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Original Article

PHARMACOKINETIC INVESTIGATION OF REMOGLIFLOZIN IN RAT PLASMA SAMPLES BY HIGH-THROUGHPUT HPLC-MS-MS

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ABSTRACT

Objective: Remogliflozin (REMO), a selective inhibitor of the renal sodium-dependent glucose transporter 2 channel, which could increase urine glucose excretion and lower plasma glucose in humans. To establish a simple, sensitive and completely validated HPLC-MS-MS approach for the analysis of Remogliflozin in rat plasma samples.

Methods: The method was developed after simple step protein precipitation by acetonitrile and Empagliflozin (EMPA) was used as internal standard. Separation was done on an CORTECS C_{18}, 90 Å, 2.7 µm, 4.6 mm X 150 mm with an isocratic mobile phase consisting of 0.1% Formic acid: acetonitrile (20:80%, v/v) and pumped at a flow stream of 0.8 ml/min at ambient temperature.

Results: The approach developed showed fine calibration curve in the quantity range of 5-1000 pg/ml with correlation coefficient (r^2) of ≥ 0.9997 and the intra-run accuracy and precision was 99.91 to 109.07% and 0.17 to 1.34, inter-run accuracy and precision was 99.8 to 101.54 and 0.17 to 1.66 according to FDA guidelines.

Conclusion: The newly designed and validated approach was simple, fast and applied effectively for single-dose oral pharmacokinetic investigation in Wistar male rats for the quantification of REMO in biological matrix.

Keywords: Bio-analytical, Empagliflozin, HPLC-MS-MS, Internal standard, Remogliflozin, Wistar male rat

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INTRODUCTION

Remogliflozin etabonate (REMO) chemically (5-Methyl-4-[4-(1-methylethoxy) benzyl]-1-(1-methylethyl)-1H-pyrazol-3-yl-6-O-(ethoxycarbonyl)-β-D-glucopyranoside) is a pro-drug of Remogliflozin. It was belongs to the class of glifozin category [1-5]. This drug will primarily be used in the treatment of non-alcoholic steato hepatitis and type 2 diabetes mellitus (TDM2), REMO inhibits the sodium-glucose transport proteins (SGLT), which are responsible for glucose re-absorption in the kidney [6-10].

Although there are many reports was published for regular Quality control checking of REMO combination with MET in both alone and formulation dosage forms using UPLC/PDA detection [11]. Utilization of full factorial Box–Behnken design model developed RP-HPLC and Green Second-Derivative UV Spectroscopic Method [12, 13]. For Greenness Evaluation established Eco-friendly manipulated UV spectroscopic techniques, semi-quantitative and quantitative methods and the employments of modification of UV spectra, three smart, reproducible and non-sophisticated spectroscopic techniques were developed [14].

Several analytical estimations have been reported for the Quantification of REMO in its Pharmaceutical tablet dosage forms under UV absorbance maxima of 229 nm [15], reverse phase C_{18} column was used as stationary phase along with mixture of water: methanol (30:70%, v/v) as a mobile phase [16] and High-Performance TLC-densitometric estimation was done using HPTLC plates pre-coated with Silica gel 60 F254 were used as the stationary phase; toluene: ethyl acetate: methanol: ammonia (4:4:2:0.1, v/v/v/v) was used as mobile phase [17].

The screened literature revealed that the described HPLC-MS-MS methods were lack of poor sensitivity and reproducibility and long retention time. To the best of our knowledge, there is no published report in the literature that demonstrates validation of a sensitive assay for the determination of REMO in rat plasma samples using EMPA as Internal standard by HPLC–MS-MS. EMPA was used as an internal standard in order to improve the method's accuracy, precision and recovery and reliability in evaluating it against matrix effects.

Through this investigation, a simple, rapid and sensitive HPLC–MS-MS approach for the estimation of REMO concentration in rat plasma samples of was developed and validated as per FDA guidelines and successfully applied to pharmacokinetic study.

MATERIALS AND METHODS

Standards and reagents

The reference standards of REMO (99.99% purity) and EMPA (99.97% purity) standards was obtained from Cadila Healthcare Ltd, Ahmedabad, India. HPLC grade acetonitrile and Formic acid were got from Merck chemical division (Mumbai, India). Purified HPLC mark water was obtained by Milli-Q (Milli Q system, USA) water purification system.

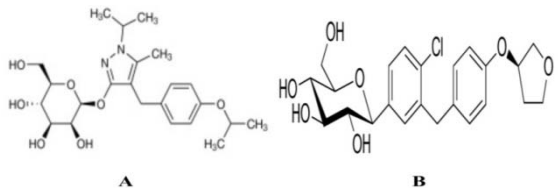

Fig. 1: Chemical structures of A) Remogliflozin B) Empagliflozin

Animals

The Clinical investigation was conducted using Wistar male rats (n=20, Wt: 200-350 g) was obtained from Department of Pharmacology, Raghavendra Institute of Pharmaceutical Education and Research, Anantapur, A. P., India. The study protocol was approved by the Institutional Animal Ethical Committee and conducted as per the ethical guidelines laid down by CPCSEA, New Delhi, India (Approval No.: 878/ac/05/CPCSEA/11/21).

Instrumentation

Waters, Alliance e2695 model HPLC (Waters Corporation, Milford, USA) equipped with an auto sampler, column oven and degasser were operated for the analysis. The HPLC system was coupled to SCIEX QTRAP 5500 mass spectrometer (SCIEX, Canada) provided with electrospray ionization interface. To interpret chromatographic data, the software Analyst 1.6 was used [18]. CORTECS C_{18}, 90 Å, 2.7 µm, 4.6 mm X 150 mm analytical column was used for separation and analysis.

HPLC conditions

Mobile phase was a mixture of 0.1% Formic acid and Acetonitrile (20:80 v/v). Isocratic elution was done at 0.8 ml/min flow stream. The temperature of column and sample were maintained at ambient temperature. The volume of sample injection was 10 µl.

Mass spectrometer conditions

The mass spectrometer was managed in positive ion electrospray ionization interface mode [27]. Multiple reaction monitoring (MRM) mode has been applied to quantify REMO and EMPA. Mass parameters such as source temperature, IS, heater gas, nebulizer gas, curtain gas, and CAD gas (purged all gas channels with ultra-high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized (table 1). The ion transitions observed were m/z 451.11→289.13 for REMO and m/z 451.15→177.09 for EMPA (internal standard).

Standard solutions and internal standard

The stock standard solutions of REMO and EMPA (1000µg/ml) were prepared using diluents (Acetonitrile: Water, 50:50% v/v). Working standard solutions of the REMO were made through serial dilution of the stock standard solution with diluent, with REMO ranging from 5.00 to 1000 pg/ml. Working standard solution of the EMPA (100 pg/ml) was made by diluting aptly stock standard solution of EMPA with diluent. All prepared standard solutions are stored at 4 °C and bring to ambient room temperature before using it.

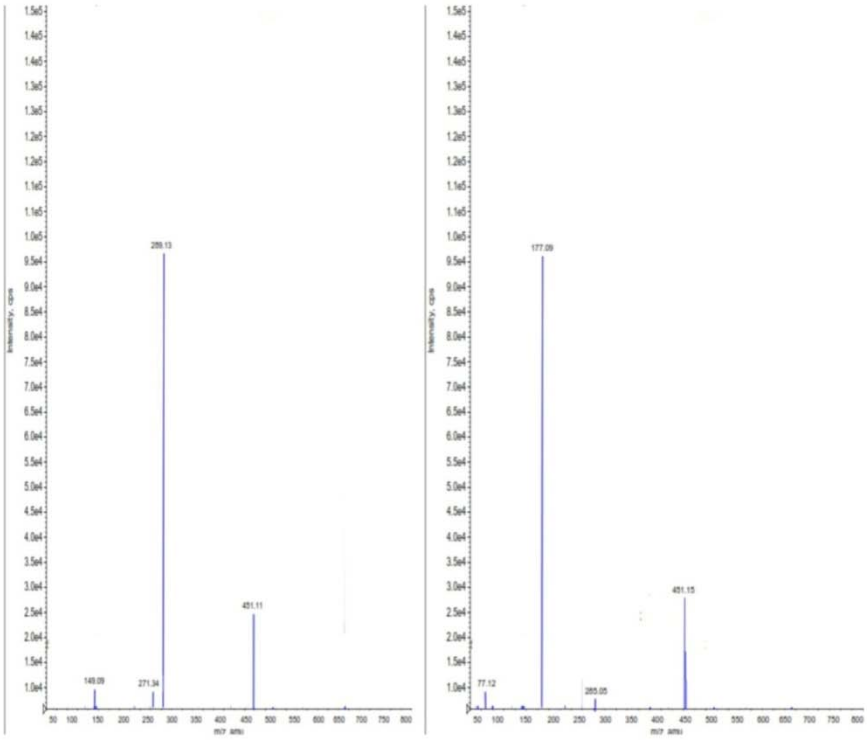

Fig. 2: First and third (Q1 andQ3) mass scans of remogliflozin and empagliflozin

Table 1: Optimized mass parameters of remogliflozin and empagliflozin

Molecular ion to product ion transitions used for quantification								
Compound	Molecular ion (m/z)				Product ion (m/z)			
Remogliflozin	451.11[M+H]+				289.13+			
Empagliflozin	451.15[M+H]+				177.09+			
	Source dependent parameters [Optimized sing ultra-high pure nitrogen] (psi)				Compound dependent parameters (volts)			
	CUR gas	CAD gas	Nebulizer gas	Heater gas	CB	DP	CE	CXP
Remogliflozin	10	10	10	40	10	50	10	10
Empagliflozin	10	10	15	40	10	50	10	10
Common mass parameters								
Ion spray voltage	4500 volts							
Source temperature (°C)	400 °C							
Scan type	MRM							
Dwell time	350 msec							
Mode	Positive ion							
Type of ionization	Electro spray ionization (ESI)							

Calibration standard solutions

Spiked calibration standard solutions of REMO (5.00, 10.00, 50.00, 100.00, 200.00, 400.00, 600.00, 800.00 and 1000.00 pg/ml) were made in rat plasma (100 µl). To every calibration standard solution, 50 µl of internal standard solution with concentration of EMPA (100 pg/ml) was added. All the solutions were stored at-80 °C and prior to analysis they are brought to ambient temperature [26].

Quality control samples

Samples of quality control were made as explained above in the similar way at concentrations corresponding to 5 pg/ml (LLOQ), 15 pg/ml (LQC), 500 pg/ml (MQC) and 800 pg/ml (HQC). All the solutions were stored at-80 °C and prior to analysis they are brought to ambient temperature [26].

Preparation of sample for analysis

In a 1.5 ml centrifuge tube, to each standard and sample, 50µl of internal standard solution with concentration of EMPA (100 pg/ml) was added. All the spiked standards rat samples were extracted by protein precipitation with-1000 µl of acetonitrile was added and mixed using vortex cyclomixer. The mixture was centrifuged for 15 to 20 min at 4000 rpm. The supernatant solution was collected in a HPLC vial and 10 µl of prepared sample was injected to the HPLC-MS-MS system.

Optimized method

Choice of stationary phase

Preliminary development trials have performed with octadecyl columns with different types, configurations and from different manufacturers. Finally the expected separation and shapes of peak was succeeded Analytical column CORTECS C_{18}, 90 Å, 2.7 µm, 4.6 mm X 150 mm.

Selection of mobile phase

Several systematic trials were performed to optimize the mobile phase. Different solvents like Acetonitrile, methanol, water and acetonitrile in different mobile phase ratios, by using different buffer solutions in order to get sharp peak and base line separation of the components and without interference of the excipients and plasma matrices. Satisfactory peak symmetry, resolved and free from tailing was obtained in mobile phase 0.1% Formic acid: acetonitrile (20:80%, v/v) in isocratic condition.

Selection of the mobile phase flow rate

Flow rates of the mobile phase were changed from 0.5-1.0 ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments that 0.8 ml/min flow rate was ideal for the successful elution of the analyte. After completion of several systematic trials to optimize the chromatographic conditions, a sensitive, precise and accurate HPLC-ESI-MS-MS method was developed for the analysis of Remogliflozin in spiked rat plasma samples.

Validation of method

Following the FDA bio-analytical method validation principles, the approach was validated to reveal the system appropriateness, auto sampler carryover, sensitivity, specificity, matrix consequence, linearity, precision, accuracy, extraction recovery, stability and ruggedness [19].

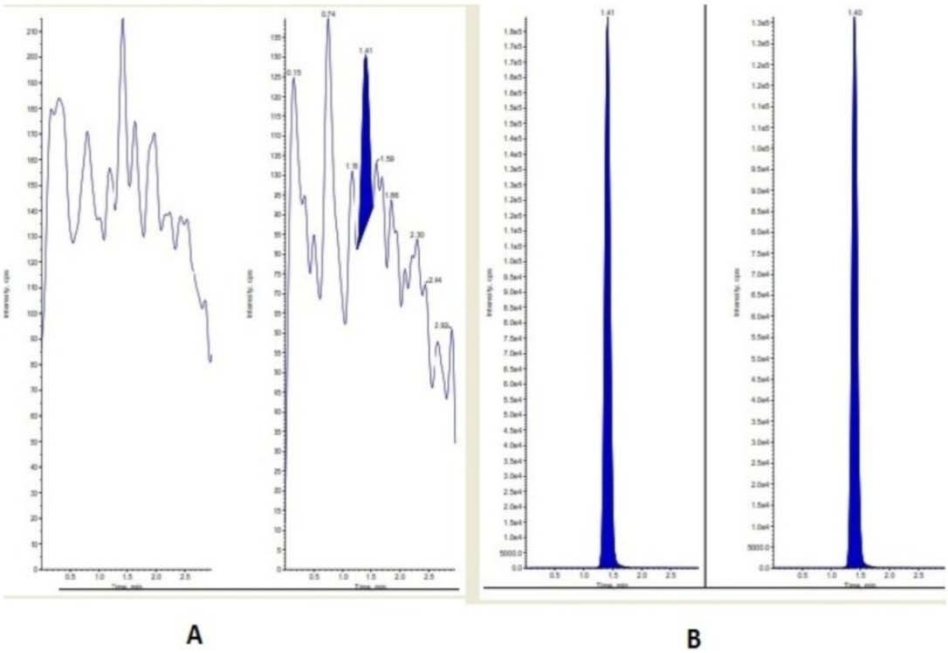

Fig. 3: Chromatogram of A) Plasma blank B) Analyte and internal standard

System suitability

System suitability was done to ensure that the HPLC-MS-MS system performs well by producing accurate and precise results. For this, MQC sample was injected in five replicates. The percent coefficient of variation (CV) was computed for the retention time and area response of REMO and EMPA.

Auto-sampler carryover

The auto-sample carryover was evaluated by injecting blank rat plasma sample after ULOQ and HQC samples. The carryover of REMO and EMPA should be lower than 20% mean peak area of REMO in LLOQ sample. The carryover of internal standard should be lower than 1% of mean peak area.

Screening of biological matrix (specificity)

This test was done to indicate that the blank endogenous plasma components were not chromatographically interacted with REMO and internal standard EMPA. Interference from endogenous rat plasma components was assessed through evaluating 6 individual blank rat plasma samples and LLOQ sample [25].

Sensitivity

LLOQ was the limit to quantify the molecule accurately and precisely. Signal to noise ratio should be at least 10. The LLOQ value was evaluated by analyzing LLOQ level samples in six replicates.

Matrix effect

The matrix consequence of rat plasma on the simultaneous analysis of REMO and EMPA was evaluated through comparison of peak areas of REMO and EMPA in extracted blank plasma with that of obtained from REMO and EMPA standard solutions. The matrix effect was studied at levels of LQC and HQC in 3 replicates.

Linearity

The linearity of REMO was evaluated in the series of 5 pg/ml–1000 pg/ml concentrations, respectively. Calibration curve of REMO was plotted by plotting peak area ratios (analyte peak area/internal standard peak area) against the different concentrations of analytes. The linearity was checked by linear regression analysis using 1/x as weight.

Table 2: Outcomes of linearity test

Spiked plasma concentration (pg/ml)	Concentration measured (mean) (pg/ml)	Precision (CV %)
5.0	5.52	5.46
10.0	10.25	1.40
50.0	48.45	6.74
100.0	100.40	0.89
200.0	212.93	2.12
400.0	408.47	0.44
600.0	596.40	0.09
800.0	792.50	0.23
1000.0	10014.3	0.05

CV-Coefficient of Variation; n=5

Limit of quantization

Six LOQ standards were prepared in screened rat plasma lot along with IS (100.00pg/ml) and signal to noise ratio (S/N) was calculated using instrument software.

Precision and accuracy

We ran six replicates in a single set with samples from the HQC, MQC, LQC and LLOQ concentration levels to measure intra-day precision and accuracy. By analyzing HQC, MQC, LQC and LLOQ concentration samples on 3 separate batches, the inter-day precision and accuracy were tested. The Precision and accuracy are reported as percent CV and percent recovery, respectively.

Recovery of internal standards and analytes

REMO recovery was assessed by comparing peak areas of extracted LQC, MQC and HQC samples with spiked LQC, MQC and HQC samples after extraction. EMPA recovery was analyzed by equating peak areas of the extracted samples to spiked samples after extraction.

Ruggedness

Ruggedness was conducted by repeating the analysis of HQC, MQC, LQC and LLOQ samples in two different columns by two different analysts using the same bio-analytical procedure. The percent CV of recovery of REMO and EMPA were determined.

Stability studies

The stability of REMO in rat plasma was evaluated by analyzing HQC, and LQC samples under different storage conditions like room temperature stability, freeze thaw at-80 °C, auto-sampler at 2-8 °C, 24 h and long term stability (30 d)-80 °C.

Pharmacokinetic study

In the present study, bioavailability test formulation examined by applying the ethical guidelines laid down by CPCSEA, New Delhi, India, and the protocol approved by the institutional animal ethics committee (IAEC).

Twenty Wistar male rats were obtained from Department of Pharmacology, Raghavendra Institute of Pharmaceutical Education and Researach, Anantapur, A. P, India. These 20 rats were randomly grouped into four groups I, II III and IV. Pharmacokinetic study was conducted in harmony with international animal care and maintenance standards. The rats were kept under controlled environment: temperature of 22±2 °C, 50±10% relative humidity and 12h dark and light cycle. Rats were allowed water and food. After fasting for at least 12h with free access to water, all the rats were given REMO orally at dose 10 mg per kg body weight, respectively (US Department of Health and Human Services, Food and Drug Administration 2003). From all the rats, blood samples (0.2 ml) were collected via tail vein in heparinized (Sodium heparin was used as anti-coagulant) tubes at 0.15, 0.30, 0.60, 1.0, 2.0, 3.0, 6.0, 6, 8, 12 and 24 h after dose. Blood samples were centrifuged at 3000 rpm for 10 min to attain plasma sample. The prepared plasma samples were stock up at −80 °C. The plasma samples were analyzed within a month. The curve of mean plasma concentration versus time is shown in table 7. Pharmacokinetic parameters have been computed using non-compartmental statistics, using Win Non-Lin 5.1 software (Pharsight, USA).

The pharmacokinetic parameters determined are given as-maximum plasma concentration (C_{max}), time to reach peak concentration (T_{max}), terminal elimination half-life ($t_{1/2}$), plasma clearance (CL), area below the curve from zero to time of last sampling (AUC_{0-t}) and AUC_{0-t} extrapolated into infinity ($AUC_{0-\infty}$)

The plasma concentration-time profiles were examined visually, and the C_{max} and T_{max} values were calculated. The AUC_{0-t} was calculated using the trapezoidal method. The $AUC_{0-\infty}$ max was computed up to the last measurable concentration and extrapolations were conducted based on that concentration and the terminal elimination rate constant (Ke) of plasma was derived from the slope of the last concentration–time curve (by means of the linear regression method). The terminal elimination half-life ($t_{1/2}$) was then calculated as 0.693/Ke

RESULTS AND DISCUSSION

Method establishing

The chromatography and mass spectrometer conditions were optimized to yield sensitive and efficient detection and quantification of REMO. EMPA recovery was chosen as internal standard. EMPA is chromatographically equivalent (fig. 1) and they will prone to the same matrix effect. Both analytes were chemically extract from the matrix. Therefore, the accuracy of the method will be enhanced and matrix effects are avoided.

In order to optimize electrospray ionization interface conditions for REMO and EMPA, triple quadrupole mass scan was done in positive and negative ion detection mode. Good response was attained in positive mode of ionization. The ion transitions observed for quantification were m/z 451.11 →289.13 forREMO and m/z 451.15 →177.09 for EMPA. Fig. 2 shows the mass spectra of REMO and EMPA.

To achieve good peak shape and mass spectrometer response for REMO and EMPA various chromatographic conditions were optimized. Finally, CORTECS C_{18}, 90 Å, 2.7 µm, 4.6 mm X 150 mm was chosen for separation and analysis. The mobile phase with 0.1% Formic Acid: acetonitrile (20:80, v/v) was selected and directed at an isocratic flow stream of 0.8 ml/min with 10 µl of sample injection volume. The retention times of REMO and EMPA were 1.41 min and 1.40 min, correspondingly, with 3 min run time (fig. 3).

System suitability

The percentage CV values were less than 2.0% for retention times of REMO and EMPA. Also, the percentage CV for area ratio was less than 2%. Hence, the system passed the system suitability test.

Auto sample carryover

Peak area response of REMO and EMPA was not observed in the blank rat plasma sample after successive injections of HQC and LLOQ at the retention times of REMO and EMPA. Therefore, this method does not exhibit auto sample carryover.

Specificity and limit of detection (LOD)

Interfering peaks were not observed at REMO and EMPA retention times in the chromatogram of blank rat plasma. Thus, proved specificity of the method to analyze REMO and EMPA, simultaneously, the limit of detection of this method was shows at 0.80 pg/ml, which can be quantifiable by this method.

Sensitivity

The LLOQ value for REMO was 5 pg/ml. The accuracy and precision (%CV) determined at LLOQ quantity level and found to be within the approved limits.

Matrix effect

The percent CV of matrix factor for at MQC level of REMO was found to be 1.96. The percent CV value indicated that there was no significant effect of the matrix on the bio-analytical methodology for simultaneous evaluation of REMO [22].

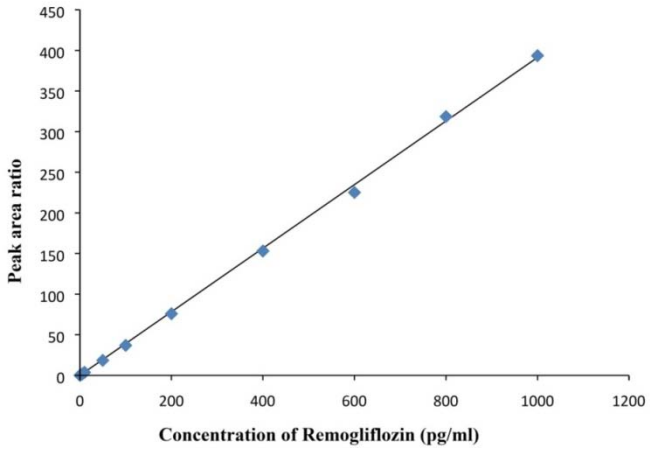

Fig. 4: Calibration curve

Linearity

The REMO and EMPA calibration curves were linear through the concentration series of 5 to 1000 pg/ml and assayed in five replicates on five different days, which was depicted in fig. 4. The calibration curve had a coefficient correlation (r^2) was>0.9998. The results were depicted in table 2.

Precision and accuracy

The intra-day and inter-day accuracy was ranged from 99.91% to 101.54%. The intra-day and inter-day precision was ranged from 0.17 to 1.66 % (table 3). The accuracy and precision results met the acceptable bio-analytical criteria [23].

Recovery of internal standard and analyte

The extraction recoveries and percent CV for REMO (93.06% to 93.91%) and EMPA (97.33 to 102.62%) at LQC, MQC and HQC levels are summarized in table 4 and fig. 5. The percent CV ranged from 0.69% to 1.68% for REMO**.** The results demonstrated that the bio-analytical method had good extraction efficiency. This also showed that the recovery was not dependent on concentration.

Table 3: Outcomes of precision and accuracy test

Sample	Intra-day		Inter-day	
	Percent accuracy	Percent RSD	Percent accuracy	Percent RSD
LLOQ	109.07	1.13	101.54	0.80
LQC	100.82	1.34	99.80	1.66
MQC	99.91	0.17	99.84	0.61
HQC	100.30	0.30	100.10	0.17

RSD-relative standard deviation, n=6

Table 4: Outcomes of recovery test

Statistical values	LQC sample	MQC sample	HQC sample
Remogliflozin (Analyte)			
% Recovery	93.06	93.08	93.91
%CV	1.68	1.26	0.69
Empagliflozin (Internal standard)			
% Recovery	99.70	97.33	102.62
%CV	1.11	1.63	0.94

CV-Coefficient of Variation, n=6

Ruggedness

The percent recoveries and percent CV of REMO determined with two different analysts and on two different columns were within acceptable criteria in HQC, MQC, LQC and LLOQ samples (table 5). The percent Accuracy ranged from 99.88% to 102.80%. The percent CV values ranged from 0.09% to 1.64%. The results proved method was rugged.

Table 5: Outcomes of ruggedness test

Sample	Different columns		Different analysts	
	Percent accuracy	Percent CV	Percent accuracy	Percent CV
LLOQ	100.81	0.79	101.12	1.64
LQC	100.73	1.75	102.80	0.73
MQC	99.88	0.09	100.15	0.03
HQC	100.34	0.17	100.38	0.12

CV-Coefficient of Variation, n=6

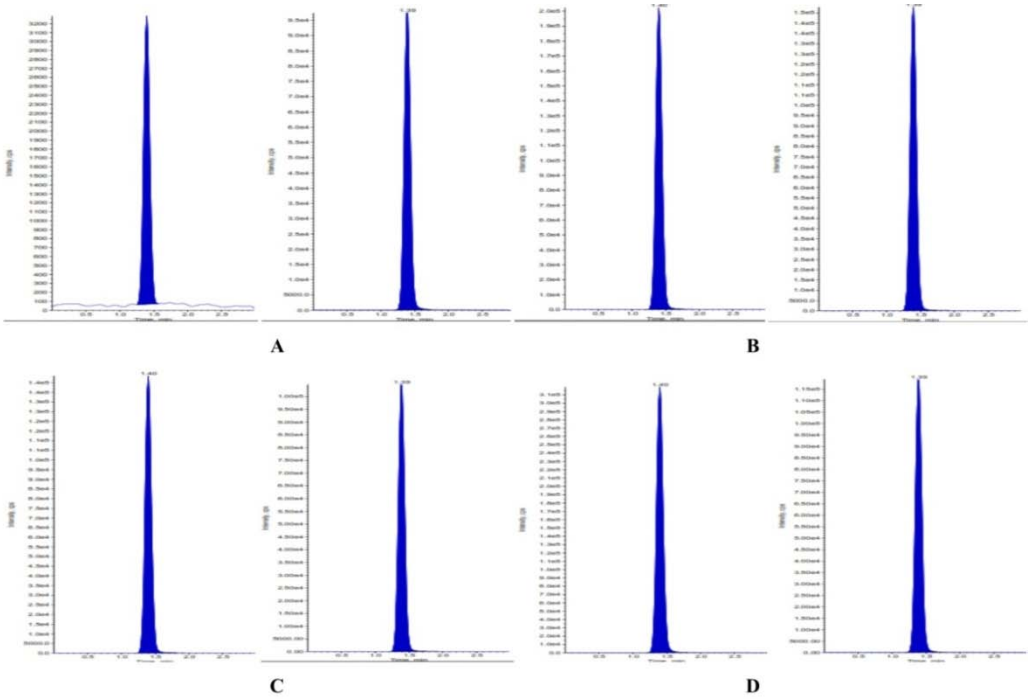

Fig. 5: Chromatograms of A) LLOQ B) LQC C) MQC D) HQC

Table 6: Outcomes of stability tests

Statistical value	Remogliflozin	
	LQC	HQC
Bench top stability		
% Recovery	101.94	100.70
% RSD	1.62	0.28
Freeze and thaw stability		
% Recovery	100.47	100.59
% RSD	0.56	0.23
Long-term stability at −80 °C		
% Recovery	100.91	99.84
% RSD	0.95	0.45
Auto-sampler stability (2-8 °C, 24h)		
% Recovery	103.23	99.95
% RSD	2.33	0.23

RSD-Relative Standard Deviation, n=6

Stability studies

The results of REMO stability tests were summarized in table 6. The findings showed that REMO was durable in rat plasma under various storage conditions studied, including stored at Freeze-thaw at-80 °C, Bench top (normal room temperature) for 48 h, Auto-sampler at 2-8 °C for 24 h and Long term at–80 °C for 30 d [24].

Developed method applied to pharmacokinetic studies

The established and validated bio-analytical approach was productively applied to study the pharmacokinetics following oral administration of 10 mg per kg body weight to twenty rats [20, 21]. The average plasma quantity Vs time profiles subsequent to oral dose administration of REMO are depicted in fig. 6. From the

curve it was found that REMO was detected up to 24 h after dosing. All plasma concentrations of Analyte were within the standard curve region and above 5 pg/ml (LOQ) throughout the sampling period. The method was adequate during the 24 h sampling period to determine the plasma concentration profile of REMO.

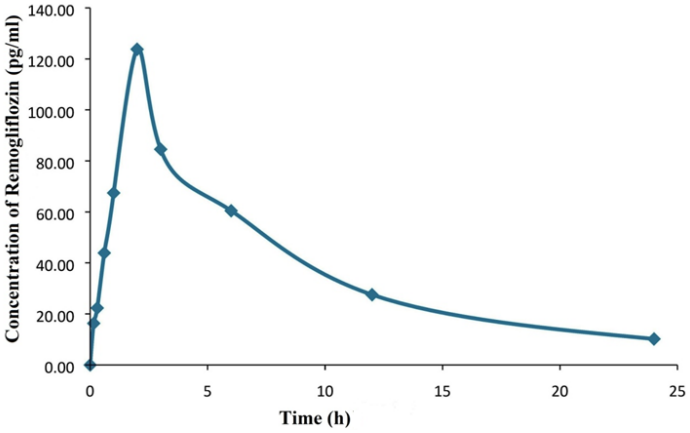

Fig. 6: Concentration versus time profile

Table 7: Outcomes of pharmacokinetic parameters

Parameter	PK value
C_{max} (pg/*ml*)	123.41(5.2)
t_{max} (h)	1.89 (0.21)
AUC_{0-t} (pg/ml/h)	1977.69 (238.13)
$AUC_{0-\infty}$ (pg/ml/h)	2136.154 (256.8)
$t^{1/2}$ (h)	5.93 (0.53)

n=9

CONCLUSION

In this investigation, a simple and sensitive HPLC–MS-MS method was established and validated to quantify Remogliflozin in the sample plasma of rat was five folds higher sensitive and more significant to other previously published methods. The method involved simple single step precipitation method using acetonitrile for sample preparation. The results showed satisfactory recovery as well as a lack of major matrix effects. The validation results are well within the criteria of acceptance. This method was extended effectively to the Remogliflozin pharmacokinetic study in rats.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Applicable

FUNDING

No funding and self-financed.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

No conflicts of interest present in this research

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