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Research Article

A SIMPLE AND SENSITIVE METHOD FOR DETERMINATION OF METFORMIN AND SITAGLIPTIN IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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ABSTRACT

Objective: To develop and validate a simple and rapid liquid chromatography/tandem mass spectrometry (LC–MS/MS) method for the simultaneous quantitation of antidiabetic drugs metformin and sitagliptin in human plasma using metformin-d6 Hcl and sitagliptin-d4 HCl as internal standard (IS) respectively.

Methods: After acetonitrile - induced protein precipitation of the plasma samples, metformin, sitagliptin and IS were chromatographed on reverse phase C18 (50mm×4.6mm i.d., 5 um) analytical column. Quantitation was performed on a triple quadrupole mass spectrometer employing electrospray ionization technique and operating in multiple reaction monitoring (MRM) and positive ion mode. The total chromatographic run time was 2.0 min and calibration curves were linear over the concentration range of 25–3000 ng/ml for metformin and 5–800 ng/ml for sitagliptin. The method was validated for selectivity, sensitivity, recovery, linearity, accuracy and precision and stability studies.

Results: The recoveries obtained for the Metformin and its IS was ≥39% and Sitagliptin and its IS was ≥64%. Recoveries obtained were consistent and reproducible. Inter-batch and intra-batch coefficient of variation across five validation runs (LLOQ, LQC, MQC1, MQC and HQC) was less than 7.5% for both metformin and sitagliptin.

Conclusion: A simple and sensitive analytical method was developed and validated in human plasma. This method is suitable for measuring accurate plasma concentration in bioequivalence study and therapeutic drug monitoring as well, following combined administration.

Keywords: Metformin; Sitagliptin; LC–MS/MS; Protein precipitation; Human plasma

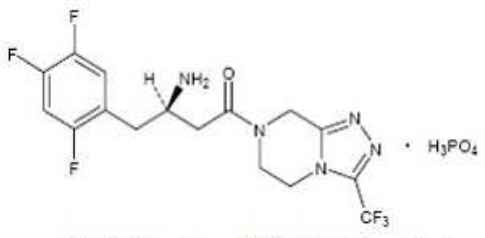
INTRODUCTION

Sitagliptin, 7 - [(3R) - 3 - amino - 1 - oxo - 4 - (2,4,5 - trifluorophenyl)butyl] - 5,6,7,8 - tetrahydro - 3 - (trifluoromethyl) - 1,2,4 - triazolo[4,3 - a]pyrazine phosphate (Fig. 1) and Metformin, Imidodicarbonimidic diamide,*N,N*-dimethyl-,monohydrochloride (Fig. 1) are two well known hypoglycemic drugs. Sitagliptin is a novel oral hypoglycemic drug of the dipeptidylpeptidase 4 inhibitor class [1].Dipeptidyl peptidase-4 (DPP-4) inhibitors are a new class of oral antihyperglycemic agents that enhance the body's ability to regulate blood glucose by increasing the active levels of incretins, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). There are numerous DPP-4 inhibitors in development with sitagliptin as the first approved agent for the treatment of patients with type 2 diabetes [2]. Sitagliptin is used as a single therapy or in combination with Metformin. Metformin is a biguanide drug effective in patients who lack functioning islet cells as it act by simulations of glycolysis in peripheral tissues [3, 4]. Metformin exerts its glucose- lowering effects primarily through increased hepatic insulin sensitivity and the resultant suppression of hepatic glucose output. Metformin may also modestly enhance glucose uptake in peripheral tissues and increase glucose metabolism in the splanchnic bed [5].

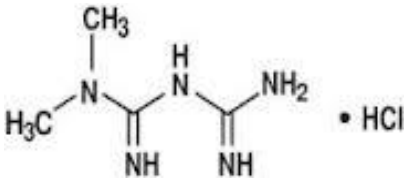
Literature survey reveals several methods for the determination of Sitagliptin in pharmaceutical preparation or biological fluids including spectrophotometry [6-8], liquid chromatography-tandem mass spectroscopy (LC-MS/MS) [9-12] and only one capillary electrophoresis method [13].

Several methods have also been described for the determination of Metformin either alone or in combination with various drugs, such as spectrophotometry [14-17], LC [18-20], LC/MS–MS [21-23] and capillary electrophoresis [24-26].

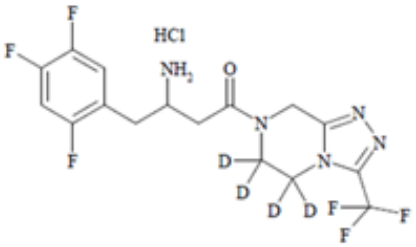
Regarding Sitagliptin and Metformin simultaneous analysis, two spectroscopic methods have been reported for the simultaneous determination of both drugs in pharmaceutical preparations [27,28] and only three chromatographic methods have been reported for their determination in pharmaceutical preparations [29-31]. Capillary zone electrophoresis method is reported for simultaneous analysis of Sitagliptin and Metformin in pharmaceutical preparations [32].



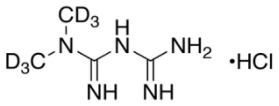
Sitagliptin Phosphate



Metformin Hydrochloride



Sitagliptin d4 HCl



Metformin d6 HCl

Fig. 1: It shows chemical structure of Sitagliptin Phosphate, Metformin Hydrochloride, Sitagliptin d4 HCl (Internal Standard), Metformin d6 HCl (Internal standard).

We felt that this simultaneous estimation method will help the researchers as the two drugs used in this method are available on the market with a fixed dose combination. The present work describes a simple, rapid and sensitive method that employs protein precipitation technique for sample preparation and liquid chromatography with electrospray ionization–tandem mass spectrometry for simultaneous quantitation of sitagliptin and metformin in human plasma. The application of this assay method to a clinical pharmacokinetic study in healthy male volunteers following oral administration of sitagliptin is described. To the best of our knowledge, no LC-MS/MS method was reported yet for the simultaneous determination of the studied drugs neither in pharmaceutical preparations nor in biological fluids.

MATERIALS AND METHOD

Chemicals and reagents

Sitagliptin phosphate was procured from Teva API India Limited, New Delhi and Metformin Hydrochloride was procured from Wanbury Limited, Maharashtra. Sitagliptin D4 HCl (Fig. 1) was procured from Vivan Lifesciences pvt. Ltd. and Metformin D6 HCl (Fig. 1) were procured from Clearsynth Labs (P) Ltd. Methanol and Acetonitrile of HPLC grade was obtained from J.T.Baker INC (Phillipsburg, NJ, USA). Purified water was obtained from Milli Q gradient water purification system (Millipore, Bangalore, India). Ammonium formate AR and Formic acid AR were purchased from SD Fine-Chem Ltd. (Mumbai, India). Human plasma containing K_2EDTA anticoagulant was purchased from Delta Laboratories (Bangalore, India).

Liquid chromatography and mass spectrometric condition

The liquid chromatography system (Shimadzu, Kyoto, Japan) consisted of a binary LC-20AD prominence pump, an autosampler (SIL–HTc), an online solvent degasser (DGU-20A3 prominence) and a temperature-controlled compartment for column (CTO10AS VP). Chromatographic separation was performed on Hypurity C18 (50mm×4.6mm i.d., 5µ) analytical column (Thermo Electron Corporation, Cheshire, UK) maintained at 40ºC temperature. The mobile phase consisted of Acetonitrile: 10mM ammonium formate (pH 3.5 adjusted with formic acid) (60:40 v/v). The flow rate of the mobile phase under isocratic condition was kept at 1.0 ml/min, split ratio 50:50, 50% to drain and 50% to MS/MS. The auto sampler temperature was set at 5°C and the injection volume was 10µL. The total LC run time was 2.0 min. Detection of analytes and IS was performed on a triple quadrupole mass spectrometer, API-3200, (MDS SCIEX, Toronto, Canada) equipped with turbo ion spray source in the positive ion mode. Analyst software version 1.6.1 was used to control all parameters of LC and MS. Quantitation was performed using multiple reaction monitoring (MRM) mode, based on parent→ product ion transitions for metformin (130.0→71.0), sitagliptin (408.1→235.1) and IS metformin D6 (136.0→77.0), sitagliptin D4 (412.5→239.5). Source dependent parameters optimized were gas 1(Nebuliser gas): 60 psi; gas 2(heater gas): 55 psi; ion spray voltage (ISV): 5000V; temperature (TEM): 500° C. Compound dependent parameters declustering potential (DP) and collision energy (CE) were set at 40 and 20, respectively for metformin; 44 and 27, respectively for sitagliptin; 33 and 35, respectively for metformin D6; 44 and 28, respectively for sitagliptin D4. Entrance potential (EP) and cell exit potential (CXP) were set at 10 and 6V, respectively for both the analytes and IS. Nitrogen was used as collision activated dissociation (CAD) gas and was set at 5 psi. Quadrupole 1 and quadrupole 3 were maintained at unit resolution and dwell time was set at 200 ms.

Preparation of stock and working dilution and spiking solutions

The standard stock solutions of metformin, Sitagliptin and IS were prepared by dissolving their accurately weighted compounds in methanol to give a final concentration of 1000 µg/ml. The combined working solutions of analytes in the desired concentration range were prepared by appropriate dilution of standard stock solutions with methanol: water (80:20 v/v). All the solutions were stored at 2–8° C and were brought to room temperature before use.

The calibration standards (CS) and quality control (QC) samples were prepared by spiking blank plasma (5% of total volume of blank plasma) with respective working solutions. Calibration standards were made at concentration of 25.000, 50.000, 100.000, 300.000, 600.000, 1200.000, 1700.000, 2400.000 and 3000.000 ng/ml for metformin; 5.000, 10.000, 25.000, 50.000, 100.000, 200.000, 400.000, 600.000 and 800.000 ng/ml for sitagliptin. Quality controls were prepared at 25.000 ng/ml (low limit of quality control, LLOQ QC), 75.000 ng/ml (low quality control, LQC), 500.000 ng/ml (middle quality control, MQC1), 1200.000 ng/ml (middle quality control, MQC) and 2400.000 ng/ml (high quality control, HQC) for metformin; 5.000 ng/ml (LLOQ QC), 15.000 ng/ml (LQC), 150.000 ng/ml (MQC1), 400.000 ng/ml (MQC) and 600.000 ng/ml (HQC) for sitagliptin. Spiked plasma samples were aliquoted in microcentrifuge tubes and stored at −20 ºC until use.

Sample preparation

To an aliquot of 0.1mL plasma sample in a 5.0mL RIA Vials, add 20µL of IS working solution (mixture of 3000 ng/mL metformin-d6 HCl and 800ng/mL sitagliptin-d4 solution) and vortex-mixed for 10 secs. The samples were precipitated using 1mL acetonitrile, followed by vortexing for 5mins and centrifugation at 4000rpm for 10min. Withdraw 0.8 mL of the upper clear layer solution and evaporate at 40°C under nitrogen gas for 30 mins and reconstitute with 500µL of mobile phase and was transferred to an auto-sampler vial for LC–MS/MS analysis and inject 10µL of volume.

Bioanalytical method validation

The method was validated for selectivity, sensitivity, linearity, precision and accuracy, recovery, stability, matrix effect and dilution integrity following the USFDA guidelines [33]. The specificity of the method was tested by screening six different batches of blank human plasma containing K_2EDTA as anticoagulant. Each blank sample was tested for interferences in the MRM channels using the proposed extraction procedure and chromatographic/MS–MS conditions, and the results were compared with those obtained for the analytes at a concentration near to the lower limit of quantification (LLOQ).

The linearity of the method was determined by analysis of standard plots associated with a nine-point standard calibration curve. Three linearity curves containing nine non-zero concentrations were analysed. Best-fit calibration curves of peak area ratio versus concentration were drawn. The concentration of the analytes were calculated from calibration curve ($y = mx + c$; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor $1/x^2$ for metformin and sitagliptin. The regression equation for the calibration curve was also used to back-calculate the measured concentration at each QC level. The peak area ratio values of calibration standards were proportional to the concentration of the drug in plasma over the range tested.

Inter-batch and intra-batch accuracy and precision was evaluated at five different concentrations levels (LLOQ, LQC, MQC1, MQC and HQC) in six replicates for both the analytes. Mean and standard deviation (S.D.) values were obtained for calculated drug concentration over these batches. The accuracy and precision was calculated and expressed in terms of Percent Accuracy and coefficient of variation (% CV), respectively.

Recovery of the extraction procedure was performed at LQC, MQC1, MQC and HQC levels using the proposed extraction procedure. It was evaluated by comparing peak area of extracted samples (spiked

before extraction) to the peak area of unextracted samples (quality control working solutions spiked in extracted plasma).

Stability experiments were performed to evaluate the analyte stability in plasma samples under different conditions, simulating the same conditions, which occurred during study sample analysis. Bench top stability, Autosampler stability, short-term stability was performed at LQC and HQC levels using six replicates at each level.

RESULTS AND DISCUSSION

Method Development

It was important to optimize chromatographic conditions, mass spectrometry parameters and extraction technique to develop and validate a selective and rapid assay method for simultaneously quantitation of metformin and sitagliptin in human plasma. MS parameters were optimized by infusing standard analyte solution of 200 ng/ml into the mass spectrometer having electrospray as the ionization source and operating in the multiple reaction monitoring (MRM) mode. The signal intensities obtained in positive mode were much higher than those in negative ion mode since the analytes and internal standards have the ability to accept protons. Metformin, Sitagliptin, metformin D6 and Sitagliptin D4 gave predominant protonated $(M+H)^+$ parent ions at m/z 130.0, 408.1, 136.0 and 412.5 ions, respectively in Q1 MS full scan spectra. Fragmentation was initiated using sufficient nitrogen for collision-activated dissociation and by applying 20V collision energy to break the parent ions. The most abundant ions found in the product ion mass spectrum were m/z 71.0, 235.1, 77.0 and 239.5 for metformin, Sitagliptin, metformin D6 and Sitagliptin D4 respectively. Declustering potential and collision energy were determined by observing maximum response of the product ion. Electrospray ionization (ESI) was selected as the ionization source as it gave high spectral response for both the analytes and the regression curves obtained were linear. Also, ESI source provided reliable data on method validation and for quantitation of samples from human volunteers.

Since metformin and sitagliptin have different physicochemical properties, it was difficult to set chromatographic conditions that produced sharp peak shape and adequate response. This included mobile phase selection, flow rate, column type and injection volume. Methanol, acetonitrile were tried in different ratio with buffers like ammonium acetate, ammonium formate as well as acid additives like formic acid and acetic acid in varying strength. It was observed that 10mM ammonium formate (pH 3.0): acetonitrile (40:60 v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Acidic buffer helped to improve the peak shape and spectral response. 40% aqueous part was adequate to retain the polar compound metformin. The use of a short chromatography column C18, hypurity (50mm×4.6mm i.d., 5µ) helped in the separation and elution of all four compounds in a very short time. The total chromatographic run time was 2.0 min for each run.

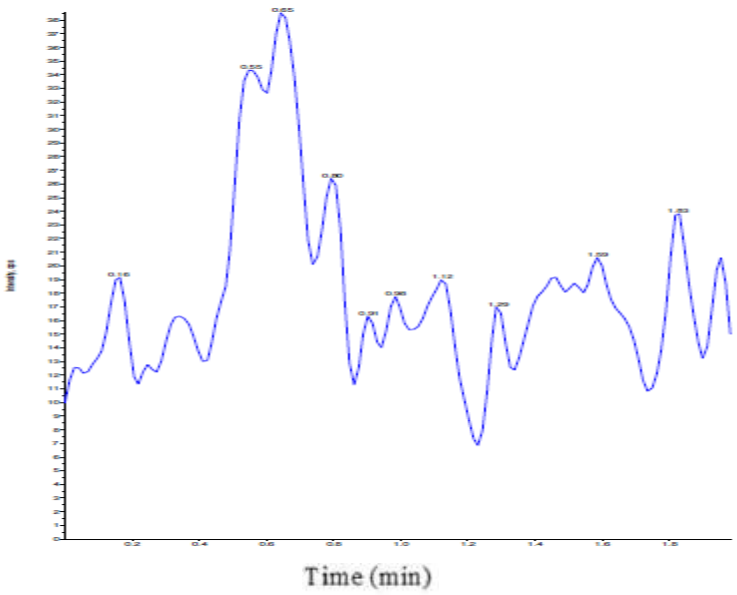
Simultaneous recovery of both the analytes from plasma was difficult as metformin is highly polar while sitagliptin is comparatively a less polar compound. Simple protein precipitation technique was tried with acetonitrile and methanol to recover both the analytes and IS. After precipitation with acetonitrile and direct injection of supernatant gave poor peak shape for both analytes as well as IS and also the response was less for LLOQ sample on Sitagliptin side. The method was modified by evaporating the supernatant layer and drying the sample at 40°C in evaporator. The samples were reconstituted with 0.5 mL of mobile phase. Peak shape was better for both analytes as well as IS and also response increased for LLOQ of Sitagliptin. The major advantage of this method was its efficiency in extracting both the analytes and IS. Moreover, there was no significant matrix effect of IS on both the analytes.

Specificity and Selectivity

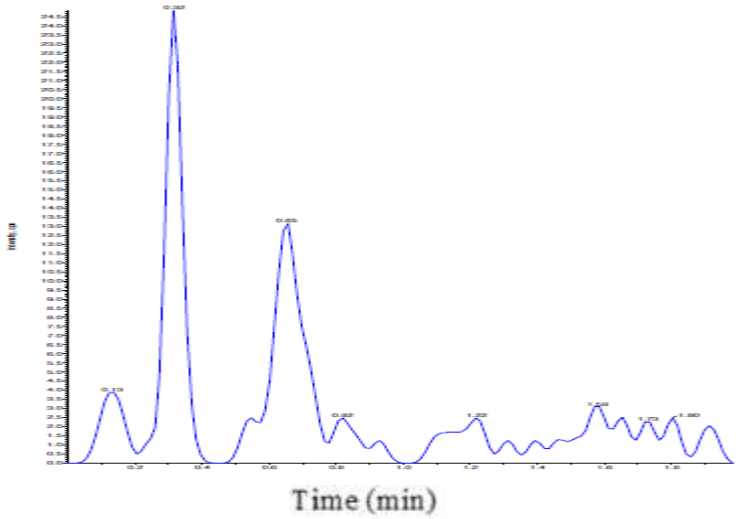
Fig. 2 [A-F], showed the typical chromatograms of blank plasma, spiked plasma sample with metformin, sitagliptin and the IS (Metformin D6 & Sitagliptin D4). The retention time for Metformin and its IS was at 0.60 min and retention time for Sitagliptin and its IS was at 0.65 min. No significant interference in the blank plasma traces was observed from endogenous substances in drug free human plasma at the retention time of analytes or the IS.

Linearity, accuracy and precision

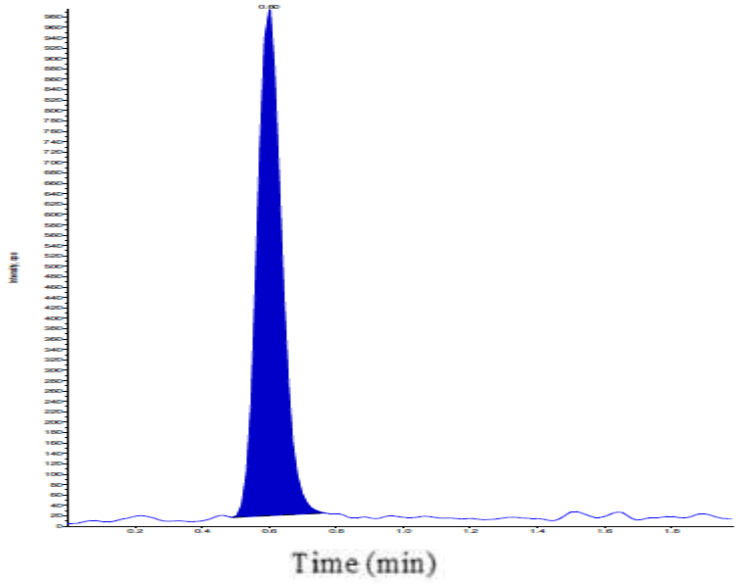
Calibration curves were linear from 25.000–3000.000 ng/ml with correlation coefficient $r \geq 0.9998$ for metformin and 5.000–800.000 ng/ml with $r \geq 0.9985$ for sitagliptin. The r values, slopes and intercepts were calculated using linear regression $(1/x^2)$ weighing analysis. The observed mean back-calculated concentration of calibration standards with accuracy (%) and precision (% CV) of three linearity are given in Table 1A and 1B. Accuracy expressed in terms of % Accuracy was within ±2.5% for metformin and ±4.0% for sitagliptin of their nominal concentration. The intra- and inter-run precision and accuracy of the assay were assessed by running a single batch of samples containing a calibration curve and six replicates at each QC levels. Concentrations were calculated from calibration curve and the intra-day precision was less than 7.25% and 6.87% for metformin and sitagliptin respectively and inter-day was less than 6.75% and 7.33% for metformin and sitagliptin respectively as shown in Table 2A and 2B.



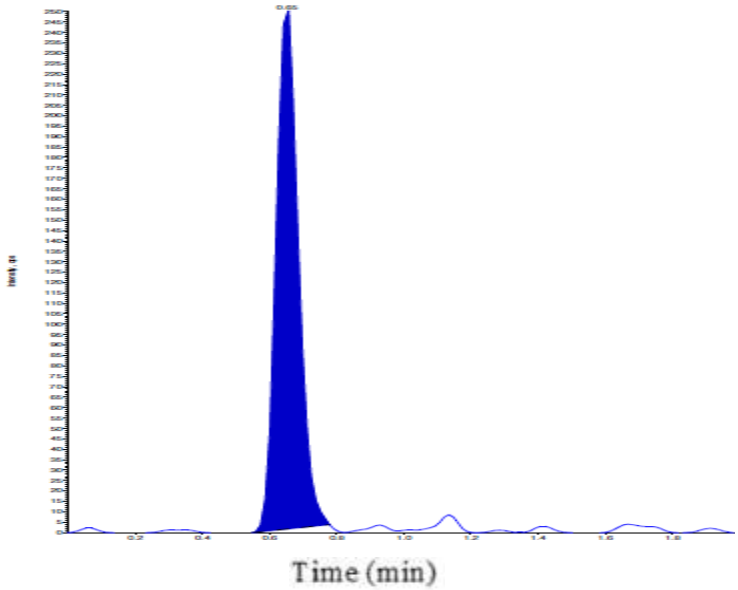
Typical chromatogram of blank plasma - Metformin [A]



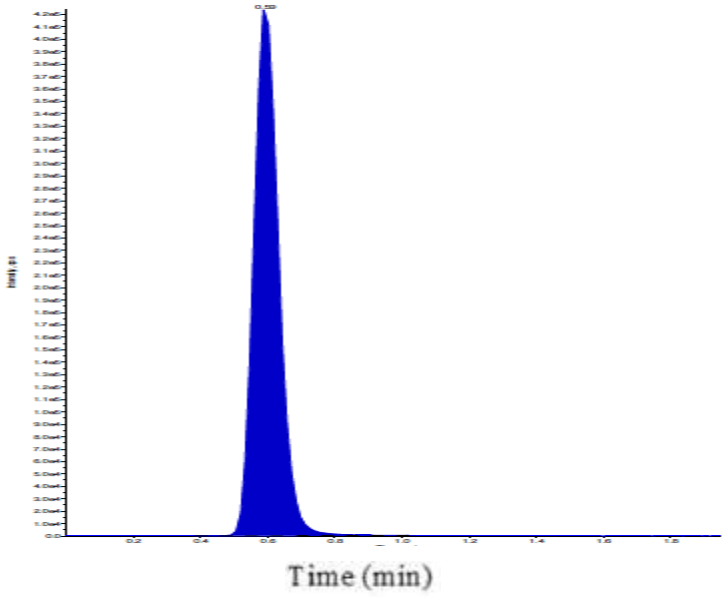
Typical chromatogram of blank plasma - Sitagliptin [B]



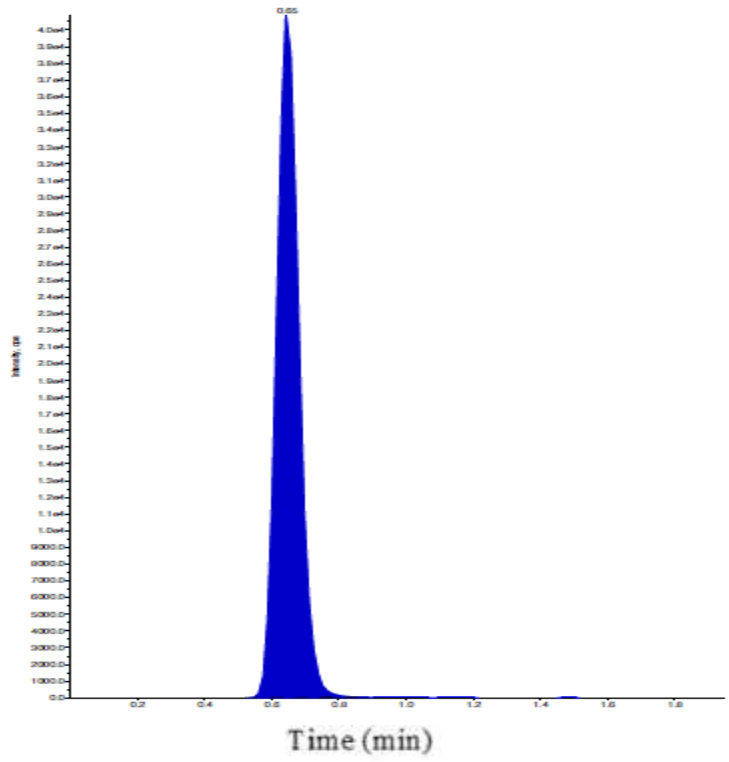
Typical chromatogram of LLOQ - Metformin [C]



Typical chromatogram of LLOQ - Sitagliptin [D]



Typical chromatogram of Metformin d6 (IS) [E]



Typical chromatogram of Sitagliptin d4 (IS) [F]

Fig. 2: It shows the typical chromatograms of blank plasma, spiked plasma sample with metformin, sitagliptin and the IS (Metformin D6 & Sitagliptin D4).

Table 1A: Concentration of Metformin in calibration standards prepared in human plasma

	Nominal Conc. (ng/mL)	Mean conc. (ng/mL)	% CV	Accuracy %
CC1	25.000	25.461 ± 0.70	2.76	101.84
CC2	50.000	50.953 ± 1.08	2.13	101.91
CC3	100.000	100.054 ± 1.33	1.32	100.05
CC4	300.000	303.072 ± 4.70	1.55	101.02
CC5	600.000	608.264 ± 6.69	1.10	101.38
CC6	1200.000	1204.610 ± 17.67	1.47	100.38
CC7	1700.000	1733.962 ± 48.09	2.77	102.00
CC8	2400.000	2449.735 ± 61.90	2.53	102.07
CC9	3000.000	3046.122 ± 63.01	2.07	101.54

Table 1B: Concentration of Sitagliptin in calibration standards prepared in human plasma

	Nominal Conc. (ng/mL)	Mean conc. (ng/mL)	% CV	Accuracy %
CC1	5.000	5.045 ± 0.08	1.62	100.91
CC2	10.000	10.256 ± 0.27	2.68	102.56
CC3	25.000	24.687 ± 0.57	2.31	98.75
CC4	50.000	49.377 ± 1.98	4.00	98.75
CC5	100.000	101.806 ± 2.71	2.66	101.81
CC6	200.000	206.203 ± 3.84	1.86	103.10
CC7	400.000	407.368 ± 18.01	4.42	101.84
CC8	600.000	600.684 ± 10.84	1.80	100.11
CC9	800.000	826.016 ± 39.54	4.79	103.25

[a]Data represent the mean ± S.D. of three observations.

Table 2A: Data of Intra-day and Inter-day precision and accuracy for Metformin in human plasma

	Intra Day			Inter Day		
	Mean conc. (ng/mL)	% CV	Accuracy %	Mean conc. (ng/mL)	% CV	Accuracy %
LLOQ QC (25.000 ng/mL)	25.907 ± 1.45	5.61	103.63	25.671 ± 1.41	5.48	102.68
LQC (75.000 ng/mL)	75.254 ± 2.59	3.44	100.34	75.196 ± 2.63	3.50	100.26
MQC1 (500.000 ng/mL)	522.236 ± 32.85	6.29	104.45	514.316 ± 30.77	5.98	102.86
MQC (1200.000 ng/mL)	1210.340 ± 87.71	7.25	100.86	1213.711 ± 81.92	6.75	101.14
HQC (2400.000 ng/mL)	2487.948 ± 136.11	5.47	103.66	2453.095 ± 150.33	6.13	102.21

[a]The intra- and inter-assay data represent the Mean ± S.D. of 12 and 18 observations, respectively.

Table 2B: Data of Intra-day and Inter-day precision and accuracy for Sitagliptin in human plasma

	Intra Day			Inter Day		
	Mean conc. (ng/mL)	% CV	Accuracy %	Mean conc. (ng/mL)	% CV	Accuracy %
LLOQ QC (5.000 ng/mL)	5.011 ± 0.17	3.46	100.22	5.026 ± 0.20	3.97	100.52
LQC (15.000 ng/mL)	14.949 ± 0.97	6.50	99.66	14.917 ± 0.99	6.61	99.45
MQC1 (150.000 ng/mL)	154.136 ± 7.30	4.73	102.76	152.110 ± 7.99	5.25	101.41
MQC (400.000 ng/mL)	398.055 ± 27.34	6.87	99.51	391.424 ± 24.33	6.22	97.86
HQC (600.000 ng/mL)	607.714 ± 36.74	6.05	101.29	598.295 ± 43.85	7.33	99.72

[a]The intra- and inter-assay data represent the Mean ± S.D. of 12 and 18 observations, respectively.

Recovery and matrix effect

Six replicates at LQC, MQC1, MQC and HQC levels were prepared for recovery determination. Mean recovery found was 40.5% and 64.5% and the precision (% CV) was 6.31 and 4.04 for metformin and sitagliptin, respectively. Recovery of metformin D6 and sitagliptin D4 was 39.1% and 64.6 with a % CV of 1.54 & 1.71 respectively. Recovery data were shown in Table 3A and 3B for Metformin and Sitagliptin respectively.

Stabilities

Stock solution of both analytes and IS were stable at room temperature for 22 hrs and at 2–8ºC for 30 days. Both analytes in control human plasma at room temperature were stable at least for 8 hrs (bench top stability), stable in autosampler for 44 hrs and for minimum of five freeze and thaw cycles. Spiked plasma samples, stored at −20ºC for long term stability experiment, were stable for minimum of 30 days. Different stability experiments in plasma with values for precision and percent change are shown in Table 4A and 4B.

CONCLUSIONS

The developed LC–MS/MS assay for metformin and sitagliptin is selective, rapid and rugged, suitable for routine measurement of subject samples. This method has significant advantages in terms of simple precipitation procedure and a shorter chromatographic run time (2.0 min). The method gave consistent and reproducible recoveries for analytes and IS from plasma, with minimum interference. The extract (10 µL) can be directly submitted for LC–MS analysis after drying and reconstitution to give high throughput. The established LLOQ is sufficiently low to conduct a pharmacokinetic study with test formulation of metformin and sitagliptin.

Table 3A: Extraction recovery of Metformin from plasma

Metformin spiked Concentration (ng/mL)	Metformin concentration found (n=6)	Recovery (Mean ± S.D.)%
75.000	29.937	39.92 ± 0.75
500.000	208.324	41.66 ± 2.11
1200.000	510.783	42.57 ± 7.73
2400.000	913.508	38.06 ± 9.13

Table 3B: Extraction recovery of Sitagliptin from plasma

Sitagliptin spiked Concentration (ng/mL)	Sitagliptin concentration found (n=6)	Recovery (Mean ± S.D.)%
15.000	9.832	65.55 ± 1.51
150.000	97.683	65.12 ± 2.91
400.000	267.003	66.75 ± 4.03
600.000	365.085	60.85 ± 8.47

Table 4A: Stability data for Metformin under different storage conditions

Sr. No.	Storage conditions	Concentration (ng/mL)		% CV	% Mean Change
		Nominal	Mean found Conc. (±SD)		
1	Bench Top Stability for 8 Hrs	75.000	70.874 ± 3.08	2.17	-1.67
		2400.000	2140.112 ± 43.996	1.96	-4.43
2	Short Term Stability at -20.0°C	75.000	71.703 ± 2.28	1.49	10.17
	for 6 Days	2400.000	2210.266 ± 79.75	3.45	2.71
3	Auto Sampler Stability for 44 Hrs	75.000	75.265 ± 6.32	4.20	5.41
		2400.000	2356.603 ± 56.90	2.32	1.59
4	Freeze and Thaw Stability	75.000	69.815 ± 4.73	3.39	-6.89
	at -20.0°C (5th Cycle)	2400.000	2251.327 ± 109.93	4.88	1.91
5	Long Term Stability at -20.0°C	75.000	77.542 ± 8.28	5.37	2.86
	for 30 Days	2400.000	2320.721 ± 153.19	6.60	5.04

Table 4B: Stability data for Sitagliptin under different storage conditions

Sr. No.	Storage conditions	Concentration (ng/mL)		% CV	% Mean Change
		Nominal	Mean found Conc. (±SD)		
1	Bench Top Stability for 8 Hrs	15.000	15.170 ± 0.81	1.06	2.62
		600.000	611.304 ± 19.90	2.45	-9.01
2	Short Term Stability at -20.0°C	15.000	15.928 ± 1.93	2.33	8.82
	for 6 Days	600.000	663.811 ± 22.57	2.61	-0.58
3	Auto Sampler Stability for 44 Hrs	15.000	14.848 ± 2.35	3.52	-1.21
		600.000	601.940 ± 19.26	2.40	-0.71
4	Freeze and Thaw Stability	15.000	13.833 ± 0.28	2.08	-2.02
	at -20.0°C (5th Cycle)	600.000	626.177 ± 11.18	1.79	1.57
5	Long Term Stability at -20.0°C	15.000	13.414 ± 0.66	4.91	-4.98
	for 30 Days	600.000	554.926 ± 13.59	2.45	-9.99

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