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Original Article

Novel and Simple LC-MS/MS Technique for the Simultaneous Quantitation of Metformin and Remogliflozin in Rat Plasma

Smit Jayantilal Patel[1,2,*], Hiralben Mehta[1], Basheer Ahmmad Shaik[2], Nadeem Khan[2]

[1]Department of Quality Assurance, Parul Institute of Pharmacy and Research, Parul University, Vadodara, Gujarat, INDIA.
[2]Department of Chemistry, Jai Research Foundation, Gujarat, INDIA.

ABSTRACT

Background: Metformin and remogliflozin in fixed dose combination prescribe to control blood sugar and avoid long term complication associated with diabetes mellitus. **Aim:** The aim is to establish a straightforward and sensitive bioanalytical method as well as to validate LC/MS-MS technique for the concurrent quantitative determination of metformin and remogliflozin in rat plasma. **Materials and Methods:** The analytical separation of analytes with contrast physicochemical properties was successfully performed with C_{18} column (BDS Hypersil, 150 x 4.6 mm, 3.5 micron) at 40°C on API 4000 Mass Spectrometer coupled with Nexera X2 HPLC. Mobile phase was composed of 10 mM ammonium acetate dissolved in Milli-Q water (pH 7) (25%) and acetonitrile (75%) (v/v) at 0.7 mL/min flow through isocratic elution. A 10 µL injection volume was utilized in a brief run time of 4 min. **Results and Discussion:** Developed LC-MS/MS technique has been validated as per ICH guidelines (ICH M10). The validation data showed a precise, accurate, selective method and recovery and stability falling within the acceptable limits. **Conclusion:** A meticulous, precise, and repeatable LC-MS/MS method employing liquid-liquid extraction has been developed for concurrent quantitation of metformin and remogliflozin in rat plasma. The benefits provided by this method in terms of plasma sample requirement for processing as well as improved both sensitivity and reproducibility. The established method is set to be employed for the simultaneous pharmacokinetic analysis of both analytes within a biological matrix.

Keywords: Acetonitrile, LC-MS/MS, ICH, Metformin, Remogliflozin, Simultaneous quantitation.

Correspondence:
Mr. Smit Jayantilal Patel
Department of Quality Assurance, Parul Institute of Pharmacy and Research, Parul University, Vadodara, Gujarat, INDIA.
Department of Chemistry, Jai Research Foundation, Gujarat, INDIA.
Email: patelsmit87@gmail.com

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INTRODUCTION

Millions of people worldwide suffer with diabetes mellitus, a common and complicated chronic illness marked by elevated blood glucose level. Improper management of diabetes may results in significant health. The disorder is caused by either inadequate insulin synthesis or the body's inability to utilize insulin, which is crucial for controlling blood glucose levels. Type 1 diabetes, which is primarily diagnosed in children and young adults, is brought on by the immune system erroneously targets and damages pancreatic beta cells that produce insulin. As a results, little or no insulin is produced, necessitating lifelong insulin therapy. Type 2 is prominent in adults and largely associated with lifestyle factors like obesity, poor dietary habits, and less physical activity. In this type of diabetes mellitus, the body fails to produce sufficient insulin or develops resistance to insulin to maintain normal glucose levels. Management of diabetes involves a sophisticated approach that includes regular monitoring of blood sugar levels, medication, dietary changes and regular physical activity. Over the years, multiple discoveries of oral hypoglycemic agents and management strategies have made it possible to manage and understand diabetes.[1]

The main way that metformin, also known as 3-(diaminomethylidene)-1,1-dimethylguanidine (Figure 1) lowers blood sugar in the treatment of diabetes is by preventing the liver from releasing glucose. It has also been demonstrated to have number of cardioprotective effects.[2,3] Remogliflozin, a SGLT-2 inhibitor, chemically 5-methyl-1-(1-methyl ethyl)-4-({4-[(1-methyl ethyl)oxy]phenyl}methyl) -1H-pyrazole -3-yl-β-D-glucopyranoside) (Figure 2) is used to treat progressive diabetes[4,5] and acts by decreasing the kidneys ability to reabsorb glucose, hence increases excretion of glucose. When administered in combination, it helps to control blood sugar levels.[6]

The review of literature identified several analytical techniques for quantification of metformin either alone[7,8] or alongside other antidiabetic drugs like teneligliptin,[9] sitagliptin,[10,11] dapagliflozin,[12]

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glyburide,[13] glipizide.[14] Similarly, various analytical methods for remogliflozin quantification are reported individually[15,16] or with other antidiabetic drugs.[17-19] While previous LC-MS/MS technique has documented simultaneous measurement of remogliflozin etabonate and metformin in rabbit plasma,[17] present investigation introduces a new, sensitive and high-throughput LC-MS/MS approach for the simultaneous measurement of metformin and remogliflozin in rat plasma, featuring lower LLOQ, reduced sample volume and rapid processing, making it ideally suited for preclinical pharmacokinetic applications. Consequently, this work was undertaken as per ICH guidelines (ICH M10)[20] by using bixafen as internal standard. The structure of bixafen is given in Figure 3.

MATERIALS AND METHODS

Chemicals, reagents and instrumentation

Metformin (98.56%) and Remogliflozin (99.97%) were provided by Sun Pharmaceutical Industries Limited (Gujarat, India) and Glenmark Pharmaceuticals (Maharashtra, India), respectively. Bixafen was bought from Sigma Aldrich (99.3%). Acetonitrile, ammonium acetate and ethyl acetate were procured from Merck and deionized water was supplied through the Millipore Milli-Q system. 10 mM ammonium acetate solution was prepared by dissolving 0.77 g ammonium acetate in 1 L of Milli-Q Water and pH adjusted to 7.

The API 4000 Mass Spectrometer, integrated with the Nexera X2 HPLC System from AB Sciex coupled with Electrospray Ionization (ESI) in positive mode (ionization). Chromatographic separation was conducted on C18 column (BDS Hypersil, 150 x 4.6 mm, 3.5 µ) at 40°C. Composition of mobile phase was 10 mM ammonium acetate in Milli-Q water (pH 7) (25%) and acetonitrile (75%), at flow rate of 0.7 mL/min. The method employed isocratic elusion mode with an injection of 10 µL. Short run time of 4 min was maintained for each injection. Analytes were detected by the Channel Electron Multiplier (CEM) detector. The settings for the ion source in Electrospray Ionization (ESI) operating in positive mode; Curtain gas (CUR) was set at 40 psi; Collision gas (CAD) to 10 psi, ion spray voltage (ISV) to 5500 V, nebulizer gas (GS1) to 50 psi, turbo gas to (GS2) 60 psi at 400°C. Multiple Reaction Monitoring (MRM) transition for quantification: m/z 130.1→60 and 130.1→71.1 for metformin, m/z 451.2→289.0 for remogliflozin, and m/z 414.0→394.0 for bixafen were used. The analyst software was utilized for data acquisition and instrument control (version number 1.6.3).

Preparation of standard solutions for calibration

Standard solution of metformin was prepared by dissolving 13.80 mg of metformin in 10 mL of diluent (aq. acetonitrile, 50%) to obtain 1360128 ng/mL. In contrast, standard solutions of remogliflozin was prepared by dissolving 10.16 mg of remogliflozin in 10 mL of acetonitrile to obtain 1015695 ng/mL. Bixafen stock solution was also separately formulated in acetonitrile. These initial standard solutions further mixed with diluent to prepare a single mixture of standard working solution of 2720.26 ng/mL and 2132.96 ng/mL for metformin and remogliflozin, respectively. This prepared working solution was diluted with diluent to obtain spiking solution of 6.86, 13.71, 27.42, 54.84, 156.69, 391.72, 652.86, 816.08 ng/mL for metformin and 5.38, 10.75, 21.50, 43.00, 122.86, 307.15, 511.91, 639.89 ng/mL for remogliflozin. This spiking solution then spiked into blank plasma to obtain standard solution in the range of 0.69 to 81.61 ng/mL for metformin and 0.54 to 63.99 ng/mL for remogliflozin. In the same manner, four QCs samples at 0.69, 2.03, 36.86 & 61.43 ng/mL (metformin) and 0.54, 1.60, 28.99 & 48.32 ng/mL (remogliflozin) were prepared and considered as LLOQC, LQC, MQC and HQC.

Sample extraction

Initially, 200 µL of the diluent (50% acetonitrile) added to plasma samples and vortexed for a short period. This was followed by 2 mL addition of ethyl acetate and centrifugation at 4500 rpm for 5 min at 15°C. Approximately, 1.6 mL of supernatant was separated and the contents evaporated in a nitrogen evaporator till the samples were completely dried. Subsequently, dried samples were reconstituted with 300 µL diluent and samples were transferred to the designated auto-sampler vials for analysis.

Validation

The current methodology method was validated as per ICH guideline (ICH M10) by assessing selectivity, conducting ASCOT, determining linearity, establishing LLOQ and evaluating precision and accuracy. Additionally, stability, reinjection reproducibility, matrix effect, and recovery were also established.

Selectivity

Six separate drug free rat plasma samples were selected for investigation of interference of analytes at retention times. Selectivity of method was based on the chromatograms of blank plasma, spiked plasma with remogliflozin and metformin along with IS. The criteria for acceptance of the experiment were successfully achieved.

Autosampler carryover test

Autosampler carryover was determined by processing and analyzing standard blank, LLOQ, and ULOQ (upper limit of quantification) standard in sequence as standard blank, LLOQ, ULOQ, and reinjection of standard blank (to determine carryover). Standard blank reinjection of the initial sample was performed to check if any carryover was detected. Second injection in series (LLOQ standard) was injected to find if there was any interference at a retention time of remogliflozin and metformin (analytes) in blank (standard).

Linearity

Eight calibration standards, including ULOQ and LLOQ were utilised to prepare calibration standard curves. The concentration range for calibration standard solution was 0.69 to 81.61 ng/mL and 0.54 to 63.99 ng/mL for metformin and remogliflozin, respectively. The regression equation was generated by plotting peak area ratio of the remogliflozin and metformin relative to internal standard (y-axis) against nominal concentrations of remogliflozin and metformin by employing a $1/X^2$ weighted linear regression model.

LLOQ determination

LLOQ determination performed to evaluate sensitivity of the developed method. LLOQ samples were processed by spiking metformin and remogliflozin into six individual lots of rat plasma at LLOQ level with a working internal standard solution. These samples were analyzed with precision & accuracy samples alongside a calibration curve and batch QC's. Three batches of LLOQ determination were evaluated.

Precision and accuracy

Six replicates of QCs (LLOQC, LQC, MQC, and HQC) representing entire calibration curve range with concentrations at LLOQC slightly higher than LLOQ, LQC was approximately 3 times LLOQ concentration, MQC approximately 40-60% of ULOQ and HQC was at approximately 75-85% of ULOQ were used for determination of precision and accuracy. To assess the ruggedness and potential transferability of the developed LC-MS/MS method, additional evaluations were carried out under deliberately varied conditions. The method was tested using two different analysts and columns on different days. Quality control samples at low, medium, and high concentration levels were analyzed under each condition.

Reinjection reproducibility

To verify the validity of the processed samples and to ensure proper sample storage before injection, reinjection reproducibility was evaluated. Accepted precision and accuracy batch samples were stored for a period of 20 hr in an autosampler at 2 to 8°C and the entire batch was re-injected for estimation of reinjection reproducibility.

Matrix effect

The matrix effect for the metformin and remogliflozin was investigated (LQC and HQC). Six lots of rat plasma were extracted, and extract was spiked at LQC and HQC separately. Analytes peak area in the samples was compared with neat standard solutions at the same theoretical concentration (LQC and HQC). IS normalized factor and matrix factor were calculated.

Recovery

The recovery of metformin and remogliflozin was assessed by analyzing six replicates of extracted samples (LQC, MQC, and HQC). The results were compared with mean analytes response from post-extracted samples along with internal standard at their respective working concentrations.

Stability

Stability of the metformin and remogliflozin in rat plasma was established under the following storage conditions: (1) samples maintained at ambient temperature (bench top stability), (2) samples stored in autosampler for 20 hr (15°C for autosampler stability), (3) samples after five freeze and thaw cycles, (4) samples stored for 30 days at -70±10°C. Stability determinations were performed by using freshly prepared standards of the calibration curve and batch QCs.

DISCUSSION

Design and optimization of method

A dependable analytical method utilizing liquid chromatography has been established for the identification of metformin and remogliflozin in rat plasma. Amongst APCI (Atmospheric Pressure Chemical Ionization) and ESI (Electrospray Ionization) techniques, acceptable ionization and sensitivity for both drugs were obtained with ESI. Bixafen was used as an internal standard. The primary goal of internal standards is to minimize errors (experimental & processing) and to enhance the robustness developed analytical method. The choice of bixafen as an internal standard is motivated by the presence of pyrazole linkage and structure, which it shares with remogliflozin, as well as to reduce the costs associated with using deuterated compounds.

Numerous LC-MS/MS technique have been previously reported for the quantification of Metformin in various biological matrices, often employing C18-based stationary phases, protein precipitation and/or liquid-liquid extraction, and relatively high LLOQ values. Many of these methods either focus solely on metformin or lack sensitivity and throughput required for

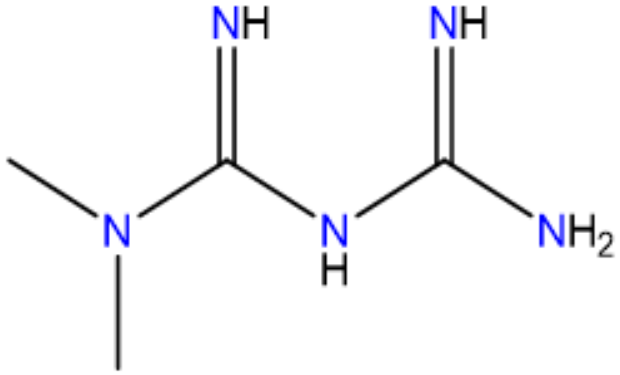

Figure 1: Structure of metformin.

simultaneous drug quantification in low volume preclinical samples. In previously published methods, certain challenges were highlighted due to the structural and physicochemical nature of metformin and remogliflozin. With smaller molecular size and high polarity of metformin, it is always challenging to quantitatively analyze it in biological fluids.[21] Smaller size and high polarity metformin leads to poor retention while developing analytical methods on reversed-phase chromatography columns.[10] On the other hand, remogliflozin is moderately nonpolar therefore can be adequately retained on C18 columns.[22]

In the course of optimization process, different stationary phases (Zorbax Eclipse Plus C18 (50 × 4.6 mm, 1.8 µ), Phenomenex Kinetex C18 (100 × 4.6 mm, 2.6 µ), Synergi Polar-RP (100 × 4.6 mm, 4 µ)

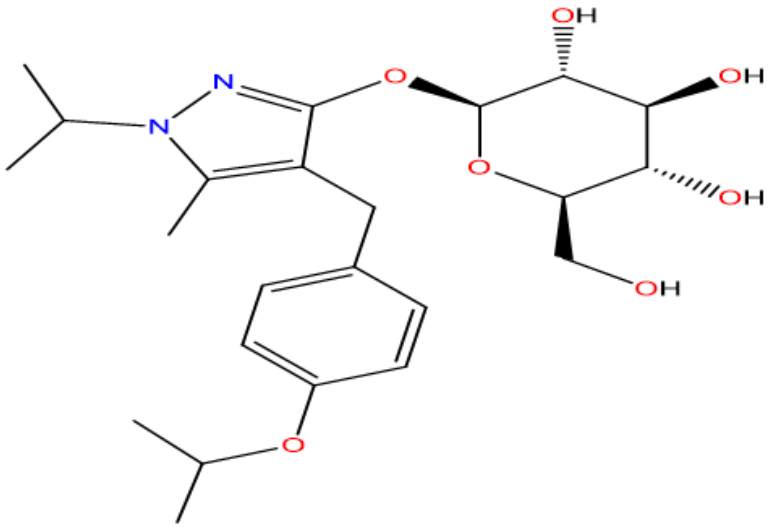

Figure 2: Structure of remogliflozin.

Table 1: Linearity data of metformin and remogliflozin.

Metformin			
Nominal Concentrations (ng/mL)	**Recovered Concentrations (ng/mL)**	**Accuracy (%)**	**Linear Regression Equation**
0.69	0.68	99.1	y = 0.0352 x + (-0.00311) (r^2=0.998)
1.37	1.28	93.2	
2.74	2.77	101	
5.48	5.38	98.2	
15.67	14.9	94.8	
39.17	38.0	98.0	
65.29	69.9	107	
81.61	83.6	103	
Remogliflozin			
Nominal Concentrations (ng/mL)	**Recovered Concentrations (ng/mL)**	**Accuracy (%)**	**Linear Regression Equation**
0.54	0.55	101	Y= 0.0334 x + (-0.002) (r^2=0.999)
1.08	1.02	94.3	
2.15	2.18	102	
4.30	4.14	96.4	
12.29	11.9	94.3	
30.72	30.4	99.1	
51.19	55.0	104	
63.99	64.4	101	

Table 2: Data of Precision and Accuracy.

Metformin					
Intervals	**QC Level**	**Fortified Concentrations (ng/mL)**	**Recovered Concentrations (ng/mL)**	**% Accuracy**	**%CV**
Day 1	LLOQ	0.69	0.68	98.55	5.88
	LQC	2.03	1.83	90.15	5.10
	MQC	36.86	36.62	99.35	5.35
	HQC	61.43	62.82	102.26	4.60
Day 2	LLOQ	0.69	0.77	111.27	5.19
	LQC	2.03	1.84	90.63	6.52
	MQC	36.86	34.78	93.97	5.41
	HQC	61.43	58.20	94.74	2.58
Day 3	LLOQ	0.69	0.65	94.40	5.38
	LQC	2.03	1.96	96.55	4.77
	MQC	36.86	34.99	90.40	3.09
	HQC	61.43	55.45	90.27	4.60
Remogliflozin					
Intervals	**QC Level**	**Fortified Concentrations (ng/mL)**	**Recovered Concentrations (ng/mL)**	**% Accuracy**	**%CV**
Day 1	LLOQ	0.54	0.59	109.26	5.08
	LQC	1.60	1.50	93.75	6.00
	MQC	28.99	28.02	96.65	1.17
	HQC	48.32	43.63	90.29	1.42
Day 2	LLOQ	0.54	0.52	96.29	5.06
	LQC	1.60	1.45	90.63	2.49
	MQC	28.99	27.25	93.97	2.11
	HQC	48.32	46.47	96.21	2.36
Day 3	LLOQ	0.54	0.57	105.66	3.51
	LQC	1.60	1.62	101.30	3.70
	MQC	28.99	28.60	98.66	3.04
	HQC	48.32	47.75	98.81	3.12

evaluated using a mobile phase consisting of acetonitrile, buffers (ammonium acetate in formic and acetic acids) and methanol. To ensure adequate separation, an initial mobile phase containing an equal ratio of buffer and solvents (acetonitrile/methanol) was evaluated. Metformin was initially eluted near the column dead time which promoted the requirement of modification of the mobile phase composition i.e., increased proportion of the organic phase (70-80%). This change significantly altered elution behaviour with remogliflozin eluting before metformin. Further optimization was carried out by adjusting buffer concentrations (2 to 10 mM) to improve response and to ensure proper peak shape. It was observed that higher buffer concentrations (8-10 mM) enhanced peak shape, particularly remogliflozin. After several trials, an optimal equilibrium achieved among the retention time, resolution, analyte response, and peak shape by utilizing 10 mM ammonium acetate in milli-Q water (pH 7) and acetonitrile (25:75, v/v) on BDS Hypersil C18 column. The retention times recorded were 2.17 for metformin and 1.78 for remogliflozin within a total 4-min run time.

Extraction procedures or pre-concentration techniques are crucial in bioanalysis, for the removal of unwanted interferences. It is mandatory to clean samples before injecting on LC-MS/MS or any other hyphenated technique. Various methods have evolved in the last few decades, some are versatile, and few are specific and selective. These methods have been used depending on the nature of matrices to be cleaned. Considering simplicity, easy-to-use conventional approaches viz., Liquid-Liquid Extraction (LLE),[23]

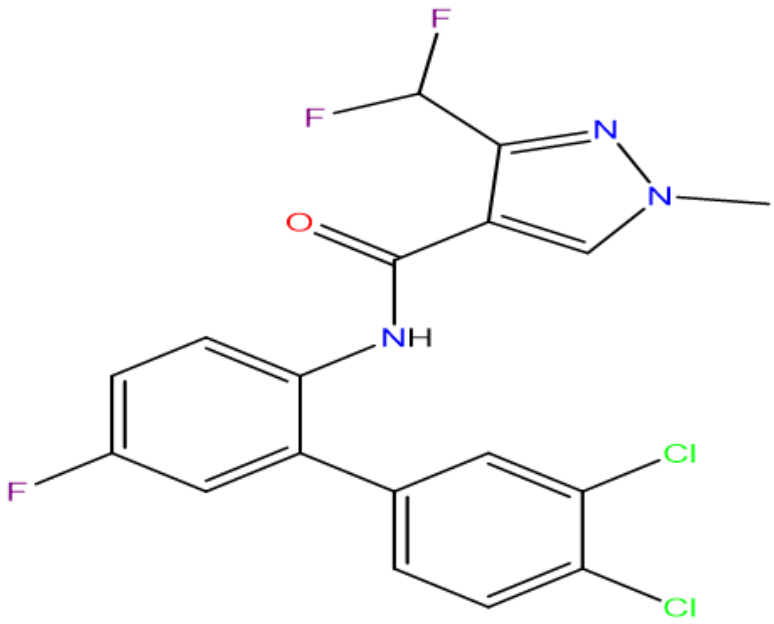

Figure 3: Structure of bixafen.

Table 3: Results of % recovery of metformin and remogliflozin.

QC level	Metformin		Remogliflozin	
	Concentrations (ng/mL)	%Recovery	Concentrations (ng/mL)	%Recovery
LQC	2.03	98.32	1.60	101.30
MQC	36.86	100.62	28.99	98.66
HQC	61.43	101.28	48.32	98.81

Solid Phase Extraction (SPE),[24] and Protein Precipitation (PPT)[25] are still popular among the researchers and these methods are useful for extracting the drug moiety from biological matrices. The selection of the diluted acetonitrile as an extracting agent makes the method simple, effective and offers a high recovery for metformin, remogliflozin and bixafen.

RESULTS

Method validation

Selectivity

The current analytical method demonstrated selectivity without any interference from the plasma matrix. Chromatograms clearly indicate that there was no co-elution at retention time of analytes and IS. Spiked LLOQ concentration was found to be within ±20% of the nominal concentration. Simultaneous chromatogram with the both analytes along internal standard is presented in Figure 4.

Autosampler carryover test

The autosampler carryover experiment showed no carryover of analytes in the standard blank and reinjected the standard blank after injection of LLOQ and ULOQ samples throughout the experiment.

Linearity

Linear regression equation was obtained by plotting the peak area ratio of metformin/bixafen and remogliflozin/bixafen and their corresponding equation were, y = 0.0352 x + (-0.00311) and y = 0.0334 x + (-0.002) for metformin and remogliflozin, respectively. The linearity data is presented in Table 1. The correlation coefficient (r^2) value was 0.998 and 0.999 for metformin and remogliflozin, respectively. Linearity chromatogram is presented in Figure 5.

LLOQ determination

LLOQ of metformin in rat plasma determined to be 0.69 ng/mL and 0.54 ng/mL for remogliflozin. The S/N ratio exceeded 5 with recovered concentrations obtained from the samples were within ±20% of expected values. Additionally, %CV for the samples was below 20%.

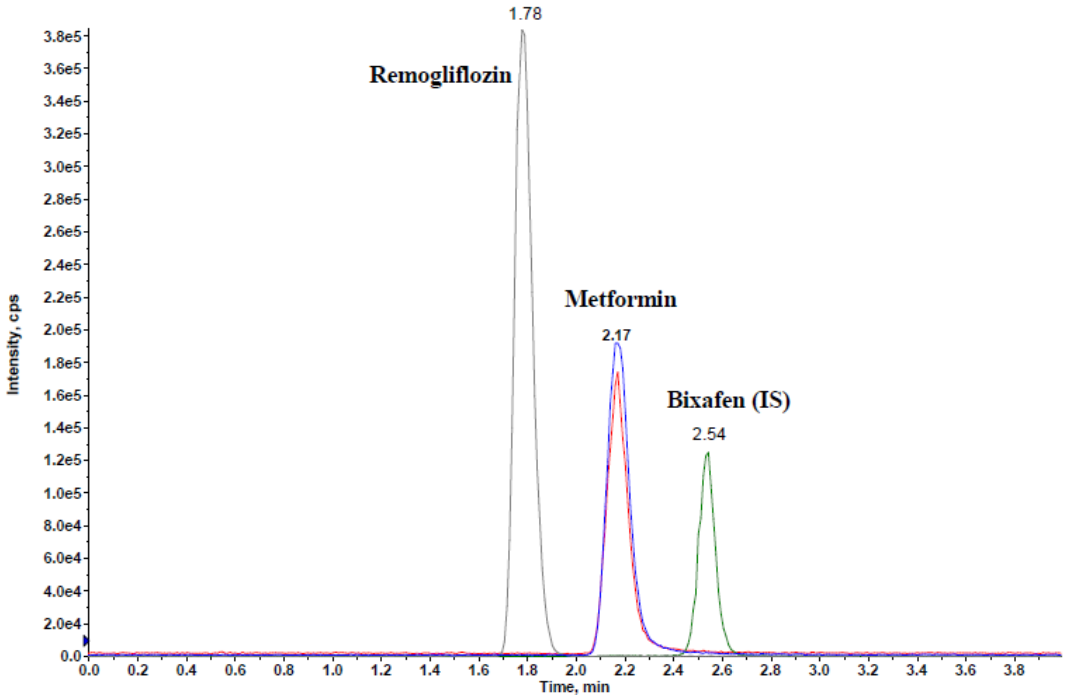

Figure 4: Simultaneous chromatogram with both the analytes along internal standard.

Table 4: Results of stability.

Metformin					
Condition	**Recovery Level**	**Fortified Concentrations (ng/mL)**	**Recovered Concentrations (ng/mL)**	**% Accuracy**	**%CV**
Room Temperature	LQC	2.03	1.89	93.10	2.19
	HQC	61.43	58.20	94.74	2.11
Autosampler	LQC	2.03	1.90	93.60	4.19
	HQC	61.43	60.25	98.08	3.78
Freeze-thaw (5 cycles)	LQC	2.03	1.88	92.69	2.29
	HQC	61.43	61.43	98.19	3.26
Frozen -70°C	LQC	2.03	2.10	102.10	5.12
	HQC	61.43	62.29	101.40	3.45
Remogliflozin					
Condition	**Recovery Level**	**Fortified Concentrations (ng/mL)**	**Recovered Concentrations (ng/mL)**	**%Accuracy**	**%CV**
Room Temperature	LQC	1.60	1.57	98.13	2.19
	HQC	48.32	47.14	97.56	2.11
Autosampler	LQC	1.60	1.41	88.60	4.96
	HQC	48.32	44.89	92.90	5.12
Freeze-thaw (5 cycles)	LQC	1.60	1.47	91.68	2.29
	HQC	48.32	48.12	99.59	1.45
Frozen -70°C	LQC	1.60	1.64	102.51	1.19
	HQC	48.32	49.49	102.42	2.01

Precision and accuracy

Results of precision and accuracy for metformin and remogliflozin are detailed in Table 2 with data provided for each day separately for both analytes. The Coefficient of Variation (%CV) was determined to be less than 6.52 for metformin and less than 6.00 for remogliflozin. Accuracy levels ranged from 90.15% to 111.27% for metformin and 90.29% to 109.26% for remogliflozin, indicating acceptability.

Reinjection reproducibility

Reinjection reproducibility was estimated over a 20 hr period, showing accuracy between 97.24% and 103.21%, with precision values below 2.41 for both analytes.

Matrix effect

Matrix-dependant signal suppression and enhancement is a common challenge in the quantification using Liquid chromatography coupled with mass spectrometry. These effects can influence the quality of signals and exert a deleterious impact on method parameters like LOD, LOQ, linearity, accuracy, precision, etc. To understand these effects better and to minimize their influence, matrix match blanks have been used to validate analytical method parameters. The results of matrix effect results indicate that there were no significant matrix effects. IS-normalized matrix factor for the both analytes varied in the range of 0.91 to 1.09 which suggest the minimal influence of components of matrix on the analyte response.

Recovery

A straight forward liquid extraction technique yielded highly accurate (%CV ≤2.1) and quantitative recovery rates for polar metformin (98.32-101.28%) and moderately non-polar remogliflozin (98.66-101.30%) across QC levels depicted in Table 3. The extraction recovery for the internal standard was 96.52% and 98.29% with metformin and remogliflozin, respectively.

Stability

It is requisite to understand the stability of drug moieties during experimental conditions and tenure. The percentage recovery values for the stability samples, at various stability conditions in plasma - namely, bench top (6 hr) autosampler (20 hr) freeze-thaw (five cycles) and long-term storage (-70ºC, 30 days) ranged from 92.69% to 102.51% for both analytes. Detailed stability results for metformin and remogliflozin in plasma can be found in Table 4.

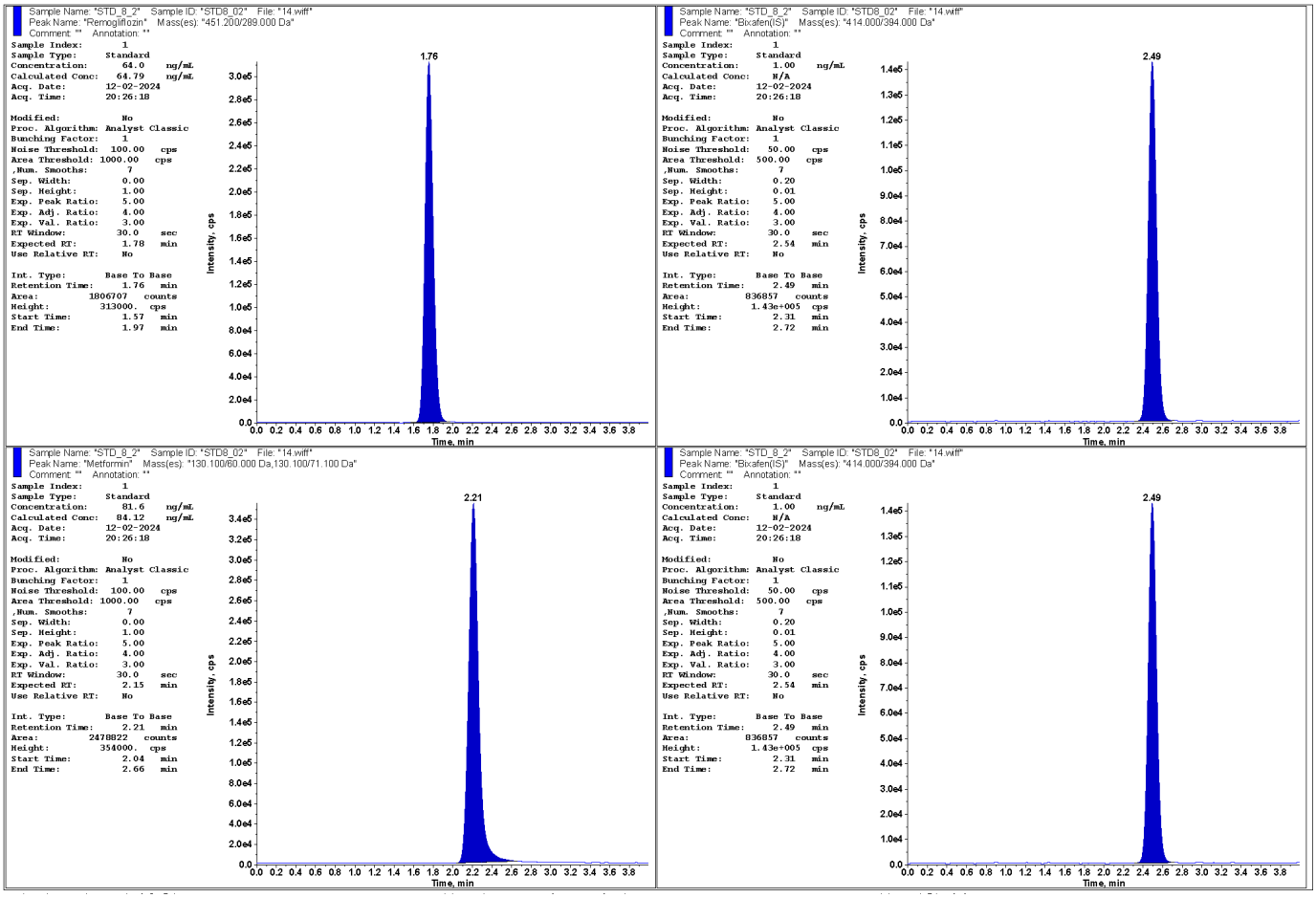

Figure 5: Chromatogram of calibration standard solution of metformin and remogliflozin.

CONCLUSION

A meticulous, precise and reproducible LC-MS/MS method employing liquid-liquid extraction has been developed and validated for concurrent measurement of metformin and remogliflozin in rat plasma in accordance with ICH guidelines. Importantly, satisfactory accuracy and precision were achieved throughout the range of linearity. The method emphasizes simplicity and rapidity with high accuracy, precision and also gives information about control solution's stabilities. The proposed method offers advantages in terms of plasma sample volume required for processing as well as improved sensitivity and reproducibility. The C18 column provided adequate retention of metformin and remogliflozin, having different physicochemical properties without using gradient elution but with an isocratic mode of short minutes run time. Outcome of validation results indicated that developed method is precise, accurate and unaffected by matrix effects and can be used for pharmacokinetics estimation.

ACKNOWLEDGEMENT

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ABBREVIATIONS

LLOQC: Lower limit of Quantification; **IS:** Internal Standard; **ICH:** International Council on Harmonization; **MRM:** Multiple Reaction Monitoring; **CEM:** Channel Electron Multiplier; **ULOQ:** Upper Limit of Quantification; **LQC:** Low Quality Control; **QC:** Quality Control; **MQC:** Middle Quality Control; **HQC:** High Quality Control; **SGLT-2:** Sodium Glucose Co-transporter 2; **LC/MS-MS:** Liquid Chromatography and Mass Spectroscopy/ Mass Spectroscopy; **ESI:** Electrospray Ionization; **ASCOT:** Autosampler Carryover Test; **APCI:** Atmospheric Pressure Chemical Ionization.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

LC-MS/MS method developed and validated for simultaneous quantification of metformin and remogliflozin in rat plasma adhering to ICH M10. Method validation included tests for various validation parameters like matrix effect, linearity, precision, accuracy, reinjection reproducibility, recovery and stability. Linear response was observed between 0.69 to 81 ng/mL and 0.54 to 64 ng/mL for metformin and remogliflozin, respectively with r^2>0.999. Precision values of the quality control samples remained within acceptable limits (<15%) and extraction recoveries were accurate. The validation outcomes indicated that method developed is precise, accurate and devoid of matrix effects, thereby confirming appropriateness for pharmacokinetic estimation.

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