acid, belongs to meglitinide class of potent insulin secretagogue. Nateglinide act as hypoglycemic agent by binding to β cells of the pancreas and restore the early phase insulin secretion.[6] Mechanism of action involved in this is nateglinide inhibits ATP sensitive K^+ channels by binding competitively to sulphonylurea receptors present in the membrane of pancreatic β cells. This results depolarization of the pancreatic β cells and open the voltage dependent L type calcium channels, which promotes influx of calcium ions into insulin

containing secretory granules and causes subsequent insulin release.[7] In this way it is more effective at lowering the postprandial blood glucose levels and useful for the treatment of type 2 diabetes mellitus with impaired insulin secretion. Nateglinide have a neutral effect on weight or cause a slight increase in weight compared to sulphonylureas.[8]

The long term complications of type 2 diabetes mellitus are reduced by glycemic control. Insulin resistance and loss of early insulin secretion are major defects for progression of type 2 diabetes mellitus. Same defects are the major causes for postprandial hyperglycemia. In this type of patients early component of hyperglycemia is postprandial hyperglycemia. So combination therapy is required to treat patients having type 2 diabetes mellitus with postprandial hyperglycemia. Monotherapy of nateglinide and metformin improve glycemic control by different mechanisms, but in combination they had complementary effects like improving HbA1C and improve overall glycemic control by reducing mealtime glucose levels in type 2 diabetes mellitus.[9] Currently only one manufacturer providing this combination and available for this treatment, examples are Glinate-MF (Metformin 500mg+ Nateglinide 60mg) from Glenmark. So there is a need for the method to estimate the plasma concentrations of metformin and nateglinide simultaneously.

Literature survey reveals that several methods have been reported for the quantitative determination of metformin[10-20] and nateglinide.[21,22] individually in biological fluids. However, till date no LC-MS/MS method has been reported in literature for the simultaneous determination of metformin and nateglinide in human plasma. Hence, authors attempted to develop a simple and reproducible LC-MS/MS method for simultaneous quantification of these two analytes which helps the researchers for therapeutic drug monitoring and pharmacokinetics.

The aim of this work is, to develop a simple, selective and sensitive method, which employs liquid-liquid extraction technique for sample preparation and liquid chromatography with electrospray ionisation-tandem mass spectrometry for simultaneous quantitation of metformin and nateglinide in human plasma. The proposed method has significant advantages over earlier reported methods like 1) simultaneous quantification of two analytes, 2) Shorter run time, 3) Wider linearity range with a more sensitivity, 4) Simple reproducible extraction. The present method has been validated as per the current USFDA guidelines.[23] The application of this assay in a clinical pharmacokinetic study following oral administration of metformin and nateglinide are described.

EXPERIMENTAL

Materials and reagents

Reference standards of metformin, nateglinide (Figure. 1) and internal standard (repaglinide) were purchased from Sigma-Aldrich (Hyderabad, India). HPLC grade ammonium formate was procured from Thermo Fisher Scientific (Mumbai, India). HPLC grade methanol and acetonitrile were procured from J.T Baker (Phillipsburg, USA). Milli-Q water (18.2 mΩ and TOC≤50 ppb) from Milli-Q purification system, Millipore (Bangalore, India) was used throughout the study. Methyl tert - butyl ether was purchased from Rankem (Hyderabad, India). Drug free human plasma was procured from Biochemed (Winchester, USA).

Chromatographic and mass spectrometric conditions

Waters Acquity UPLC system (Waters corporation, Milford, USA) consisting of binary solvent manager, sample manager and column manger was used for solvent and sample delivery. Mobile phase A consisted of 5 mM ammonium formate in Milli-Q water and mobile phase B was acetonitrile. The analytes and IS were separated by using the following gradient (minutes, % mobile phase B) (0.01, 10) (0.20, 10) (0.90, 90) (1.10, 90) (1.20, 10) (1.50, 10) delivered with a flow rate of 0.5 mL/min on a Hypersil Gold C_{18} (50 mm×3.0 mm, 1.9 µm; Thermo scientific, India) column maintained at 40°C.The sample manager was maintained at 5°C and injection volume was 5 µL. The total chromatographic run time was 1.50 min. The analytes and IS were detected using a Waters XEVO TQ mass spectrometer (Waters corporation, Milford, USA) equipped with Z spray source. The quantification of analytes and IS were achieved by operating the mass spectrometer in positive ion ESI with multiple reaction monitoring (MRM) mode. Nitrogen gas was used as both cone gas and desolvation gas with a flow rate of 50 L/Hr and 800 L/Hr respectively. The source dependent parameters capillary voltage, extractor voltage, source temperature and desolvation temperature were set at 3.50 KV, 3 V, 150°C and 400°C respectively. The precursor to product ion transitions along with the cone voltage and collision energy for each analyte and IS were as follows: Metformin m/z 130.1→ 60.1, 16 V, 12 eV; Nateglinide m/z 318.2→ 166.2, 21 V, 18 eV; Repaglinide m/z 453.2→ 230.2, 26 V, 22 eV with dwell time 100 ms. Data acquisition and calculations were performed using Masslynx software, version 4.0.

Preparation of calibration standards and quality control samples

Individual standard stock solutions of metformin and nateglinide were prepared by dissolving requisite amounts in methanol to obtain final drug concentration of 1mg/ml respectively.

Respective working stock solutions of metformin with concentrations in the range of 1 to 20000 ng/mL and nateglinide with concentrations in the range of 0.01 to 200 ng/mL were prepared by serial dilutions with acetonitrile: water (50:50). Combined Calibration standards were prepared by spiking (2% of total plasma volume) in blank human plasma with respective working stock solutions. A nine point calibration curve standards were prepared for metformin and nateglinide. The combined quality control (QC) samples were similarly prepared for metformin and nateglinide at concentration of 15000 ng/mL and 150 ng/mL (high quality control, HQC), 7500 ng/ml and 75ng/mL (middle quality control), 3 ng/mL and 0.03ng/mL (low quality control) and 1 ng/ml and 0.01ng/mL (lower limit of quantification quality control, LLOQ QC) respectively with blank human plasma by a separate weighing of standards. Stock solution (1 mg/mL) of the internal standard (repaglinide) was prepared by dissolving appropriate amount in methanol and its working stock solution (250 ng/mL) was prepared by diluting the stock solution in acetonitrile: water (50:50).

Sample preparation

A simple liquid-liquid extraction method was developed for extraction of analytes and IS from human plasma. Prior to analysis all frozen subject samples, calibration standards and quality control samples were thawed at ambient temperature. In the following order 5 µL of IS working stock solution (250 ng/mL repaglinide) was added into each 1.5 mL eppendorf tube except for blank plasma. 100 µL of standards, QCs, study samples and blank plasma were transferred into eppendorf tubes. After vortexing for 30 seconds, 100 µL of Milli-Q water was added to each tube and vortexed to mix. The analytes and IS were extracted with 1mL of methyl *tert* - butyl etherby vortexing for 10 min and followed by centrifugation at 5000 g for 10 min. The organic layer was transferred into a clean test tube and evaporated to dryness at 40°C under a gentle stream of nitrogen in the Turbo vap evaporator (Caliper life sciences, USA). The dried extract was reconstituted with 0.2 mL of mobile phase and 5 µL of aliquot was injected onto the LC-MS/MS system for analysis.

Method validation

The bioanalytical method was thoroughly validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation (US Food and Drug Administration, 2001).The method was validated for selectivity, linearity, precision and accuracy, recovery, matrix effect, dilution integrity and stability. Selectivity is the ability of analytical method to differentiate and quantify the analytes in the presence of other expected

components in the sample. This was evaluated by comparing the chromatograms of six different blank human plasma with corresponding spiked plasma at LLOQ QC level. Peak areas of endogenous compounds co eluting with the analytes should be less than 20% of the peak area of LLOQ response. Peak areas of endogenous compounds co eluting with IS should be less than 5% of the mean response of internal standard in LLOQ samples. Linearity was tested for metformin and nateglinide in the concentration range of 1 to 20000 ng/mL and 0.01 to 200 ng/mL respectively. For the determination of linearity five standard calibration curves containing at least nine non-zero standards were constructed by a weighed ($1/x^2$) least squares linear regression method through the measurement of the peak area ratio of analyte to IS. In addition blank and blank+IS were analyzed to conform the absence of direct interferences, these data were not included to construct calibration curves. The acceptance limit of accuracy for each of the back calculated concentrations were ±15% except for LLOQ, where it was ±20%. For a calibration run to be accepted at least 75% of the standards, including the LLOQ and ULOQ were required to meet the acceptance criterion, otherwise the calibration curve was rejected. Intra-day precision and accuracy were determined by analyzing six replicate analysis of each quality control (LLOQ, LQC, MQC and HQC) samples of two different batches on same day. Inter-day precision and accuracy were determined by analyzing six replicate analysis of each quality control (LLOQ, LQC, MQC and HQC) samples of five different batches.The acceptance criteria included accuracy with in ±15% deviation from the nominal values, except the LLOQ where it should be ±20% and a precision of ±15% coefficient of variance (%CV), except for LLOQ, where it should be ±20%. The extraction recovery of each analyte was estimated at three different QC levels (six replicates of each LQC, MQC and HQC) by comparing the peak area response of extracted analytes with unextracted analytes (extracted blank sample spiked with the analytes) that represents 100% recovery. Similarly recovery of IS was estimated by comparing the mean peak area of extracted QC samples (n=18) with mean peak area of unextracted QC samples. Recovery of the analytes and IS need not be 100% but it should be precise and reproducible at all QC levels. The matrix effect was evaluated by comparing the peak area ratios (analyte/IS) obtained from post extraction spiking with mean peak area ratio (analyte/IS) obtained at the same concentration in neat solution. The matrix effect was determined at all three QC levels using six replicates at each level for each analyte, where IS was determined at a single concentration of 250 ng/mL. Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. The dilution integrity experiment was carried out for each analyte by analysing six replicate samples at

concentration of two times the ULOQ concentration was prepared and diluted to 2- and 4-fold with blank plasma.Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8°C) was determined by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench-top stability (8 h), processed sample stability (autosampler stability for 24 h), dry extract stability (8 h), freeze–thaw stability (four cycles) and long-term stability (60 days) were tested at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15%) and precision (±15% CV).

Pharmacokinetic study

The pharmacokinetic study was carried out in healthy male volunteers (n = 6). Six volunteers were administered a single oral dose of metformin (500 mg) and nateglinide (60 mg) combination tablet. Blood samples were collected at pre-dose and 0.18, 0.33, 0.50, 0.67, 0.83, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 hours in K2-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA).The tubes were centrifuged (Thermo Scientific, Germany) at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –80°C till their use. This study was carried out as per the approval and guidelines of the local ethical committee. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples at low, middle and high concentration levels were also assayed in triplicate. Plasma concentration-time profile of metformin and nateglinide was analyzed by non-compartmental method using WinNonlin Version 5.3 (Pharsight Corporation, CA, USA).

RESULTS AND DISCUSSION

Method development and optimization

Optimization of the mass spectrometric conditions

Mass spectrometric conditions were optimized so as to achieve the maximum stable response of the precursor ions and the major product ions of the analytes. Both analytes in this study were basic in nature so mass spectrometer was operated in positive ion mode to get good response. The inherent selectivity of MRM mode for quantification of analytes was expected to be beneficial in developing a selective and sensitive method. All analytes showed the singly charged protonated ions $[M+H]^+$ as the prominent ion in the full scan of Q1 spectrum

and was used as the precursor ion to obtain Q3 product ion spectra. The cone voltage and collision energy was optimized to get highest intensity for precursor ion and product ion respectively. The mass transition ion pair was selected as m/z 130.1→ 60.1 for metformin, m/z 318.2→ 166.2 for nateglinide. The product ion mass spectra for each analyte were presented in Figure. 2(A and B).

Optimization of the chromatographic conditions

Chromatographic conditions, especially the composition of mobile phase and column were optimized inorder to achieve good chromatographic resolution; symmetric analyte peak shapes within a short run time. The feasibility of various mixtures of solvents such as methanol and acetonitrile with different buffers such as ammonium acetate, ammonia solution with altered flow rates on different types of columns such as C_{18} and C_8 were tested for complete chromatographic resolution of analytes and IS from interfering biological matrix. Finally the gradient mobile phase system consisting of 5 mM ammonium formate-acetonitrile mixture (minutes, % mobile phase B) (0.01, 10) (0.20, 10) (0.90, 90) (1.10, 90) (1.20, 10) (1.50, 10) delivered with a flow rate of 0.5 mL/min on a Hypersil Gold C_{18} column achieved the good chromatographic separation of analytes and IS with desired response. The retention times of metformin, nateglinide and IS were 0.82, 1.07 and 0.99 min respectively. Several compounds were investigated to find a suitable IS and finally repaglinide was found to be compatible with targeted analytes in terms of extraction efficiency, chromatographic behavior and ionization yield.

Optimization of the sample extraction procedure

Due to the complex nature of plasma, a sample pre treatment was often needed to remove protein and potential interferences prior to LC-MS/MS analysis. Currently, the most widely employed biological sample preparation methodologies were protein precipitation (PPT), solid phase extraction (SPE) and liquid-liquid extraction (LLE). LLE procedure gives more clean and matrix free sample compared to other techniques. Hence liquid-liquid extraction was used for sample preparation in this study. It produces a clean chromatogram of a blank sample and yield good, reproducible recovery for analytes from the plasma.

METHOD VALIDATION

Selectivity

Representative chromatograms obtained from blank plasma sample and blank plasma spiked with LLOQ standard of each analyte and IS were presented in Figure. 3(A and B) and Figure.

4(A and B). There is no significant endogenous interferences observed in the respective MRM channel at the retention time of each analyte and IS in blank plasma sample.

Linearity

Linearity of each calibration curve was determined by plotting the peak area ratio of analyte to IS (y) versus the nominal concentration(x) of the calibration points, and fitted to the y = mx+c using a regression factor ($1/x^2$). The nine point calibration curve was found to be linear over the concentration range of 1 to 20000 ng/mL for metformin and 0.01 to 200 ng/mL for nateglinide. Correlation coefficients were in the range $0.980<r^2<0.995$ for all the analytes. The percentage accuracy values ranged from 97.00% to 102.52%, while the precision (%CV) values ranged from 1.28 to 10.60 for both the analytes. Table 1 summarizes the calibration curve results for both the analytes.

Precision and Accuracy

The intra- and inter-day precision and accuracy values were within the acceptance limit for all the analytes and summarized in Table 2. The intra-day accuracy ranged between 93.33% and 100.68% with a precision of 1.48% to 10.55%, the inter-day accuracy between 94.00% and 101.72% with a precision of 1.65% to 10.67%.

Recovery

The extraction recoveries of all the analytes and IS were good and reproducible. The mean overall recoveries (with the precision) of all the analytes were summarized in Table 3.

Matrix effect

There was no effect of endogenous contribution from blank plasma in the measurement of both the analytes and IS. The average matrix factor values ranged from 0.97 to 1.05, while the precision (%CV) values ranged from 0.46 to 3.12 for both the analytes.

Dilution integrity

The upper concentration limit was extended to 40000 ng/mL for metformin and 400 ng/mL for nateglinide by a half and quarter dilution with screened human blank plasma. The mean back calculated concentrations for half and quarter dilution samples within 85-115% of nominal value, while precision values ranged from 1.60% to 2.34% for both the analytes.

Stability studies

Analysis of stock solution stability was performed at 20000 ng/mL for metformin and 200 ng/mL for nateglinide. After storage for 24 days at 2-8°C and at room temperature for 6 h, more than 98% of metformin and nateglinide remained unchanged. The results of bench-top stability (8 h), processed sample stability (Auto sampler stability for 24 h), dry extract stability (8 h), freeze–thaw stability (four cycles) and long-term stability (60 days) were summarized in Table 4 and found to be within the acceptance limit.

APPLICATION OF THE METHOD IN PHARMACOKINETIC STUDY

The established UPLC-MS/MS method was successfully applied to the determination of metformin and nateglinide concentrations in human plasma samples collected from healthy volunteers (n=6). The mean plasma concentrations vs time profiles of metformin and nateglinide were shown in Figure. 5. In addition, the pharmacokinetic parameters were presented in Table 5.

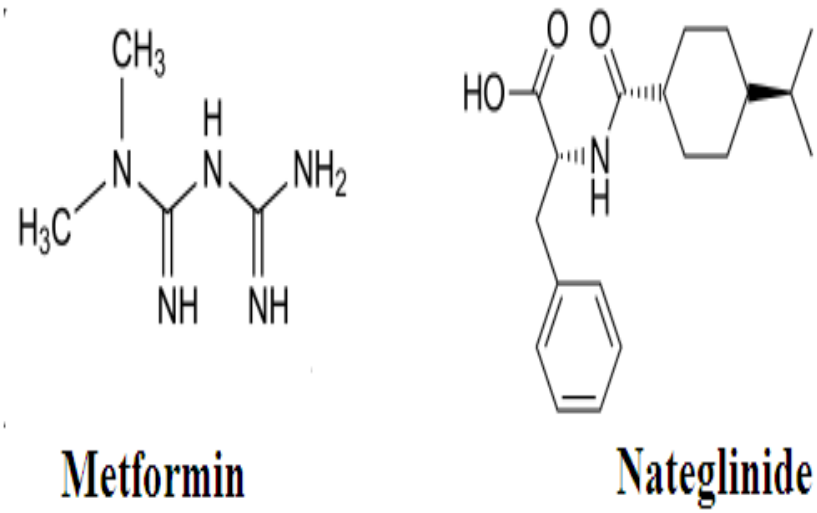

Figure 1: Chemical structures of metformin and nateglinide.

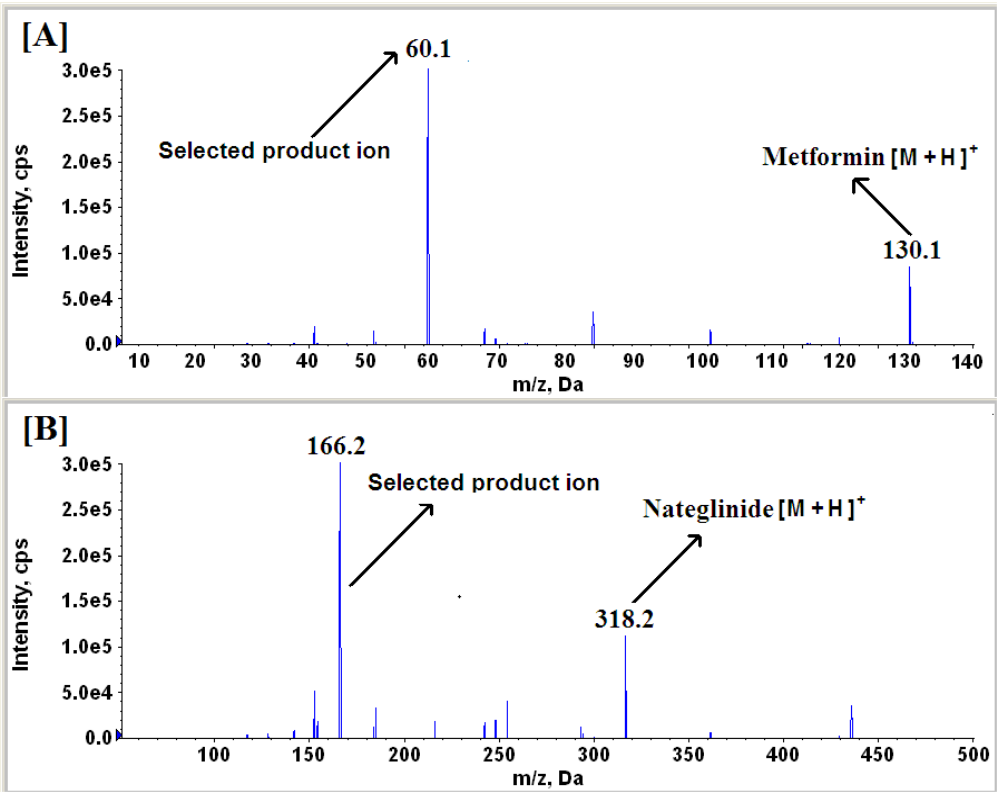

Figure 2: Product ion mass spectra of [M+H] $^{+}$ of [A] metformin and [B] nateglinide.

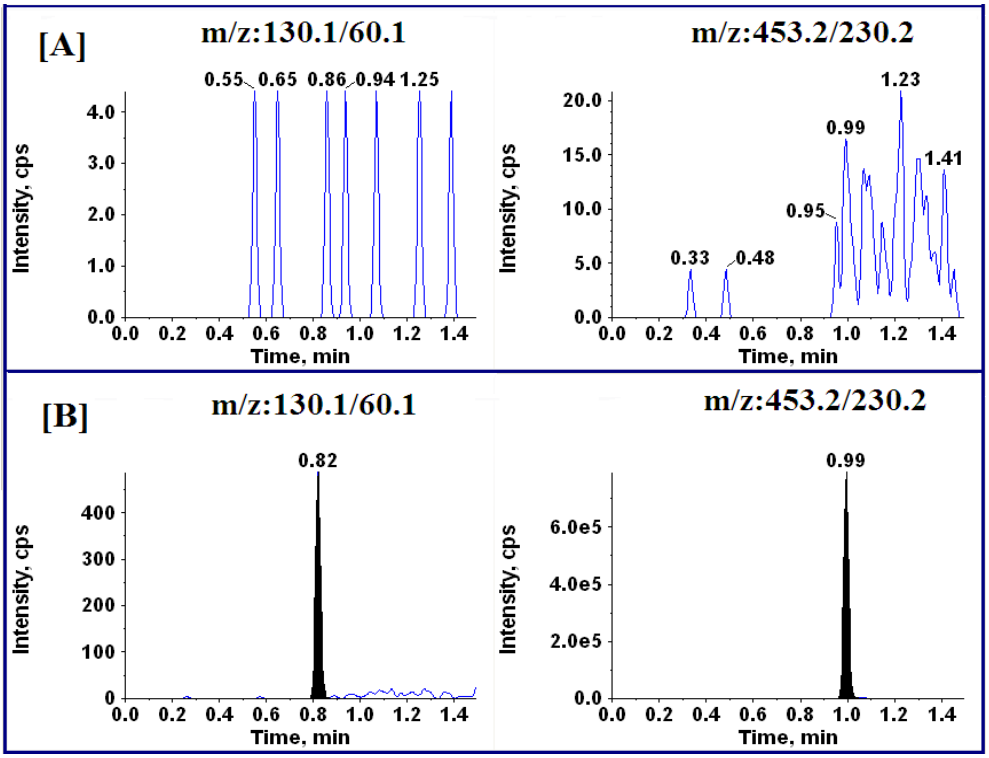

Figure 3: Typical multiple reaction monitoring mode chromatograms [A] Blank chromatograms of metformin and IS, and [B] Human plasma spiked with metformin at LLOQ level and IS.

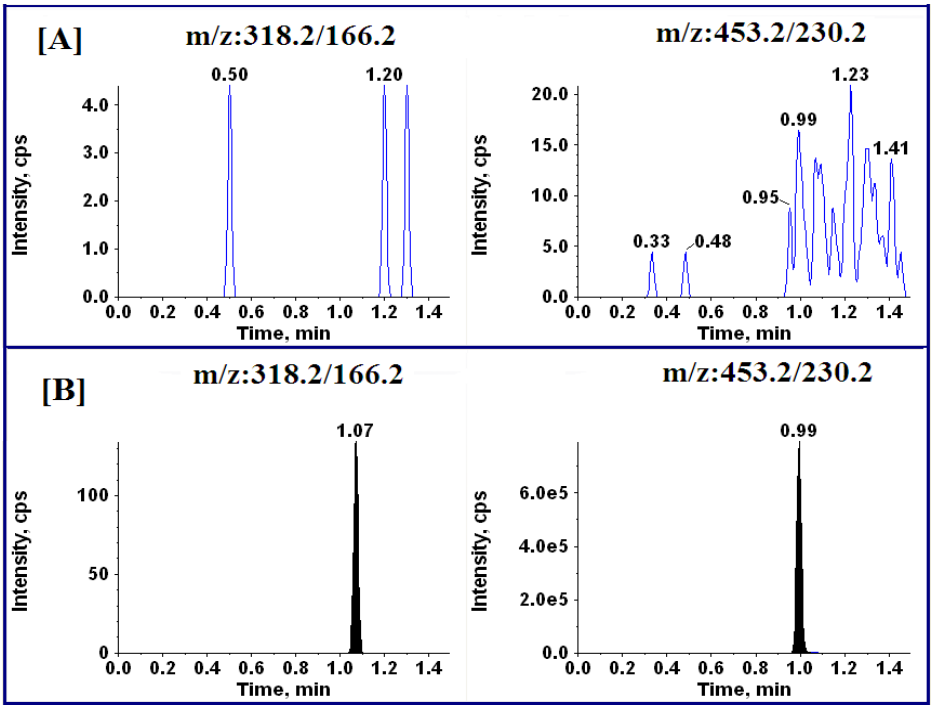

Figure 4: Typical multiple reaction monitoring mode chromatograms [A] Blank chromatograms of nateglinide and IS, and [B] Human plasma spiked with nateglinide at LLOQ level and IS.

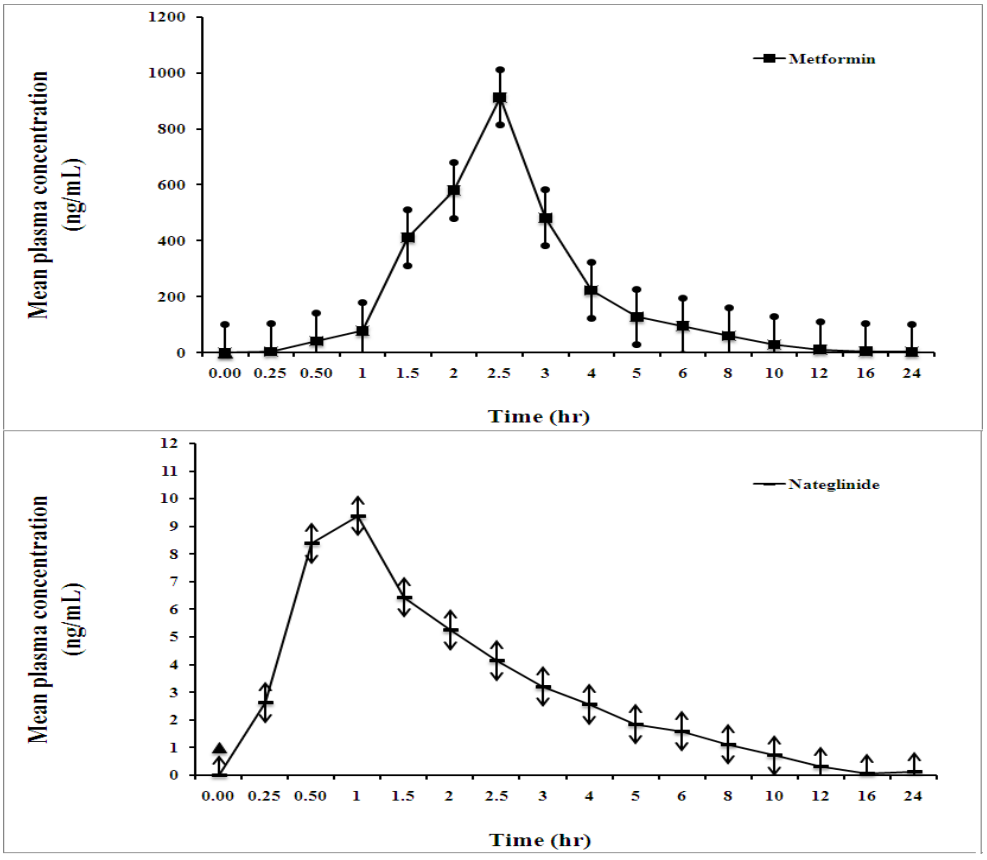

Figure 5: Mean plasma concentration-time profile of metformin and nateglinide.

Table 1. Precision and accuracy data for back - calculated concentrations of calibration standards.

Concentration added (ng/ml)	Metformin			Concentration added (ng/ml)	Nateglinide		
	Mean (n=5)	CV (%)	Accuracy (%)		Mean (n=5)	CV (%)	Accuracy (%)
1.000	1.020	10.60	102.02	0.010	0.010	10.00	100.00
2.000	2.050	5.79	102.52	0.020	0.019	5.88	97.00
10.000	9.785	8.17	97.85	0.100	0.101	5.03	100.80
50.000	50.559	4.88	101.12	0.500	0.508	5.21	101.68
500.000	498.018	3.44	99.60	5.000	4.970	4.71	99.41
5000.000	4960.771	2.34	99.22	50.000	50.719	1.91	101.44
10000.000	9918.605	1.66	99.19	100.000	101.956	2.83	101.96
16000.000	15952.992	1.28	99.71	160.000	158.682	2.54	99.18
20000.000	19826.696	1.55	99.13	200.000	198.725	3.41	99.36

Table 2. Intra -day and inter -day accuracy and precision of metformin and nateglinide.

Concentration added (ng/mL)	Metformin				Nateglinide			
	LLOQ 1.000	LQC 3.000	MQC 7500.000	HQC 15000.000	LLOQ 0.010	LQC 0.030	MQC 75.000	HQC 150.000
Intra-day (n=6)								
Mean	1.007	3.002	7349.027	15060.516	0.009	0.030	74.565	150.060
CV (%)	9.36	8.66	5.56	3.23	10.55	5.93	8.36	1.48
Accuracy (%)	100.68	100.07	97.99	100.40	93.33	99.17	99.42	100.04
Inter-day (n=30)								
Mean	0.984	2.970	7271.773	15257.646	0.009	0.030	75.111	150.568
CV (%)	9.00	8.03	5.92	3.31	10.67	5.13	7.21	1.65
Accuracy (%)	98.37	99.00	96.96	101.72	94.00	98.78	100.15	100.38

Table 3. Mean overall recoveries of analytes and IS.

Analyte name	Sample concentration (ng/mL)	Response unextracted (Mean ± CV (%)	Response extracted (Mean ± CV (%)	Recovery	Overall recovery (Mean ± CV (%)
Metformin	3	1556 ± 2.52	1444 ± 2.54	92.83	94.79 ± 1.88
	7500	3860099 ± 0.95	3675869 ± 0.39	95.23	
	15000	7658421 ± 0.34	7376187 ± 0.20	96.31	
Nateglinide	0.030	576 ± 2.45	522 ± 2.69	90.67	89.89 ± 1.73
	75	1544263 ± 1.17	1360473 ± 1.59	88.10	
	150	2930049 ± 0.73	2663592 ± 0.69	90.91	
IS	500	1755398 ± 1.30	1459204 ± 1.76	83.13	

Table 4. Summary of stability data of metformin and nateglinide in human plasma.

Stability	Metformin		Nateglinide	
	LQC	HQC	LQC	HQC
Bench top (27°C, 8 h) Mean (n=6) CV (%) Change (%)	2.966 8.92 0.88	14940.702 1.94 -0.45	0.030 6.34 -0.56	150.573 1.57 0.17
Auto sampler (4°C, 24 h) Mean (n=6) CV (%) Change (%)	3.026 7.53 2.91	15067.236 1.01 0.40	0.031 6.45 4.49	149.909 1.33 -0.27
Dry extract (4°C, 24 h) Mean (n=6) CV (%) Change (%)	2.961 5.06 0.71	14975.918 1.72 -0.21	0.030 5.16 1.12	150.680 1.92 0.24
Freeze-thaw (-80°C, After 4th cycle) Mean (n=6) CV (%) Change (%)	2.979 8.24 1.32	14995.241 1.47 -0.08	0.030 7.89 1.12	149.273 1.35 -0.69
Long term (-80°C, 60 days) Mean (n=6) CV (%) Change (%)	2.900 8.51 1.62	15060.123 1.61 1.02	0.030 6.34 -3.28	149.625 1.97 -0.53

Table 5. Pharmacokinetic data of metformin and nateglinide (n-6, Mean ± SD).

Parameter	Estimated value	
	Metformin	Nateglinide
C_{max} (ng/ml)	913.519±34.37	11.162±1.07
T_{max} (h)	2.50±0.00	0.75±0.27
$T_{1/2}$ (h)	1.58±0.20	3.20±0.80
AUC_{0-t} (ng.h/mL)	2106.557±138.35	30.750±9.23
$AUC_{0-\alpha}$ (ng.h/mL)	2110.995±140.12	30.917±9.18

CONCLUSION

In summary, a selective, sensitive and rapid LC-MS/MS method for simultaneous quantification of metformin and nateglinide in human plasma was developed and fully validated as per FDA guidelines. Till date there was no reported LC-MS/MS method for simultaneous quantification of metformin and nateglinide in any biological matrix. This method offers significant advantages, in terms of wide range of linearity, recovery, rapid extraction and shorter run time. Finally the shorter chromatographic runtime gives the method capability for high sample throughput. From the results of all the validation parameters we can conclude that the present method can be useful for pharmacokinetic/bioequivalence studies with desired precision and accuracy.

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