Development and Validation of a New HPLC Method for the Simultaneous Estimation of Saxagliptine and Dapagliflozin and Its Application in Pharmacokinetic Studies

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Abstract— *A New HPLC method for the simultaneous estimation of Saxagliptine and Dapagliflozin in their combine dosage form was developed and validated as per the ICH guidelines. The method involves separation on XTerra C_{18} column (150mm x 4.6mm x5µm particle size). The optimized mobile phase consists of phoaphate buffer (pH 4) and Acetonitrile (50:50v/v) with a flow rate of 1ml/min and UV detection at 225nm. Retention time was 2.1min (Saxagliptine), 2.8min (Dapagliflozin). Linearity was observed in the range of 20-60µg/ml for Saxagliptine and 10-120µg/ml for Dapagliflozin with correlation coefficients (r^2 =0.999). The percentage recoveries of Saxagliptine and Dapagliflozin were in the range of 99.99-100.50% which was with in the acceptance criteria. The percent RSD was NMT 2% which proved the precision of the developed method. The developed method is simple, specific, sensitive, precise and accurate and was found suitable for estimation of Saxagliptine and Dapagliflozin in bulk and dosage forms. The developed HPLC method was also found suitable for application in pharmacokinetic studies for the estimation of Saxagliptine and Dapagliflozin in plasma samples.In the real in vivo pharmacokinetic study the biological half-lives ($t_{1/2}$) of Saxagliptine and Dapagliflozin estimated by the proposed method are in good agreement with the literature values. The good agreement of the absorption and elimination parameters estimated using HPLC method developed with those of literature values indicated that newly developed HPLC method is suitable and could be used in pharmacokinetic studies.*

Keywords— *Saxagliptine, Dapagliflozin, Simultaneous estimation, ICH guidelines, Application in Pharmacokinetic studies.*

I. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a chronic progressive metabolic disorder characterized by absolute or relative insulin deficiency [1,2]. Several new drug molecules were introduced in recent years for effective control of diabetes mellitus by using them alone or in combination. One such combination is Saxagliptin and Dapagliflozin combination introduced in to market in 2017.

Saxagliptin is chemically known as (1S,3S,5S)-2-[(2S)-2-amino-2-(3-hydroxy-1- adamantyl)acetyl]-2 azabicyclo hexane-3-carbonitrile) with molecular formula of C18H25N3O2 and molecular weight of 315.41g/mol [3]. Saxagliptin is a selective and potent dipeptidyl peptidase (DPP)-4 inhibitor, approved as an adjunct to diet and exercise to improve glycemic control in type 2 diabetes mellitus (T2DM). In patients with T2DM, once-daily administration of saxagliptin before breakfast achieves sustained inhibition of plasma DPP-4 activity and reduction of postprandial hyperglycemia, including after dinner, associated with an increase in plasma glucagon-like peptide-1 levels [4,5,6].

Dapagliflozin is chemically known as (1s)-1, 5- anhydro-1-C-[4-chloro-3-[(4-ethoxyphenyl) methyl] phenyl]-D-glucitol. It has a molecular formula of C24H33ClO8 with molecular weight 408.98 [7]. Dapagliflozin is selective Sodium Glucose Co-Transporter 2 inhibitor(SGLT 2). It acts by reducing the reabsorption of glucose by the kidney, leading to excretion of excess glucose in the urine, there by improving glycemic control in patients with type 2 diabetes mellitus [8].

Though several methods are reported in literature for the estimation of Saxagliptine [9-22] and Dapagliflozin [23-30] individually and in combination with other drugs.

No HPLC methods are reported for estimation of Saxagliptine and Dapagliflozin in combination. The objective of the present study was to develop and validate a new RP-HPLC method for the simultaneous estimation of Saxagliptine and Dapagliflozin in bulk ,dosage forms and to evaluate its application in pharmacokinetic studies for estimation of Saxagliptine and Dapagliflozin in plasma samples.

II. MATERIALS AND METHODS

Materials and Reagents

HPLC grade Acetonitrile (Lichrosol**R,** Merck Lifesciences Pvt. Ltd., Mumbai, India), HPLC water (Lichrosolv**R**MerckLifesciencesPvt.Ltd., Mumbai, India) Potassium Dihydrogen phosphate (Thermo Fischer Scientific Pvt Ltd., Mumbai, India), and Ortho phosphoric acid (S D Fine –Chem. Ltd., Mumbai, India) were used in the study. The working standards of Saxagliptine and Dapagliflozin were generous gift obtained from HiQ Pharma Labs Pvt

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Ltd.,Hyderabad, India. Qtern tablet containing Saxagliptine 5mg and Dapagliflozin 10mg (Astrazeneca pharmaceutical limited) was procured from UK market.

Instrumentation

Chromatography was performed on a WATERS 2695 HPLC column (waters corporation, Mildord, USA) with an autosampler and equipped with a 2996 series of PDA detector with a spectral bandpass of 1.2nm. Components were detected using UV and that processing was achieved by Empower 2 software.Ultrasonic bath (Toshcon by Toshniwal), digital P^H meter (Adwa – AD 1020), UV/VIS spectrophotometer (Labindia UV 3000) were used in the study.

Chromatography Conditions

The chromatographic separation was performed on XTerra C $_{18}$ (4.6 x 150mm, 5μm particle size) at an ambient column temperature. The samples were eluted using Phosphate buffer (pH adjusted to 4 with OPA): Acetonitrile(50:50v/v) as the mobile phase at a flow rate of 1ml/min the mobile phase and samples were degassed by ultrasonication for 20 min and filtered through 0.45μm Nylon (N66) 47mm membrane filter. The measurements were carried out with an injection volume of 10μL, flow rate was set to 1 mL/min, and UV detection was carried out at 225 nm. All determinations were done at ambient column temperature (30°C). The chromatograms of the prepared standard stock solutions of Saxagliptine, Dapagliflozin and Glimiperide were recorded under optimized chromatographic conditions.

Preparation of Buffer and Mobile Phase

Preparation of 0.025M Phosphate buffer

3.4g of potassium dihydrogen ortho phosphate was weighed and taken in a 1000ml volumetric flask and P^H was adjusted to 4 with dilute OPA, finally the solution was filtered by using 0.45 micron membrane filter and sonicated for 10 min.

Preparation of mobile phase

500 ml (50%) of phosphate buffer and 500 ml of Acetonitrile (50%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 μ filter under vacuum filtration.

Diluent

Mobile phase was used as diluent.

Preparation of Standard Solutions

Stock solution of Saxagliptine

Standard stock solution of Saxagliptine was prepared by dissolving 10 mg of Saxagliptine in 10 ml of diluent (Buffer: Acetonitrile, 50:50 v/v) in a 10 ml clean dry volumetric flask and the standard solutions was filtered through 0.45μm nylon membrane filter and degassed by sonicator to get the concentration of 1000μg/ml of Saxagliptine. The above standard stock solution suitably diluted with diluents to obtain various concentrations of Saxagliptine.

Stock solution of Dapagliflozin

Standard stock solution of Dapagliflozin was prepared by dissolving 10 mg of Dapagliflozin in 10 ml of diluent (Buffer: Acetonitrile, 50:50 v/v) in a 10 ml clean dry volumetric flask and the standard solutions was filtered through 0.45μm nylon membrane filter and degassed by sonicator to get the concentration of 1000μg/ml of Dapagliflozin. The above standard stock solution suitably diluted with diluents to obtain various concentrations of Dapagliflozin.

Working Standard Solution of Saxagliptine:

Working standard solution of Saxagliptine was prepared by taking 0.4 ml of stock solutions of Saxagliptine in to clean dry 10ml volumetric flask and make up volume with diluent to get a concentration of 40μg/ml of Saxagliptine.

Working Standard Solution of Dapagliflozin:

Working standard solution of Dapagliflozin was prepared by taking 0.8 ml of stock solutions of Dapagliflozin in to clean dry 10ml volumetric flask and make up volume with diluent to get a concentration of 80μg/ml of Dapagliflozin.

Preparation of Sample Solutions of Saxagliptine and Dapagliflozin

Twenty tablets were accurately weighed and powdered and tablet powder equivalent to 262 mg of Saxagliptine and Dapagliflozin was taken into 10 ml clean dry volumetric flask, diluent was added and sonicated to dissolve completely and volume was made up to volume with the diluent. The above sample solution was filtered and suitably diluted to get a concentration of 40 μg/ml of Saxagliptine and 80 μg/ml of Dapagliflozin.

Application of the method developed in pharmacokinetic studies

The new HPLC method developed was evaluated for its application in pharmacokinetic studies for the estimation of Saxagliptine and Dapagliflozin in plasma samples. For this purpose a calibration curve was initially constructed using plasma samples spiked with drugs.

Calibration curve

Plasma samples obtained from rabbits were spiked with different amounts of Saxagliptine and Dapagliflozin to obtain various concentrations in the range 20ng/ml-300ng/ml in the case of Saxagliptine and 100ng/ml-1500ng/ml in the case of Dapagliflozin along with Canaglifozin(IS) at a concentration of 5000ng/ml in each case. For HPLC analysis 0.5ml of plasma from each were taken in to dry centrifuge tubes, Acetonitrile (5ml) were added and mixed thoroughly for 5 min. The mixtures were then centrifuged at 5000 RPM for 10min.The supernatant organic phase (4ml) was collected in to dry test tubes and dried in a hot water bath at 60°C. To each tube 1.0ml of mobile phase was added and mixed thoroughly to dissolve the residue. The resulting solution (30μl) was then injected into column for chromatography.

Pharmacokinetic study

The HPLC method developed was also evaluated for its application in real *In vivo* Pharmacokinetic study. For this purpose the Pharmacokinetic study was performed in healthy rabbits weighing 2.0 -2.5 Kg of either sex (n=6) at a dose of 0.2mg/kg of Saxagliptine and 0.4mg/kg of Dapagliflozin togheher. Institutional Animal Ethics Committee (No. CPCSEA/CH/ORG/2017-045) approved the protocols.

After collecting the blank blood sample, the product in the study was administered orally with 10 ml of water. Blood

samples (1.0 ml) were collected from marginal ear vein at different times (0.5,1,2,4,8 and 12hrs) after administration. Blood samples were collected into heparinized test tubes and were centrifuged for 15 min at 15,000 rpm to separate plasma. The plasma samples were stored under refrigerated conditions at 4-8°C prior to assay for drug content on the same day. The plasma concentrations of Saxagliptine and Dapagliflozin were determined by the HPLC method developed. In each case 0.5ml of plasma was subjected to HPLC analysis as described above under calibration curve.

Estimation of Pharmacokinetic Parameters

Pharmacokinetic parameters of absorption and elimination namely Cmax, Tmax, elimination rate constant (Kel) and biological half-life (t½) were estimated from the time versus plasma concentration data.

III. RESULTS AND DISCUSSION

Optimization of Chromatographic Conditions

During the optimization cycle, different columns with different lengths and internal diameters were tried namely, Waters C18 column, hypersil column, lichrosorb, and XTerra column but finally satisfactory separation was obtained on XTerra C 18 (4.6 x 150mm, 5µm) column. Methanol and acetonitrile were examined individually and simultaneously as organic modifiers and acetonitrile was found to be more suitable, individually, as it allowed better separation of the three analytes under investigation. Isocratic mode of elution with different ratios of organic to aqueous phases was tried in order to achieve proper separation of the cited analytes in a reasonable run time. The use of 0.025M Phosphate buffer was necessary in this method in order to influence the ionization of the analytes and to help in their co-elution. Also pH was constant as each of SAXA, DAPA and GMP is obviously affected by the mobile phase composition and pH. The effect of pH on the separation of the analytes was studied. It was found that pH higher than 5.59 was not suitable as due to improper separation of the analyzed compounds. pH was adjusted at 4 for the best separation of the three analytes in a reasonable run time (<10 min) and with good resolution between all peaks. Different flow rates were studied and flow rate of 1 mL min-1 was found to be optimum.

Quantitation was achieved with UV-detection at 225 nm. The column temperature was set at 30°C. Optimized method was providing good resolution and peak shape for SAXA, DAPA and GMP. Under above described experimental conditions, all the peaks were well defined and free from tailing. The concern of small deliberate changes in the mobile phase composition, flow rates, and column temperature on results were evaluated as a part of testing for methods robustness.

Validation of Method Developed

The proposed method was validated according to the ICH guidelines for system suitability, specificity, recovery, precision, linearity, robustness, limit of detection (LOD) and limit of quantification (LOQ). Under the validation study, the following parameters were studied.

System Suitability Test

HPLC system was optimized as per the chromatographic conditions. 10 µl of standard solutions of drugs were injected in triplicate into the chromatographic system. To ascertain the system suitability for the proposed method, the parameters such as retention time, theoretical plates, and tailing factor were calculated.

Specificity

The specificity of the method was carried out to check whether there is any interference of any impurities with the retention time of analyte peaks. The specificity was performed by the injecting blank, Placebo and standard solutions of drugs.

Precision

Precision is expressed as the closeness of agreement between a series of measurements obtaining from multiple sampling of the same homogeneous sample. Six replicate injections of a known concentration of Saxagliptine (40 µg/mL) and Dapagliflozin (80 µg/mL), have been analyzed by injecting them into a HPLC column on the same day. The intermediate precision was estimated by injecting samples prepared at the same concentrations on three different days by different operators. The peak area ratios of all injections were taken and standard deviation, % relative standard deviation (RSD), was calculated.

Accuracy

Accuracy is tested by the standard addition method at different levels: 50, 100 and 150%. A known amount of the standard drug was added to the blank sample at each level. The mean recovery of Saxagliptine and Dapagliflozin were calculated and accepted with 100±2%.

Linearity

Appropriate volumes of Saxagliptine and Dapagliflozin stock 100(mg/ml) standard solutions were diluted with mobile phase to yield 20,30,40,50,60 µg/mL of Saxagliptine and 40,60,80,100,120 µg/mL Dapagliflozin respectively .Six replicates of each concentration were independently prepared and injected in to HPLC system. The linearity was determined by calculating a regression line from plot of peak area ratio of drug and IS versus concentration of the drug. Regression analysis were computed for Saxagliptine and Dapagliflozin. The method was evaluated by determination of correlation coefficient and intercept values according to ICH guidelines.

Limit of Detection and Limit of Quantification

Limit of detection (LOD) and limit of quantification (LOQ) of Saxagliptine and Dapagliflozin were determined by calibration curve method. Solutions of Saxagliptine and Dapagliflozin were prepared in linearity range and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using the following equations:

LOD= 3 x N/ B
LOQ= 10 x N/ B

where N is residual variance due to regression; B is the slope.

Robustness:

HPLC conditions were slightly modified to evaluate the analytical method robustness. These changes included the flow rate, column temperature and the Acetonitrile proportion in the mobile phase.

Validation of Method Developed

The proposed method was validated according to the ICH guidelines[31] for system suitability, specificity, recovery, precision, linearity, robustness, limit of detection (LOD) and limit of quantification (LOQ). Under the validation study, the following parameters were studied.

System Suitability

The Retention time of Saxagliptine and Dapagliflozin using optimum conditions was 2.10min and 2.81min respectively. For two of them, the peak symmetries were <1.5 and the theoretical plates numbers were >2000 and %RSD of areas of six standard injections of Saxagliptine and Dapagliflozin was less than 2. These values are within the acceptable range of United States pharmacopoeia definition and the chromatographic conditions. The results obtained are shown in Table 1.

TABLE 1. System suitability results of Saxagliptine and Dapagliflozin

Parameter	Saxagliptine	Dapagliflozin	Glimiperide
Peak area	50614 ± 0.1*	97232 ± 0.2*	36692 ± 0.8*
Theoretical plates	3728.32±0.432**	2371.38±0.815**	6446.98±0.629**
Retention time	2.10±0.01**	2.81±0.01**	8.87±0.04**
Tailing factor	1.36±0.04**	1.25±0.03**	1.18±0.03**

*RSD (%)
**Mean of SD of six determinations

Specificity

The specificity of the method was evaluated by assessing interference from excipients in the pharmaceutical dosage form prepared as a placebo solution. Optimized Chromatogram of Saxagliptine and Dapagliflozin is shown in Fig. 1 clearly shows the ability of the method to assess the analyte in the presence of other excipients.
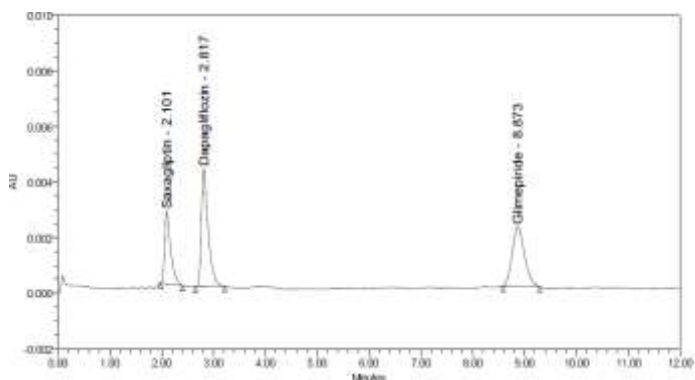

Fig. 1. Optimized Chromatogram of Saxagliptine and Dapagliflozin

Precision

System precision:

One dilution of both the drugs in six replicates was injected into HPLC system & was analyzed and the results were found within the acceptance limits (RSD<2) as shown in the Table 2 below.

TABLE 2. System Precision data for Saxagliptine and Dapagliflozin

	Saxagliptine (40µg/mL)			Dapagliflozin (80µg/mL)		
S. No	Retention time (min)	Peak Area	P/A Ratio	Retention time (min)	Peak Area	P/A Ratio
1	2.093	50634	1.38	2.806	97035	2.6446
2	2.094	50543	1.3775	2.806	97154	2.6478
3	2.096	50653	1.3805	2.806	97046	2.6449
4	2.098	50634	1.38	2.806	97534	2.6582
5	2.101	50537	1.3773	2.809	97254	2.6506
6	2.102	50682	1.3813	2.814	97368	2.6537
Avg	2.14	50614	1.4	2.8	97231.83	2.6499
SD	0.004	59.9	0	0	194.768	0.0053
%RSD	0.19	0.1	0.1	0.1	0.2	0.2

Method Precision (Repeatability):

Six replicate injections of a known concentration of sample preparation of Saxagliptine (40 µg/mL) and Dapagliflozin (80 µg/mL) have been analyzed by injecting them into a HPLC column on the same day. From the results obtained, %RSD was calculated and was found to be within the limits (<2). The results of precision are given in Table 3.

TABLE 3. Method Precision data for Saxagliptine and Dapagliflozin

	Saxagliptine (40µg/mL)			Dapagliflozin (80µg/mL)		
S No	Peak Area	P/A Ratio	% Assay	Peak Area	P/A Ratio	% Assay
1	50852	1.394	100.27	97290	2.668	99.86
2	50988	1.398	100.54	96467	2.645	99.01
3	50586	1.387	99.74	97515	2.674	100.09
4	50824	1.394	100.21	97230	2.666	99.8
5	50107	1.374	98.8	96629	2.650	99.18
6	50076	1.373	98.74	97372	2.670	99.94
Avg	50572	1.387	99.72	97083	2.662	99.65
SD	394.3	0.011	0.777	428.9	0.011	0.442
%RSD	0.779	0.780	0.780	0.441	0.441	0.443

Ruggedness

Intermediate precision was accessed injecting sample preparation of Saxagliptine (40 µg/mL) and Dapagliflozin (80 µg/mL) in six replicates in to HPLC column on the same day and on consecutive days and in different laboratories by different analysts. Results were found within the acceptance limits (RSD<2) as shown in the Tables 4, 5 below.

Accuracy

A known amount of the standard drug was added to the blank sample at each level. Good recovery of the spiked drugs was obtained at each added concentration, and the mean percentage recovery of Saxagliptine and Dapagliflozin was achieved between 100.21–100.50 ± 0.148% and 99.99 – 100.13±0.74.The results are given in Tables 6,7.

TABLE 4. Ruggedness Data for Saxagliptine

Concentration (µg/ml)	Laboratory-1 (% Assay)-HPLC-1				Laboratory-2 (% Assay)-HPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
40	100.27	99.27	99.83	99.41	99.86	99.83	100.19	99.28
40	100.54	99.86	99.01	99.98	99.01	99.93	99.68	99.45
40	99.74	100.53	100.01	100.24	100.09	99.96	100.44	100.41
40	100.21	100.51	99.8	100.18	99.8	100.28	99.34	100.65
40	98.8	99.54	99.78	100.11	99.18	100.52	100.18	98.95
40	98.74	99.95	99.94	99.87	99.94	99.82	99.13	100.22
Average	99.72	99.94	99.65	99.97	99.65	100.06	99.83	99.83
SD	0.777	0.508	0.442	0.303	0.442	0.282	0.525	0.690
%RSD	0.780	0.508	0.443	0.303	0.443	0.282	0.526	0.691

TABLE 5. Ruggedness Data for Dapagliflozin

Concentration (µg/ml)	Laboratory-1 (% Assay)-HPLC-1				Laboratory-2 (% Assay)-HPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
80	99.86	99.41	100.12	99.43	99.11	100.11	99.13	100.18
80	99.01	99.98	100.54	100.23	100.24	100.13	99.41	99.13
80	100.09	100.24	99.54	100.58	99.15	99.27	99.61	99.45
80	99.8	100.18	99.21	99.1	99.41	100.02	99.38	100.41
80	99.18	100.11	100.64	99.45	100.53	100.5	100.12	100.65
80	99.94	99.87	99.85	99.69	100.8	99.14	100.71	99.41
Average	99.65	99.97	99.98	99.75	99.87	99.86	99.73	99.87
SD	0.442	0.303	0.561	0.555	0.741	0.536	0.585	0.622
%RSD	0.443	0.303	0.561	0.556	0.742	0.537	0.586	0.622

TABLE 6. Recovery data of Saxagliptine

Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S1:50%	5	5.04	100.72	Mean = 100.50%(n=3) SD =0.2 %RSD =0.2
S2:50%	5	5.02	100.4	
S3:50%	5	5.02	100.4	
S4:100%	10	10.03	100.3	Mean = 100.3%(n=3) SD =0.2 %RSD =0.2
S5:100%	10	10.05	100.5	
S6:100%	10	10.01	100.1	
S7:150%	15	15.05	100.37	Mean = 100.21%(n=3) SD =0.2 %RSD =0.2
S8:150%	15	15.03	100.2	
S9:150%	15	15.01	100.07	

TABLE 7. Recovery data of Dapagliflozin

Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S1:50%	10	9.97	99.7	Mean = 100.1%(n=3) SD =0.4 %RSD =0.4
S2:50%	10	10.04	100.4	
S3:50%	10	10.02	100.2	
S4:100%	20	20.03	100.15	Mean = 100.13%(n=3) SD =0.1 %RSD =0.1
S5:100%	20	20.01	100.05	
S6:100%	20	20.04	100.2	
S7:150%	30	29.96	99.87	Mean = 99.99%(n=3) SD =0.1 %RSD =0.1
S8:150%	30	30.02	100.07	
S9:150%	30	30.01	100.03	

values of coefficient of determinations (Fig. 2 and Fig. 3). The results were tabulated in Table 8.

TABLE 8. Linearity data of Saxagliptine and Dapagliflozin

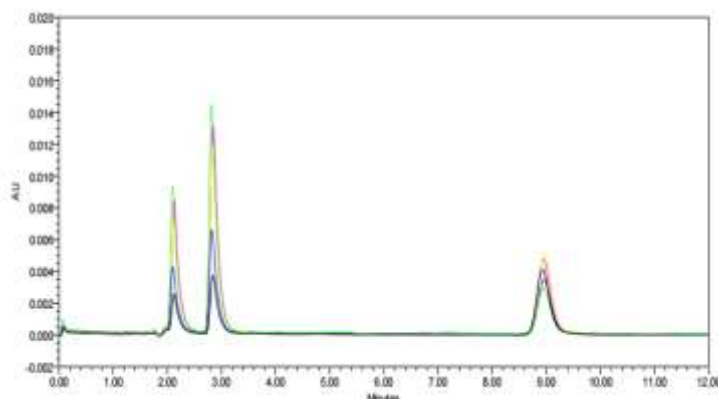| Concentration of Saxagliptine (µg/ml)	Peak area	P/ A Ratio	Concentration of Dapagliflozin (µg/ml)	Peak area	P/ A Ratio
20	25569	0.330	40	49258	0.063
30	37880	0.489	60	72958	0.094
40	50852	0.657	80	97847	0.126
50	62833	0.812	100	120646	0.155
60	75278	0.972	120	146776	0.189

Fig. 2.Overlay of Linearity Chromatograms

Linearity and Range

Linearity was assessed for the two oral anti diabetic drugs at concentration ranges 20-60µg/ml for Saxagliptine and 40-120µg/ml for Dapagliflozin. A linear relationship was established at these ranges between Area under the peak (AUP) and concentration. Good linearity was proved by high
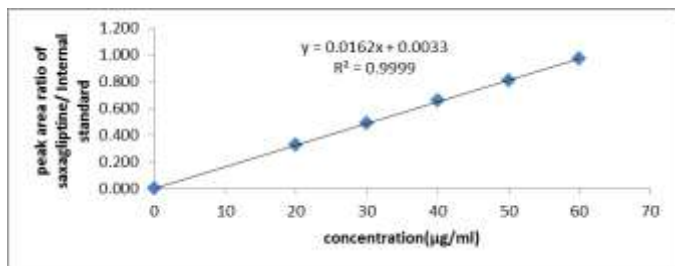
Vinutha Kommineni, K. P. R. Chowdary, and S. V. U. M. Prasad, "Development and Validation of a New HPLC Method for the Simultaneous Estimation of Saxagliptine and Dapagliflozin and Its Application in Pharmacokinetic Studies," *International Research Journal of Pharmacy and Medical Sciences (IRJPMS)*, Volume 1, Issue 6, pp. 16-24, 2018.

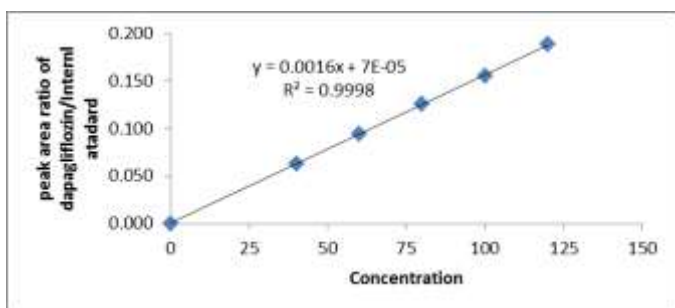Fig. 3. Linearity graph of Saxagliptine

Fig. 4. Linearity graph of Dapagliflozin

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The limit of detection and limit of quantification were evaluated by serial dilutions of Saxagliptine and Teneligliptine stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ. The LOD value for Saxagliptine and Dapagliflozin was found to be 1.63 μg/mL and 1.94 μg/mL, respectively, and the LOQ value 2.39 μg/mL and 3.50μg/mL, respectively.

Robustness

The result of robustness study of the developed assay method was established in Tables9,10,11. The result shown that during all variance conditions, assay value of the test preparation solution was not affected and it was in accordance with that of actual. System suitability parameters were also found satisfactory; hence the analytical method would be concluded as robust.

TABLE 9. Robustness (change in flow rate) for Saxagliptine and Dapagliflozin

Drug	Change in Flow rate (ml/min)	Change in flow Rate (0.8ml/min to 1.2 ml/min)		
		%Assay	SD	% RSD
Saxagliptine	0.8	100.3	0.412	0.41
	1	100.16	38	0.1
	1.2	99.86	0.2	0.2
Dapagliflozin	0.8	99.32	0.08	0.081
	1	100.35	80	0.1
	1.2	99.32	0.1	0.1

TABLE 10. Robustness (change in Mobile phase composition) for Saxagliptine and Dapagliflozin

Drug	Change in mobile phase	Change in Mobile phase (10% less organic phase & 10%more organic phase)		
		%Assay	SD	% RSD
Saxagliptine	10% less organic phase	100.1	0.3	0.3
	Actual	100.16	38	0.1
	10% more organic phase	100.1	0.5	0.5
Dapagliflozin	10% less organic phase	99.32	80	0.1
	Actual	100.35	80	0.1
	10% more organic phase	99.7	0.7	0.7

TABLE 11. Robustness (change in column Temparature) for Saxagliptine and Dapagliflozin

Drug	Change in column temperature	Change in column temperature		
		%ASSAY	SD	%RSD
Saxagliptine	25°C	**99.9**	**0.408**	**0.408**
	30°C	100.16	38	0.1
	35°C	99.59	0.242	0.243
Dapagliflozin	25°C	99.73	0.129	0.129
	30°C	100.35	80	0.1
	35°C	99.03	1.27	1.29

Summary of Results

The retention time of Saxagliptine and Dapagliflozin was found to be 2.10 min and 2.81 min respectively with resolution of 3.26. Linearity was observed for Saxagliptine and Dapagliflozin in the range of 20-60μg/ml for Saxagliptine and 40-120μg/ml for Dapagliflozin with correlation coefficients (r^2=0.999). The percentage recoveries were between 100.21 % to 100.50% and 99.99% to 100.13% for Saxagliptine and Dapagliflozin respectively, which indicate accuracy of the proposed method. The percent RSD values of accuracy for Saxagliptine and Dapagliflozin were found to be < 2 %. The percent RSD values of method precision are 0.780% and 0.443% for Saxagliptine and Dapagliflozin respectively and percent RSD values of system precision are 0.1% and 0.2% for Saxagliptine and Dapagliflozin. The percent RSD values of reproducibility for Saxagliptine and Dapagliflozin were found to be < 2 % indicating that the proposed method is precise. LOD values for Saxagliptine and Dapagliflozin were found to be 0.72μg/ml and 0.15μg/ml respectively and LOQ values for Saxagliptine and Dapagliflozin were found to be 2.40μg/ml and 0.51μg/ml respectively. The percent RSD values of robustness studies were found to be < 2% which revealed that the method is robust. These results indicate that the proposed method is specific, sensitive, accurate and precise for the determination of Saxagliptine and Dapagliflozin in bulk and in dosage forms.

Application of the HPLC method developed in Pharmacokinetic study

The new HPLC method developed was evaluated for its application for the estimation of Saxagliptine and Dapagliflozin in plasma samples in pharmacokinetic studies. When plasma samples spiked with the drugs were analysed by

the proposed method good linearity was observed in theconcentration range of 20-300ng/ml in the case of Saxagliptine and 100-1500ng/ml in the case of dapagliflozin. The chromatograms of plasma analysis are shown in Fig. 5-6. The linearity curves are shown in Fig. 7-8.
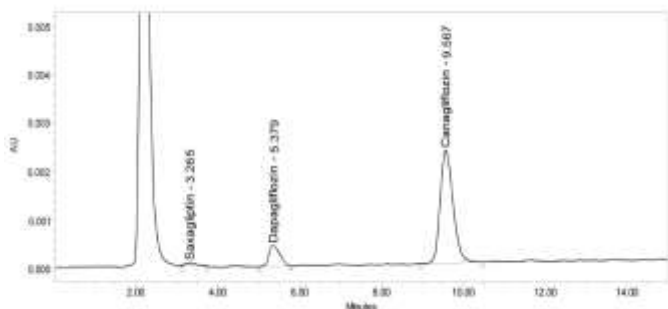

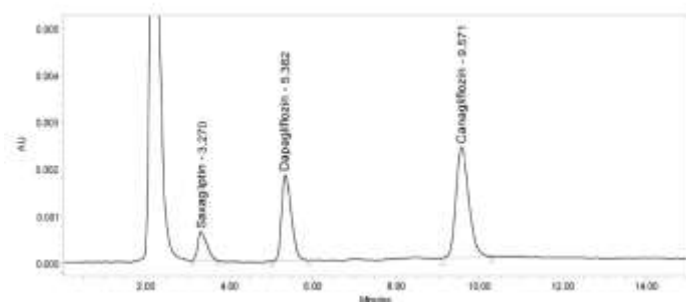Fig. 5. Chromatogram of plasma sample containing saxaglptine (20ng/ml) and (100ng/ml) of dapagliflozin

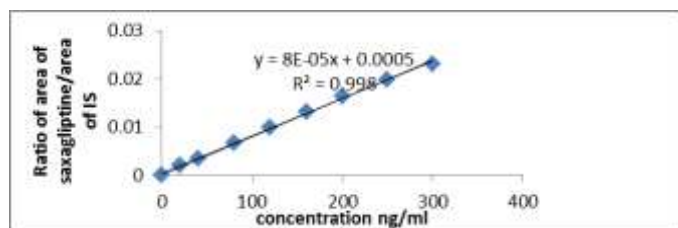Fig. 6. Chromatogram of plasma sample containing saxaglptine (300ng/ml) and (1500ng/ml) of dapagliflozin

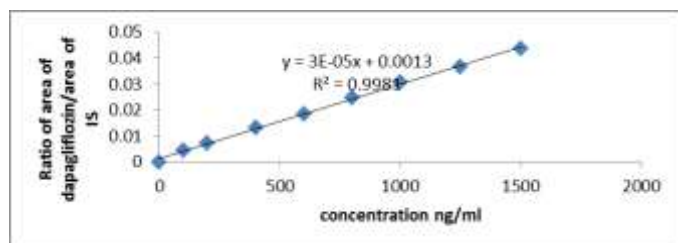Fig. 7. Linearity graph of Saxagliptine in plasma analysis

Fig. 8. Linearity graph of Dapagliflozin in plasma analysis

The precision (Percent RSD) of the method in plasma analysis was ranging from 0.45% to 2.26% for Saxagliptine and Dapagliflozin respectively indicating good reproducibility of the method. The HPLC method was also evaluated in a real pharmacokinetic study. The pharmacokinetic study was performed in healthy rabbits. The drugs were orally administered at a dose of 0.2mg/kg of Saxagliptine and
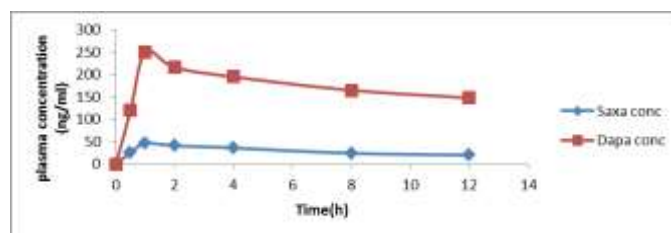
0.4mg/kg of Dapagliflozin. Blood samples were collected at different times after drug administration and subjected to HPLC analysis by the new method developed. The plasma concentrations of Saxagliptine and Dapagliflozin are determined by the HPLC method developed. Time versus Plasma concentration profiles are shown in Fig. 9.

Fig. 9. Plasma Concentrations of Saxagliptine and Dapagliflozin following their oral administration in rabbits.

Pharmacokinetic parameters (Cmax,Tmax,Kel,t1/2) are estimated from plasma concentration data. Log plasma concentration versus time plot was found to be a biphasic curve. A linear straight line was fitted to the points in the elimination phase. From the slope of the elimination regression line the elimination rate constant (Kel) was calculated. The biological half-live ($t_{1/2}$) was estimated by the equation:

$$t_{1/2} = 0.693/K_{el}$$

The biological half-live ($t_{1/2}$) was found to be 9.49h and 18.83h for Saxagliptine and Dapagliflozin respectively. The estimated biological half-lives are in good agreement with those reported in literature [31] for Saxagliptine (7.2h) and Dapagliflozin(15.0h). Both the drugs are absorbed rapidly.In the case of Saxagliptine, a Cmax of 46ng/ml was observed at 1hafter administration. In the case of Dapagliflozin, a Cmax of 250ng/ml was observed at 1hr after administration. This result also agreed with the literature.

The good agreement of the absorption and elimination parameters estimated using HPLC method developed with those of literature values indicated that newly developed HPLC method is suitable and could be used in pharmacokinetic studies.

IV. CONCLUSIONS

1. A New HPLC method for the simultaneous estimation of Saxagliptine and Dapagliflozin in their combine dosage form was developed and validated as per the ICH guidelines.
2. Linearity was observed in the range of 20-60µg/ml for Saxagliptine and 10-120µg/ml for Dapagliflozin with correlation coefficients (r^2=0.999).
3. The percentage recoveries of Saxagliptine and Dapagliflozin were in the range of 99.99-100.50% which was with in the acceptance criteria.
4. The percentage RSD was NMT 2% which proved the precision of the developed method.
5. The developed method is simple, specific, sensitive, precise and accurate and was found suitable for

estimation of Saxagliptine and Dapagliflozin in bulk and dosage forms.

6. The developed HPLC method was also found suitable for application in pharmacokinetic studies for the estimation of Saxagliptine and Dapagliflozin in plasma samples.

7. In the real in vivo pharmacokinetic study the biological half-lives ($t_{1/2}$) of Saxagliptine and Dapagliflozin estimated by the proposed method are in good agreement with the literature values.

8. The good agreement of the absorption and elimination parameters estimated using HPLC method developed with those of literature values indicated that newly developed HPLC method is suitable and could be used in pharmacokinetic studies.

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