Development and Validation of Bio-analytical method for Dapagliflozin in human plasma by LC-MS/MS

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Abstract
When samples are biological fluids such as plasma, serum or urine, this technique is described as Bioanalytical sample preparation. This technique is used to clean up a sample before analysis and/or to concentrate a sample to improve its detection. In the present work development and validation of Bioanalytical method determination of drug from dosage form by HPLC and biological matrices by LC-MS/MS. In this paper Bioanalytical technique for the quantitation of CAN, DAP and OND in human plasma along with stability studies by LC-MS/MS method.

Key words: Analytical method development, Bioanalytical, Dapagliflozin

Introduction

Bioanalytical methods play a major part in separation of drugs and metabolites from sample matrix. In a sample preparation technique, cleaning of sample was done before analysis in order to improve its detection. [1]

Qualitative and quantitative determination of medication and biological samples can be performed by analytical and bioanalytical methods. In recent years bioanalytical method validation plays a key part in determining pharmacokinetic, bioavailability and bioequivalence studies. In demand to get accurate and precise method, Pharmaceutical analysis plays a key part in dosage form and in biological matrices. To ensure the safety of drug to humans, quality control and quality assurance are essential. So, progress of new analytical approaches is vital for drugs and in biological fluids. [2-3]

The purpose of current effort incorporated in the thesis was to develop high performance liquid chromatography (HPLC) and liquid Chromatography Tandem mass spectrometry (LC-MS/MS) intended for estimation of drugs such as antidiabetic,. Validation of analytical methods was done as per ICH guidelines and Bioanalytical validation was done by USFDA and EMA guidelines.

Objective of proposed methods

- To develop and validate appropriate sensitive and specific Bioanalytical technique for the quantitation of CAN, DAP and OND in human plasma along with stability studies by LC-MS/MS method.
- Bioanalytical HPLC technique for MET and CAN in human plasma.
- Analytical methods with stability studies for DAP & SAX, MET & DAP, MET & TEN and SOF &VEL by HPLC

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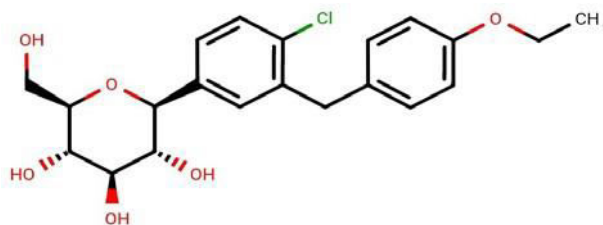**E.mail:** akansharawat8046@gmail.com

Dapagliflozin
Molecular Weight: 408.873 g/mol
Molecular Formula: $C_{21}H_{25}ClO_6$
Chemical Structure:

Synonym : (2S, 3R, 4R, 5S, 6R)-2-(4-chloro-3-(4-ethoxybenzyl) phenyl)-6- (hydroxymethyl) tetrahydro-2H-pyran-3, 4, 5-triol
Chemical name: Dapagliflozin; 461432-26-8; Forxiga; BMS-512148; Farxiga; BMS 512148
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.
Biological Half Life: 12.9 hours.
Bioavailability: 78% (after 10 mg dose)
PKa: 12.57 (strongest acid), 3 (strongest basic).

Material and Method [4-7]
Preparation of Solution and Reagents Diluent [Methanol/Water (50/50, V/V)]
Measured and transferred separately 500 ml of Methanol and 500 ml of water to an appropriately sized container. Assorted well, labelled the solution and stored at room temperature.
Mobile Phase Buffer [0.1% Ammonia in 5mM Ammonium Formate]:
Using a calibrated balance, weighed exactly around 315.15 mg of NH_4HCO_2 quantitatively to 1000 ml flask and small amount of HPLC grade water was mixed, then 1.0 ml of NH_4OH was added, finally it was ended up to final volume with the same solvent.
Reconstitution Solution [Acetonitrile/Mobile Phase Buffer (70/30, V/V)]:
Transferred separately 700 ml of Acetonitrile and 300 ml of mobile phase buffer to an appropriately sized container. The solution was mixed well and kept at room temperature.
Autosampler Rinsing Solution [Acetonitrile / Water (50/50, V/V)]:
Transferred separately 500 ml of Acetonitrile and 500 ml of HPLC grade Water to an appropriately

sized container. The solution was mixed well and stored at room temperature.
Internal Standard Solution Preparation of ISTD stock solution (1.000 mg/ml):
Transferred 1.000 mg of **Dapagliflozin** D4 into one ml vol. flask, dissolved in DMSO and methanol. The solution was assorted well and kept in refrigerator on 2-8 °C, protected from light.
Preparation of ISTD Working Solution (200.000 ng/ml):
By using a calibrated pipette, pipetted out 20 µl of ISTD stock (1.000 mg/ml) into a 100ml vol. flask with diluent, stored in refrigerator at 2-8 °C, protected from light.
Linearity Standards Preparation
Preparation of Calibration Curve stock &spiking solution (1.000 mg/ml):
Weighed and transferred 10 mg of Dapagliflozin into 10 ml vol. flask. The solutionadded with 2.5 ml of DMSO and made up with methanol. The solution stored in refrigerator at 2-8 °C, protected from light. From the CC stock solution (1.000 mg/ml), spiking solution was set as described in the tab below

Table 1: Preparation of 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

The solutions were mixed well, labelled with the batch numbers and kept at 2-8 °C.

Calibration Curve standards preparation:
Spiking solution of CC were described in the following table.

Table 2: CC Standard preparation

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	STD10
16000.000	0.100	4.900	5.000	320.000	STD9
10000.000	0.100	4.900	5.000	200.000	STD8
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	STD5
400.000	0.100	4.900	5.000	8.000	STD4
200.000	0.100	4.900	5.000	4.000	STD3
100.000	0.100	4.900	5.000	2.000	STD2
50.000	0.100	4.900	5.000	1.000	STD1

Distributed 0.400 ml of to each std, kept at -70 ± 15°C, protected from light.

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 2.5ml 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: Preparation of QC spiking solution samples

Stock Conc (ng/ml)	Volume Occupied(ml)	Volume of Dil (ml)	Fin 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	646.800	QCM3
646	1.121	3.879	5.000	145.013	QCL
1000000	0.100	1.900	2.000	50000.000	DIQC

The solutions were mixed well, labelled with the batch numbers and kept at 2-8 °C.

Preparation of spiked QC samples:
QC Samples were prepared as designated in the following table:

Table 4: QC spiked sample preparation:

Stock Conc (ng/ml)	Stock Volume(ml)	Plasma Volume(ml)	Last Volume(ml)	Last Conc (ng/ml)	Spiked QC ID
15400	0.1	4.9	5	308	QCH
9240	0.1	4.9	5	184.800	QCM1

1293.600	0.1	4.9	5	25.872	QCM2
646.800	0.1	4.9	5	12.936	QCM3

Spiked solution	Spiked Volume	Plasma Volume	Final Volume (ml)	Spiked ID
Diluent	0.20	9.800	1.000	Std bulk
145.013	0.1	4.9	5	2.900	QCL

50000.	0.1	4.9	5	1000.000	DIQC

Distributed 0.400 ml of each standard, it was stored at -70 ± 15°C, protected from light.

Preparation of Standard blank samples:
The standard blank was prepared by spiking the Diluent in screened human K2EDTAplasma as designated in the following table

Distributed 0.400 ml of standard, it was stored at -70 ± 15°C, protected from light.
Results and Discussion

Fig. 1: Mass spectra of Dapagliflozin

Fig. 2: Mass spectra of Dapagliflozin D5

In the current study, LC-MS/MS assay was established for Negative ionization was evaluated. The full scan mass spectrum of Dapagliflozin and internal standard in the negative multiple reactions monitoring (MRM) is presented Fig. The consistency of the technique was evaluated on the basis of linearity, precision, selectivity, accuracy, recovery and carryover test. Finally, the chromatographic separation was achieved on a mixture of acetonitrile: buffer (70:30v/v) at a flow

proportion 1.2 ml per mts, bring about separation period of 1.70 min for analyte and internal standard.

Representative chromatogram of aqueous standard analyte was shown below.
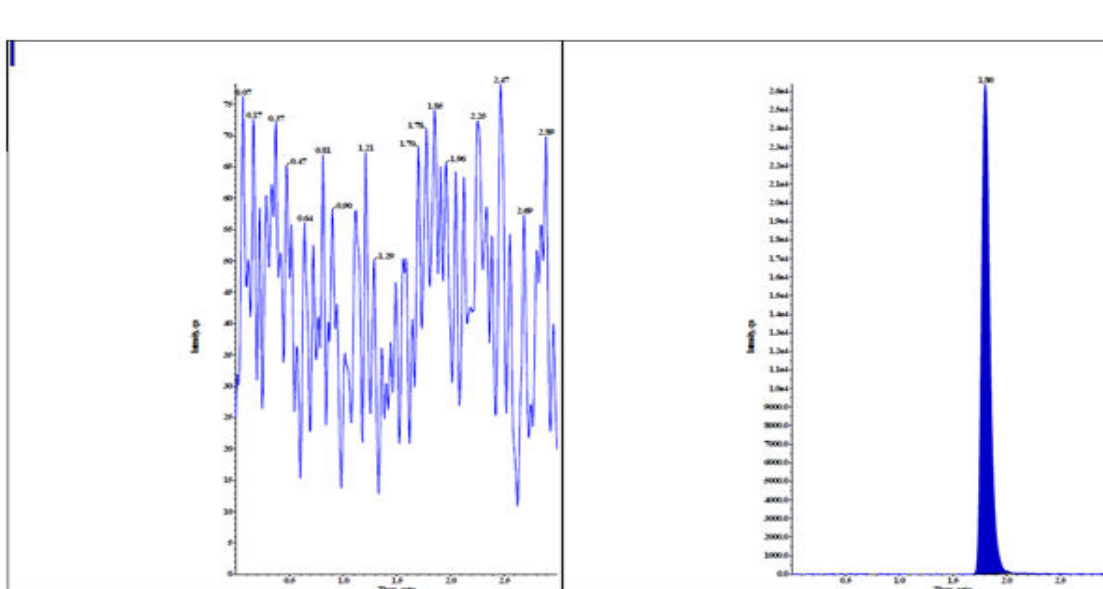
Fig. 3: Representative chromatogram of Standard

Typical chromatograms of extracted, blank plasma from the batches of plasma screened, it was found that there was no intrusion from endogenous components detected at the mass transitions of dapagliflozin and internal standard are given. The results were displayed in Fig.
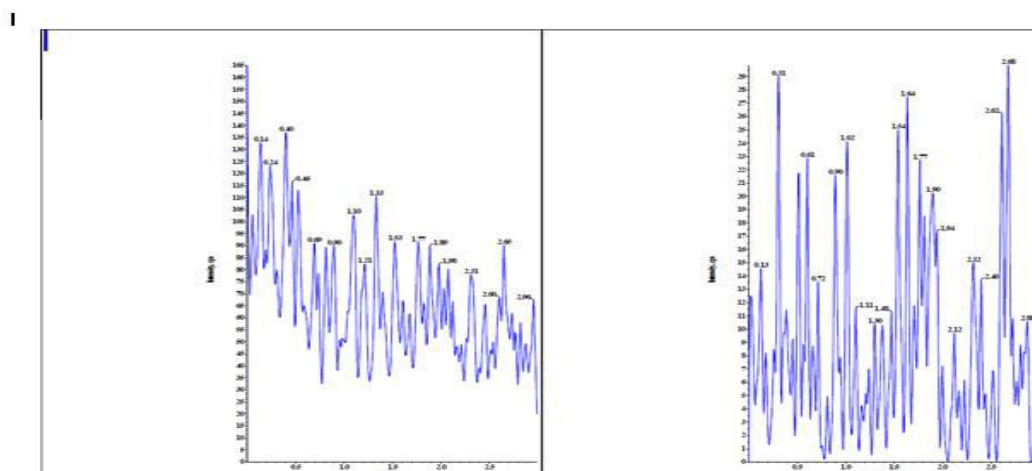
Fig. 4: Blank plasma Chromatogram

The calibration curve for regression analysis was shown in Fig. Correlation coefficient was greater than 0.99 in the concentration range of 1-402 ng/ml for Dapagliflozin. The results are shown in Table.

Table 5: 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	0.140100	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	102.777	98.95	101.19	101.86	106.48	103.85	106.12	97.22

Table 6: Linearity data for Dapagliflozin

S. No	Slope	Intercept	R^2
1	0.0658	-0.007	0.9996
2	0.0568	0.00589	0.9954
3	0.0578	0.00236	0.9978

No substantial consequence was found in altogether eight batches for Dapagliflozin. The precision of internal standard normalized matrix at each level (QCH & QCL) was originated to be 3.84 and 6.63 correspondingly. The precision of ISTD at each level shouldbe less than 15.00. The stated method displayed that no matrix effect was found for plasma and shown in Table.

Table 7: Effect of matrix on Dapagliflozin

S. No	QCL			QCH		
	Analyte area	ISTD area	IS matrix factor	Analytearea	ISTD area	IS matrix factor
1	22068.5	149904.5	0.90	3165546.5	143076.5	0.95
2	24147.0	145927.0	0.99	2992331.5	127977.0	1.01
3	20966.5	146110.0	0.87	3076372.5	134214.5	0.98
4	24148.0	141914.5	1.02	3147586.0	129955.0	1.04
5	23581.0	136064.0	1.04	2930063.0	130955.0	0.97
6	24035.5	139955.0	1.04	3198085.5	133109.0	1.03
7	24143.0	140461.5	1.03	3113176.5	137275.5	0.98
8	23142.5	141957.5	0.98	3414875.0	138600.0	1.06
Mean			0.984	Mean		1.003
SD			0.0652	SD		0.0385
%CV			6.63	%CV		3.84

The concentration of LLOQ for Dapagliflozin was set at 1.010 ng/ml. The exactness andaccurateness for Dapagliflozin was originate to be 8.13% and 102.59%

Table 8: Sensitivity of Dapagliflozin

S. no	Conc (ng/ml)
	LLOQ
	1.010
1	1.150
2	0.998
3	1.050
4	1.102
5	0.911
6	1.006
Avg	1.0362
SD	0.08428
%CV	8.13
%Mean accuracy	102.59

Accuracy defined as ratio of calculated mean values of the LLOQ, low, middle and high-quality control samples to their corresponding nominal values, stated in percentage

Within precision of Dapagliflozin for LLOQ QC, QCL, QCM-1, QCM-2, QCM-3 and QCH extended from 2.59% to 5.11%, 3.98% to 7.54%, 5.70% to 6.79%, 5.57% to 5.69%, 2.19% to 2.92%, 1.90% to 3.02% correspondingly

Within batch accuracy 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% correspondingly.

Table 9: Precision and Accuracy for Dapagliflozin

S. no	Conc (ng/ml)					
	LLOQQC	**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
Mean	**1.0147**	**2.8293**	**12.5057**	**27.4242**	**212.0980**	**304.7780**
S. D	**0.02780**	**0.21336**	**0.71253**	**1.56159**	**4.63672**	**5.77846**
C.V%	**2.74**	**7.54**	**5.70**	**5.69**	**2.19**	**1.90**
%Nominal	**100.47**	**95.91**	**96.01**	**99.88**	**110.26**	**98.23**
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
Mean	**1.0707**	**2.8170**	**12.7567**	**27.4868**	**206.0185**	**315.3825**

S. D	0.05467	0.13062	0.86639	1.54442	6.01429	6.29778
C.V%	5.11	4.64	6.79	5.62	2.92	2.00
%Nominal	106.01	95.49	97.94	100.11	107.10	101.65
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	1.0632	3.0560	12.7853	27.5440	209.4913	318.9658
S. D	0.02750	0.12175	0.80675	1.53413	5.98513	9.64833
C.V%	2.59	3.98	6.31	5.57	2.86	3.02
%Nominal	105.27	103.59	98.16	100.32	108.91	102.81

It was passed out at 7 days 14 hrs for Dapagliflozin (19958.425 ng/ml) and internal standard (101.365ng/ml). The precision for Dapagliflozin was 0.39% and 0.84% correspondingly and % stability was originated to be 100.67%. The precision for Internal standard was 2.45% and 1.91% correspondingly and % stability was originated towards 101.63%

Table 10: Long Term Stock Solution 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**	

Study was carried out at 6 days 19 hrs for Dapagliflozin and internal standard by injectingsix repeats of LLOQ (10ng/ml), ULOQ (7505ng/ml), and internal standard (5000ng/ml). The precision ranged for LLOQ, ULOQ and ISTD was 9.09% to 10.56, 2.99% to 4.78% and 1.51% to 1.68% correspondingly. The % of stability was originated towards 101.62%, 106, 04 % & 99.16% respectively.

**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	**3593925.5**	**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**	

It was carried out by injecting six repeats of QCL (2.950 ng/ml) and QCH (310.256 ng/ml). The precision ranged for QCL & QCH was 2.51 % to 3.78% and 0.81% to 1.00% correspondingly. The % stability for QCL and QCH was originate towards 95.92 % and 108.17% correspondingly.

Table 12: Freeze thaw stability

S. no	QCH		QCL	
	Comp Sample	**Stab Sample**	**Comp Sample**	**Stab Sample**
1	309.456	312.256	2.963	3.021
2	310.265	318.564	2.888	2.955
3	308.564	313.256	2.958	2.889
4	315.123	315.265	3.214	2.956
5	309.654	320.562	3.069	3.110
6	308.265	315.265	2.995	3.001
Mean	**310.2212**	**315.8613**	**3.0145**	**2.9887**
SD	**2.51037**	**3.15905**	**0.11402**	**0.07490**
% CV	**0.81**	**1.00**	**3.78**	**2.51**
%Mean Stability	**100.17**		**95.92**	
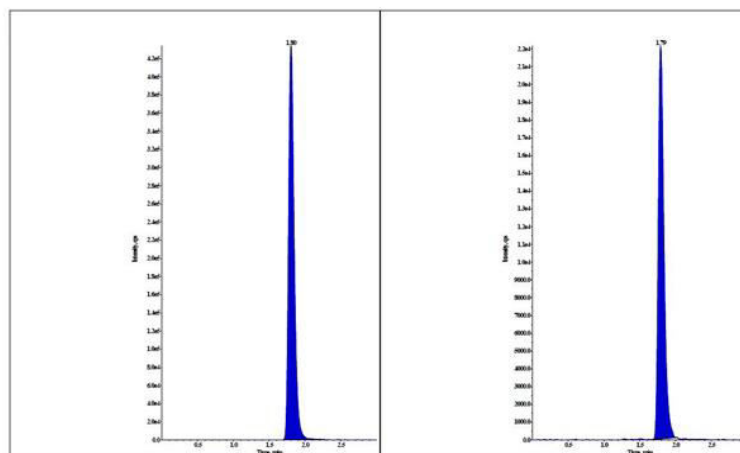
Fig. 5: Chromatogram of Plasma QCH Sample

Conclusion

The present thesis compiled with Bioanalytical method for drugs namely Dapagliflozin in human plasma by LC-MS /MS and Bioanalytical HPLC method for MET Analytical HPLC method for MET & DAP, For Bioanalytical technique both HPLC & LC –MS/MS are used where LC-MS /MS technique have advantage due to shorter runtime. For LC- MS/MS method, Extraction techniques like SPE & LLE are used because of its cost effective. Sensitivity was more in LC –MS /MS and in HPLC it requires more clean-up process. Internal standard such as Dapagliflozin are usedto match with analyte so that matrix effect will be controlled. Due to selectivity and sensitivity LC –MS/MS have more advantage when compared to separation of HPLC. The reported methods can be used further for quality control analysis and pharmacokinetic studies respectively.

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