RAPID, METHOD FOR DETERMINATION OF METFORMIN AND CANAGLIFLOZIN IN PLASMA BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETER, APPLICATION TO BIOEQUIVALENCE STUDY

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ABSTRACT

Objective: The objective of the study was to develop and validate a rapid selective bioanalytical method for the simultaneous determination of metformin and canagliflozin in plasma by liquid chromatography-tandem mass spectrometer (LC-MS/MS) to facilitate bioequivalence study sample analysis. Stock solutions and spiking solutions were prepared accurately in methanol and 80% methanol in water, respectively.

Methods: Chromatography monitored using Analyst 1.6.2 software. Method was found selective, no matrix effect, reproducible and consistent recovery, accuracy, precision, and stable in aqueous as well as extracted/matrix samples. Method found linear over the range 10–2000 ng/ml for metformin and 30–6000 ng/ml for canagliflozin. The method is successfully applied to analyze samples collected in a bioequivalence study after administration of metformin/canagliflozin 1000/150 mg to 24 healthy male volunteers.

Results: Extraction was carried out using a simple solid phase extraction using mobile phase elution. 5 μl sample delivered as injection volume in 1.000 ml/min isocratic mobile phase flow on turbo ion electron spray technique for positive mode on API 4000 MS.

Discussion: Chromatography achieved within 3 min using a mobile phase containing acetonitrile and 10 mM ammonium formate buffer in a ratio of 70:30 on chromolith C18 analytical column. Q1/Q3 are 130.1/70.1, 136.3/77.1, 462.2/267.2 (with ammonium adduct), and 466.4/267.1 for metformin, metformin D6, canagliflozin, and canagliflozin D4, respectively.

Conclusion: Rapid, sensitive method for the simultaneous estimation of metformin and canagliflozin in plasma by LC-MS/MS is successfully developed, validated and applied to analyze 960 unknown samples of a bioequivalence study. Incurred sample reanalysis was revealed great reproducibility.

Keywords: Metformin+canagliflozin, Metformin and canagliflozin in plasma, Bioequivalence of metformin and canagliflozin, Canagliflozin+metformin by liquid chromatography-tandem mass spectrometer.

INTRODUCTION

Metformin hydrochloride is chemically N, Ndimethylimidodicarbonimidic diamide hydrochloride (1, 1- dimethylbiguanide hydrochloride). Canagliflozin is chemically named as (2S,3R,4R,5S,6R)-2-[3-{[5-(4-fluorophenyl)thiophen-2-yl]methyl}-4-methylphenyl)-6-(hydroxymethyl) oxane-3,4,5-triol. Molecular weight of metformin is 129.2 g/mol and molecular weight of canagliflozin is 444.5 g/mol [1].

Metformin and canagliflozin are fixed dose combination antihyperglycemic agents to improve glycemic control in diabetes patients; it has million dollars market worldwide. Metformin and Canagliflozin both are old drugs, and many formulations are available in the market along with fixed-dose combination formulations. When we checked at published methods, there are plenty of methods reported for the estimation of either metformin or canagliflozin in plasma by liquid chromatography-tandem mass spectrometer (LC-MS/MS) or high-performance liquid chromatography (HPLC) or gas chromatography-MS, but no method is published for the simultaneous estimation in plasma by LC-MS/MS, development of single method is very difficult due to different polarities of drugs and other chemical properties. When we see at the structures of both, metformin ionizes in positive mode, whereas canagliflozin ionizes in negative mode. Devineni *et al.* reported method individual estimation methods for metformin and canagliflozin [2], Attimara *et al.* reported LC-MS method for the simultaneous determination of metformin and miglitol in human plasma: Application to pharmacokinetic studies [3], Kobuchi *et al.* reported method for canagliflozin by LC-MS/MS [4] and

many others have reported either metformin in plasma or canagliflozin in plasma [5-21].

Simultaneous estimation one hand reduce the time of analysis to half so that the end results will be available early and regulatory submission can be done so early to capture the market share, and the other hand it reduces the cost of analysis significantly.

The objective of the current work is to develop, validate a simultaneous estimation method for the estimation of metformin and canagliflozin in human plasma by LC-MS/MS and its application to bioequivalence study.

METHODS

Metformin, metformin d6, canagliflozin, and canagliflozin D4 were purchased from Vivan life sciences and Clearsyth Labs Pvt. Ltd., Mumbai, India. HPLC grade solvents and chemicals were used during analysis, whereas the water is taken from the in-house water purification system. Hydrophobic lipophilic balance (HLB) 1 cc/30 mg cartridges are from Phenominex, USA. LC-MS/MS system consists of ABSciex, API4000 with a front-end HPLC from Shimadzu. Analytical column is purchased from Merck Millipore, USA. A bioequivalence study was conducted based on a Independent Ethics Committee approved the protocol.

Stock solutions were prepared by accurately weighing respective working reference standard (STD) on balance, dissolved in methanol, labeled,

and stored at refrigerated conditions. Working stock dilutions were made using 50% methanol in water as diluent. Eight point calibration curve (CC) STDs are prepared in screened plasma for the CC range 10–2000 ng/ml for metformin and 30–6000 ng/ml for canagliflozin. Quality control (QC) samples at the lower limit of quantitation (LLOQ), lower QC, middle QC, and high QC levels are prepared at 102% to STD 1, 3 times to STD1, 45% to STD8, and 76% to STD8 in plasma. Required buffers, mobile phase, rinsing solutions are prepared as required and used up to 3 days. CC STDs and QC samples are bulk spiked and stored at −70°C until analysis. Fresh CC STDs are prepared for the evaluation for Stability evaluation.

Extraction procedure

To a 0.200 ml plasma sample, 0.050 ml internal STD is added and vortexes for proper mixing; then the sample is diluted with 0.500 mL water to prevent clogging of cartridges; cartridges are conditioned with 1 ml methanol followed by equilibration with 1 ml water; above prepared sample is then loaded onto cartridge; cartridges are washed with 1 ml water followed by 1 ml 10% Acetonitrile in water; and then samples are eluted with 0.500 ml mobile phase.

Instrument conditions

ABSCIEX API4000 Mass spectrometer coupled with Shimadzu LC system was utilized for reverse phase chromatography with an Isocretic flow at 1.000 mL/min on RP18 Analytical column. Mass spectrometer conditions are presented under Table 1.

Integration and quantitation

Data acquired on Sciex analyst 1.6.2 software; automatic integration was done for a base to base and optimum integrations, CC is plotted with area ratio versus nominal concentrations of CC STDs, unknown sample concentrations are back-calculated from plotted curve using the formula $y=mx+c$, weighing factor was $1/x^2$.

RESULTS AND DISCUSSION

Method development

About 100 ng/ml tuning solution in 90% methanol was prepared from stock solution for analyte and internal STD; mass parameters were optimized using tuning solution, metformin ions found intense in positive mode and no ions found in negative mode, canagliflozin ions found intense in negative mode and no ions found in positive mode, but our objective is to get both in a single mode; hence, we decided to go for

adduct form for canagliflozin as it can form ammonium adduct easily, adduction of canagliflozin found intense and reproducible. Labeled internal STDs are chosen as internal STDs to eliminate matrix effects and to get the highest reproducible results; matrix effects are nullified when labeled STDs are used. Based on the properties of the drugs correlating with ionization, the mobile phase, column conditions were optimized, metformin can easily extracted with protein precipitation technique but canagliflozin is difficult to extract; hence, we have tested for liquid-liquid extraction using polar to non-polar organic solvents but failed to good more than 50% recovery for both analytes, so finally we moved to solid phase extraction on HLB cartridges, and we got good recovery with reproducibility. Interference at retention times, carryover, matrix effect, precision, and accuracy tested and few alterations were done, and method is finalized. Pre-method validation was conducted before initiating method validation; this includes selectivity, carry over, precision, and accuracy, linearity, matrix effect, and bench-top stability. Weighing and regression method is finalized based on pre-method validation results.

Method validation

Method validation is conducted as per in-house STD operating procedure which is developed based on the European Medical Agency and United States Food and Drugs Administration guidelines on method validation. Autosampler carryover, selectivity, sensitivity, recovery, precision, accuracy, linearity, ruggedness for column, and analyst change, hemolysis and lipemic effect, aqueous, and extracted sample stability were evaluated. Method validation parameters, procedures, and acceptance are predefined.

Sensitivity

Sensitivity is performed to check the signal difference from baseline noise. LLOQ sample from 6 individual plasma lots was analyzed, signal-to-noise ratio is calculated using software. Sensitivity found always >5. Limit of detection is not evaluated due to non-requirement in the current study.

Selectivity

Selectivity is performed to check the endogenous/exogenous interferences. Selectivity of the method is tested using 10 plasma lots including 6 normal, 2 hemolytic, and 3 hyperlipidemic by processing one blank, one blank+Internal STD, and one LLOQ sample from each individual lot. Percentage of interference at analyte/IS
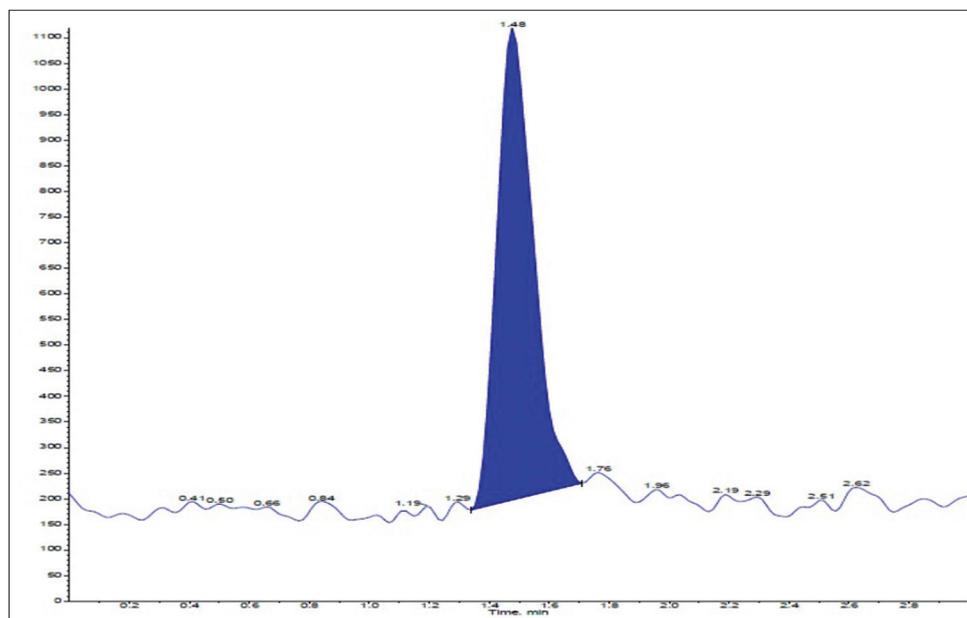

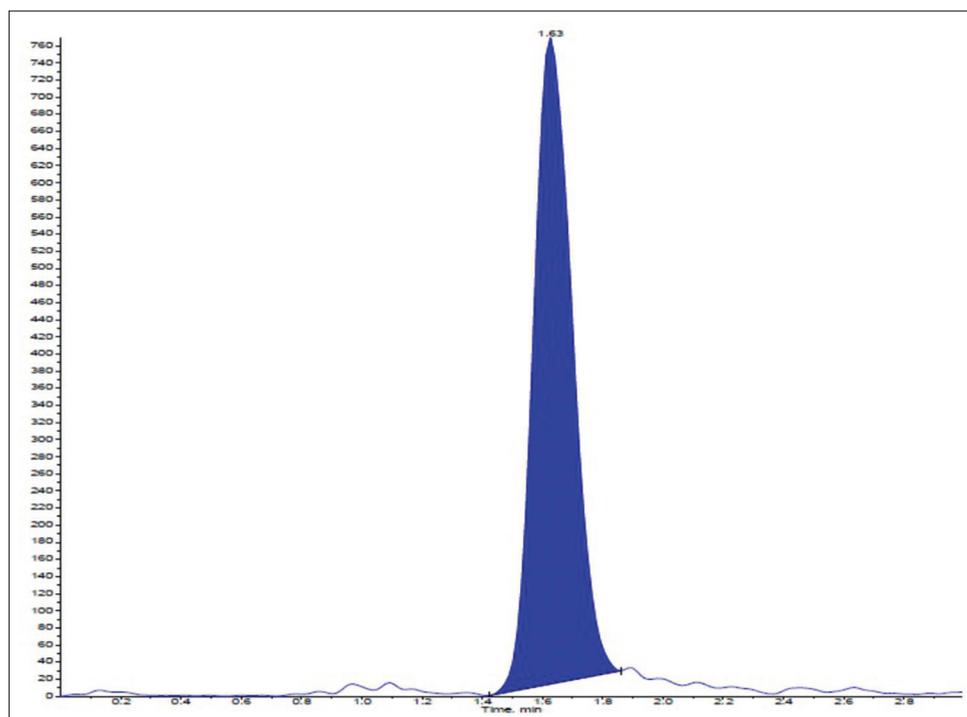**Fig. 1: Metformin lower limit of quantitation**

Fig. 2: Canagliflozin lower limit of quantitation

Table 1: Mass conditions

Description	Q1 Mass	Q3 Mass	Dwell	DP	EP	CE	CXP
Metformin	130.100	71.100	200	60.00	10.00	20.00	13.00
Metformin D_6	136.300	77.100	200	32.23	10.00	33.12	6.41
Canagliflozin (ammonium adduct)	462.250	267.200	200	54.00	10.00	24.81	6.00
Canagliflozin D_4 (ammonium adduct)	466.400	267.100	200	60.00	10.00	25.00	15.00
Source temperature: 500°C		IS voltage: 5000 kV		Polarity: Positive		Scan type: MRM	

DP: Declustering potential, EP: Entrance potential, CE: Collision energy, CXP: Collision exit potential

Table 2: Global statistics of precision and accuracy of metformin

Between-batch precision and accuracy				
Description	**LLOQQC**	**LQC**	**MQC**	**HQC**
Mean±SD	10.1988±0.80233	25.2631±1.07795	807.3190±26.91706	1583.0191±76.30179
Precision (% CV)	7.87	4.27	3.33	4.82
Nominal concentration (ng/ml)	10.533	26.492	817.656	1566.390
% Accuracy	96.83	95.36	98.74	101.06

CV: Coefficient of variation, SD: Standard deviation, LLOQQC: Lower limit of quantitation quality control, LQC: Lower quality control, MQC: Middle-quality control, HQC: High-quality control

retention times is calculated considering LLOQ area 100. Selectivity found acceptable. Sample LLOQ chromatograms are presented under Figs. 1 and 2.

Recovery

Recovery of extraction efficiency was tested. Absolute recovery using three levels of QC s was tested. QC samples are processed and injected along with equivalent pure samples prepared in reconstitution solution; mean areas at each QC level are compared. Recovery found >70% with consistent and reproducible.

Linearity, precision, and accuracy

Precision and accuracy batch consists of one cc and 24 qC samples at four different levels were analyzed. A total of 4 precision and accuracy batches were analyzed in two different days for intra- and inter-day calculations. CC for both analytes found linear all the times with r>0.99. Global statistics are tabulated in Tables 2 and 3.

Ruggedness

Ruggedness of the method is tested for the changes column, analyst, and instrument. One precision and accuracy were analyzed by a different analyst on different column/different instrument. Results found acceptable. Precision found 2–7% whereas accuracy found between 86% and 109%.

Stability

Stability of analytes and internal STDs is tested in aqueous solutions for short-term as well as long-term by injecting a sample from stability solution along with comparison solution prepared freshly. Stability of processed samples for different storage conditions such as evaporation stability, wet extract stability, autosampler stability, and wet extract bench top stability was done along with the stability of drugs in plasma at different conditions such as benchtop stability, freeze-thaw stability, whole blood stability, and long-term stability. Stock solution found stable up to 6 h; benchtop stability found acceptable for 6 h, freeze-

Table 3: Global statistics of precision and accuracy of canagliflozin

Between-batch precision and accuracy				
Description	LLOQQC	LQC	MQC	HQC
Mean±SD	29.9213±1.30406	74.6727±2.19700	2405.5809±89.89150	4702.9788±165.34826
Precision (% CV)	4.36	2.94	3.74	3.52
Nominal concentration (ng/ml)	31.187	78.437	2420.894	4637.728
% Accuracy	95.94	95.20	99.37	101.41

CV: Coefficient of variation, SD: Standard deviation, LLOQQC: Lower limit of quantitation quality control, LQC: Lower quality control, MQC: Middle-quality control, HQC: High-quality control

thaw stability found stable up to IV cycles, autosampler stability found stable up to 52 h, whole blood stability found acceptable for 2 h, and long-term stability in plasma found stable for 28 days.

Miscellaneous experiments such as dilution integrity, hemolysis effect, lipemic effect, selectivity in the presence of concomitant drugs were conducted and found all experiments acceptable.

Application of the method

Validated method has been applied to a bioequivalence study involved 24 volunteers, fixed-dose combination formulation of metformin and canagliflozin was administered as per independent Ethics Committee approved protocol to 24 volunteers in a two way crossover design with 7 days washout period, volunteers whoever meeting inclusion and exclusion criteria of protocol and whoever given informed consent were recruited after successful completion of screening tests. Dosing was done under fasting conditions, regular blood sampling was done, vitals checked, blood samples were centrifuged, and plasma was separated then stored at −70°C until sample transfer to the bioanalytical department for analysis. No adverse events were reported, and all 24 volunteers completed the clinical phase of the study. All samples of a given subject were analyzed using 8 point CC and interspersing QC samples. Batch evaluation was conducted and samples selected for repeat analysis for analytical reasons, no batch failures found, 22 individual samples were reanalyzed for an improper internal STD response. Incurred sample reanalysis (ISR) was conducted for 10% samples, 95% samples ISR values are matching with original assay value. After completion of the analysis, quality assurance department reviewed that subject concentration data are submitted to biostatistician in a locked Microsoft Excel file. Statistician conducted pharmacokinetic analysis on win online, and biostatistician analysis is on SAS software. Non-compartmental analysis is done to check 90% confidence intervals for bioequivalence review. 90% confidence intervals found between 80% and 125% for a test when compared to reference formulation. Mean C_{max} found is 1256 ng/ml for metformin and 390 ng/ml for canagliflozin.

CONCLUSION

Rapid, sensitive method for the simultaneous estimation of metformin and canagliflozin in plasma by LC-MS/MS is successfully developed, validated and applied to bioequivalence study sample analysis. Chromatography achieved on the C18 column on acetonitrile, ammonium formate mobile phase, extraction was conducted using solid phase extraction on HLB cartridges. Positive MRM detection was performed on API 4000 LC-MS/MS. The analysis is rapid with 3 min run time for each sample, around 200 samples can be analyzed daily, the method found selective from endogenous/exogenous interferences; all validation parameters proved that the method is accurate, precise, and stable. Method found linear over the range 10–2000 ng/ml for metformin and 30–6000 ng/ml for canagliflozin. The method is applied to analyze 960 unknown samples of a bioequivalence study after administration of a fixed-dose combination of metformin and canagliflozin tablets. ISR was revealed great reproducibility with over 90% ISR sample concentrations matching with original assay concentrations.

AUTHORS' CONTRIBUTIONS

The research work has been jointly carried out by the authors under the guidance of Dr. G. Nithya, the Corresponding author.

CONFLICTS OF INTEREST

Nil.

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