

**SIMULTANEOUS ESTIMATION OF SAXAGLIPTIN AND DAPAGLIFLOZIN IN
HUMAN PLASMA BY VALIDATED HPLC-UV METHOD**

**Saxagliptin ve dapagliflozin tarafında doğrulanmış HPLC-UV
yöntemi insan plazma eş zamanlı tahmin**

ESTIMATION OF SAXAGLIPTIN AND DAPAGLIFLOZIN IN HUMAN PLASMA

SAXAGLIPTIN ve DAPAGLIFLOZIN insan plazma tahmini

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Abstract:

Objective: The fixed dose combination of saxagliptin and dapagliflozin, recently approved antidiabetic medication. It is marketed with a brand name Qtern. The intend method aim to develop a simple, rapid, sensitive and validated isocratic reversed phase high performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of saxagliptin and dapagliflozin in human plasma by using linagliptin as internal standard as per US-FDA guidelines.

Materials and methods: The method was performed on Waters 2695 HPLC equipped with quaternary pump. The analyte separation was achieved using Eclipse XDB C18 (150×4.6µ×5mm) column with a mobile phase consisting of 0.1% ortho phosphoric acid and acetonitrile (50:50) with pH adjusted to 5.0 at 1ml/min flow rate.

Results: The analyte was detected at 254nm. Retention time of the internal standard, saxagliptin and dapagliflozin was found at 2.746, 5.173 and 7.218minutes, respectively. The peaks were found to be free of interference. The method is validated over a dynamic linear range of 0.01 to 0.5µg/ml and 0.05 to 2µg/ml for saxagliptin and dapagliflozin, respectively, with a correlation coefficient of 0.998. The precision and accuracy of samples of six replicate measurements at LLOQ level was within limit. The analytes were found to be stable in human plasma at -28°C for 37 days.

Conclusion: The stability, sensitivity, specificity and reproducibility of this method make it appropriate for the determination of saxagliptin and dapagliflozin in human plasma.

Key words: Saxagliptin, Dapagliflozin, Linagliptin, Human plasma, Isocratic.

Özet:

Amaç: Saxagliptin ve dapagliflozin'in sabit doz kombinasyonu antidiyabetik ilaç tedavisi için onaylanmıştır ve Qtern markası ile pazarda yer almaktadır. Bu çalışmada amaç, insan plazmasındaki Saxagliptin ve dapagliflozin'in eş zamanlı tayini için US-FDA klavuzlarına uygun şekilde linagliptin iç standardı kullanarak ve basit, hızlı, hassas ve validasyonu yapılmış izokratik ters faz yüksek performanslı sıvı kromatografi (RP-HPLC) yöntemi geliştirmektir.

Gereç ve Yöntem: Method 4'lü akış pompasına sahip Waters 2695 marka HPLC cihazı ile gerçekleştirilmiştir. Analitin ayrılmasında Eclipse XDB C18 kolon (150 X 4.6µm x

5mm) kullanılmıştır. Kullanılan mobil fazın bileşimi ise pH 5.0' e ayarlanmış %0.1 orto fosforik asit ve asetonytril (50:50) şeklinde olup akış hızı analiz süresince 1 ml/dk'dır.

Bulgular: Analit 254nm'de tayin edilmiştir. İç standart, saxagliptin ve dapagliflozin'in alıkonma zamanları sırasıyla 2.746, 5.173 ve 7.218dk olarak tespit edilmiştir. Pikler interferanslar gözlenmeden elde edilmiştir. Metot validasyonu saxagliptin ve dapagliflozin için sırasıyla 0.01 ile 0.5µg/ml ve 0.05 ile 2µg/ml doğrusal derişim aralığında 0.998 korelasyon katsayısı ile gerçekleştirilmiştir. Numunlere ait 6 ölçüme ait kesinlik ve doğruluk tayin sınırları içerisinde bulunmuştur. Analitlerin insan plazması içinde -28°C sıcaklıkta 37gün boyunca kararlı halde kaldığı belirlenmiştir.

Sonuç: Bu yöntemle elde edilen kararlılık, duyarlılık, özgüllük ve tekrarlanabilirlik sonuçları, geliştirilen bu yöntemin insan plazmasında saxagliptin ve dapagliflozin'in belirlenmesi için uygun olduğunu ortaya koymuştur.

Anahtar kelimeler: Saxagliptin, Dapagliflozin, Linagliptin, İnsan plazma, Isocratic.

INTRODUCTION

The combination of saxagliptin and dapagliflozin has the potential to confer significant benefits in glycaemic control without the risk of weight gain and hypoglycaemia, which may be associated with other medications used to treat type 2 diabetes(1). The fixed dose combination containing 10mg dapagliflozin and 5mg saxagliptin was recently approved by the FDA for adults with type-2 diabetes. The combination was available with a brand name Qtern(2). Dapagliflozin belongs to the sodium glucose co-transporter-2 (SGLT2) inhibitors with a chemical name (2*S*,3*R*,4*R*,5*S*,6*R*)-2-[4-chloro-3-(4-ethoxybenzyl)phenyl]-6-(hydroxymethyl) tetrahydro-2*H*-pyran-3,4,5-triol (fig.1a)(3-6).

Saxagliptin belongs to the class of drugs inhibiting the enzyme dipeptidyl-peptidase-4 (DPP-4). This class of compound works by stimulating glucose-dependent insulin release. Chemically, it is (1*S*,3*S*,5*S*)-2-((2*S*)-Amino(3-hydroxytricyclo(3.3.1.1^{3,7})dec-1-yl)acetyl)-2-azabicyclo(3.1.0) hexane-3-carbonitrile (fig.1b) and with a molecular formula C₁₈H₂₅N₃O₂(7-9). A review of the literature revealed that a few analytical methods like LC-MS(10-11), HPLC (12-19), and spectroscopic methods(20-21) are available for the estimation of these drugs, either individually or in combination with other diabetic drugs like metformin(22-26). The present work aimed to develop a simple, rapid and accurate method for the estimation of dapagliflozin and saxagliptin in human plasma, as per USFDA guidelines (27). Also, the present method is the first for the estimation of this combination in a biological matrix.

MATERIALS AND METHODS

Reagents and chemicals

The pure drug samples of saxagliptin and dapagliflozin were purchased from Selleckchem.com LLC, supplied by Pro lab marketing. HPLC grade acetonitrile, HPLC grade methanol and all other chemicals were obtained from Merck Chemical Division, Mumbai. HPLC grade water obtained from the Milli-Q water purification system was used throughout the study.

Instrumentation

Chromatography was performed with Waters 2695 HPLC provided with a quaternary pump, auto-sampler, column oven, degasser and & 2996 PDA detector to provide a compact and with class Empower-2 software.

Chromatographic conditions

The separation was achieved by isocratic elution using an Eclipse XDB C18 column (150×4.6µm×5mm) with a mobile phase consisting of 0.1% orthophosphoric acid and acetonitrile (50:50) with a pH adjusted to 4.5. The separation was achieved within 10min at 254nm using 1ml/min flow rate. The sample dilution was carried by using a water:acetonitrile (50:50) ratio as a diluent.

Preparation of internal standard

The working standard of internal standard was prepared by transferring 10mg of linagliptin to a 10ml volumetric flask; the final volume was completed using the diluent. From the resulting stock, 10µg/ml solution was prepared by further dilutions.

Preparation of calibration and quality control solutions

The stock solutions of saxagliptin and dapagliflozin were prepared individually by dissolving 10mg of the drug in 10ml of diluent to obtain a 1mg/ml concentration of each analyte. The stock solution was further diluted to prepare working standards. The calibration and quality control samples were obtained by spiking 10µl of the above-prepared solutions of each analyte into 250µL of plasma containing 50 µL of the internal standard. This resulted in final concentrations of saxagliptin and dapagliflozin of 0.01µg/mL to 0.50µg/mL and 0.05µg/mL to 2.00µg/mL, respectively, after spiking them into the plasma.

Sample preparation and extraction

The standard plasma samples, containing 10µl of each analyte and 50 µl of internal standard (10µg/mL), were mixed with 2mL of acetonitrile. The sample tubes were vortexed for 2min. and were then centrifuged at 3200 rpm for 3min. The resultant

organic layer was used for analysis. The resultant organic layer was used for analysis.

Method validation

A thorough and complete method validation was performed following the USFDA guidelines. The method was validated for system suitability, auto-sampler carryover, specificity and screening of the biological matrix, sensitivity, matrix effect, linearity, precision and accuracy, recovery of the analyte and internal standard, ruggedness, reinjection reproducibility and stability. The stability studies include bench top, freeze-thaw and long-term stability at -28°C and -80°C.

Specificity

Specificity of the biological matrix was assessed and screening was performed using six blank standards and lower limit of quantification (LLOQ) level samples. All of the samples were checked to determine the extent of interference by the plasma components with the analyte and internal standard.

Calibration curve

The linearity of the method was determined by analysis of the standard plots associated with an eight-point standard calibration curve. Eight concentrations of saxagliptin and dapagliflozin range from 0.01 to 0.50 µg/ml and 0.05 to 2.00 µg/ml, respectively, were used, which included the LLOQ, LQC, MQC, HQC and ULOQ. The calibration curve was constructed by plotting the peak area ratio of the analytes to the internal standard against standard concentrations. The percentage difference of back-calculated concentrations to the nominal concentration (distribution of the residuals) was determined to validate the correlation. The acceptance criteria for the calibration model applied if residuals were within 15% for all calibration levels except LLOQ. Correlation coefficient, slope and intercept were determined to evaluate the calibration curve.

Accuracy and Precision

Intra-day precision and accuracy were assessed at the lower, middle, high and lower limit of quantification quality control samples LQC, MQC, HQC and LLOQ in six

replicates for both of the analytes, while inter-day precision and accuracy were assessed for three consecutive days by using quality control samples. Mean values were obtained for calculated drug concentration over these batches. The accuracy of the analytical method describes the closeness of the mean test results obtained by the method to the actual value of the analyte and was determined by replicate analysis of the analyte. The accuracy and precision were calculated and expressed in terms of % mean accuracy and coefficient of variation (% CV), respectively.

Recovery

Recovery of the analytes from the extraction procedure was performed at LQC, MQC, and HQC levels. It was assessed by comparing the peak area of the extracted samples (spiked before extraction) to the peak area of the unextracted samples (quality control working solutions spiked in extracted plasma).

Ruggedness

Ruggedness refers to the ability of an analytical method to remain unaffected by small variations. Parameters were used to evaluate the constancy of the results when external factors such as analyst, laboratory, instrument, reagents and days are varied. The ruggedness of the method assessed was determined using different analysts and on different instruments of the same make.

Sensitivity

Sensitivity is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision. Sensitivity experiments were carried out by using six replicates of the LLOQ level sample to determine the lowest limit of detection, the % mean accuracy and the % coefficient of variation.

Stability

Stability studies were performed by keeping replicates of plasma samples at room temperature for 24hrs. The freeze-thaw stability of the drugs in plasma samples were studied over three freeze-thaw cycles, by thawing at room temperature for 2-3 h and

refrozen for 12-24 h. The stability of the drugs in plasma was also tested after storage at -80°C. The concentration of the drugs after each storage period was related to the initial analyte concentrations of freshly prepared samples. Samples were considered stable if the assay values were within the acceptable limits of accuracy and precision.

RESULTS

Method optimisation

To obtain the best results, different mobile phase compositions containing phosphate buffer systems with varied pH and organic solvents like methanol and acetonitrile were used to provide adequate sensitivity and selectivity in a short separation time. The best results were obtained with the mobile phase consisting of 0.1% phosphoric acid (pH 4.5) and acetonitrile (50:50) with a flow rate of 1ml/min. The detection was monitored at 254nm. With these conditions, the retention times of linagliptin, saxagliptin and dapagliflozin were found to be 2.784, 5.295 and 7.204minutes, respectively.

Method validation

System suitability and auto-sampler carry over

System suitability was assessed by using the MQC level sample as six homogenous injections. The %coefficient of variation for retention time and response was calculated. The results were presented in table 1. The obtained values are lower than 1%, which shows the suitability of the system for the analysis of the selected combination in human plasma.

Auto-sample carryover was assessed by ULOQ (upper limit of quantification) and LLOQ levels to ensure that it did not affect the accuracy and precision. There was no carryover observed.

Linearity

The ratio of peak area of analyte to internal standard was used for construction of the calibration curve. The linearity of saxagliptin and dapagliflozin was established by an eight-point calibration curve. The most variable regression equation of the calibration

curve for saxagliptin and dapagliflozin was $y = 0.126(\pm 0.02)x + 0.003$ & $y = 0.53(\pm 0.02)x - 0.002$, respectively. The linearity of the calibration graph was validated by the high value of the correlation coefficient with an average value of 0.996 and 0.998 for saxagliptin and dapagliflozin, respectively. The standard curves of saxagliptin and dapagliflozin are presented in fig. 2.

Precision & Accuracy

The precision and accuracy of the methods were assessed by analysing six replicates of LLOQ, LQC, MQC and HQC levels. The accuracy of the method was determined by calculating the % mean accuracy and the precision by calculating relative standard deviation (RSD). The data regarding precision and accuracy are summarised in table 2. The chromatogram of quality control samples is shown in fig. 3. The % mean accuracy of saxagliptin and dapagliflozin ranged from 98.33 to 101.29 and from 98.43 to 103.28, respectively.

Recovery

Recovery of saxagliptin and dapagliflozin was determined by comparing the mean peak areas of six replicates of three quality control samples (HQC, MQC & LQC) with the mean peak areas of unextracted quality control samples at the same level. The results of the recovery study are given in table 3. The results are within the acceptance limits.

Ruggedness

The present method showed good ruggedness when the method was performed using different analysts and on different instruments of the same make. The results of the ruggedness study were found to be within acceptable limits, proving no significant analyst-to-analyst and instrument-to-instrument variation and hence the ruggedness of the method. The results are presented in table 4.

Stability

The stability of the analytes in human plasma was assessed by the analysis of six replicates of quality control samples at low and high concentration levels at room

temperature over 24 hours (bench top stability). The measured concentrations were compared with those of freshly prepared and processed samples. The results obtained indicated that the two drugs saxagliptin and dapagliflozin were stable for at least 24 hours in human plasma when retained at room temperature. On the other hand, the results obtained for quality control samples subjected to long-term stability at -28°C for 37 days and at -80°C indicate the stability of analytes in human plasma. In contrast, the freeze-thaw stability determined by using LLOQ, LQC, MQC and HQC level of samples also indicated the stability of analytes in human plasma. The results obtained are compiled in table 5.

DISCUSSION

Since there is no reported sensitive method for the estimation of saxagliptin and dapagliflozin as a combination, the validated LC-UV method was developed for routine analysis in a biological matrix. Moreover, the available methods were developed to assess drugs either individually or in combination. Therefore, there is a need to develop an analytical method for the estimation of this combination. The current method aims to develop a simple, accurate and reliable method for the simultaneous estimation of saxagliptin and dapagliflozin in human plasma. Good resolution and minimum tailing was achieved by using this method. The method used a simple single step protein precipitation with acetonitrile and provided good selectivity when tested for peak interference from endogenous sources by comparing the blank chromatogram with quality control samples. The retention times of the internal standard, saxagliptin and dapagliflozin were found to be 2.746, 5.173 and 7.218 minutes, respectively. The developed method proved to be rugged and had adequate recovery and no matrix effect. The recovery was determined by comparing the extracted sample with the unextracted samples at three quality control sample levels i.e., LQC, MQC, LLOQ. The results were found to be within acceptable limits. The linearity of the method was tested by developing an eight-point calibration curve which included all quality control sample concentrations. The linear range for saxagliptin and dapagliflozin was found to be 0.01 to 0.50µg/ml and 0.05 to 2.00µg/ml, respectively. The regression coefficient and equation for saxagliptin and dapagliflozin was 0.996 and 0.998, respectively. The linear

range and statistical parameters prove that the developed method is more sensitive than the reported liquid chromatography coupled with a PDA detector. Using the stability studies, it was found that analytes were stable in plasma throughout the analysis period. The stability data were built by comparing the stability samples with freshly prepared samples. On the other hand, long-term stability was established by subjecting quality control samples to -28°C for 37 days and to -80°C . The results obtained indicate that the method is sensitive, reliable and a cost-effective method. Furthermore, the method can be made applicable for pharmacokinetic estimation.

CONCLUSION

The proposed method for the estimation of a saxagliptin and dapagliflozin binary mixture in human plasma is simple, accurate and reliable. The single-step protein precipitation, short runtime of 10 minutes and isocratic elution make the method economical and suitable for the analysis of a large number of samples. The method has been validated as per the requirements of the US-FDA. It can therefore be concluded that the method is suitable for the routine quantification of saxagliptin and dapagliflozin in human plasma.

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Table 1: Statistical analysis of system suitability parameters

Parameter	Internal Standard	Saxagliptin	Dapagliflozin	Acceptance
Retention time (t_R)	0.50	1.07	0.49	% RSD \leq 2
Area under the peak	0.88	0.35	0.98	% RSD \leq 5
Resolution ($R_s=2[t_{R2}-t_{R1}] / [W_1+W_2]$)	-	10.95	7.11	$R_s > 2$
Number of theoretical plates ($N=16 \times [t_r/W]^2$)	7238	9817	9758	Increases with efficiency of the separation.
Tailing Factor (T)	1.13	1.12	1.08	$T \leq 2$
HETP (H= L/N cm/plate)	0.005	0.015	0.0038	Smaller the value, higher the column efficacy
Capacity factor ($K'=[t_R-t_M]/t_M$)	1.11	2.98	4.55	1-10

Table2: Intra and inter day precision and accuracy summary *n=18

Added conc. (µg/ml)	Saxagliptin				Dapagliflozin			
	0.40	0.02	0.040	0.010	1.60	1.00	0.2	0.050
Between-batch (n=18)								
Mean	0.4038	0.0199	0.0394	0.0098	1.6127	1.0017	0.2019	0.0496
SD	0.0196	0.0015	0.0021	0.0008	0.0743	0.0763	0.0103	0.0037
%RSD	4.85	7.29	5.31	7.99	4.61	7.62	5.12	6.97
% CV	100.94	99.44	98.61	98.33	100.79	100.17	100.95	99.26
Day 1 (n=6)								
Mean	0.4030	0.0197	0.0395	0.0098	1.6128	1.0085	0.2018	0.0493
SD	0.0217	0.0012	0.0016	0.0008	0.1138	0.0915	0.0097	0.0037
%RSD	5.40	6.16	4.16	7.66	7.05	9.08	4.83	7.44
% CV	100.75	98.33	98.75	98.33	100.80	100.85	100.92	98.67
Day 2 (n=6)								
Mean	0.4052	0.0200	0.0395	0.0098	1.6117	1.0328	0.2062	0.0492
SD	0.0107	0.0017	0.0027	0.0008	0.0534	0.0525	0.0114	0.0029
%RSD	2.65	8.37	6.93	7.66	3.31	5.08	5.52	5.90
% CV	101.29	100.00	98.75	98.33	100.73	103.28	103.10	98.43
Day 3 (n=6)								
Mean	0.4032	0.0200	0.0393	0.0098	1.6135	0.9637	0.1977	0.0503
SD	0.0267	0.0017	0.0022	0.0010	0.0545	0.0755	0.0098	0.0042
%RSD	6.61	8.37	5.49	10.00	3.38	7.83	4.94	8.40
% CV	100.79	100.00	98.33	98.33	100.84	96.37	98.83	100.67

Table 3: Extraction recovery data from human plasma * n=6

Analyte	Nominal concentration (µg/ml)	% Recovery	% RSD*
Saxagliptin	0.04 (LQC)	76.40	1.58
	0.20 (MQC)	88.07	0.21
	0.4 (HQC)	71.60	2.08
	Across mean	78.689	8.469
Dapagliflozin	0.2 (LQC)	82.82	0.17
	1.0 (MQC)	78.76	0.93
	1.6 (HQC)	82.79	0.96
	Across mean	81.458	2.86
Internal standard	0.1	82.22	0.46

Table-4: Ruggedness data * n=6

Parameter	Saxagliptin				Dapagliflozin			
	HQC	MQC	LQC	LLOQ	HQC	MQC	LQC	LLOQ
Different Column								
Mean	0.401	0.195	0.039	0.010	1.597	1.008	0.198	0.050
SD	0.027	0.011	0.001	0.008	0.119	0.052	0.011	0.004
% CV	6.66	5.38	2.98	7.66	7.47	5.12	5.31	7.90
% Mean Accuracy	100.17	97.50	97.92	98.33	99.79	100.77	98.83	100.00
Different Analyst								
Mean	0.404	0.195	0.040	0.010	1.614	1.006	0.201	0.049
SD	0.018	0.0164	0.002	0.001	0.070	0.071	0.009	0.002
% CV	4.39	8.43	3.84	8.94	4.35	7.02	4.45	4.71
% Mean Accuracy	101.08	97.50	98.75	100.00	100.86	100.63	100.33	98.33

Table5: Stability data of saxagliptin and dapagliflozin in human plasma

Storage conditions	Saxagliptin		Dapagliflozin	
	LQC	HQC	LQC	HQC
Bench top stability				
Mean calculated concentration (µg/ml) ± SD	0.0402±0.0017	0.4033 ± 0.0175	0.1952 ± 0.0111	1.5783 ± 0.0686
%CV	4.29	4.34	5.67	4.35
% Mean accuracy	100.42	100.83	97.58	98.65
Freeze thaw stability (after 3 cycles)				
Mean calculated concentration (µg/ml) ± SD	0.0379 ± 0.0023	0.3998 ± 0.0188	0.1903 ± 0.0273	1.5639 ± 0.0904
%CV	3.76	8.56	7.34	4.35
% Mean accuracy	98.78	99.47	97.89	99.90
Stability at -28°C (long term stability)				
Mean calculated concentration (µg/ml) ± SD	0.0398±0.0012	0.4013±0.01328	0.1920±0.01279	1.6110±0.0718
%CV	2.93	3.31	6.66	4.46
% Mean accuracy	99.58	100.33	96.00	100.69
Stability at -80°C (long term stability)				
Mean calculated concentration (µg/ml) ± SD	0.0400±0.0015	0.4018±0.0098	0.1968±0.01347	1.6120 ± 0.0830
%CV	3.87	2.43	6.84	5.15
% Mean accuracy	100.00	100.46	98.42	100.80

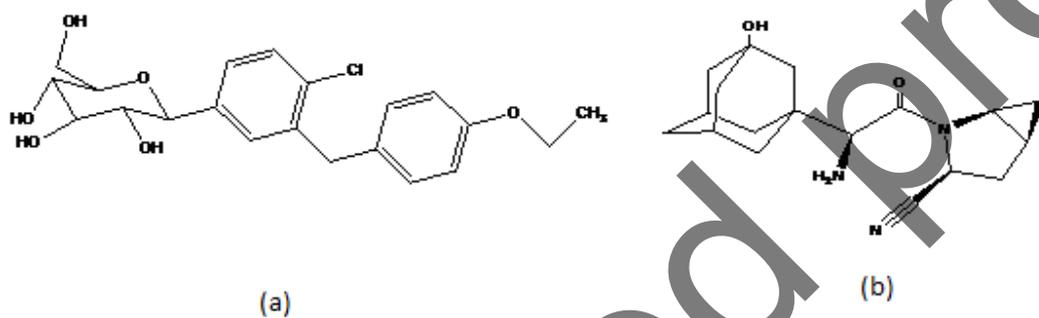


Fig. 1: Chemical structure of dapagliflozin (a) and saxagliptin (b).

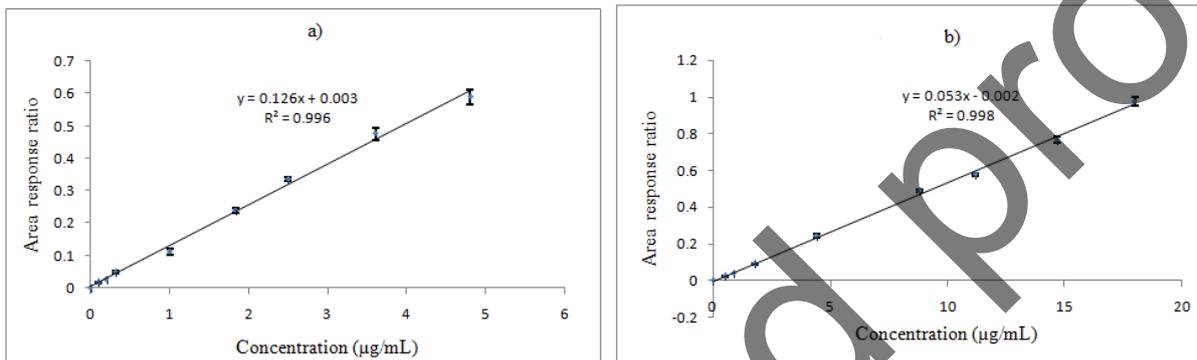


Fig. 2: Standard curves of saxagliptin (a) and dapagliflozin (b).

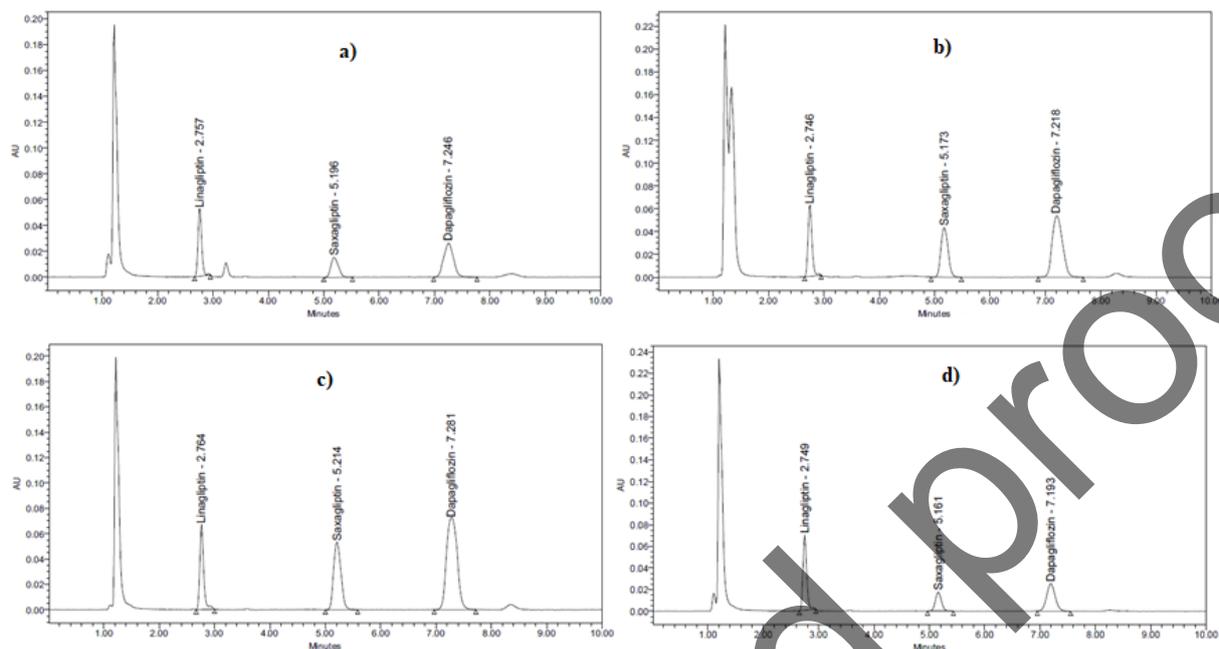


Fig. 3: Chromatograms of Low QC Sample(a), Middle QC Sample (b), High QC Sample (c) and Lower Limit QC sample (d).