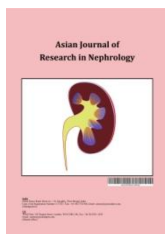

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Comparison of Blood and Urine Profiles of Remogliflozin in Rat PK Study Using a Single and Simple UPLC Method

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Authors' contributions

This work was carried out in collaboration among all authors. All authors contributed to the study conception and design. All authors read and approved the final manuscript.

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ABSTRACT

This study describes a comparison between blood and urine profiles of Remogliflozin simultaneously in a rat pharmacokinetic study using a simple UPLC method. Quantitative determination of Remogliflozin is performed in rat blood with the recovery values in the range of 85-115%. A reversed phase Ultra-performance liquid chromatography (UPLC) with UV detector is used. The method utilizes an Acquity UPLC CSH Fluoro-Phenyl column (150x1mm, 1.7 µm) and a gradient elution with mobile phase consisting of acetonitrile and 0.1% formic acid (15:85) at a flow rate of 0.8 mL min^{-1}. The method adequately resolves the analyte and internal standard (metformin) within a run time of 5 min. The UV detection carried out at 275 nm. In both blood and urine, the

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detection limit of Remogliflozin is found to be 2 µg mL^{-1} and the LOQ as 10 µg mL^{-1}. Two profiles were compared by conducting single oral dose in vivo rat PK study after complete validation of the UPLC method.

Keywords: Antidiabetic; remogliflozin; pharmacokinetics; UPLC; rat blood and Urine.

1. INTRODUCTION

Remogliflozin etabonate (Fig. 1) is a pro-drug of remogliflozin. Remogliflozin etabonate is a drug of the gliflozin [1] class for the treatment of non-alcoholic steatohepatitis ("NASH") and type 2 diabetes [2]. As the drug exhibits anti-diabetic property, the sugar level in blood decreases while the same increases in urine. So both blood and urine samples need to be analyzed simultaneously. Remogliflozin inhibits the (SGLT) sodium-glucose transport proteins [3] which are responsible for glucose reabsorption in the kidney. Blocking this transporter causes blood glucose to be eliminated through the urine [4]. Remogliflozin is selective for SGLT2 that causes a concentration-dependent increase in urine glucose excretion [5,6]. Metformin is not metabolized. It is cleared from the body by tubular secretion and excreted unchanged in the urine; it is undetectable in blood plasma within 24 hours of a single oral dose. Metformin is a dimethyl biguanide that primarily reduces elevated blood glucose levels by reducing hepatic glucose production and improving peripheral tissue sensitivity to insulin. So metformin is selected as internal standard for the present study. Although remogliflozin etabonate is an *O*-glycoside susceptible to β-glucosidase, the PK profile and potency of remogliflozin etabonate may have been advanced. Indeed, renal excretion of glucose in response to remogliflozin etabonate given as a 50-mg dose twice daily to type 2 diabetic patients was ~70 g/day [7], which was similar to that of dapagliflozin given as a 25-mg dose once daily in a 2-week study [8]. Furthermore, remogliflozin etabonate caused a decrease in not only hyperglycemic parameters and body weight but also blood pressure [9].

Very few studies have been conducted on Remogliflozin and not a single rapid bioanalytical method by UPLC has been published till date. UPLC brings dramatic improvements in sensitivity, resolution and speed of analysis can be calculated. The sample extraction procedures need to be verified with different procedures to get maximum recovery. A simple UV spectroscopic method [10], stability indicating TLC method [11] and HPLC method [12] were published in which the prodrug was estimated in bulk and pharmaceutical dosage form at 2-10 µg mL^{-1} concentration range. A UPLC method [13,14], was reported for combining remogliflozin and metformin hydrochloride in bulk and in the formulation with a sensitivity range 10-100 ng mL^{-1}. As of today, no research has revealed about the bioanalysis of Remogliflozin in blood and urine samples using an UPLC method.

The objective of the current research is to develop and validate a rapid, reliable, sensitive and simple ultra-performance liquid chromatography method for the quantification of Remogliflozin in rat blood and urine After complete validation, the method was applied to analyze study sample analysis in rats by giving a single oral dose at 3 mg kg^{-1} body weight. In this study we focused on comparing the drug profiles of Remogliflozin in blood as well as urine in the same time points.

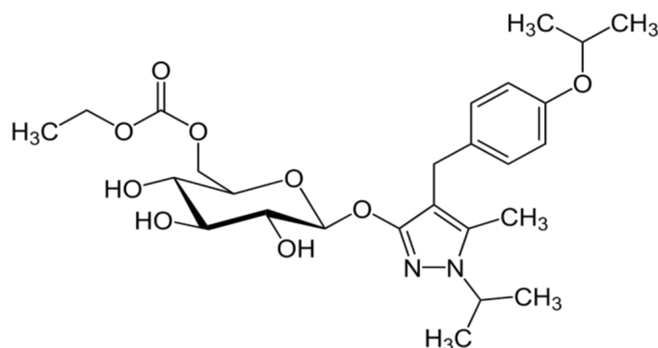

Fig. 1. Chemical structure of remogliflozin etabonate

2. EXPERIMENTAL

2.1 Instrumentation and Chromatographic Conditions

UPLC–UV Analysis

The LC system consisted of a Waters Acquity UPLC with Empower software equipped with a photodiode array detector. A Acquity UPLC CSH Fluoro-Phenyl column (150x1 mm, 1.7 µm) from Waters was used as stationary phase and temperature maintained at 20°C. The mobile phase consisted of Acetonitrile and 0.1% formic acid at 15:85 ratio pumped at a flow rate of 0.8 mL min^{-1}. Analysis was performed using isocratic mode about 5 min. The detection wavelength was 275 nm and the injection volume was 10 µL. The autosampler maintained at 4°C

2.2 Chemicals

Remogliflozin and internal standard (Metformin) are purchased from Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). Acetonitrile and DMSO of HPLC grade and all other chemicals were obtained from Merck (Mumbai, India). Formic acid (GR grade) was purchased from Merck Chemicals Ltd., Mumbai. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Milford, MA, USA). Biological matrices (blood and urine) were obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until use.

2.3 Preparation of Calibrators and QC Samples

A standard stock solution of Remogliflozin was prepared by dissolving standard 50 mg of Remogliflozin into 10 ml volumetric flask, to this added 5 ml of DMSO and sonicated for 10 minutes at a temperature not exceeding 20°C. Allowed the solution to attain room temperature and then diluted to the volume with DMSO to have a solution with a concentration of 5000 µg mL^{-1}. Calibration standard and quality control (QC) samples were prepared by adding corresponding working solutions with drug-free rat blood and drug-free rat urine. A volume of 10 mL of appropriate diluted stock solution at different concentrations and 10 mL of IS at a fixed concentration were spiked into 200 µL of rat blood/urine to yield final concentrations of calibration samples 10, 25, 50, 100, 200, 300, 400 and 500 µg mL^{-1}. The final concentration of IS was 100 µg mL-1. Similarly, QC samples were prepared at four concentration levels LLOQ (10 µg mL^{-1}), LQC (50 µg mL^{-1}) MQC (200 µg mL^{-1}) and HQC (400 µg mL^{-1}).

2.4 Sample Preparation

0.1 mL of blood/urine sample was placed into a 2-mL eppendorf tube, and 50 µL of IS and 150 µL of methanol were added. The samples were vortexed for 1 min, kept at −20°C for 10 min and then vortexed for 1min and then centrifuged at 10,000 rotations per minute for 10 min on refrigerated centrifuge at 4°C (Fig. 2). Then, 150 µL of the extract was transferred to a 200-µL glass injection vial and an aliquot of 10 µL was injected into the UPLC system.

2.5 Analytical Validation

All validation experiments were performed according to the Bioanalytical Method Validation Guidance for Industry [15] and the ICH guidelines [16] on validation of bioanalytical methods.

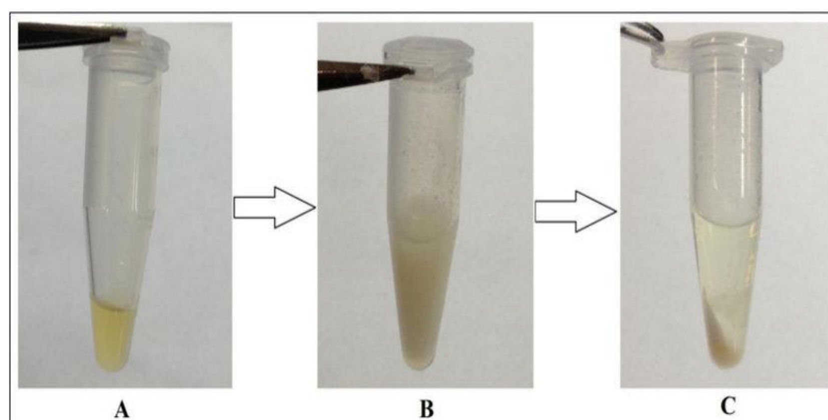

Fig. 2. Protein precipitation of sample

2.5.1 Assay specificity and selectivity

Specificity was assessed by verifying the absence of significant interference in the biological control medium with regard to the retention time of the compound (s) to be assayed. The specificity of the method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around the retention time.

2.5.2 Linearity

A calibration curve was prepared within the range of 10 to 500 µg mL^{-1} Remogliflozin in each run. Half of the calibration samples were analyzed at the beginning of the run and half at the end. The simplest calibration model and weighting procedure were used. The calculations of the curve's parameters were based on the ratio of the peak areas of Remogliflozin/IS versus the concentration of Remogliflozin. Remogliflozin concentrations for samples were calculated from the curve's equation obtained by means of linear regression.

Accuracy of back-calculated calibration samples should be within ±15% of the corresponding nominal concentration, except at the lowest concentration level, where the accuracy should be within ±20%. Per calibration curve, a maximum of 33% of the calibration samples, except the LLOQ and upper limit of quantification (ULOQ, 500 µg mL^{-1}), may differ from these specifications. At least 6 concentration levels were represented in each curve.

2.5.3 Matrix effect, extraction recovery, and process efficiency

The influence of the matrix on the quantification of Remogliflozin was monitored using a comparison of: (1) the instrument response for the low, medium, and high QCs (n = 4 per level) injected directly in mobile phase (neat solutions), (2) the same amount of analyte added to extracted blank samples (post extraction spiked samples), and (3) the same amount of analyte added to the biological matrix before extraction (pre extraction spiked samples). Total process efficiency was calculated from the ratio of mean peak areas of Remogliflozin in extracted validation samples versus neat unextracted samples. This term accounts for any loss in signal attributable to the extraction process or matrix effect. Extraction recovery was calculated from the ratio of mean peak areas of Remogliflozin in extracted validation samples versus blank samples spiked after extraction. The absolute matrix effect was calculated from the ratio of mean peak areas of Remogliflozin in blank samples spiked after extraction versus neat unextracted samples. If the ratio was 85% or 115%, an exogenous matrix effect was inferred.

2.5.4 Matrix variability

To confirm that the biological matrix would not interfere with the assay, the selectivity of the developed method was tested by analyzing 6 different lots of blank blood samples and also 6 different lots of blank urine samples spiked with IS at the LLOQ level (n = 3 per lot), and blank blood samples with no IS (n = 3 per lot) against a calibration curve. The results for the LLOQ samples were considered acceptable if the precision from each matrix lot was ±20% and the accuracy was within the range of 80%–120%. The acceptance criterion for the analysis of the blank samples from the 6 individual lots was based on the raw peak areas found at the retention times of Remogliflozin and IS. No more than 10% of the blank samples could have peak areas greater than 20% of the average peak area of Remogliflozin in the LLOQ QCs.

2.5.5 Stability studies

Stability evaluations were performed in both aqueous and matrix based samples. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). Remogliflozin stability in blood and urine was evaluated by performing bench top stability, long-term stability, short term stability and freeze-thaw stability. The processed samples were studied for stability in auto sampler at 10°C. Stability in blood and urine was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples.

3. RESULTS AND DISCUSSION

3.1 Chromatographic and Detection Parameters

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed-phase C18 column. The different columns tried were Symmetry C18, Luna C18

and Zorbax C18. The best results were observed with the Acquity UPLC CSH Fluoro-Phenyl column (150x1 mm, 1.7 µm) using 0.1% formic acid and acetonitrile (85:15) as mobile phase in isocratic mode. Variation of the column temperature between 20 and 30°C did not cause significant change in the resolution, however changes in retention time were observed. The column was used at 20°C at a flow rate of 0.8 mL min^{-1}. The method allowed the separation of analyte with IS in 5 min (Fig. 3) runtime.

3.2 Specificity, Linearity, Accuracy and Precision

The specificity of both methods was confirmed by comparing chromatograms of blank plasma, spiked plasma with analytes at LOQ concentrations. No interfering endogenous peaks were observed around their retention times. The eight point calibration curves for 3 analytes showed a linear correlation between concentration and peak area. Calibration data (Table 1) indicated the linearity (r^2 > 0.99) of the detector response for all standard compounds from 10 to 500 µg mL^{-1} The limits of detection by UPLC was found to be 2 µg mL-1 and LOQ was found to be 10 µg mL-1. All standards and samples were injected in triplicate. Multiple injections showed that the results are highly reproducible and showed low standard error. A recovery experiment was performed to confirm the accuracy of the method. Blank blood/urine was spiked with Low QC, Mid QC and High QC levels of the standard stock solution and then extracted and analyzed under optimized conditions. The extraction recoveries of all samples from rat blood were in the range of 94.1-109.9% and from urine samples in the range of 90.1-114.3% with relative standard deviations less than 10.0%, which indicates the sample preparation technique is suitable for extracting. Intra- and inter-day precision of the method was determined by analyzing QC samples on two consecutive days and the obtained intra-day accuracies were in the range of 93.9–107.0% and inter-day accuracies were in the range of 92.5–109.2%. The recovery results are displayed in Table 2 and Table 3. To investigate carry-over from one sample to the other in the autosampler, each validation run containing a calibration curve included a blank sample analyzed directly after the sample at the ULOQ calibration level. The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration. To demonstrate that the method is suitable for blood and urine sample with test compound concentration higher than the ULOQ, the dilution integrity was assessed using validation samples spiked with the test compound at 2-, 4-, and 10-fold the concentration of the high QC. The dilution test was performed by increasing the concentration of IS by the appropriate dilution factor. After extraction, the dry extract was taken up with a volume of injection solvent also multiplied by the same factor. Accuracy of the calculated concentrations within the range of 85%–115% of the nominal values would suggest that samples containing Remogliflozin at a higher concentration than the ULOQ can be diluted using the above tested dilution method.

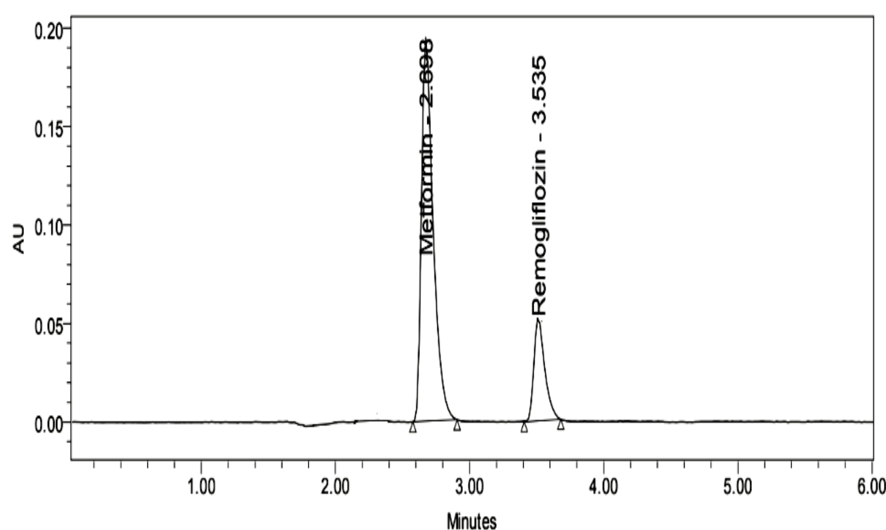

Fig. 3. LLOQ chromatogram showing the separation of the analyte from IS

Stability evaluations were performed in both aqueous and matrix-based samples. The stock solutions were stable for a period of 24 h at room temperature and for 60 days at 1–10°C. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The processed samples were stable up to 36 h in auto sampler at 10°C. The long-term matrix stability was evaluated at −20°C over a period of 60 days. No significant degradation of analytes was observed over the stability duration and conditions. The long-term stability results presented in Table 4 were within 85–115%. Stability in blood and urine was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples. The short-term stability of analyte at room temperature was within 85–115% upto 24 h. The stability results presented in Table 5 and Table 6. Remogliflozin was stable upto 10 h on bench top at room temperature and over 4 freeze–thaw cycles. In both blood and urine, the freeze-thaw study was carried out and the results are presented in Table 7 and Table 8. The variability of the matrix effect in both blood and urine has resulted a very minute changes in the recovery of middle concentration of calibration curve. The results of Matrix effect area presented in Table 9.

3.3 Application of the Method to Pharmacokinetic Study

Albino rats (220±20 g) used were maintained in a clean room at a temperature between 22±2°C with 12 h light/dark cycles and a relative humidity rate of 50±5%. Rats were housed in cages with a supply of normal laboratory feed with water ad libitum. For all of the studies, the animals (n=6) were deprived of food 12 h before dosing, but had free access to water. In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the developed UPLC method was successfully applied to a pharmacokinetic study by administration of Remogliflozin as single solution to six male albino rats by oral route using BD syringe attached with oral gavage needle (size 18) at the dose of 3 mg/kg body weight. Approximately, 0.2 mL of blood samples from each anesthetized (isoflurane) rat at pre-determined time intervals was collected using a capillary tube into pre-labeled eppendorf tubes containing 10% of K_2EDTA anticoagulant (20 µL). The time intervals for the sample collection were 0 (predose), 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h (postdose). The total blood volume collected from each rat was approximately 1.7 to 1.9 mL which does not exceed the maximal recommended blood volume of 20% (2.0 mL for a 200 g body weight). Similarly urine samples also collected at the same time points. All the samples were extracted by protein precipitation (using methanol) method and the obtained supernatant samples were transferred into pre-labeled micro vials.

The blood/ urine samples thus obtained were stored at –30°C till analysis. Post analysis the pharmacokinetic parameters were computed using WinNonlin® software version 5.2 and SAS® software version 9.2. All the samples were analyzed by the developed method and the mean concentrations vs time profile of Remogliflozin is shown in Figs. 4 & 5. The pharmacokinetic parameters estimated are shown in Table 10.

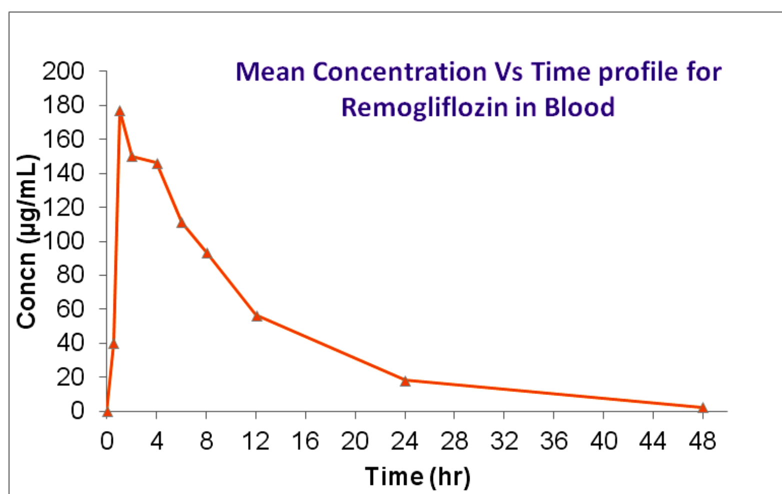

Fig. 4. Mean plasma concentration–time profile curve in Blood

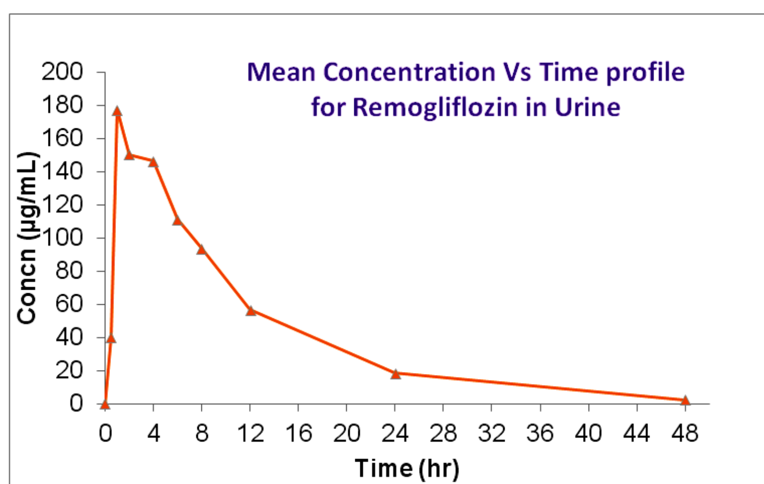

Fig. 5. Mean plasma concentration–time profile curve in Urine

Table 1. Linearity data of remogliflozin

Concn in µg mL^{-1}	Area in blood	Area in Urine
10	15098	1111
25	30574	2301
50	60576	5062
100	120143	10528
200	232582	21542
300	316451	31247
400	416451	40127
500	547136	49736

Table 2. Intra-day precision & accuracy results in blood and urine

Matrix	Intra-day (% Recovery ± SD)			
	LLOQ QC	Low QC	Mid QC	High QC
Blood	98.165±3.983	99.699±1.443	101.677±3.541	100.617±3.225
Urine	98.586±3.483	100.732±3.736	98.223±3.625	100.772±0.820

Table 3. Inter-day precision & accuracy results in blood and urine

Matrix	Inter-day (% Recovery ± SD)			
	LLOQ QC	Low QC	Mid QC	High QC
Blood	100.241±5.178	100.110±3.614	99.513±2.626	100.222±2.143
Urine	101.998±4.625	99.207±2.292	98.757±5.451	100.774±3.511

Table 4. Long-term Stability studies of Remogliflozin in blood and urine at two QC levels (n=6)

	In Blood				In Urine			
	LQC- 50 µg mL^{-1}		HQC- 400 µg mL^{-1}		LQC- 50 µg mL^{-1}		HQC- 400 µg mL^{-1}	
	Day 0		Day-60		Day 0		Day-60	
Long-term stability	49.500	48.141	398.018	387.831	48.000	47.401	392.248	399.047
	45.552	51.024	398.861	380.001	44.555	50.744	396.745	391.214
	51.960	48.521	397.826	377.382	49.960	49.923	396.684	382.589
	45.998	49.245	390.701	391.392	47.478	47.545	392.256	396.235
	51.104	48.128	394.742	383.211	50.236	49.336	390.111	385.741
	49.459	47.729	394.710	396.289	51.189	52.258	399.710	399.258
Mean	48.929	48.798	395.810	386.018	48.570	49.535	394.626	392.347
SD	2.628	1.205	3.056	7.160	2.417	1.875	3.639	7.041
CV(%)	5.370	2.468	0.772	1.855	4.976	3.786	0.922	1.795

Table 5. Short-term Stability studies of Remogliflozin in blood and urine at Low QC level (n=6)

	Remogliflozin LOW QC 50 µg mL^{-1}					
	0 Hour		Blood (24 Hours)		Urine (24 Hours)	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
Short term stability	49.500	99.000	52.225	104.450	47.500	95.000
	45.552	91.104	51.125	102.250	49.501	99.001
	51.960	103.920	48.247	96.494	48.130	96.259
	45.998	91.996	51.370	102.740	48.790	97.580
	51.104	102.208	50.879	101.758	50.897	101.794
	49.459	98.918	48.257	96.515	48.225	96.451
Mean	48.929	97.858	50.351	100.701	48.840	97.681
SD	2.628		1.687		1.212	
CV(%)	5.370		3.351		2.482	
%Change	n/a		2.906		-0.18061	

Table 6. Short-term Stability studies of Remogliflozin in blood and urine at High QC level (n=6)

	Remogliflozin High QC 400 µg mL^{-1}					
	0 Hour		Blood (24 Hours)		Urine (24 Hours)	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
Short term stability	398.018	99.505	400.141	100.035	394.214	98.554
	398.861	99.715	397.588	99.397	399.025	99.756
	397.826	99.457	405.699	101.425	399.587	99.897
	390.701	97.675	395.874	98.969	405.879	101.470
	394.742	98.686	388.238	97.060	398.987	99.747
	394.710	98.678	399.588	99.897	399.215	99.804
Mean	395.810	98.952	397.855	99.464	399.485	99.871
SD	3.056		5.766		3.721	
CV(%)	0.772		1.449		0.931	
% Change	n/a		0.517		0.928	

Table 7. Freeze thaw stability (after IV cycle) study Results (n-6) conducted below -20°C & -50°C for Blood samples

	Remogliflozin in Blood							
	Freeze Thaw Cycle-IV below -20°C				Freeze Thaw Cycle-IV below -50°C			
	LOW QC 50 µg mL^{-1}		HIGH QC 400 µg mL^{-1}		LOW QC 50 µg mL^{-1}		HIGH QC 400 µg mL^{-1}	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
Freeze Thaw Cycle-IV	49.587	99.174	385.478	96.370	48.129	96.258	402.215	100.554
	52.548	105.095	395.876	98.969	49.258	98.516	403.203	100.801
	49.741	99.482	402.148	100.537	50.699	101.398	399.897	99.974
	52.987	105.975	395.478	98.870	47.123	94.247	399.015	99.754
	51.472	102.943	392.587	98.147	52.125	104.249	400.580	100.145
	50.258	100.516	386.125	96.531	49.202	98.405	406.587	101.647
Mean	51.099	102.198	392.949	98.237	49.423	98.845	401.916	100.479
SD	1.459		6.357		1.787		2.751	
CV(%)	2.855		1.618		3.616		0.684	

Table 8. Freeze thaw stability (after IV cycle) study Results (n-6) conducted below -20°C & -50°C for Urine samples

	Remogliflozin in Urine							
	Freeze Thaw Cycle-IV below -20°C				Freeze Thaw Cycle-IV below -50°C			
	LOW QC		HIGH QC		LOW QC		HIGH QC	
	50 µg mL^{-1}		400 µg mL^{-1}		50 µg mL^{-1}		400 µg mL^{-1}	
	Conc found		% Recovery		Conc found		% Recovery	
Freeze Thaw Cycle-IV	50.216	100.432	399.147	99.787	50.141	100.283	398.874	99.719
	51.477	102.954	398.259	99.565	51.987	103.975	393.258	98.315
	48.241	96.483	400.691	100.173	52.588	105.175	396.875	99.219
	50.148	100.296	399.255	99.814	46.874	93.748	392.874	98.219
	52.587	105.175	399.686	99.921	51.587	103.174	402.157	100.539
	48.369	96.739	397.871	99.468	47.587	95.174	401.622	100.405
Mean	6	6	6	6	6	6	6	6
SD	50.173	100.346	399.152	99.788	50.127	100.255	397.610	99.402
CV(%)	1.704		1.010		2.395		4.007	

Table 9. Matrix effect results

Unit No.	In Blood		In Urine	
	200 µg mL^{-1}		200 µg mL^{-1}	
	Neat standard sample peak area	Extracted blank plus spiked sample peak area	Neat standard sample peak area	Extracted blank plus spiked sample peak area
1	137926	152646	179586	186810
2	142389	159061	183349	192377
3	133019	148960	175195	180298
4	135686	153468	173026	188998
5	139885	134553	182667	158888
6	136729	158312	177287	201461
Mean	137605.667	151166.667	178518.333	184805.333
SD	3276.261	8963.751	4108.848	14480.074
CV(%)	2.381	5.930	2.302	7.835
Matrix effect	0.099		0.035	

Table 10. Pharmacokinetic parameters of Remogliflozin in rat (n=6, Mean ± SD)

Parameter	Remogliflozin
C_{max} (µg mL^{-1})	205.2147 ± 1.224
T_{max} (h)	1.00 ±.021
$t_{1/2}$ (h)	1.69 ± 0.1604
K_{el} (h^{-1})	0.41005 ± 0.0128

Cmax: maximum analyte concentration.
Tmax: time point of maximum concentration.
$t_{1/2}$: half life of drug elimination during the terminal phase.
K_{el}: elimination rate constant

The pharmacokinetic parameters evaluated were Cmax (maximum observed drug concentration during the study), AUC_{0-48}(area under the blood concentration–time curve measured 48 hours, using the trapezoidal rule), Tmax (time to observe maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the blood concentration versus time curve, using the method of least square regression) and $t_{1/2}$ (terminal half-life as determined by quotient 0.693/Kel).

4. CONCLUSION

As an anti-diabetic drug, remogliflozin is given at an oral dose of 100 mg which is a high amount to get the bioavailability. Anyhow the plasma

concentration levels fall in the range of micrograms, then this UPLC method can be used with high precision and accuracy which is somewhat less expensive than LC-MS. The methodology used in this experiment may very well be used to test remogliflozin in both blood and urine at a time. The overall pharmacokinetic parameters were within the range of 80-125% therefore it can be concluded that, the present study provides firm evidence to support in clinical pharmacokinetic studies for further research of selected drug. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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