Journal of Pharmaceutics and Drug Research
JPDR, 6(2): 672-683
www.scitcentral.com
ISSN: 2640-6152
Original Research Article: Open Access

LC-MS/MS Bioanalytical Method of Dapagliflozin in Human Plasma

Sunitha N*, Ramya Sulakshna M and Manohar Babu S

SIMS College of Pharmacy, Mangaldas nagar, Guntur, Andhra Pradesh, India.

Received October 20, 2022; Revised November 20, 2022; Accepted November 23, 2022

ABSTRACT

Dapagliflozin is an anti-diabetic type 2 drug inhibits (SGLT-2) inhibitor. The aim of study is to develop and validate a rapid LC-MS/MS assay of Dapaglifozin in human plasma. The technique was evaluated on the basis of precision, linearity, accuracy, recovery, selectivity and carry over test. The final chromatographic separation was done on a mixture of acetonitrile: buffer (70:30 v/v) with 1.2ml flow rate per mts. The time period for separation is about 1.7 min for analyte and internal standard.

Keywords: Dapagliflozin, LC-MS/MS, Validation, Human plasma

INTRODUCTION

Bioanalytical method plays an important role in separation of drugs and metabolites from sample matric. In preparation technique, cleaning was done before analysis to improve the detection process. The foremost goal of sample preparation is to separate unwanted component in the matrix component that can cause interference during analysis.

The following are the sample preparation techniques

i) Precipitation of proteins

ii) Extraction of liquids

iii) Extraction in solid phase

Optimization of Extraction Procedure [1,2]

The technique cleans the sample before analysis and biological fluids and to improve the detection. Three objectives are followed before separation

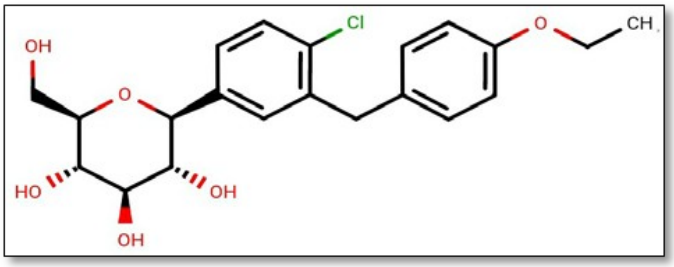
- Analyte extraction with a suitable solvent
- Removal of compounds which are interfered
- Concentration of analyte before.

Dapaglifozin [3,4]

Molecular Weight: 408.873 g /mol

Molecular Formula: $C_{21}H_{35}Cl\ O_6$

Chemical Structure:



Synonym: (2S, 3R, 4R, 5S, 6R)-2-(4-chloro-3-(4-ethoxybenzyl) phenyl)-6- (hydroxymethyl) tetrahydro-2H-pyran-3, 4, 5-triol

IUPAC name: (2S, 3R, 4R, 5S, 6R)-2-{4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl}-6-(hydroxymethyl) oxane-3, 4, 5-triol.

Solubility: Soluble in water

Description: Drug remains pale yellow in color. It prevents glucose re-absorption in kidney.

Storage: Stored at 20˚C. Kept in a firmly locked container.

Molecular Weight: 413.9 g/mol

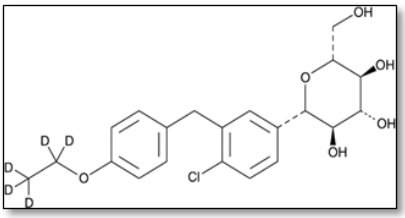
Corresponding author: Sunitha N, Associate Professor SIMS College of Pharmacy Mangaldas Nagar Guntur- 522002, Andhra Pradesh, India, Tel: 09966166153; E-mail: suniadikarb4@gmail.com

Citation: Sunitha N, Sulakshna MR & Babu SM. (2023) LC-MS/MS Bioanalytical Method of Dapagliflozin in Human Plasma. J Pharm Drug Res, 6(2): 672-683.

Dapagliflozin D_5 [5]

Molecular Formula: $C_{21}H_{20}CID_5O_6$

Chemical Structure:



Synonym: (1S)-1, 5-anhydro-1-C- [4-chloro-3- [[4-(Ethoxy-1, 1,2,2,2, d5) Phenyl] methyl] phenyl]-D-glucitol

Chemical Name: Dapagliflozin D5

Solubility: To some extent Solvable in methanol also DMSO.

Description: White solid

Storage: Stored at -20ºC in inert condition

Preparation of reagents: Dissolve 1:1 ratio of methanol and water in appropriate container. Label and store at room temperature.

Mobile Phase Buffer:

Weigh about 315.5 mg of NH_4CO_2 to 1000 ml volumetric flask with a small amount of HPLC grade water and mix well. Then 1 ml of NH_4OH was added and finally make up with the same solvent.

Reconstitution Solution [Acetonitrile/Mobile Phase Buffer (70:30 v/v): 7:3 ratio of Acetonitile and mobile phase buffer to 1000 ml volumetric flask was prepared and kept at room temperature.

Auto sampler Rinsing solution: 1:1 ratio of Acetonitrile and HPLC grade water was mixed and stored at room temperature.

Internal Standard Solution: 1 mg of Dapagliflozin into 1ml DMSO and methanol. Store at 2-8ºC. Protected from light.

ISTD Working solution (2ng/ml): 20 ul of ISTD stock (1 mg/ml) into 100ml volumetric flask with diluent. Store at 2-8ºC.

Linearity determination:

Preparation of stock and spiking solution (1mg/ml)

10 mg of Dapagliflozin is transferred into 10 ml vol. flask, add 2.5ml of DMSO and make up to mark with methanol. Store at 2-8ºC **(Table 1)**.

Table 1. Calibration Curve Samples.

Stock Conc (ng/ml)	Taken Volume	Diluent Volume	Final Volume	Final Conc (ng/ml)	Prepared Spiked Solution
1000000	0.100	4.900	5	20000	STD10
20000	4.000	1.000	5	16000	STD9
16000	3.125	1.875	5	10000	STD8
10000	2.500	2.500	5	5000	STD7
5000	2.000	3.000	5	2000	STD6
2000	2.500	2.500	5	1000	STD5
1000	2.000	3.000	5	400	STD4
400	2.500	2.500	5	200	STD3
200	2.500	2.500	5	100	STD2
100	2.500	2.500	5	50	STD1

Table 2. Standard Calibration Curve (Table 2)

Stock Conc (ng/ml)	Stock Volume	Plasma Volume	Final Volume	Final Conc(ng/ml)	Spiked CC
20000.000	0.100	4.900	5.000	400.000	STD 10
16000.000	0.100	4.900	5.000	320.000	STD 9
10000.000	0.100	4.900	5.000	200.000	STD 8
5000.000	0.100	4.900	5.000	100.000	STD7
2000.000	0.100	4.900	5.000	40.000	STD6
1000.000	0.100	4.900	5.000	20.000	STD 5
400.000	0.100	4.900	5.000	8.000	STD 4
200.000	0.100	4.900	5.000	4.000	STD 3
100.000	0.100	4.900	5.000	2.000	STD 2
50.000	0.100	4.900	5.000	1.000	STD 1

Preparation of Quality Control and Stabilization Samples Preparation of QC stock solution (1.000 mg/ml):

Weighed and transferred 10 mg of Dapagliflozin into 10 ml vol. flask, dissolved in 2.5 ml of DMSO, and made up through methanol. It was mixed well, labelled and kept at 2-8°C, protected from light.

Preparation of QC Spiking Solutions

Quality Control spiking solutions was set (1.000 mg/ml) as designated in the following **Table 3**.

Table 3. Preparation of QC spiking solution samples.

Stock Conc	Volume occupied (ml)	Volume of Diluted (ml)	Final volume (ml)	Final Conc (ng/ml)	Spiked CC
1000000	0.077	4.923	5.000	15400.000	QCH
15400	3.000	2.000	5.000	9240.000	QCM1
9240	0.700	4.300	5.000	1293.600	QCM2
1293	2.500	2.500	5.000	646.300	QCM3
646	1.121	3.879	5.000	145.013	QCL
1000000	0.100	1.900	2.000	50000.000	DIQC

Optimized chromatographic condition (Table 4)

Table 4. Optimized HPLC Parameters.

S No	Equipment/Software	Model
1	Column	Intersil C18, 4.6 × 150mm, 5µm
2	Mobile Phase	Acetonitrile: Mobile phase buffer [70:30]
3	Flow rate	1.2 ml per mts
4	Column temperature	30 ± 5 ºC
5	Vol of injection	10 µl
6	R_t of Analyte	At about 1.70 mts
7	R_t of ISTD	At about 1.70 mts
8	Run time	3.00 mts

Mass Parameters (Table 5)

Ion Source	Turbo spray Ionization
Scan	MRM
Polarity	Dapagliflozin (Negative) Dapagliflozin D5 (Negative)
Dapagliflozin	407.400/329.100 (m/z)
Dapagliflozin D5	412.500/334.30 (m/z)

Table 5. Mass states file parameters.

ESI Source Parameter	Settings
Curtain Gas (CUR)	30 psi
Nebulizer Gas (GS1)	45 psi
Auxiliary Gas (GS2)	45 psi
Ion spray voltage (IS)	4000 psi
Collision Gas (CAD)	6 psi
Temperature (TEM)	500ºC

Compound dependent parameters (Table 6)

Table 6. Compound Depend parameters.

Parameter	Analyte	ISTD
Decl. Potential (DP)	-80 V	-80 V
Colli. Energy (CE)	-20V	-20V
Colli. Pot (CXP)	-10V	-10V
Ent Potential (EP)	-5V	-5V
Dwell Time (m sec)	200	200

Mass spectra's (Figures 1 & 2)

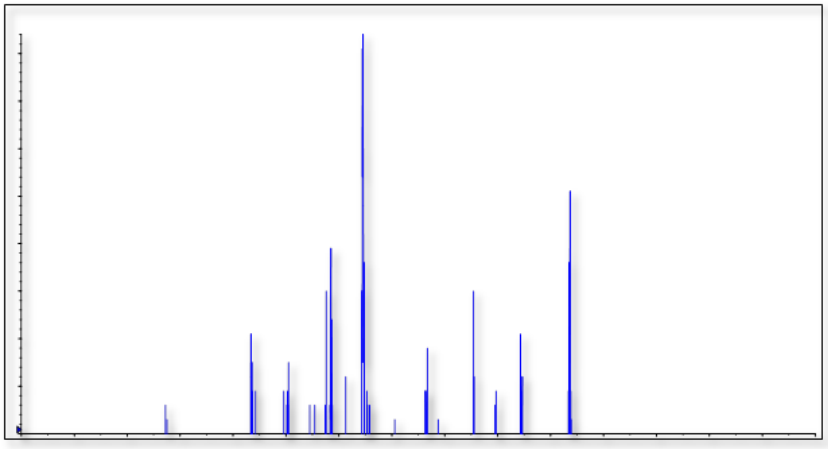


Figure 1. Mass spectra of Dapagliflozin.

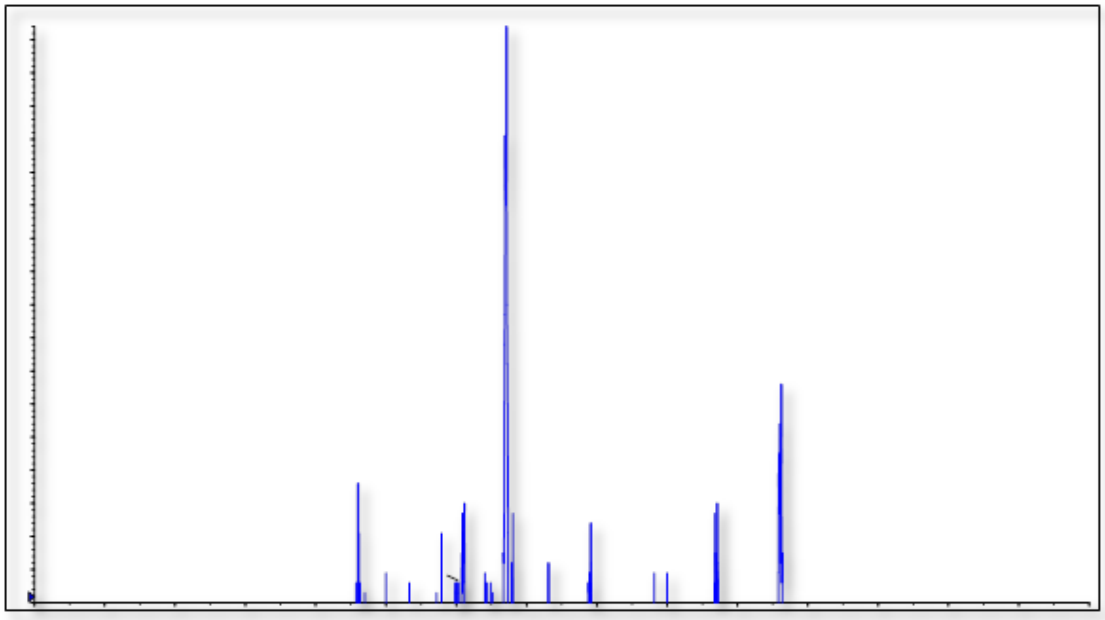


Figure 2. Mass spectra of Dapagliflozin D5.

Extracted Sample Preparation:

Extraction Technique: SPE

Arranged the pre-labeled unfilled tubes as per the lot order and added 50 µl of ISTD working solution, except in STD Blank. Transferred 300.000 µl of plasma from Step-1 into altogether tubes and vortexed for about 10 sec. Then added 200.000 µl of HPLC grade water into all the tubes and vortexed for around 05 sec. Arranged the mandatory number of OASIS HLB cartridges (30 mg/1ml, 1CC) on solid phase extraction manifolds. Conditioned the cartridges with methanol (1ml), 1ml HPLC grade water at low manifold pressure. Loaded about 550.000 µl of prepared samples on conditioned cartridges carefully then washed the cartridges with 1ml HPLC grade water. Dried at high manifold pressure for 2 min eluted the contents with 1.000 ml of Acetonitrile and collected the elution into prelabelled vials. Evaporated all the samples to aridness beneath nitrogen gas at 40 ± 5ºC. Then 200 ul of reconstituted solution is injected into LC-MS/MS.

Method Validation (Figures 3 & 4)

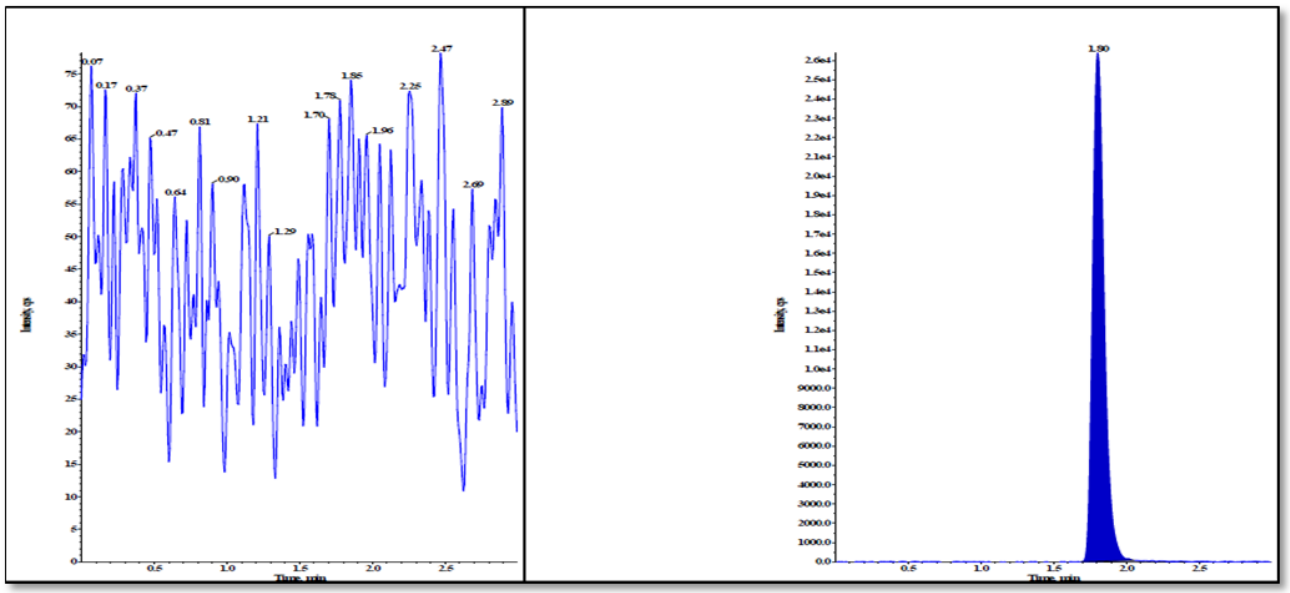


Figure 3. Representative chromatogram of Standard.

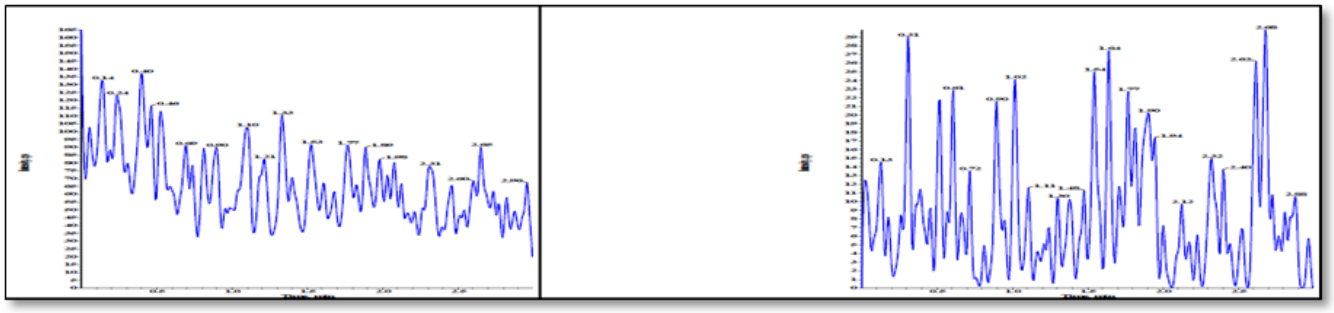


Figure 4. Blank plasma Chromatogram.

Linearity (Table 7)

Table 7. Linearity for Dapagliflozin.

S No	Conc (ng/ml)									
	1	2	3	4	5	6	7	8	9	10
CC	1.010	2.020	4.015	8.100	20.200	40.050	100.520	201.251	350.258	401.289
1	1.119	2.010	4.125	7.560	19.365	39.652	105.365	205.369	350.369	401.258
2	1.056	2.123	4.268	7.965	20.365	40.369	106.358	210.354	345.369	402.175
3	1.023	2.154	3.986	8.520	21.589	42.369	109.365	211.268	355.687	366.987
Mean	1.0660	2.0957	4.1263	8.0150	20.4397	40.7967	107.0293	208.9970	350.4750	390.1400
SD	0.04877	0.07579	0.14100	0.48195	1.11388	1.40808	2.08279	3.17500	5.15982	20.05633
% CV	4.58	3.62	3.42	6.01	5.45	3.45	1.95	1.52	1.47	5.14
% Nominal	105.54	103.75	102.77	98.95	101.19	101.86	106.48	103.85	106.12	97.22

Calibration curve (Figure 5)

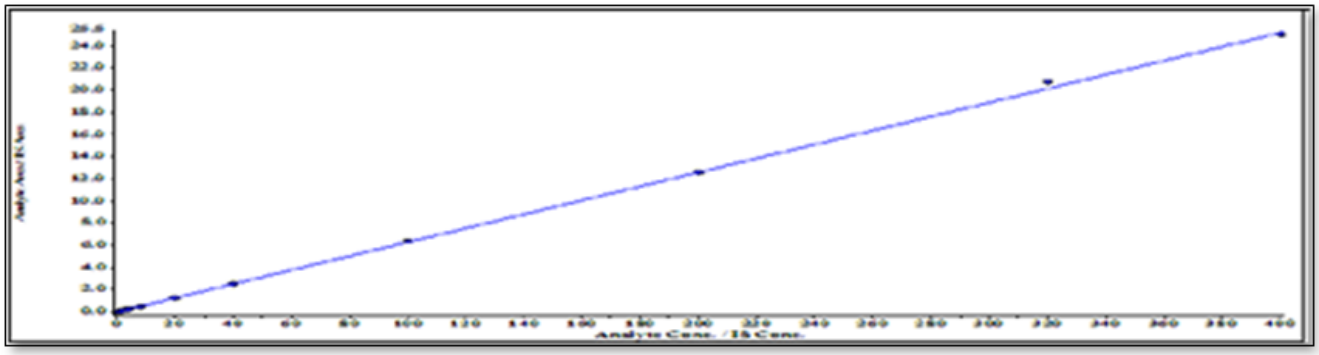


Figure 5. Representative Analyte Calibration Curve in Human Plasma.

Sensitivity

The concentration was kept at 1.010 min for Dapagliflozin was found to be 8.13 % and 102.59 % **(Table 8)**.

Table 8. Sensitivity of Dapagliflozin.

S No	Concentration (ng/ml)
1	1.150
2	0.998
3	1.050
4	1.102
5	0.911
6	1.006
Average	1.0362
SD	0.0828
% CV	8.13
% Mean Accuracy	102.59

Precision and Accuracy

Batch Precision and Accuracy

Precision of Dapaglifozin within for LLOQ QC, QCL, QCM-1, QCM-2, QCM-3 and QCH extended from 2.59% to 5.11%, 3.98% to 7.54%, 5.7% to 6.79%, 5.57% to 5.69%, 2.19% to 2.92%, 1.90% to 3.02% respectively. Accuracy within batch for LLOQ QC, QCL, QCM-1, QCM-2, QCM-3 and QCH extended from 100.47% to 106.01%, 95.49% to 103.59%, 96.01% to 98.16%, 99.88 % to 100.32%, 107.10% to 110.26%, 98.23% to 102.81% respectively.

Stability studies

Extended period stock solution stability for Analyte and ISTD (2-8ºC)

It was done for 7 days 14 h with 19958.425 ng/ml of Dapagliflozin and 101.365ng/ml internal standard **(Table 10)**.

Long term spiking solution stability for Analyte and ISTD (2-8ºC)

Study was carried at 6 days 19 **(Table 11)**.

Reinjection stability

For this study, six sets of QC samples (QCL, QCM 2 and QCH) were analyzed. The % stability at 48 h extended from 98.14 % to 101.33% and CV extended from 1.18% to 8.30% correspondingly **(Table 12)**.

Interday Precision and Accuracy (Table 9)

Table 9. Between batch/Interday results for Dapagliflozin.

S. No	Concentration (ng/ml)					
	LLOQC	QCL	QCM-1	QCM-2	QCM-3	QCH
QC	1.010	2.950	13.025	27.456	192.356	310.256
1	1.023	2.888	12.354	28.654	205.689	306.235
2	0.999	2.564	12.584	26.305	209.365	301.256
3	1.065	2.555	12.985	27.580	210.584	299.654
4	1.008	2.960	13.560	29.654	212.564	302.568
5	1.009	3.010	12.001	25.365	215.689	303.268
6	0.984	2.999	11.550	26.987	218.697	315.687
7	1.023	2.850	12.950	25.365	202.364	318.568
8	1.045	2.658	13.658	27.236	199.365	312.569
9	1.112	2.950	11.964	28.654	202.658	303.987
10	1.150	2.654	13.698	27.654	205.698	320.897
11	1.009	2.930	11.580	29.654	210.368	319.687
12	1.085	2.860	12.690	26.358	215.658	316.587
13	1.020	2.998	13.654	26.354	215.333	322.598
14	1.056	3.025	11.888	27.268	212.654	328.697
15	1.084	3.111	13.560	28.984	205.684	329.687
16	1.068	3.254	13.280	29.684	210.254	315.568
17	1.099	3.058	12.365	25.654	213.658	305.698
18	1.052	2.890	11.965	27.320	199.365	311.547
Mean	1.0495	2.9008	12.6826	27.4850	209.2026	313.0421
SD	0.04451	0.18855	0.76042	1.45379	5.83647	9.34280
C.V %	4.24	6.50	6.00	5.29	2.79	2.98
% Nominal	103.91	98.33	108.76	100.11	97.37	100.90

Table 10. Long term stability for Analyte and ISTD.

S No	Dapagliflozin		ISTD	
	Comp Samples	Stab Samples	Comp Samples	Stab Samples
1	3780234	3768974	125131	132564
2	3754569	3798954	126358	131205
3	3745896	3789546	129564	129564
4	3698756	3768545	130254	130256
5	3702654	3775896	133697	132564
6	3735698	3758962	131254	125897
Mean	3736301.2	3776812.8	129376.3	130341.7
SD	31298.57	14843.60	3166.46	2489.58
% CV	0.84	0.39	2.45	1.91
% Stability	100.67		101.63	

Table 11. Long term spiking solution stability for Analyte and ISTD.

S No	ULOQ		LLOQ		ISTD	
	Comp Sample	Stab Sample	Comp Sample	Stab Sample	Comp Sample	Stab Sample
1	3434567	3612456	9012	8697	124587	130256
2	3564598	3512698	9254	8564	123466	128654
3	3654598	3456875	8987	9956	126458	126546
4	3660256	3602547	8869	9154	123964	122365
5	3612547	3615475	9365	9256	124362	121365
6	3636987	3625478	9365	8965	125698	123654
Mean	3503925.5	3570921.5	9142.0	9098.7	124755.8	125473.3
SD	85483.54	69402.15	213.29	495.56	1118.87	3577.46
% CV	2.38	1.94	2.33	5.45	0.90	2.85
% Stability	98.96		0.68		1.46	

Table 12. Reinjection Stability of Dapagliflozin.

S. No	Reinjection Stability		
	QCL	QCM 2	QCH
QC	2.950	27.456	310.256
1	2.854	28.654	312.547
2	2.774	26.354	309.564
3	2.666	27.265	308.564
4	2.698	29.654	313.254
5	3.124	26.354	315.264
6	3.254	28.654	318.564
Mean	2.8950	27.8225	312.9595
SD	0.24037	1.36828	3.68315
CV%	8.30	4.92	1.18
Mean accuracy	98.14	101.33	100.87

Recovery

Six groups of QCL, QCM-1 and QCH samples were treated and injected (extracted samples). The overall mean recovery of Dapagliflozin was 85.75% with CV extending from 1.31 % to 4.81%. The overall mean recovery of internal standard was 84.14 % with CV extending from 0.87% to 2.89% respectively **(Tables 13 & 14)**.

Table 13. Recovery of Dapagliflozin.

S No	QCH		QCM1		QCL	
	Post Ext Resp	Ext Resp	Post Ext Resp	Ext Resp	Post Ext Resp	Ext Resp
1	3112356	2998756	2056547	1698756	30223	22998
2	3004587	2889754	2105687	1702564	29584	23957
3	3025687	2903564	2069871	1725468	31256	25698
4	3125698	2895687	2096547	1735684	31258	24584
5	3102547	2915487	2115698	1758941	33564	25648
6	3156987	2935680	1993546	1714568	29666	24587
Mean	3087977.0	2923154.7	2072982.7	1722663.5	30925.2	24578.7
SD	59708.98	40460.13	44809.48	22514.67	1487.34	1027.76
% CV	1.93	1.38	2.16	1.31	4.81	4.18
% Mean Recovery	94.66		83.10		79.48	

Table 14. Recovery of Internal standard.

S No	QCH		QCM1		QCL	
	Post Ext Resp	Ext Resp	Post Ext Resp	Ext Resp	Post Ext Resp	Ext Resp
1	155365	130258	158798	130265	160235	134589
2	154879	122654	157897	131254	162543	133564
3	152365	125897	159654	132564	161256	132564
4	150235	129654	155697	131254	159564	139564
5	152897	131254	154860	132564	158635	140222
6	156987	132564	156987	129654	161258	141235
Mean	153788.0	128713.5	157315.5	131259.2	160581.8	136956.3
SD	2422.95	3720.23	1830.93	1180.69	1393.15	3799.38
% CV	1.58	2.89	1.16	0.90	0.87	2.77
% Mean Recovery	83.70		83.44		85.29	

Carryover Test (Figures 6-10)

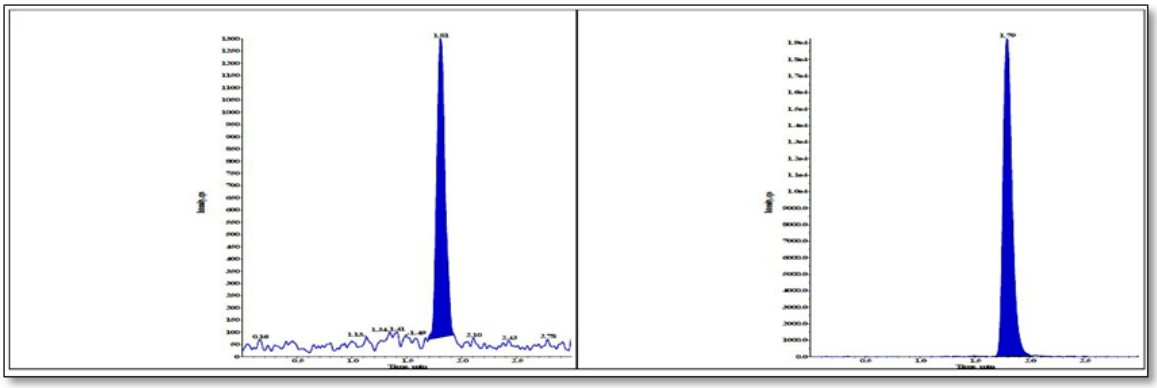


Figure 6. Chromatogram of LLOQ.

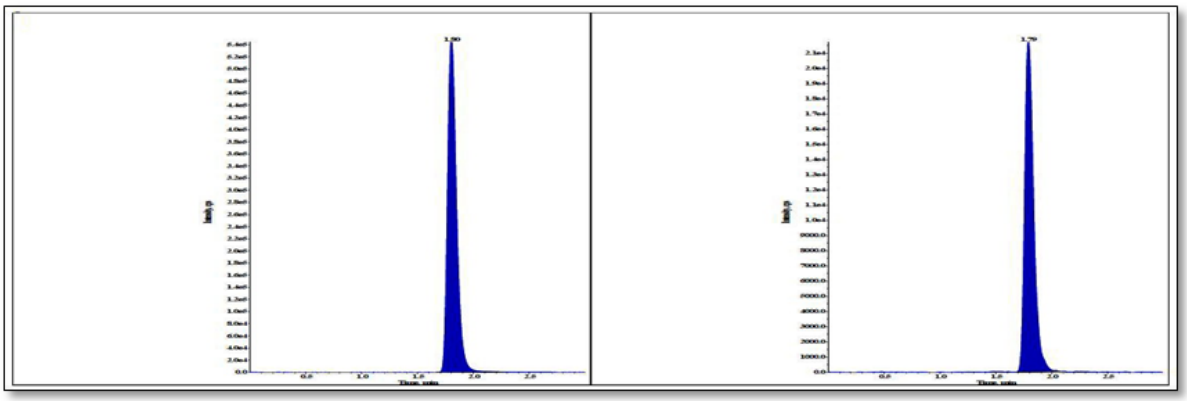


Figure 7. Chromatogram of ULOQ.

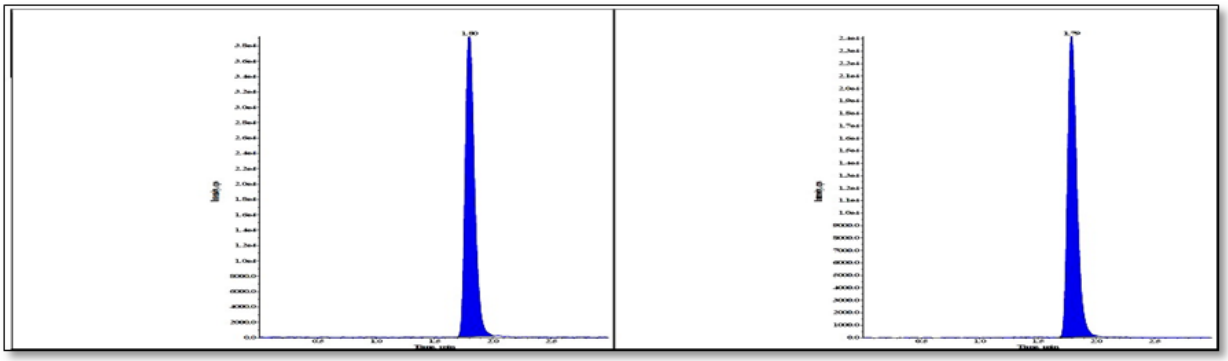


Figure 8. Chromatogram of Plasma QCL.

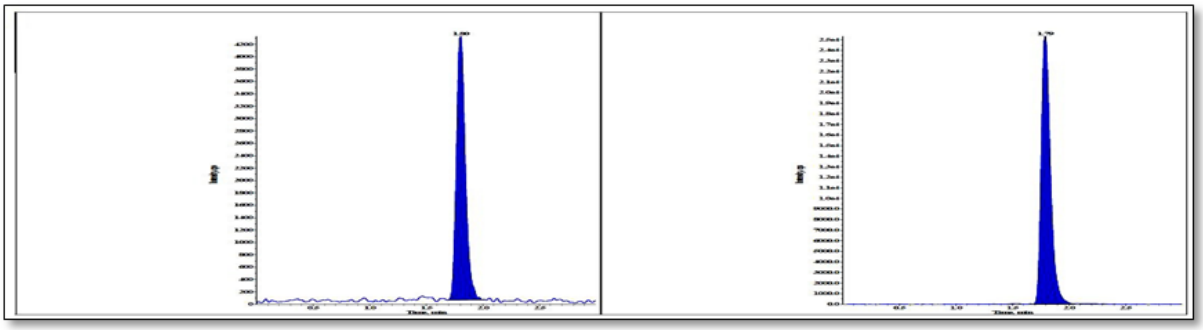


Figure 9. Chromatogram of Plasma QCM2.

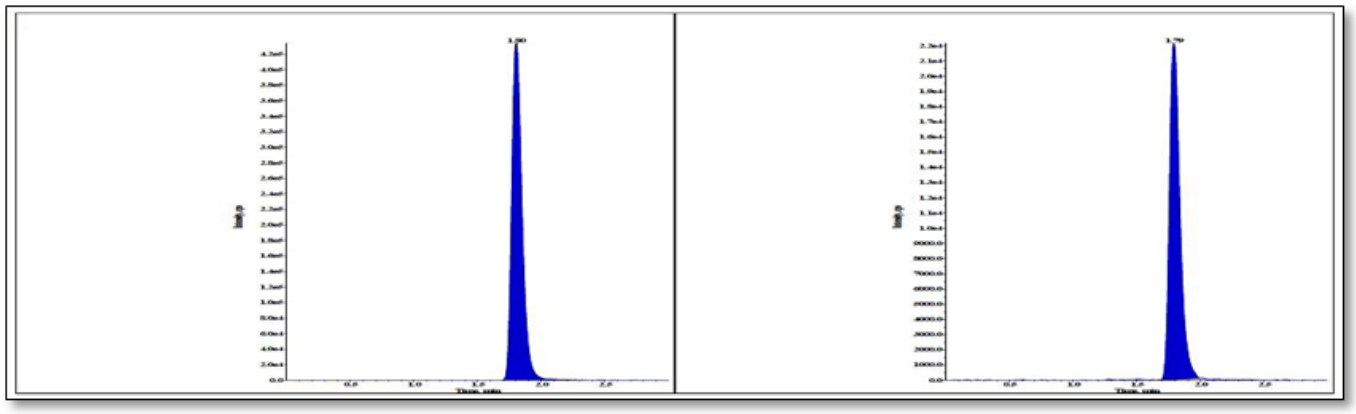


Figure 10. Chromatogram of Plasma QCH Sample.

RESULTS AND DISCUSSION

The study is to develop and validate a rapid LC-MS/MS assay of Dapaglifozin in human plasma. The technique was evaluated on the basis of precision, linearity, accuracy, recovery, selectivity and carry over test. The final chromatographic separation was done on a mixture of acetonitrile: buffer (70:30 v/v) with 1.2ml flow rate per mts. The time period for separation is about 1.7 min for analyte and internal standard.

CONCLUSION

A rapid, small volume LC-MS/MS was developed validated, with high precision, accuracy for plasma quantitation in Dapagliflozin. The simple and high quality easy automated clinically proven studies are carried. The results prove that Dapagliflozin and ISTD continue in autosampler for 67 h 15mts without any loss and the sample is analyzed within the time.

ACKNOWLEDGEMENT

I am very grateful to SIMS College of Pharmacy management for supporting me and providing everything without which it is not possible.

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