Development and Validation of a LC-ESI-MS/MS Based Bioanalytical Method for Dapagliflozin and Saxagliptin in Human Plasma

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ABSTRACT

Objective: To develop a new, rapid and sensitive LC-ESI –MS/MS method for the simultaneous estimation of Dapagliflozin and saxagliptin in human K_2EDTA plasma by Liquid –liquid Extraction method (LLE) using deutereated dapagliflozin (DGd2) and saxagliptin (SGd5). **Method:** Chromatographic separation was carried out on a reverse phase hypersil Gold C$_{18}$ (50mmx3.0mm, 5μm) column using mixture of 10 mM Ammonium acetate and methanol (20:80, v/v) at a flow rate of 0.5ml/min in isocratic mode. Quantification was achieved using an electro spray ion interface operating in positive mode, under multiple reaction monitoring (MRM) conditions. **Results:** The method showed excellent linearity over the concentration range of 50.00-10000.00 pg/mL for both the analytes. The intra-batch and inter batch precision (%CV) was ≤4.5% and Matrix effect (%CV) was1.27%, 1.20% for both the analytes. **Conclusion:** The simplicity of the method allows for application in laboratories, presents a valuable tool for bioavailability, bioequivalence, pharmacokinetic studies.

Key words: Application to pk profile studies, Method development, Validation, Dapagliflozin, Saxagliptin, LC-ESI-MS/MS.

INTRODUCTION

Type 2 diabetes is an enduring complication in which blood glucose can no longer be handled. When the cells change into insulin resistant, it feel necessity for more and more insulin to shift sugar into the cells and too much sugar stays in the blood.[1] Dapagliflozin is a sodium-glucose co transporter 2 inhibitor, which inhibits glucose retention in the kidney. Chemical name Dapagliflozin is (2S, 3R, 4R, 5S, 6R)-2-{4-chloro 3(4ethoxyphenyl)methyl]phenyl}-(hydroxymethyl) oxane-3,4,5-triol. It has a molecular formula of C21H25ClO6 and a molecular weight of 408.13. Saxagliptin is an orally effective hypoglycaemic of the advanced dipeptidylpeptidase-4 (DPP-4) inhibitor class of drugs. Chemical name for Saxagliptin is (1S, 3S, 5S)-2-[(2S)-Amino (3-hydroxytricyclo [3.3.1.1(3, 7)] dec-1-yl) acetyl]-2-azabicyclo [3.1.0] hexane-3-car-bonitrile mono hydrochloride. It has a molecular formula of C18H26ClN3O2 and a molecular weight of 351.8.[2] Few methods disclosed are dapagliflozin active pharmaceutical ingredient chromatographic method was carried out by potassium dihydrogen phosphate and acetonitrile.[3] Validated LC-MS/MS methods for the determination of dapagliflozin, a sodium-glucose co-transporter 2 inhibitor in normal and ZDF rat plasma.[4] simultaneous estimation of dapagliflozin in api and pharmaceutical dos age form by development and stability indicating HPLC method.[5] The analyte chromatography was carried by buffer and acetonitrile.[6] LC-MS/MS investigation of metformin,

saxagliptin and 5-hydroxy saxagliptin in human plasma and its pharmacokinetic study with a fixed-dose formulation in healthy Indian subjects.[7] Stability indicating RP-LC-PDA method for the quantitative analysis of saxagliptin in pharmaceutical dosage form.[8] Development of a rapid UPLC-MS/MS method for quantification of saxagliptin in rat plasma and application to pharmacokinetic study.[9] Bio analytical method validation includes all of the procedures demonstrate that method is for biological sample.[10] The method involves methanol and phosphate buffer.[11] Stability-Indicating Liquid Chromatographic Method for Determination of Saxagliptin and Structure Elucidation of the Major Degradation Products Using LC–MS.[12] A New RP-HPLC Method Development and Validation of Dapagliflozin in Bulk and Tablet Dosage Form.[13] Stability indicating RP-HPLC method development and validation for estimation of dapagliflozin and metformin hcl.[14] A mixture of acetonitrile and orthophosphoric acid is used in method.[15] Development and validation of a RP-HPLC method for the estimation of dapagliflozin in api.[16] A rapid and sensitive LC-MS/MS assay for the determination of saxagliptin and its active metabolite 5-hydroxy saxagliptin in human plasma and its application to a Pharmacokinetic study.[17] Acetonitrile and ammonium formate buffer is used for resolution of drugs.[18] Stability indicating validated RP-HPLC technique for the analysis of multi component anti-diabetic drug combos in pharmaceutical dosage forms.[19] The chromatographic separation was achieved by sodium dihydrogen phosphate buffer and acetonitrile.[20] Positive ionization with multiple reactions monitoring by mass spectrometer.[21]

Literature survey reveals that there are no methods reported for simultaneous estimation of dapagliflozin and saxagliptin by any chromatographic methods. Hence, the main objective of the present work was to develop a simple bio analytical method for simultaneous estimation of dapagliflozin and saxagliptin from human plasma and its application to bioavailability, bioequivalence and pharmacokinetics with consideration of accuracy, sensitivity, rapidity, economy, selectivity, stability studies by US-FDA guidelines.

MATERIALS AND METHODS
Chemicals and Reagents

The drugs Dapagliflozin, Saxagliptin**,** Dapagliflozind5 and Saxagliptin d2 were purchased as a gift samples from Symed labs, Hyderabad, India and Toronto research chemicals, Canada. Ethyl acetate, HPLC grade methanol and acetonitrile were obtained from J.T. Baker USA. Sodium dihydrogen phosphate (NaH_2PO_4,

reagent grade), Ammonium acetate (reagent grade) was acquired from Merck Limited, Worli, Mumbai. Human plasma was procured from Doctors labs, Hyderabad, India. Ultra-pure water from MilliQ-system (Millipore) was used throughout the investigation.

Equipment

An API 4000 HPLC-ESI-MS/MS system (Applied Bio systems), 1200 Series HPLC system (Agilent Technologies, Waldron, Germany), data acquisition and processing were adept using Analyst® Software 1.4.1. The chromatographic separation of the analytes was accomplished at 30ºC applying Hypersil Gold C_{18} (50mm x 3.0 mm, 5μm) column. A mixture of 10mM ammonium acetate: Methanol (20:80 v/v) was used as mobile phase. 0.5 mL/min and injection volume of 20 μL. Triple quadruple mass spectrometer equipped with electro spray ionization and handled in positive ionization mode for tracking down and quantification of analytes and internal standards. The intensification of the source and compound parameters are Declustering potential: 40V, entrance potential: 10V, exit Potential: 7, collision energy: 15V for Dapagliflozin and 16V for Saxagliptin .The source criteria were optimized as collision gas: 5, ion spray voltage: 5500V and temperature: 550ºC.

Preparation of calibration standards and quality control samples

Standard Stock solutions of DG (100.0 μg.mL^{-1}), DGd5 (100.0 μg.mL^{-1}), SG (100.0 μg.mL^{-1}), SGd2 (100.0 μg.mL^{-1}) were processed in methanol. From respective stock solution 100.0 ng.mL^{-1} intermediate dilution was prepared in plasma. Aliquots of 100.0 ng.mL^{-1} were used to transfix blank human plasma in order to achieve calibration curve standards of 50.0, 100.0, 500.0, 1000.0, 2000.0, 4000.0, 6000.0, 8000.0, 10000.0 pg/mL. Four levels of QC concentrations at 50.0, 150.0, 3000.0 and 7000.0 pg/mL (LLOQ, LQC, MQC and HQC) were prepared by adopting the different plasma.DGd5 and SGd2was diluted to 10.0 ng.mL^{-1} (Spiked concentration of internal standard) using 50 % methanol and stored in the refrigerator 2-8°C until analysis.

Sample preparation for analysis

Liquid-liquid extraction was carried out to extract the drug and IS for this purpose 100 μL of respective concentration of plasma sample was taken into polypropylene tubes and blended with 50μL of internal standard (10.0 ng.mL^{-1}). This was superseded by addition of 100 μL of 5mM NaH_2PO4 solution and 3.0 mL of ethyl acetate and vortexed around 10 min. Then the Samples were centrifuged at 4000 rpm for 10 min at 20°C. Further, the supernatant was conveyed into

labelled polypropylene tubes and evaporated with nitrogen gas at 40°C. Then the samples were reconstituted with the mobile phase and vortexed for 2 min.

Chromatographic and mass spectrometry conditions

The chromatographic separation of the analytes was accomplished at 30ºC applying Hypersil Gold C$_{18}$ (50mm x 3.0 mm, 5μm) column. A mixture of 10mm ammonium acetate: Methanol (20:80 v/v) was used as mobile phase. 0.5 ml/min and injection volume of 20 μl. Liquid-liquid extraction was selected to refine the drug and internal standard triple quadruple mass spectrometer equipped with electro spray ionization and handled in positive ionization mode for tracking down and quantification of analytes and internal standards. The intensification of the source and compound parameters are Declustering potential: 40V, entrance potential: 10V, exit Potential: 7, collision energy: 15V for Dapagliflozin and 16V for Saxagliptin .The source criteria were optimized as collision gas: 5, ion spray voltage: 5500V and temperature: 550ºC.The mass transitions were preferred as m/z 410.2/250.6, 415.3/250.6, 316.1/272.4 and m/z 318.2/272.3 for quantification of DG, DGd5,SG and SGd2 respectively.

Bioanalytical method validation

The method was validated according to US food and drug administration bio analytical method validation guidelines includes system suitability, selectivity and specificity, LOQ(limit of quantification or sensitivity), injector carryover, linearity, precision and accuracy, recovery, matrix effect, dilution integrity, re-injection reproducibility, ruggedness (analyst and column), sample stability studies were carried out to prove the capability of the proposed method.

Linearity

Calibration standards were prepared to achieve linearity range of 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/mL and assayed in five replicates on five different days and the outcome were depicted in Table 1, Figure 1, 2. The present method was able to quantify lower concentration of dapagliflozin and saxagliptin. The developed standard curve displays correlation coefficient (r^2) greater than 0.9993 with linearity range of 50.00-10000.00 pg/mL using the linear regression model = ax + b; Where, y= Peak area ratio of analyte, X = Concentration (pg/mL) of analyte in plasma, a = Slope, b = Intercept, r^2= Correlation coefficient.

Accuracy and precision

For intra batch and inter batch precision and one set contains four different concentrations of quality control standards of Lower limit QC (50.00 pg/mL), Low QC (150.00 pg/mL), Mid QC (3000.00 pg/mL) and High QC (7000.00 pg/mL) concentrations were prepared in screened human plasma and analyzed each quality control (QC) standard sinsix replicates on the same day. The standard deviation and % coefficient of variation (% CV) was ≤ 15% for LQC, MQC and HQC quality control standards, except LLOQ for which it is ≤ 20%. The results were depicted in Table 2.

Recovery

The extraction recovery was determined in six duplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (150.00pg/mL), medium (3000.00pg/mL), high (7000.00 pg/mL) % recovery of dapagliflozin is 95.13%

| Table 1: Calculated standard concentrations from each calibration curve for determination of DG, SG in spiked plasma.				
Spiked plasma concentration (pg/mL)	Concentration measured(mean) (pg/mL), (*n* = 5)	Precision (CV %) (*n* = 5)	Concentration measured(mean) (pg/mL), (*n* = 5)	Precision (CV %) (*n* = 5)
	Dapagliflozin (DG)		Saxagliptin (SG)	
50.0	51.0 ± 1.3	2.5	51.2 ± 1.0	2.0
100.0	96.6 ± 4.7	4.9	95.8 ± 3.4	3.5
500.0	498.4 ± 24.7	5.0	495.1 ± 26.3	5.3
1000.0	1000.0 ± 17.1	1.7	1010.5 ± 28.3	2.8
2000.0	2013.0 ± 74.6	3.7	2019.1 ± 70.0	3.5
4000.0	4008.4 ± 206.6	5.2	4067.1 ± 224.9	5.5
6000.0	5956.5 ± 190.7	3.2	5628.5 ± 735.7	13.1
8000.0	7952.2 ± 165.6	2.1	8162.8 ± 191.2	2.3
10000.0	10317.1 ± 487.6	4.7	10440.0±521.5	5.0
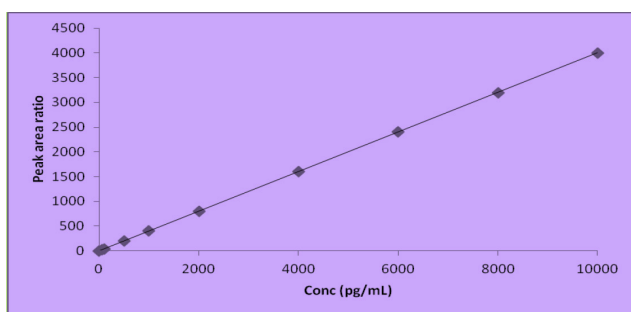
Figure 1: Calibration curve of Dapagliflozin.
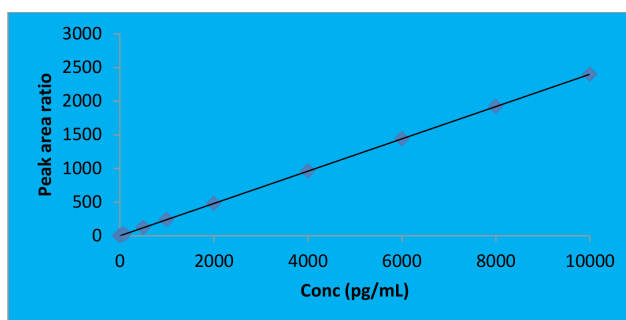
Figure 2: Calibration curve of saxagliptin.

Table 2: Intra batch (Within batch) and Inter batch (Between batches) precision and accuracy of DG and SG.						
Dapagliflozin (DG)						
Spiked plasma concentration (pg/mL)	**Within-run (n=6)**			**Between-run (n=30)**		
	Concentration measured (pg/mL) (mean± S.D.)	**Precision (CV %)**	**Accuracy %**	**Concentration measured (pg/mL) (mean± S.D.)**	**Precision (CV %)**	**Accuracy %**
50.0	51.4±2.3	4.5	102.7	55.5±4.1	7.4	110.6
150.0	152.9±1.4	2.2	105.5	151.9±1.7	1.6	102.2
3000.0	3103.8±102.0	3.3	103.2	3133.0±108.2	3.5	104.3
7000.0	7197.1±89.9	1.2	91.7	7178.7±275.5	1.8	103.9
Saxagliptin (SG)						
50.0	42.4±1.0	2.4	84.8	49.9±6.9	3.8	99.4
150.0	152.6±2.3	1.4	106.4	151.5±1.6	1.2	101.6
3000.0	3072.4±132.6	4.3	102.4	3216.1±162.6	5.1	107.1
7000.0	7160.1±105.8	1.4	98.0	7174.3±123.9	1.1	102.1

and saxagliptin is 93.51%. The standard deviation and % coefficient of variation (% CV) was ≤ 15% for LQC, MQC and HQC quality control standards, except LLOQ for which it is ≤ 20% .The results were depicted in Table 3.

Specificity and selectivity

Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity, specificity. Area response at the retention time of dapagliflozin and saxagliptin in the blank free from potential interference was less than 20% of the LLOQ peak areaof analyte retention time and less than 5% for internal standard retention time.

Sensitivity

Six LLOQ standards were prepared in screened plasma lot along with IS (500.00 pg/mL) and signal to noise ratio (S/N) was calculated using analyst software result given in Table 4.The mean S/N ratio of LLOQ is ≥5.

Injectors carry over

Injector carryover was assessed by injecting the extracted blank samples followed by extracted ULOQ, LLOQ samples and %carry over was calculated the % carry over is 0%. The results were depicted in Table 5.

Matrix effect

The blank plasma in three replicates with un-extracted mid QC (3000.00 pg/mL) were correlated with un-extracted standards of the same concentration the % CV for dapagliflozin is 1.27 and for saxagliptin is 1.20. The results were depicted in Table 6 to 7.

Stability study

Freeze and Thaw stability

The freeze-thaw stability was conducted by comparing the stability samples that had been frozen at −30°C and thawed three times, with freshly spiked quality control samples. Six aliquots each of LQC and HQC concentration levels (150.0 -7000.0 pg/mL) were used for the

Table 3: Recovery of Dapagliflozin.

Conc. (pg/mL)	Extracted DG Peak area	Unextracted DG Peak area
Low QC (150.00 pg/mL)	140596	140250
	140856	135679
	131252	141076
	130767	128436
	141955	148649
	135390	130686
N	6	6
% Recovery	99.67	
SD (±)	4.45	
%CV	4.46	
Medium QC (3000.00 pg/mL)	1997047	2357708
	1920067	2224569
	1934526	2166896
	1910517	2132067
	1892531	2044042
	1822156	2093316
N	6	6
% Recovery	88.26	
SD (±)	2.81	
%CV	3.18	
High QC (7000.00 pg/mL)	4825281	4755582
	4746815	5555112
	4841834	5048175
	4731244	5138559
	5641543	5230888
	5052374	4948616
N	6	6
% Recovery	97.47	
SD (±)	6.06	
%CV	6.37	
Mean %Recovery	95.13	
Mean % CV	6.37	

Table 4: Sensitivity of DG and SG.

Blank human Plasma B. No	GP		ES	
	LLOQ peak area	LLOQ S/N ratio	LLOQ peak area	LLOQ S/N ratio
55-17869 A (Lot.No-1)	17565	26.4	21787	46.7
	26347	27.3	18362	26.3
	21763	35.5	20536	38.5
	27688	34.5	33373	43.1
	27880	34.4	32610	43.6
	24884	33.60	30884	43.20
N	6	6	6	6
Mean		32.00		40.20

Table 5: Injector carryover of DG, SG, DGd5 and SGd2.

Inj. No	Sample	Analyte peak area				Internal standard Peak area			
		DG	SG	% Carry over		DGd5	SGd2	% carry over	
	Extracted blank	0.00		0.00		0.00		0.00	
	Extracted LLOQ	17565	21787			138477	118487		
	Extracted ULOQ	17565015	18565026			168487	128487		
	Extracted blank	0.00	0.00			0.00	0.00		

freeze-thaw stability evaluation. The % accuracy for freeze stability is 104.3%for dapagliflozin, 106.4 % for saxagliptin and thaw stability is 90.4% for dapagliflozin, 94.3% for saxagliptin .Mean % accuracy was found to be within limits 85-115 %.The results were depicted in Table 8.

Auto sampler stability for 70.0h

The auto sampler sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were reinjected after storing in the auto sampler at 20°C for 70.0 h. The reinjection reproducibility was evaluated by comparing the extracted plasma samples that were injected immediately (time 0 h), with the samples that were re-injected after storing in the auto sampler at 20°C for 70 h, each of LQC and HQC concentration levels

Table 6: Assessment of matrix effect of Dapagliflozin.

Blank human Plasma Lot. No	Blank human Plasma B. No	Mid QC (3000.00 pg/mL)		
		Extracted Peak area ratio	Unextracted Peak area ratio	Matrix factor
	55-17869 A	1.410000	1.944950	0.724954
		1.331450	1.896400	0.702093
		1.417700	1.914400	0.740545
	55-17985 A	1.421050	1.874650	0.758035
		1.387600	1.928050	0.719691
		1.334550	1.863950	0.715980
	55-17600 A	1.410000	1.894950	0.744083
		1.331450	1.945950	0.684216
		1.417700	1.875000	0.756107
	55-17975 A	1.421050	1.820900	0.780411
		1.387600	1.817200	0.763592
		1.334550	1.844050	0.723706

(150.0 -7000.0 pg/mL).The %accuracy for dapagliflozin is 108.2- 90.4% and for saxagliptin is 107.8-95.9%. Mean % accuracy was found to be within limits 85-115 %. The results were depicted in Table 8.

Bench top stability at room temperature for 9.5 h

The stability of spiked human plasma samples stored at room temperature bench top stability using standard stock solutions of DG, SG, DGd5, SGd2 (ST stability samples) were set aside on the bench up to 9.5 h and compared with newly prepared stock solutions each of LQC and HQC concentration levels (150.0 -7000.0 pg/mL). The % accuracy was found to be 98.9-81.5% for dapagliflozin and 104.2-92.6% for saxagliptin. The % accuracy was found to be within limits 85-115 %.The results were depicted in Table 8.

Long term stability studies

For long term stability evaluation the concentrations obtained after 91 days were compared with initial concentrations each of LQC and HQC concentration

Table 7: Assessment of matrix effect of Saxagliptin.

Blank human Plasma Lot. No	Blank human Plasma B. No	Mid QC (3000.00 pg/mL)		
		Extracted Peak area ratio	Unextracted Peak area ratio	Matrix factor
1	55-17869 A	1.155650	1.660350	0.696028
		1.168150	1.577550	0.740484
		1.118600	1.614800	0.692717
2.	55-17985 A	1.187000	1.595300	0.744061
		1.181600	1.582550	0.746643
		1.132300	1.556150	0.727629
3.	55-17600 A	1.155650	1.592550	0.725660
		1.168150	1.541650	0.757727
		1.118600	1.616100	0.692160
4.	55-17975 A	1.187000	1.605000	0.739564
		1.181600	1.614700	0.731777
		1.132300	1.582400	0.715559
5.	55-17978 A	1.155650	1.557500	0.741990
		1.168150	1.580400	0.739148
		1.118600	1.596150	0.700811
6.	55-18013 A	1.187000	1.715600	0.691886
		1.181600	1.599750	0.738615
		1.132300	1.667350	0.679102
N		0.722309		18
Mean		0.024		
SD (±)		1.20		
%CV				

| Table 8: Stability of Dapagliflozin and Saxagliptinin spiked human plasma samples.					
Stability experiments	Storage condition	Spiked plasma concentration (pg/ml)	Concentration measured (*n*=6) Mean ± SD	CV (%) (*n*=6)	Accuracy (%)
Dapagliflozin (DG)					
Bench top (Room temperature)	RT 61 hr	150.0	148.3 ± 8.1	5.5	98.9
		7000.0	6728.3±206.3	3.1	81.5
Processed (extracted sample)	Auto sampler 70 hr	150.0	162.3 ± 2.4	1.5	108.2
		7000.0	7536.7±294.5	3.9	90.4
Freeze and Thaw stability	-30°C Cycle-3	150.0	156.5 ± 4.0	2.5	104.3
		7000.0	7381.7±173.4	2.3	90.4
Long term stability	- 30°C, 91 days	50.0	160.3±13.2	8.2	106.9
		7000.0	7450.0±229.1	3.1	90.5
Saxagliptin(SG)					
Bench top (Room temperature)	RT 61 hr	150.0	156.3 ± 8.7	5.6	104.2
		7000.0	7411.7±213.7	2.9	92.6
Processed (extracted sample)	Auto sampler 70 hr	150.0	161.7 ± 4.9	3.0	107.8
		7000.0	7675.0±473.5	6.2	95.9
Freeze and Thaw stability	-30°C Cycle-3	150.0	159.7 ± 7.6	4.7	106.4
		7000.0	7540.0±323.0	4.3	94.3
Long term stability	- 30°C,91 days	50.0	159.0 ± 6.3	4.0	106.0
		7000.0	7608.3±297.2	3.9	95.1

levels (150.0 -7000.0 pg/mL). The % accuracy was found to be 106.9-90.5 % for dapagliflozin and 106.0-95.1 % for saxagliptin. The % accuracy was found to be within limits 85-115 %.The results were depicted in Table8.

RESULTS

Distinctive organic solvents and buffers were optimized to excerpt DG and SG from plasma samples. Optimized method is methanol and ammonium acetate buffer with concentration 10mM. After a course of trials, ethyl acetate and 5mM NaH_2PO_4 buffer were preferred as applicable due to immense recovery efficiency and matrix free interference.

The best results were obtained with ratio (20:80%) of mobile phase composition. Hypersil Gold C_{18} (50mm × 3.0 mm, 5µm) column at 30°c was used to reduce the run time and retention time Figure 3,4. Low volume of flow rate 0.5ml/min was selected to reduce the usage of mobile phase. The MS optimization was achieved by direct infusion of solutions of SG, DG, DGd5 and SGd2 into the ESI source of the mass spectrometer. The mass transitions were preferred as m/z 410.2/250.6, 415.3/250.6, 316.1/272.4 and m/z 318.2/272.3 for quantification of DG, DGd5, SG and SGd2respectively mass transi-

tions are depicted in mass spectrums Figures 5, 6. The method showed excellent linearity over the concentration range of50.00-10000.00 pg/mL for both the analytes, with correlation coefficient value ≥0.999.Intra and inter day precision and accuracy for dapagliflozin and saxagliptin standard deviation and % coefficient of variation (% CV) was ≤ 15% for LQC, MQC and HQC quality control standards, except LLOQ for which it is ≤ 20%. The extraction recovery was determined at three different concentrations of low(150.00pg/mL), medium(3000.00pg/mL), high(7000.00 pg/mL) % recovery of dapagliflozin is 95.13% and saxagliptin is 93.51%.six lots free from interference were selected for assessing the selectivity, specificity area response at the retention time of dapagliflozin and saxagliptin in the blank free from potential interference was less than 20% of the LLOQ peak area of analyte retention time and less than 5% for internal standard retention time. The sensitivity of the developed method was determined from signal to noise ratio (S/N) which was calculated using analyst software result given in Table 4. The mean S/N ratio of LLOQ is ≥ 5.Injector carryover was assessed by injecting the extracted blank samples followed by extracted ULOQ, LLOQ samples the carryover was found to be 0%.There was null or negligible matrix effect. Freeze and thaw stability studies using the
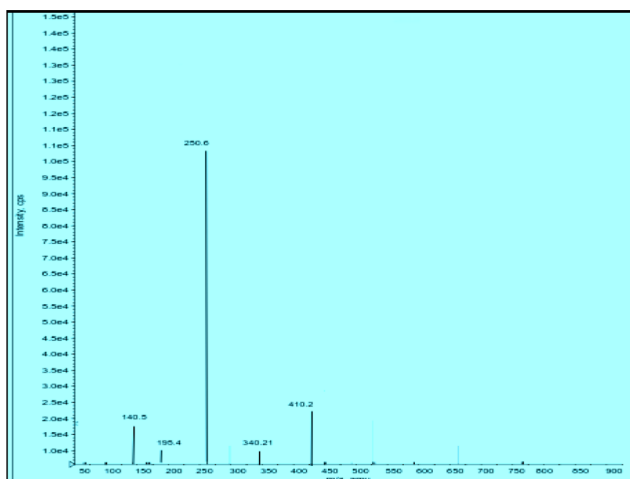
Figure 3: Parent and Product ion mass spectrum of Dapagliflozin.
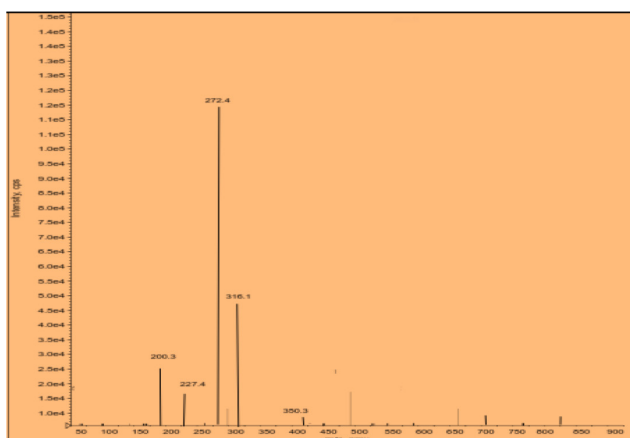
Figure 4: Parent and Product ion mass spectrum of Saxagliptin.

frozen samples at -30°C determined at low and high QC samples showed acceptable limits. The auto sampler sample stability was evaluated for the reinjection reproducibility 20°C for 70 h, each of LQC and HQC concentration levels .The % accuracy for dapagliflozin is 108.2- 90.4% and for saxagliptin is 107.8-95.9%. The bench top stability using standard stock solutions of DG, SG, DGd5, SGd2 (ST stability samples) were set aside on the bench up to 9.5 h and compared with newly prepared stock solutions each of LQC and HQC concentration levels .The % accuracy was found to be 98.9-81.5% for dapagliflozin and 104.2-92.6% for saxagliptin. Long term stability evaluation the concentrations obtained after 91 days were compared with initial concentrations each of LQC and HQC concentration levels (150.0 -7000.0 pg/mL).The % accuracy was found to be 106.9-90.5 % for dapagliflozin and 106.0-95.1 % for saxagliptin.

DISCUSSION

The optimized method showed good chromatographic separation and mass transitions with methanol and ammonium acetate buffer 10mM concentration because of its high volatility as it is required in mass spectrometry analysis. Hypersil Gold C_{18} (50mm x 3.0 mm, 5μm) column at 30°C was used for column efficiency. The basic parameters like ionization type, temperature, voltage, gas parameters such as nebulizer and heater gases, compound parameters like DP, EP, FP, CE and CXP were optimized to obtain a better spray shape and ionization to form the corresponding productions from the protonated SG, DG, DGd5 and SGd2 molecules. The best fit for calibration curve of chromatographic response verses concentration is determined by linear regression model. The data of intra and inter day precision and accuracy for dapagliflozin and saxagliptin from QC samples are summarized in Table 2. The extraction recovery was good, consistent and precise, reproducible with the optimized extraction procedure. The results were depicted in Table 3.Area response at the retention time of dapagliflozin and saxagliptin in the blank free from potential interference was less than 20% of the LLOQ peak area of analyte retention time and less than 5% for internal standard retention time. The injector carry over test showed 0% carry over for the extracted blank samples followed by extracted ULOQ, LLOQ samples. Freeze and thaw stability studies frozen at −30°C and thawed three times showed acceptable limits 85-115%.The auto sampler sample stability20°C for 70 h, each of LQC and HQC concentration levels showed Mean % accuracy within limits 85-115 %.Bench top stability using standard stock solutions of DG, SG, DGd5, SGd2 (ST stability samples) were set aside on the bench up to 9.5 h and compared with newly prepared stock solutions each of LQC and HQC concentration levels. The % accuracy was found to be within limits 85-115 %. Long term stability evaluation was performed for 91 days and compared with initial concentrations each of LQC and HQC concentration levels. The % accuracy was found to be within limits 85-115 %.

CONCLUSION

In conclusion, the recommended research work is highly specific due to the inherent selectivity of tandem mass spectrometry and has significant prevalence over other described methods in previously. The proposed method is able to simultaneously estimate dapagliflozin and saxagliptin in human plasma at very low concentration in pg/mL. High recovery with liquid –liquid extraction method and lesser retention time is time saving and

cost effective when compared with other methods. The simplicity of the method allows for application in laboratories, presents a valuable tool for bioavailability, bioequivalence, pharmacokinetic studies.

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CONFLICT OF INTEREST

No conflicts of interest.

ABBREVATIONS

LQC: Lower quality control**; HQC:** Higher quality control**; LLOQ:** Lower Limit of quantification**; ULOQ:** Upper Limit of Quantification.

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PICTORIAL ABSTRACT
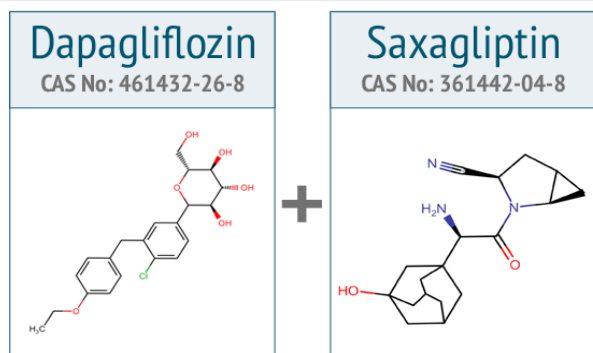
Summary

The proposed method is able to simultaneously estimate dapagliflozin and saxagliptin in human plasma at very low concentration in pg/mL. High recovery with liquid –liquid extraction method and lesser retention time is time saving and cost effective when compared with other methods. The simplicity of the method allows for application in laboratories, presents a valuable tool for bioavailability, bioequivalence, pharmacokinetic studies.

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