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Bioanalytical Method Development and Validation of Dapagliflozin in Human Plasma Using RP-HPLC Method

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Abstract

Background: Dapagliflozin is used for controlling blood glucose levels in patients with type 2 diabetes. It is a sodium-glucose cotransporter 2 inhibitor, which enhances the elimination of blood glucose through the urine by inhibiting the protein involved in the transport mechanism of SGLT2. Dapagliflozin requires a selective and sensitive bioanalytical RP-HPLC method.

Aim: Reverse phase - high performance liquid chromatography technique was used to develop and validate a bioanalytical method for the quantification of dapagliflozin (DAPA) in human plasma.

Methods: The internal standard (IS) used was azilsartan medoxomil. In isocratic mode, the mobile phase consisted of 50:50 v/v acetonitrile and 0.1% orthophosphoric acid in water at a flow rate of 1.0 mL/min. The chromatogram was recorded at 224 nm. For the chromatographic separation, a Kromasil C18 column (250 mm × 4.6 mm; 5µ) was used. The drug was extracted from plasma samples by the protein precipitation method.

Result and Discussion: The chromatographic run time was 15 min. Dapagliflozin and IS eluted at 4.6 and 5.7 min, respectively. The method was selective and sensitive, with a limit of quantification of 1.50 µg/mL. The developed method was found to be linear in the range of 1.50–60 µg/mL (r^2 = 0.9994). The accuracy and precision obtained from six sets of quality control (QC) samples ranged from 96.23% to 108.67% and 1.35% to 3.19%, respectively. The extraction recovery of dapagliflozin in three QC samples ranged from 87.39% to 90.78%. The bench-top stability, stock solution stability, stability of processed extracted samples at room temperature, and freeze-thaw stability evaluations showed no evidence of degradation of dapagliflozin.

Conclusion: The stability, selectivity, sensitivity, and reproducibility of the developed method make it suitable for the determination of dapagliflozin in human plasma.

Keywords Dapagliflozin, RP-HPLC, Internal standard, Protein precipitation, Recovery

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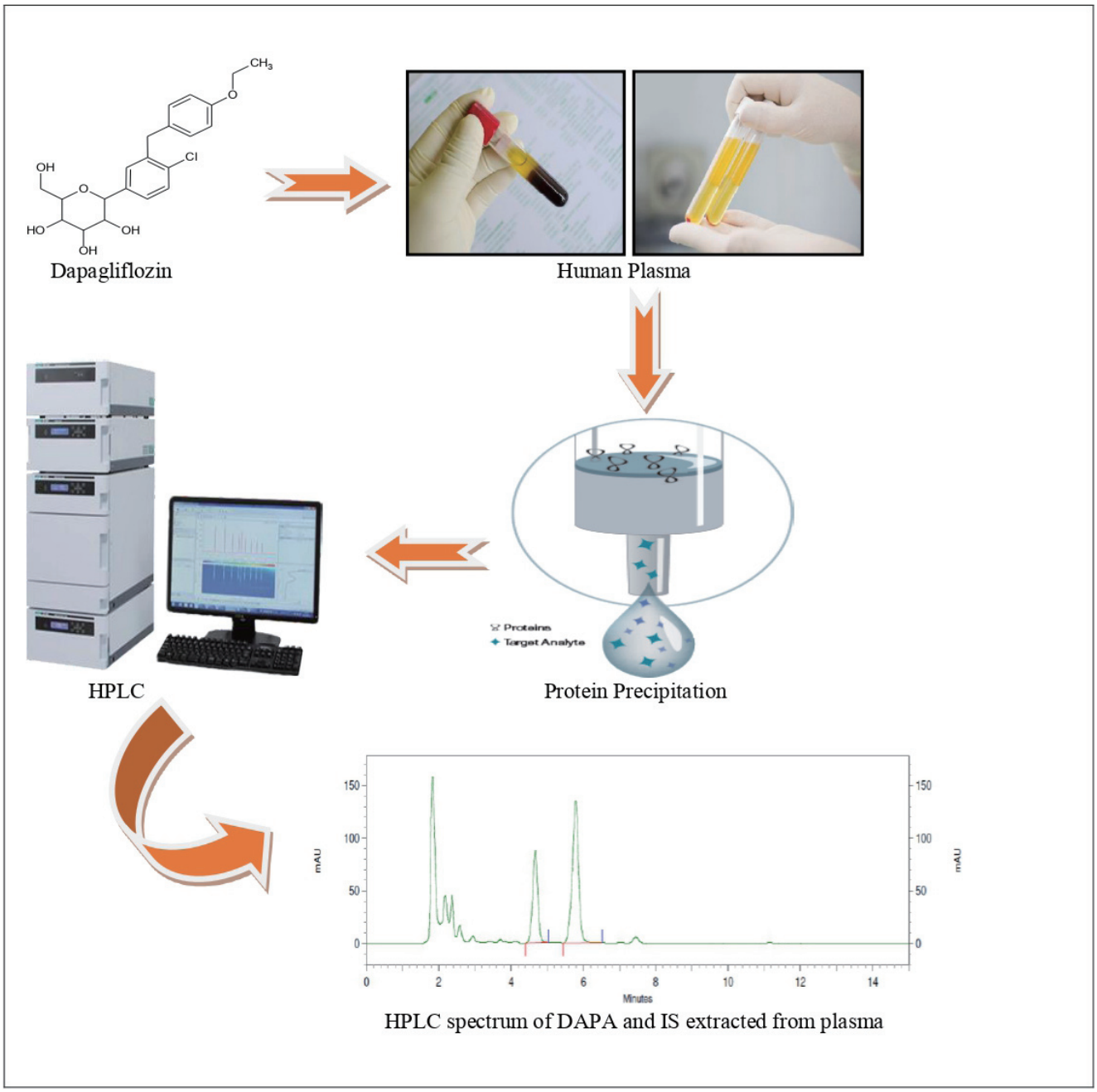

1. Introduction

A chronic condition known as diabetes mellitus (DM) arises when the pancreas is unable to produce enough insulin or when cells are unable to respond effectively to the insulin that is produced. [1]. It is characterized by various pathophysiological defects like increased peripheral insulin resistance, renal glucose reabsorption, hepatic glucose production, and defects in both insulin secretion and insulin sensitivity in adipose tissue [2, 3]. Two types of diabetes mellitus are recognized: type I diabetes mellitus, which is insulin-dependent, and type II diabetes mellitus, which is not insulin-dependent. The more common type of diabetes, known as type II diabetes, is considered to be an important health problem worldwide at the moment. Long-term, effective management of this complex and progressive illness is difficult. Type II diabetes is strongly correlated with polygenic factors and lifestyle. A diabetic patient can maintain stable blood glucose levels with the help of antidiabetic drugs, a healthy diet, and regular physical activity. Oral hypoglycemic agents are one of the treatments for type II diabetes, which lowers blood glucose levels. Dapagliflozin is a potent, highly selective, reversible, and orally active inhibitor of the SGLT2 receptor

used to treat type II diabetes. SGLT2 inhibitors are a unique adjunct to oral anti-diabetic medications that are prescribed by physicians worldwide as a second-line treatment. By promoting urinary glucose excretion, dapagliflozin lowers plasma glucose in a dose-dependent and sustained manner [4–9]. Recently, the European Commission approved the use of SGLT2 inhibitors as a supplement to insulin therapy in patients with inadequately controlled type I diabetes mellitus. The primary mechanism by which SGLT2 inhibitors reduce blood sugar levels is by inducing significant glucosuria [10]. Sodium-glucose cotransporter-2 inhibitors have been shown in large-scale randomized trials to significantly lower the risk of heart failure and the progression of renal disease in a number of patients with type 2 diabetes [11]. In the kidney's proximal tubule, the sodium-glucose cotransporter 2 (SGLT2) is selectively and reversibly blocked, allowing 90% of the filtered glucose to be reabsorbed. Inhibition of the SGLT2 transporter results in the excretion of both sodium and glucose in urine because SGLT2-mediated absorption of glucose is linked to sodium reabsorption [12]. These inhibitors are members of a new class of gliflozins, which are antidiabetic medications that lower blood glucose by preventing the kidneys from reabsorbing glucose. Their mechanism of action is insulin-independent and solely dependent on renal function and plasma glucose [13]. 90% of glucose reabsorption is carried out by the low-affinity, high-capacity Na+/K+ cotransporter SGLT2 and is found in the early part of the PCT. The first SGLT2 inhibitor approved in the world was dapagliflozin. In the liver and kidneys, dapagliflozin is converted to dapagliflozin 3-O-glucuronide by the uridine diphosphate-glucuronosyltransferase 1A9 enzyme [14]. Dapagliflozin can be taken once daily since its half-life is about 12 to 13 hours at a dose of 10 mg [15]. After oral administration, dapagliflozin absorbs rapidly, with a bioavailability of greater than 75%. Maximum plasma concentrations reach within 2 hours of administration. Only 2% of the dapagliflozin is excreted by the kidneys, and more than 90% of the plasma dapagliflozin is bound to plasma proteins [16, 17]. Figure 1 illustrates the dapagliflozin structure.

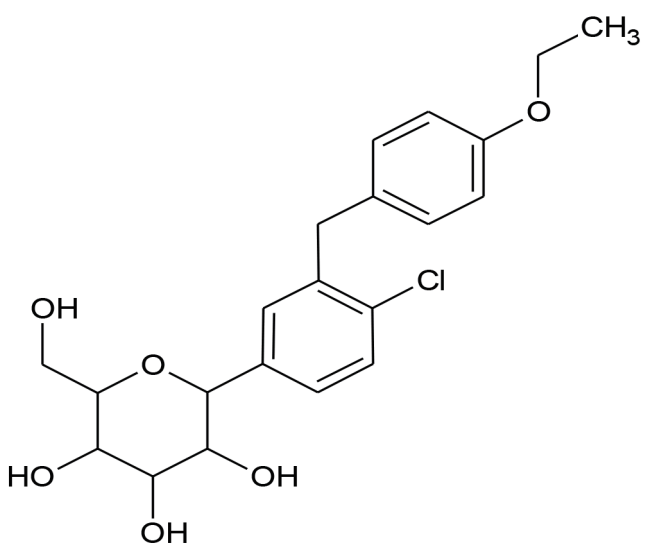

Figure 1: Chemical structure of dapagliflozin

Using HPLC, the literature review identified a number of analytical methods for the simultaneous measurement of dapagliflozin and saxagliptin [18–20], DAPA and sitagliptin [21–22], and DAPA with metformin, gliclazide, pioglitazone, empagliflozin, saxagliptin, linagliptin, and teneligliptin [23]. A bioanalytical technique was described for the simultaneous measurement of dapagliflozin and saxagliptin in human plasma using RP-HPLC [24], Ultra-performance liquid chromatography and spectrodensitometric detection in human plasma [25]. Dapagliflozin and metformin [26] and DAPA with saxagliptin [27] were simultaneously estimated in human plasma using the bioanalytical LC-MS/MS technique. Spectrofluorimetric analysis of dapagliflozin in its pure form and formulation [28], in bulk, tablet, and human plasma [29]. The stability-indicating HPLC method was reported on dapagliflozin [30]. The UHPLC-Q-Orbitrap MS method reported to determine dapagliflozin in rat plasma [31]. The determination of dapagliflozin with metformin in the presence of cyanoguanidine by HPLC was reported [32]. UV-spectrophotometric methods were reported on dapagliflozin [13], dapagliflozin and saxagliptin [33]. Currently, there is no bioanalytical technique using RP-HPLC to analyse dapagliflozin in its pure form in human plasma. For dapagliflozin, a sensitive and selective bioanalytical RP-HPLC method is thus needed.

2. Materials and Methods

2.1 Reagents and Chemicals

Dapagliflozin was obtained as a gift sample from Glenmark Pharmaceuticals Pvt. Ltd., Sinnar. Azilsartan medoxomil as an internal standard was provided by Swapnroop Drugs, Aurangabad. HPLC-grade methanol, water, and acetonitrile were purchased from Shri Ganesh Services, Nashik. Orthophosphoric acid and formic acid of AR grade were purchased from Shri Ganesh Services, Nashik. Human plasma was procured as a gift sample from Arpan Blood Bank, Sangamner, A. Nagar, India.

2.2 Instrumentation and Chromatographic Conditions

A Kromasil C18 (250 mm × 4.6 mm, 5 µm) column was used to achieve chromatographic separation in isocratic mode at room temperature on the Jasco HPLC system. At a flow rate of 1 mL/min, the mobile phase consisted of acetonitrile and 0.1% orthophosphoric acid in water (50:50 v/v). The injection volume was 20 µL. The eluent was monitored by a UV detector at a wavelength 224 nm.

2.3 Preparation of Stock Solution

Accurately weighed amounts of IS and dapagliflozin were dissolved in methanol to prepare stock solutions (2000 µg mL^{-1}). The calibration curve and quality control sample (QC) were then prepared using the stock solutions.

2.4 Preparation of Calibration Curve Standards and Quality Control Samples

The calibration curve and quality control stocks were prepared separately by dissolving 40 mg of dapagliflozin in 20 mL of methanol to get 2000 µg/mL. Aqueous linearity solutions (30, 200, 400, 600, 900, and 1200 µg/mL) were obtained by further diluting the calibration curve stock with methanol. Calibration standards in plasma (1.50, 10, 20, 30, 45, and 60 µg/mL) were prepared by diluting the above aqueous linearity solutions with blank plasma. Quality control stock (2000 µg/mL) was diluted with methanol to obtain 30, 90, 600, and 960 µg/mL in order to create aqueous quality control samples. In order to prepare quality control samples in plasma, aqueous quality control samples were diluted with blank plasma to get 1.50 (LLOQ), 4.50 (LQC), 30 (MQC), and 48 µg/mL (HQC).

2.5 Sample Preparation

The protein precipitation method was used to extract dapagliflozin from plasma. The quality control samples and calibration standards in plasma were prepared in large quantities, vortexed for one minute, and kept at -200 °C in a deep freezer. Prior to analysis, all frozen samples were thawed and allowed to equilibrate at room temperature. To 0.5 mL of spiked plasma samples, 50 µL of 320 µg/mL of internal standard was added and vigorously mixed by vortexing for 1 minute. 25 µL of 5% formic acid was added and vortexed for 1 minute. 1 mL of acetonitrile was added and vortexed for 2 minutes. The solution was centrifuged at 4000 rpm for 3 minutes. 0.5 mL of supernatant was collected and injected. Chromatogram of Blank Plasma shown in Fig. 2. Chromatograms of dapagliflozin and IS extracted from plasma are shown in Fig. 3.

2.6 Bioanalytical Method Validation

In accordance with USFDA and EMEA guidelines, the developed method was validated for selectivity, sensitivity, carry over, precision, accuracy, recovery, and stability [34, 35]. The selectivity test was ensured by individual analyses of blank plasma obtained from eight sources, of which six were normal, one was lipemic plasma, and the other was hemolyzed. These results were compared with the lower limit of quantitation (LLOQ) in the same matrix. A sensitivity test was performed by using six different LLOQ prepared from the same plasma sample. The carryover was performed by using the same blank sample but injecting it three times. One injection was made before, and two injections were made after the injection of the upper limit of quantitation (ULOQ). For precision and accuracy, extracted blank, extracted blank with internal standard (IS), calibration curve standards, and quality control (QC) samples (LLOQ, LQC, MQC, and HQC) were prepared. The % accuracy and recovered

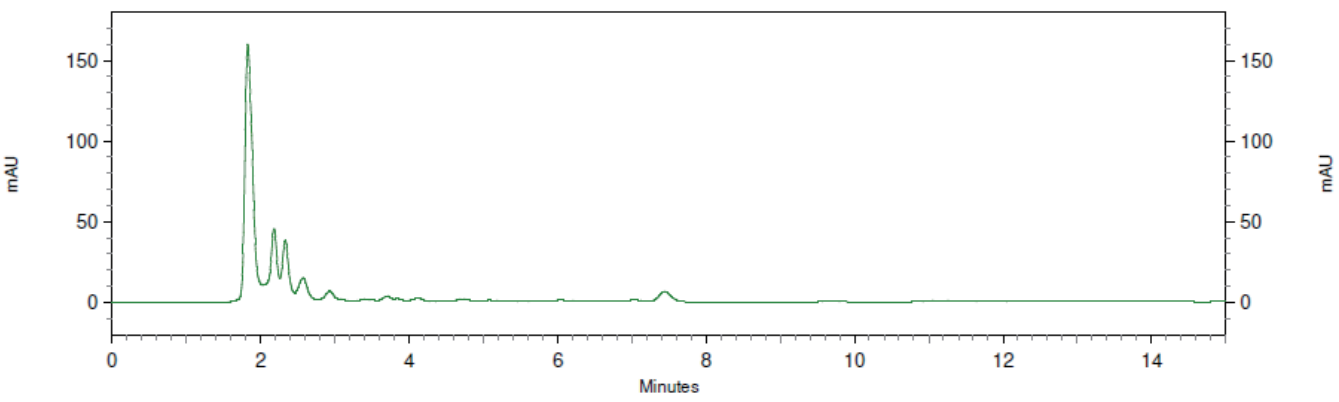

Figure 2: Chromatogram of Blank Plasma

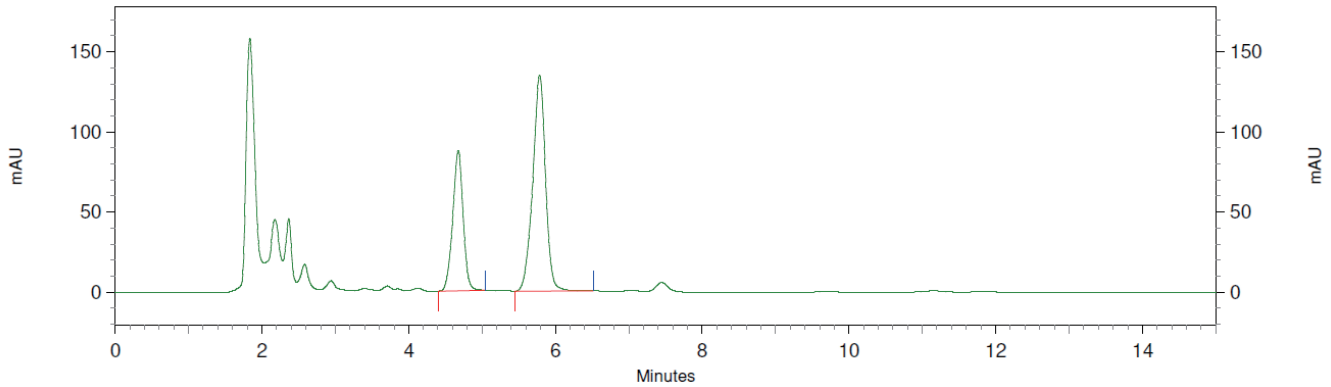

Figure 3: Chromatograms of dapagliflozin (RT. 4.6 min) and azilsartan (RT. 5.7 min) extracted from plasma.

concentration were determined. Area ratio of the analyte/IS vs. concentration was plotted to determine linearity. At LQC, MQC, and HQC, recovery was carried out. Recovery vials were prepared by extracting blanks spiked with an analyte post-extraction.

3. Results and Discussion

The developed method was validated for selectivity, sensitivity, carry over, precision, accuracy, recovery and stability according to USFDA and EMEA guidelines (34, 35).

3.1 Selectivity

Eight sources of blank plasma were analysed separately to ensure the selectivity test, of which six were normal, one was hemolyzed, and the other was lipemic plasma. Plasma lots were prepared as per the blank plasma extraction procedure. The results were compared with the LLOQ in the same matrix By comparing chromatograms of the blank plasma and the spiked plasma samples with the drugs and IS, interferences were analysed. The acceptance criteria were achieved, and as shown in Table 1, the selected plasma lots did not show interference at the retention times (RT) of the IS and the analyte.

3.2 Sensitivity

A sensitivity test was performed by using six different LLOQ prepared from the same plasma sample. The acceptance criteria for the sensitivity were achieved (Table 2), and the % CV was found to be 1.21.

3.3 Carry Over

The carry over was performed by using the same blank sample processed as per the extraction procedure but injected three times. One injection was made before, and two injections were made after the injection of the upper limit of quantitation (ULOQ). The responses of interfering peaks were within limits as compared to responses for spiked plasma samples at LLOQ for dapagliflozin and IS, as shown in Table 3.

3.4 Precision and Accuracy

Precision and accuracy (PA) were established by four QC levels per run (LLOQ, LQC, MQC, and HQC) with six replicates per QC level. The accuracy was determined as recovered concentration / actual concentration x 100. The acceptance criteria for accuracy are ± 15% for nominal concentration, except ± 20% at LLOQ. For the

Table 1: Selectivity blank & LLOQ

Plasma Lot	Area of blank plasma at R.T. of Dapagliflozin	Area of blank plasma at R.T. of IS	Area of Dapagliflozin (LLOQ)	Area of IS (LLOQ)
Lot 1	0	0	685416	9205631
Lot 2	0	0	675859	9351363
Lot 3	0	0	690428	9246503
Lot 4	0	0	679851	9312501
Lot 5	0	0	685968	9265819
Lot 6	0	0	697415	9112519
Haemolyzed	0	0	683519	9045214
Lipemic	0	0	682409	9142516

developed method, linear correlation was found in the range of 1.50–60 µg/mL (r^2 = 0.9994), as shown in Fig. 4. The % CV for the recovered concentrations obtained from six replicates per QC level (LLOQ, LQC, MQC, and HQC) was used to assess precision. LLOQ should have a % CV of NMT 20, and other QC (LQC, MQC, and HQC) should have a % CV of NMT 15. The accuracy and precision data were found to be within the accepted limits. Accuracy and precision data are summarized in Table 4,5,6.

3.5 Recovery

For the recovery study, the areas of extracted samples of dapagliflozin at LQC, MQC, and HQC were determined. Recovery vials at LQC, MQC, and HQC were prepared by extracting blanks spiked with the analyte post-extraction. The % recovery of dapagliflozin in QC samples was calculated as extracted QC samples dapagliflozin area / recovery vial QC dapagliflozin area × 100. The % recovery of IS in QC samples was calculated as extracted QC samples IS area / recovery vial QC IS area × 100. The % CV should be NMT 15. The results were found to be within the accepted limit (Table 7 and Table 8).

3.6 Stability

The stability of dapagliflozin in plasma was evaluated under different conditions. Bench-top stability, freeze-thaw stability, stability of processed extracted samples at room temperature, and stock solution stability were performed at LQC and HQC levels using three replicates at each level. The stability study results were

Table 2: Observation summary of sensitivity

LLOQ	Area of Dapagliflozin	Area of IS
LLOQ 1	675364	9245632
LLOQ 2	682510	9214521
LLOQ 3	690126	9145631
LLOQ 4	685426	9312521
LLOQ 5	699851	9285631
LLOQ 6	690428	9265819
Mean	687284	
SD	8297.238442	
% CV	1.21	

Table 3: Observation summary of carry over

Particulars	Area	% Interference
Blank 1	0	0
ULOQ	17137594	NA
Blank 2	0	0
Blank 3	0	0
LLOQ mean area from sensitivity	687284	NA

Table 4: Accuracy of calibration curve

Standards	Actual Conc. of Dapagliflozin (µg/mL)	Area of Dapagliflozin	Area of IS	Area of Dapagliflozin/ Area of IS	Recovered Conc. of Dapagliflozin (µg/mL)	% Accuracy
Blank	0	0	0	NA	NA	NA
Blank + IS	0	0	9361251	NA	NA	NA
STD A	1.50	691961	9253601	0.0748	1.56	104.00
STD B	10.00	2941897	9215814	0.3192	9.63	96.30
STD C	20.00	6003351	9305234	0.6452	20.38	101.90
STD D	30.00	8769454	9235821	0.9495	30.43	101.43
STD E	45.00	12850180	9416529	1.3646	44.13	98.07
STD F	60.00	17137594	9230108	1.8567	60.37	100.62

Table 5: Accuracy for quality control samples

Set	QC	Actual Conc. of QC	Area of Dapagliflozin	Area of IS	Area of Dapagliflozin/ Area of IS	Recovered Conc. (µg/mL)	% Accuracy
Set 1	LLOQ 1	1.50	680416	9312044	0.0731	1.50	100.00
	LQC 1	4.50	1579353	9241302	0.1709	4.73	105.11
	MQC 1	30.00	8765214	9486514	0.924	29.59	98.63
	HQC 1	48.00	13648129	9142614	1.4928	48.36	100.75
Set 2	LLOQ 2	1.50	693521	9245816	0.075	1.57	104.67
	LQC 2	4.50	1512526	9206524	0.1643	4.51	100.22
	MQC 2	30.00	8612654	9186514	0.9375	30.03	100.10
	HQC 2	48.00	13296524	9316524	1.4272	46.19	96.23
Set 3	LLOQ 3	1.50	702634	9152634	0.0768	1.63	108.67
	LQC 3	4.50	1495639	9364517	0.1597	4.36	96.89
	MQC 3	30.00	8712631	9286514	0.9382	30.05	100.17
	HQC 3	48.00	13752649	9163527	1.5008	48.62	101.29
Set 4	LLOQ 4	1.50	690129	9186524	0.0751	1.57	104.67
	LQC 4	4.50	1505324	9013284	0.167	4.60	102.22
	MQC 4	30.00	8612941	9231624	0.933	29.88	99.60
	HQC 4	48.00	13585614	9386419	1.4474	46.86	97.63
Set 5	LLOQ 5	1.50	692514	9405639	0.0736	1.52	101.33
	LQC 5	4.50	1552419	9365148	0.1658	4.56	101.33
	MQC 5	30.00	8695418	9145214	0.9508	30.47	101.57
	HQC 5	48.00	13256022	9146521	1.4493	46.92	97.75
Set 6	LLOQ 6	1.50	679854	9280136	0.0733	1.51	100.67
	LQC 6	4.50	1497856	9312504	0.1608	4.40	97.78
	MQC 6	30.00	8926517	9316504	0.9581	30.71	102.37
	HQC 6	48.00	13652143	9206527	1.4829	48.03	100.06

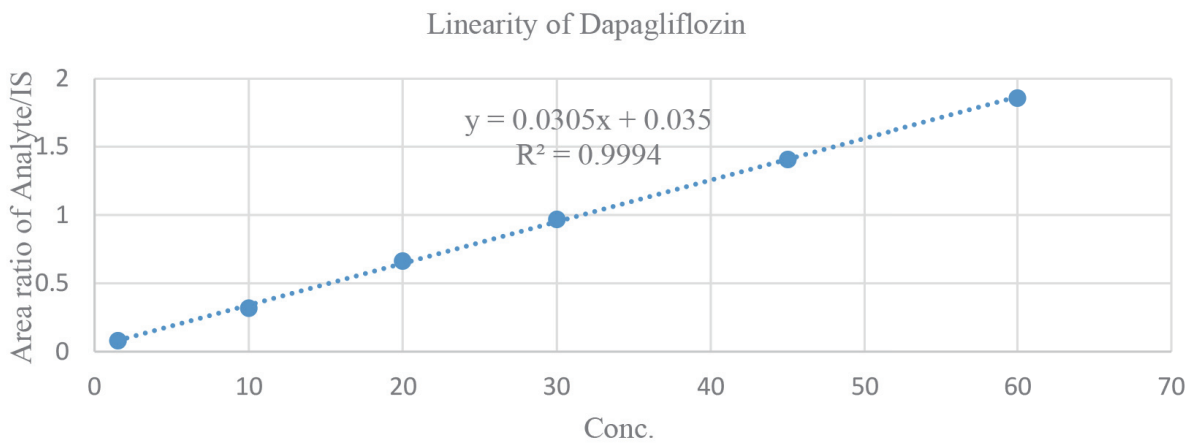

Figure 4: Linearity of Dapagliflozin

found to be within the accepted limit.

3.6.1 Bench top stability

Spiked plasma samples were kept on the bench for 6 hours and processed after 6 hours. LQC and HQC from sets 1 to 3 in the PA batch were processed after 6 hours of drug spiking and injected after processing was completed. Table 9 shows that dapagliflozin is very stable in the current matrix. Since then, nearly 99.42–100.74% mean percentage accuracy has been observed for LQC and HQC samples.

Table 6: Precision for quality control samples

Level	QC	Recovered Conc. (µg/mL)	Average Recovered Conc. (µg/mL)	% CV
LLOQ	LLOQ 1	1.50	1.55	3.19
	LLOQ 2	1.57		
	LLOQ 3	1.63		
	LLOQ 4	1.57		
	LLOQ 5	1.52		
	LLOQ 6	1.51		
LQC	LQC 1	4.73	4.53	3.00
	LQC 2	4.51		
	LQC 3	4.36		
	LQC 4	4.60		
	LQC 5	4.56		
	LQC 6	4.40		
MQC	MQC 1	29.59	30.12	1.35
	MQC 2	30.03		
	MQC 3	30.05		
	MQC 4	29.88		
	MQC 5	30.47		
	MQC 6	30.71		
HQC	HQC 1	48.36	47.50	2.05
	HQC 2	46.19		
	HQC 3	48.62		
	HQC 4	46.86		
	HQC 5	46.92		
	HQC 6	48.03		

Table 7: Recovery of dapagliflozin in QC samples

QC level	Sample no.	Rec. vial Dapagliflozin Area	Extracted QC Dapagliflozin Area	% Recovery	Mean Recovery	% CV
LQC	LQC 1	1735621	1579353	87.39	89.65	2.18
	LQC 2	1764239	1512526			
	LQC 3	1749824	1495639			
	Mean	1749895	1529173			
MQC	MQC 1	9542393	8765214	90.78		
	MQC 2	9556814	8612654			
	MQC 3	9641529	8712631			
	Mean	9580245	8696833			
HQC	HQC 1	14982515	13648129	90.77		
	HQC 2	14905362	13296524			
	HQC 3	14948513	13752649			
	Mean	14945463	13565767			

3.6.2 Freeze thaw stability

Three freeze-thaw cycles were performed on the same sample to assess the stability of the spiked plasma samples. For all the freeze-thaw samples, Low- and high-quality control samples were analyzed in triplicate. The mean percentage accuracy was determined to be 95.26% and 96.37% following three freeze-thaw cycles. (Table 9).

3.6.3 Stability of processed extracted samples at

Table 8: Recovery of IS in QC samples

QC level	Sample no.	Rec. vial IS Area	Extracted QC IS Area	% Recovery	Mean Recovery	% CV
LQC	LQC 1	10253621	9241302	89.45	89.82	1.68
	LQC 2	10386517	9206524			
	LQC 3	10452495	9364517			
	Mean	10364211	9270781			
MQC	MQC 1	10452316	9486514	88.53		
	MQC 2	10563219	9186514			
	MQC 3	10568149	9286514			
	Mean	10527895	9319847			
HQC	HQC 1	9986851	9142614	91.48		
	HQC 2	10052419	9316524			
	HQC 3	10156975	9163527			
	Mean	10065415	9207555			

Table 9: Stability tests for dapagliflozin

QC	Bench top stability		Freeze thaw stability		Stability of processed extracted samples at R.T.		Stock solution stability	
	Accuracy	% CV	Accuracy	% CV	Accuracy	% CV	Accuracy	% CV
LQC 1	105.11		97.11		102.22		98.44	
LQC 2	100.22	4.13	94.44	1.69	101.33	2.35	100.44	2.11
LQC 3	96.89		97.56		97.78		96.22	
HQC 1	100.75		93.96		97.63		97.19	
HQC 2	96.23	2.79	97.9	2.29	97.75	1.37	93.69	2.36
HQC 3	101.29		93.92		100.06		98.19	

room temperature

Processed 3 LQC and 3 HQC were kept on the bench for 6 hours and then injected.

The mean percentage accuracy of three replicates from LQC and HQC was observed at 98.48–100.44% (Table 9).

3.6.4 Stock solution stability

Aqueous LQC and aqueous HQC prepared for the PA batch were kept as such for 24 hours, and after that, new LQC and HQC were prepared from them and injected. Stock solution stability of dapagliflozin and IS showed no evidence of degradation and was recovered at 96.36–98.37% (Table 9).

4. Conclusion

A simple, selective, sensitive, and rapid bioanalytical method was developed for quantitative estimation of dapagliflozin in human plasma. For the first time, a novel bioanalytical method has been successfully developed and validated by HPLC for evaluating dapagliflozin in its pure form in human plasma over a concentration range of 1.5–60 µg/mL. Validation was performed as per USFDA and EMEA guidelines, and all parameters met the acceptable criteria. The developed method has advantages in terms of a shorter retention time (4.7 min) and simple sample pretreatment protein precipitation without drying or reconstitution. The method showed reproducible recoveries for analytes and IS from plasma. Stability studies revealed that the drug was stable. Therefore, it can be concluded that the method is suitable for the quantification of dapagliflozin in human plasma.

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Nil

Conflicts of Interest

The authors declared there is no conflicts of interest.

Abbreviations

SGLT2 - Sodium-glucose cotransporter 2
RP-HPLC – Reverse Phase - High Performance Liquid Chromatography
IS – Internal Standard
QC – Quality Control
DM - Diabetes Mellitus
PCT – Proximal Convoluted Tubule
DAPA – Dapagliflozin
LC-MS/MS – Liquid Chromatography – Tandem Mass Spectrometry
UHPLC – Ultra–High Performance Liquid Chromatography
LLOQ – Lower Limit of Quantification
LQC – Lower Quality Control
MQC – Middle Quality Control
HQC – Higher Quality Control
ULOQ – Upper Limit of Quantification
CV – Coefficient of Variation
SD – Standard Deviation
USFDA – United States Food and Drug Administration
EMEA - European Medicines Agency

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