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(RESEARCH ARTICLE)

Development and validation of simultaneous estimation of drugs in combination from pharmaceutical formulation by RP-HPLC method

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Abstract

Metformin is recommended as a first-line agent for monotherapy and combination therapy for patients with type 2 diabetes mellitus (T2DM). Patients whose glycaemic control deteriorates over time with metformin monotherapy will require additional anti-diabetic medication. The development of HPLC method for simultaneous estimation of anti-diabetic drugs in combination from solid dosage form by HPLC method. To validate the developed HPLC method as per ICH guidelines. The System suitability test ,Capacity factor, Tailing factor, Resolution, Selectivity, Separation factor, Theoretical plates, Regression coefficient, STD for intercept, LOD (limit of detection), LOQ (limit of quantification, Repeatability, Precision studies (Intra-day and Interday/Intermediate), Linearity/Calibration studies, Robustness, Force degradation/Stability indicating studies, Specificity, Drug recovery/accuracy studies are performed. The system suitability test performed for saxagliptin and metformin hydrochloride has achieved all guideline criteria; including tailing factor (*T*), separation factors (α), theoretical plates (*N*), capacity factor (*k'*), resolution (*R*) and RSD (%), force degradation studies were also performed for both these drugs. So combinedly we concluded that the proposed reverse phase chromatographic (RP-HPLC) analytical method for the simultaneous estimation in both bulk and tablet formulation have complied the ICH and US-FDA guidelines.

Keywords: Saxagliptin hydrochloride; Metformin; RP-HPLC; Diabetes mellitus

1. Introduction

According to statements by the American Diabetes Association/European Association for the Study of Diabetes and the American Association of Clinical Endocrinologists/American College of Endocrinology, metformin is recommended (unless specifically contraindicated) as a first-line agent for monotherapy and combination therapy for patients with type 2 diabetes mellitus (T2DM). This recommendation is based primarily on metformin's glucose lowering effects, absence of weight gain, generally low level of side effects, and relatively low cost [1,2]. However, many patients, particularly those with higher baseline glycated haemoglobin (HbA1c) values, may not achieve their glycaemic goals on metformin monotherapy despite titration to maximally tolerated doses, and therefore require additional medication [1, 3, 4]. Patients whose glycaemic control deteriorates over time with metformin monotherapy will require additional anti-diabetic medication. Although multiple classes of anti-diabetic agents are available, there remains a need for agents with different mechanisms of action that offer improved efficacy and/or better tolerability profiles and can be used

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either as monotherapy or in combination treatment regimens (including metformin). Dipeptidyl peptidase-4 (DPP-4) inhibitors are a class of oral anti-diabetic agents that increase circulating concentrations of the incretin gastrointestinal hormones glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide [5]. The incretins are rapidly released after meals and stimulate glucose-dependent insulin secretion. Glucagon-like peptide-1 also inhibits glucagon secretion, thereby attenuating postprandial glucose excursions [6]. The DPP-4 inhibitors improve glycaemic control by blocking the rapid inactivation of incretins, mainly glucagon-like peptide-1 [7-12]. Sitagliptin (Januvia®, Merck & Co, Inc, Whitehouse Station, NJ), the first of the DPP-4 inhibitors approved in the United States, has been used as an adjunct to diet and exercise in monotherapy and in combination regimens with other oral anti-diabetic drugs [1,11-13]. The mechanism of action of the DPP-4 inhibitors is complementary to that of metformin, which improves insulin sensitivity and reduces hepatic glucose production [5]. Hypoglycaemia, weight gain, and edema are generally not associated with DPP-4 inhibitor therapy; however, these adverse events have been associated with other anti- diabetic drug classes that are often used in conjunction with metformin (e.g. sulphonylurea, glinides, thiazolidinediones, and insulin) [6]. The low propensity for both DPP-4 inhibitors and metformin to cause hypoglycaemia or weight gain makes them an appropriate option for combination therapy in patients who are not meeting their glycaemic goals [5]. Saxagliptin (Onglyza TM, Bristol-Myers Squibb, Princeton, NJ/AstraZeneca, Wilmington, DE) is a potent, selective DPP-4 inhibitor, approved as an adjunct to diet and exercise to treat hyperglycaemia in patients with T2DM [14-16]. In phase 3 clinical trials, saxagliptin added to a stable dose of metformin, sulphonylurea, or thiazolidinedione, or given as initial therapy in combination with metformin, significantly improved glycaemic control and was well tolerated in patients with T2DM [7-9,17]. In a 24-week study in patients whose diabetes was not adequately controlled by stable metformin doses, adding saxagliptin 2.5, 5, or 10 mg daily reduced HbA1c from a baseline of 8.1%, by 0.7, 0.8, and 0.7%, respectively, compared with add-on placebo [7].

2. Material and methods

The high performance liquid chromatography (HPLC) of Shimadzu SCL-10A$_{VP}$ inbuilt with binary pump (LC- 10AT$_{VP}$), UV detector (SPD-10A$_{VP}$), Rheodyne 20µl loop capacity manual injector (P/N 77251) was used throughout the analysis. The LC-Solution software was used to interpret the HPLC reports. UltraSil-MCX®, 5µm; 100 x 2.1mm ID., column purchased from (Newcastle-UK) was used throughout the analysis. Digital weighing balance (ME-204) purchased from Mettler-Toledo (USA), ultra-sonicator Labman® purchased from UltraChrom Ltd, India. Digital pH meter from Mettler-Toledo was purchased from (Mumbai-India). 50 µ micro-syringe was purchased from Hamilton USA. 0.20µ and 0.45µ nylon membrane filters were purchased from Phenomenex® Mumbai, India. Reagents used are HPLC grade methanol and deionised water were purchased from Merck (Mumbai, India). HPLC grade ammonium formate (AF) (99%) was purchased from Merck Chemicals (Mumbai, England). 0.20 and 0.45µ nylon membrane filters were used and purchased from UltraChrom Innovatives Pvt. Ltd. (India). Saxagliptin standard was gifted from Mylan laboratories Ltd. (Hyderabad, India). Metformin standard was obtained from Sapkaal knowledge hub (Nashik, India). 20 tablets of Riax-M® XR, (saxagliptin- 5 mg and metformin-500 mg), manufactured by Dr. Reddy's Laboratories Ltd. (India) were purchased from local pharmacy store. All other chemicals and reagents were used of analytical grade.

2.1. Standard stock solution

Standard stock solutions of SXP and MET (1 mg mL-1) were prepared separately by dissolving 10 mg of the drug in methanol-water (2:1 v/v) using a 20 mL volumetric flask and completing the final volume by adjusting with either methanol or water, based on their solubility in particular solvents. Furthermore, freshly prepared sample solution was sonicated for 10-20 minutes and filtered through 0.20µ nylon filters.

2.2. Working stock solution

The saxagliptin has low UV absorption and detectivity at lower UV radiation nearly at 200-230 nm, the working stock solution of SXP; 500 µg mL-1 was prepared and by diluting 5 ml of 1000 ppm solution into 5 ml of blank volume with the mobile phase. Similarly, since the metformin has stronger UV absorbance, the working solution of MET (250 µg mL-1) was prepared by diluting 2.5 ml of 1000 ppm solution into the 7.5 ml blank of the mobile phase.

2.3. Chromatographic condition

The ultraviolet detector was operated at 228 nm for saxagliptin and 235 nm for metformin to achieve to optimum absorbance level. The buffer solution was filtered through 0.2 µm nylon membrane filter and degassed for 12 min in an ultrasonic bath prior to its use. The mobile phase was pumped through the column at a flow rate of 1 mL min-1. The column temperature was adjusted to 28 ◦ C and the injection volume was 20 µL.

2.4. Sample preparation for accuracy/drug recovery studies

Exactly 10 tablets of Riax-M® XR, were weighed separately, powdered and then mixed in a mortar. An accurately weighed 10 mg of finely powdered Riax-M® XR 5mg/500mg equivalent to 5 mg of SXP and 500 mg of MET were dissolved into 100 mL with methanol water and then sonicated until complete dissolved. The solutions were then filtered, followed by serial dilutions to the required concentrations using the mobile phase for each experiment including the standard addition technique.

2.5. Linearity/Calibration studies

Accurately measured aliquots of stock solutions, equivalent to 15.25-250 µg.mL^{-1} and 32.15-500 µg.mL^{-1} of SXP and MET, respectively were transferred separately into a series of 10 mL volumetric flasks. During each dilution, the volume was adjusted with same methanol-water; then 20 µL of each diluent were injected into HPLC.

2.6. Precision of the three methods

Three replicates of similar concentrations of the mixture of SXP (500 ppm) and MET (250 µg.L^{-1}) were analyzed three times, within the same day (intraday precision), using the procedure mentioned. Moreover, the mentioned concentrations of three replicates were analyzed on three successive days using the same procedure to investigate the intermediate 9inter-day) precision.

2.7. Robustness for the chromatographic method

The flow rate of the mobile phase was changed from 1ml.min-1 to 1.2 mL.min^{-1} and 0.8 mL.min^{-1} to evaluate the effect of the flow rate; similarly the variation of organic modifier as methanol was changed from 83% to 83±2% to monitor the system suitability parameters Finally, the effect of wavelength was monitored by making deliberate variation for saxagliptin (228±2nm) and for metformin (230±2nm) and the differences in system suitability parameters such as peak tailing, capacity factor, resolution and theoretical plates were tested and evaluated.

3. Results

The sensitivity of saxagliptin was quite negligible at 220 nm as they have preferred to separate them on C18 column [18,19]. Hence, this simultaneous estimation proved effectively the separation of saxagliptin and metformine with acceptable resolution (R) and capacity factor (k) and significantly improved UV sensitivity at 228 and 235 nm wavelengths. Moreover, the complete separation was carried out within 8 minutes.

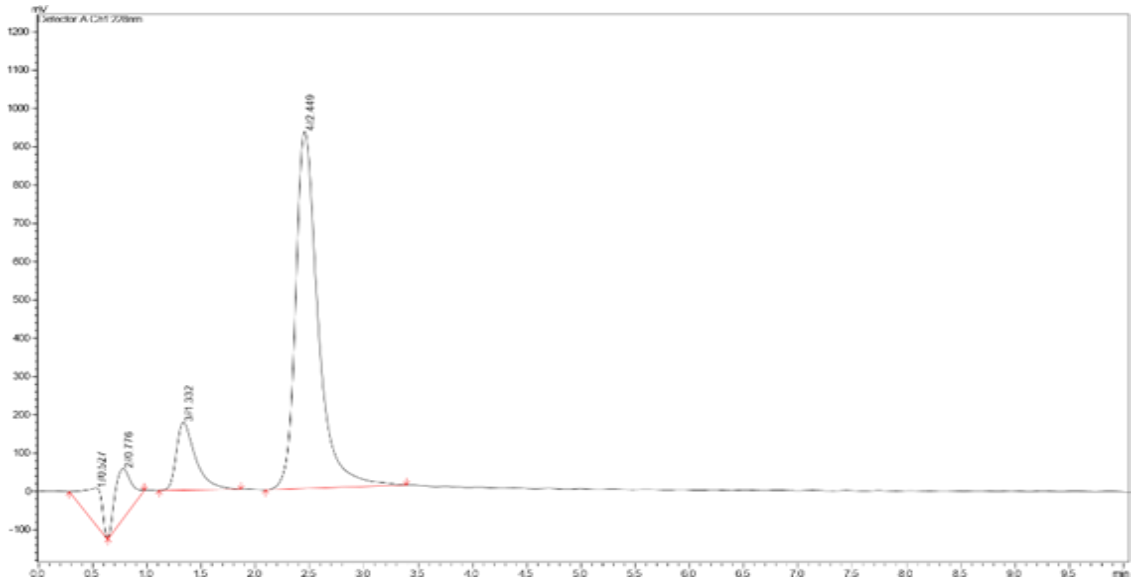

Figure 1 Method development reports of SXP (1.33) and MET (2.44) by RP-HPLC

During the optimization cycle, several columns were tried for the experiment, but the reverse phase HPLC column proved the best results with good peak sensitivity and symmetry, improved peak shape with enhanced resolution, selectivity and capacity factor, and importantly completed the whole analysis within eight minutes run time. Few

chromatogram of method development of reverse phase column were shown in (figure 1) and finally separation carried out using 10mM ammonium format-methanol (17:83 v/v) was selected.

3.1. Method validation

3.1.1. Repeatability

Implementing the procedure mentioned under section, the homologous mixture of both SXP and MET of same concentrations (500µg.mL^{-1}), were tested for six injections within the same day. The % RSD was calculated and found it is less than 2%; shown in (Table 1).

Table 1 Repeatability data of SXP and MET

S. No.	**Saxagliptin ; 228 nm**	**Metformine; 235 nm**
	Peak Area; Conc. 500 ppm	Peak Area; Conc. 250 ppm
1	2340019	8332596
2	2428726	8626274
3	2319443	8259515
4	2347548	8258027
5	2331553	8207594
6	2404107	8347476
Mean	2361899	8338580
STD. DEV.	43944.14	150186.66
RSD (%)	1.86	1.80

3.1.2. Precision

SXP and MET of three replicates of three similar concentrations; 500 ppm, 250ppm were tested and evaluated within the same day (intra-day precision). The %RSD was calculated and found less than 2%; shown in (Table 2).

Table 2 Intraday Precision data of SXP & MET

S. No.	**Concentration (ppm)**	**Area**		**Mean ± SD**		**%RSD**	
		SXP	**MET**	**SXP**	**MET**	**SXP**	**MET**
1	250 ppm	2340118	8259515	2329327± 10411.42	8241712± 29556.420 27.42	0.44	0.35
	250 ppm	2328521	8258027				
	250 ppm	2319342	8207594				
2	250 ppm	2347548	8258027	2361036± 38254.03	8271032± 70842.06	1.62	0.85
	250 ppm	2331353	8207594				
	250 ppm	2404207	8347476				
3	250 ppm	2309140	8377515	2325910± 14839.59	8310934± 73109.84	0.63	0.87
	250 ppm	2337341	8232696				
	250 ppm	2331251	8322591				
Range of %RSD						0.44 – 1.62	0.35 -0.87

The homologous mixture of both SXP and MET of three replicates of three different concentrations; 500 ppm, 250ppm were tested and evaluated in three successive days (interday/intermediate precision). The %RSD was calculated and found less than 2%; shown in (Table 3).

Table 3 Interday (intermediate) Precision data of SXP & MET

S. No.	Concentration (ppm)	Area		Mean ± SD		%RSD	
		SXP	MET	SXP	MET	SXP	MET
1	250 ppm	2440019	8454961	2419396± 26547.49	8386309± 73180	1.09	0.87
	250 ppm	2428726	8309316				
	250 ppm	2389443	8394652				
2	250 ppm	2347548	8809306	2361069± 38120.08	8882306± 139673	1.61	1.52
	250 ppm	2331553	8794260				
	250 ppm	2404107	9043353				
3	250 ppm	2309142	9161683	2315880± 6168.94	9100344± 135436	0.26	1.48
	250 ppm	2309142	8945091				
	250 ppm	2317249	9194260				
						0.26 – 1.61	0.87 – 1.52

3.1.3. Linearity

Limit of detection (LOD) which represents the concentration of analyte at S/N ratio of 3.3 and limit of quantification (LOQ) at which S/N is 10 were determined and results are given in (Figure2 & 3). Low values of LOD and LOQ indicate sensitivity of the applied method for determination of the mentioned drugs in tablets (Table 4 & 5).

Table 4 Linearity data of saxagliptin

S. No.	Concentration ($\mu g.mL^{-1}$)	Area	Average (Mean)
1	500 PPM	2533475	2508338
	500 PPM	2483201	
2	250 PPM	1262155	1257637
	250 PPM	1253119	
3	125 PPM	622160	622160
	125 PPM	-----	
4	62.5 PPM	313907	319544
	62.5 PPM	325182	
5	31.25 PPM	160770	159746
	31.25 PPM	158723	
6	Regression Equation		Y= 5011.6x + 2487.4
7	Correlation coefficient (R^2)		1
8	Std. Error of intercept		3177.71
9	Std. Dev. of intercept		7105.57
10	LOQ		4.67 $\mu g.ml^{-1}$
11	LOD		14.17 $\mu g.ml^{-1}$

Table 5 Linearity data of metformin

Name of Drug; Metformine			
S. No.	**Concentration (µg.mL^{-1})**	**Area**	**Average (Mean)**
1	250 PPM	9003119	8813682
	250 PPM	8624246	
2	125 PPM	4400212	4394017
	125 PPM	4387823	
3	62.5 PPM	1941353	1941353
	62.5 PPM	------	
4	31.25 PPM	1153925	1172291
	31.25 PPM	1190658	
5	15.62 PPM	590142	587893
	15.62 PPM	585644	
6	Regression Equation		Y= 35282x - 36065
7	Correlation coefficient (R^2)		0.9985
8	Std. Error of intercept		103116
9	Std. Dev. of intercept		230574.38
10	LOD		21.56 µg.ml^{-1}
11	LOQ		65.35 µg.ml^{-1}

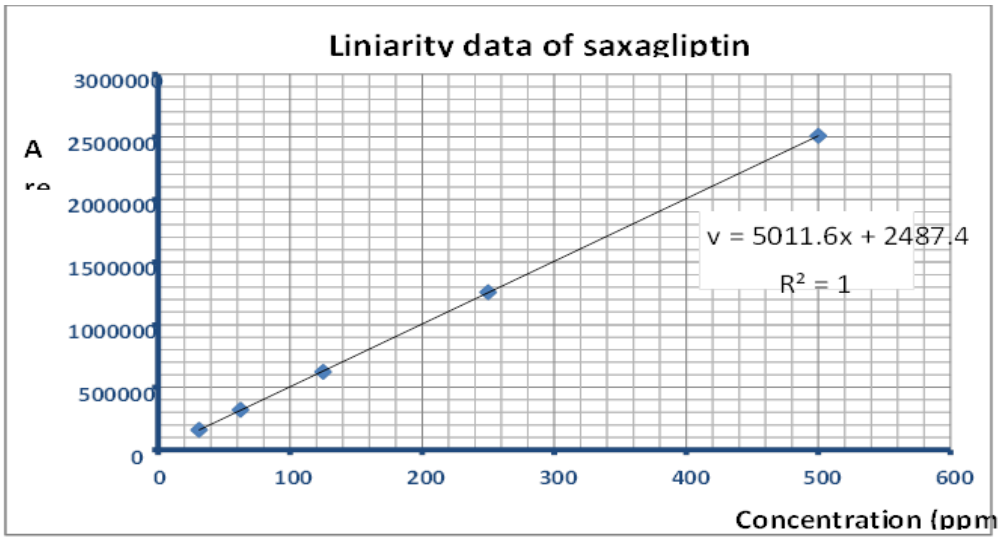

Figure 2 Calibration curve of sitagliptin

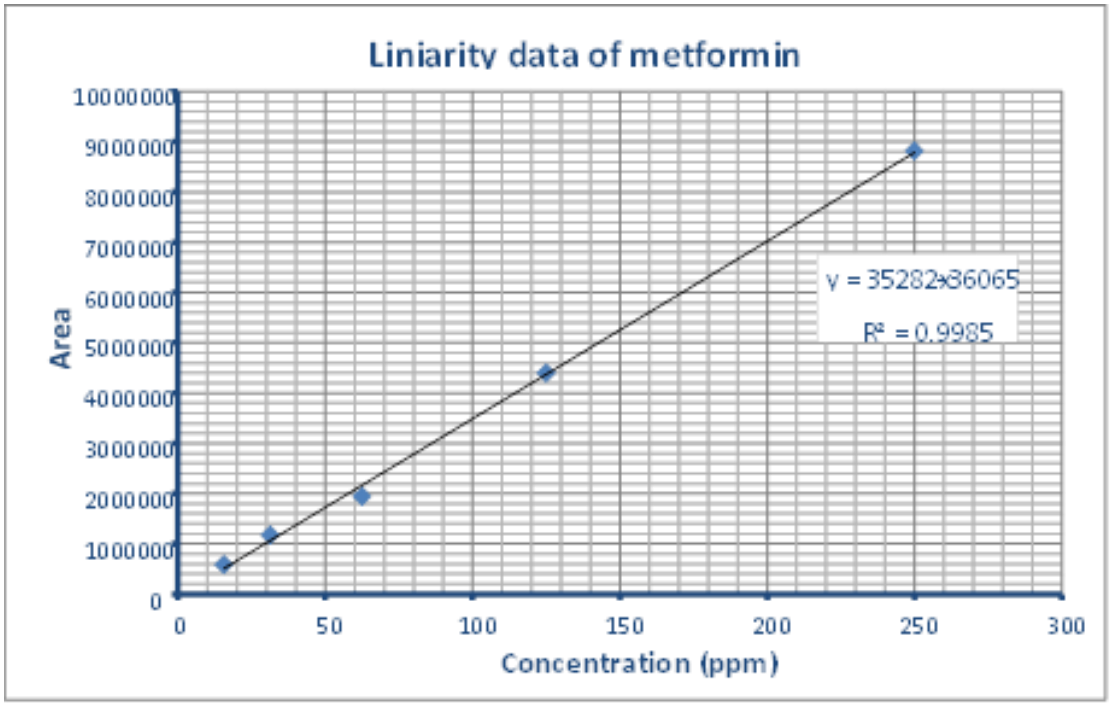

Figure 3 Calibration curve of metformin

3.1.4. Robustness for the chromatographic method

From the studies, after making deliberated changes in flow rate (± 0.2mL.min-1), organic modifier concentration; acetonitrile (±2%) and wavelength (±2nm) have not made any significant changes in resolution, capacity factor and tailing factor. Nonetheless, it seems minute changes in robustness studies makes significant changes in theoretical plate counts, Shown in (Figure 4 & 5).

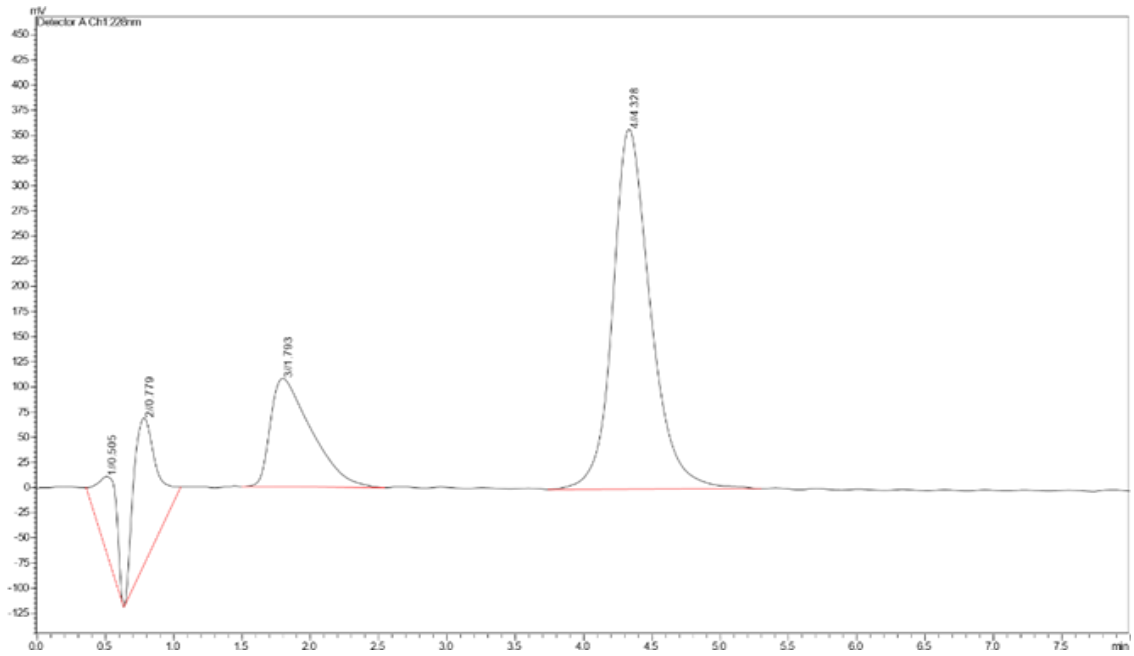

Figure 4 Robustness studies for SXP (1.79 min) and MET (4.32 min) at methanol 85%

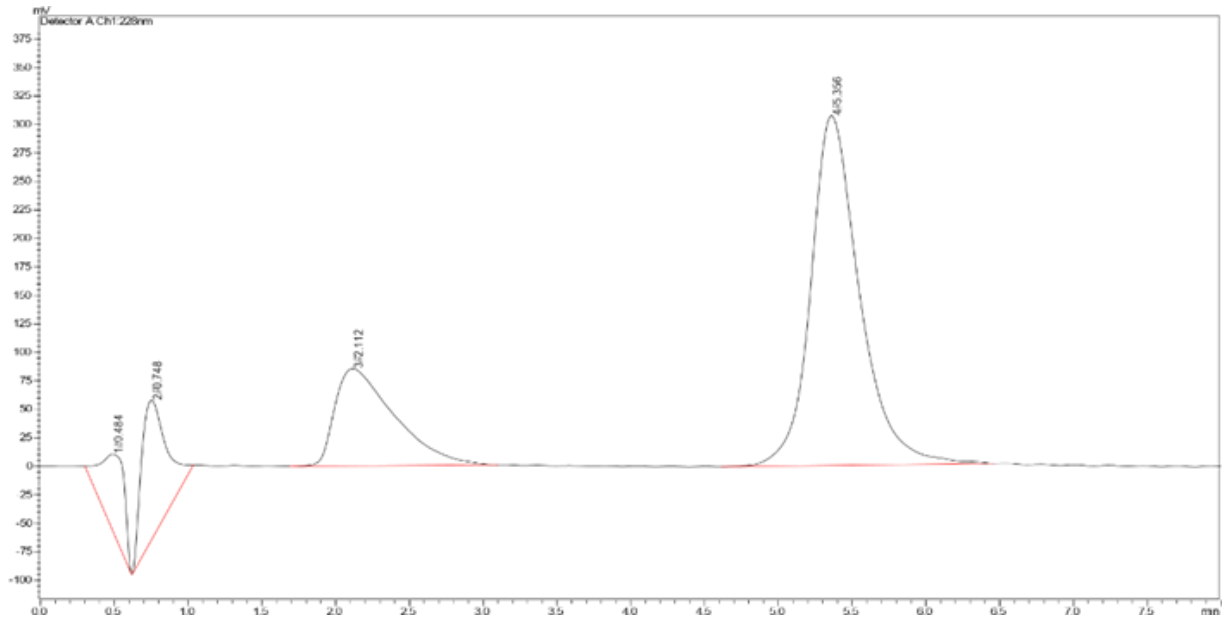

Figure 5 Robustness studies for SXP (2.11 min) and MET (5.35 min) at methanol 81%

3.1.5. Accuracy

Accuracy of the results was calculated by % recovery of 5 different concentrations of each drug. Figure 6 & 7. The results including the mean of the recovery and standard deviation are shown in (Table 6 & 7).

Table 6 Accuracy data of metformin (MET)

Conc. (%)	S. No.	S. amt. (µg.mL^{-1})	D. added (µg.mL^{-1})	Amt. rec. (µg.mL^{-1})	% recovery	Mean±SD	% RSD
80%	1	250	200	430.89	95.75	96.78±1.20	1.24
	2	250	200	434.22	96.49		
	3	250	200	445.12	98.11		
100%	1	250	250	512.27	102.45	98.70±3.30	3.35
	2	250	250	480.40	96.18		
	3	250	250	487.43	97.48		
120%	1	250	300	543.21	98.76	99.32±0.90	0.90
	2	250	300	552.01	100.36		
	3	250	300	543.67	98.84		

Table 7 Accuracy data of saxagliptin (SXP)

Conc. (%)	S. No.	S. amt. (µg.mL^{-1})	D. added (µg.mL^{-1})	Amt. rec. (µg.mL^{-1})	% recovery	Mean±SD	% RSD
80%	1	2.5	2	4.36	96.88	97.55±0.59	0.60
	2	2.5	2	4.40	97.78		
	3	2.5	2	4.41	98		
100%	1	2.5	2.5	5.10	102	99.93±2.21	2.21
	2	2.5	2.5	5.01	100.2		
	3	2.5	2.5	4.88	97.6		
120%	1	2.5	3	5.30	96.36	96.56±1.04	1.08
	2	2.5	3	5.36	97.69		
	3	2.5	3	5.26	95.63		

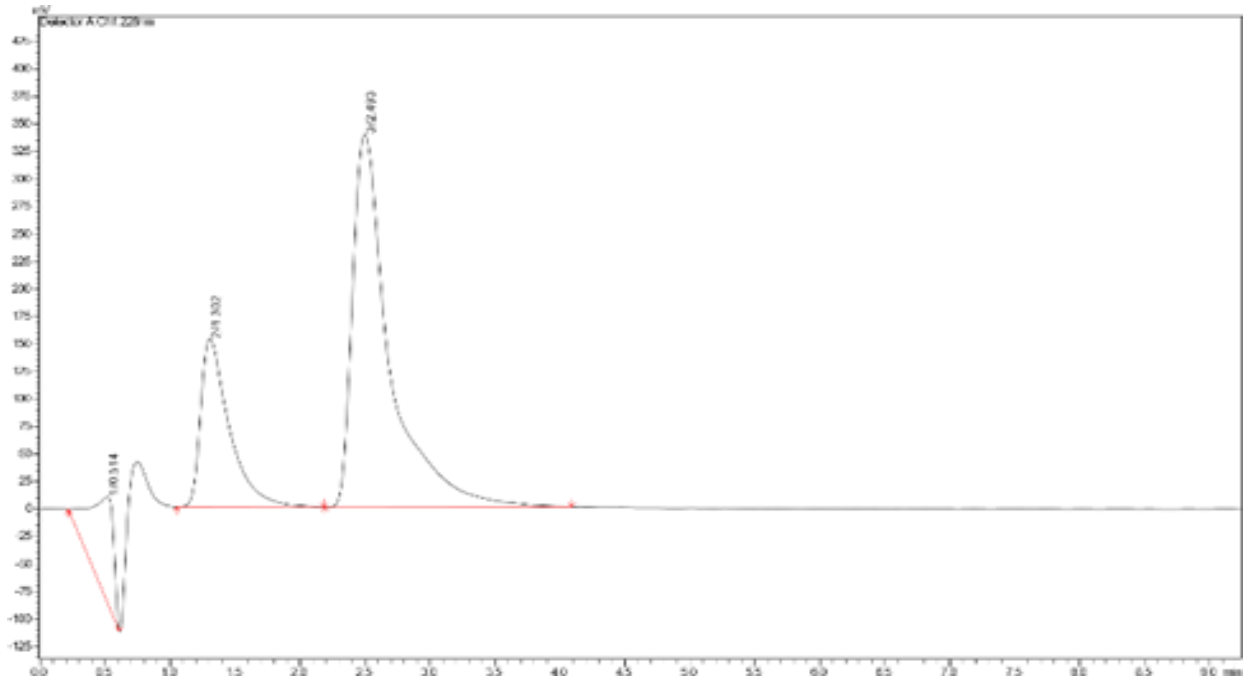

Figure 6 Accuracy data of SXP (1.30 min) and MET (2.49) at 80%

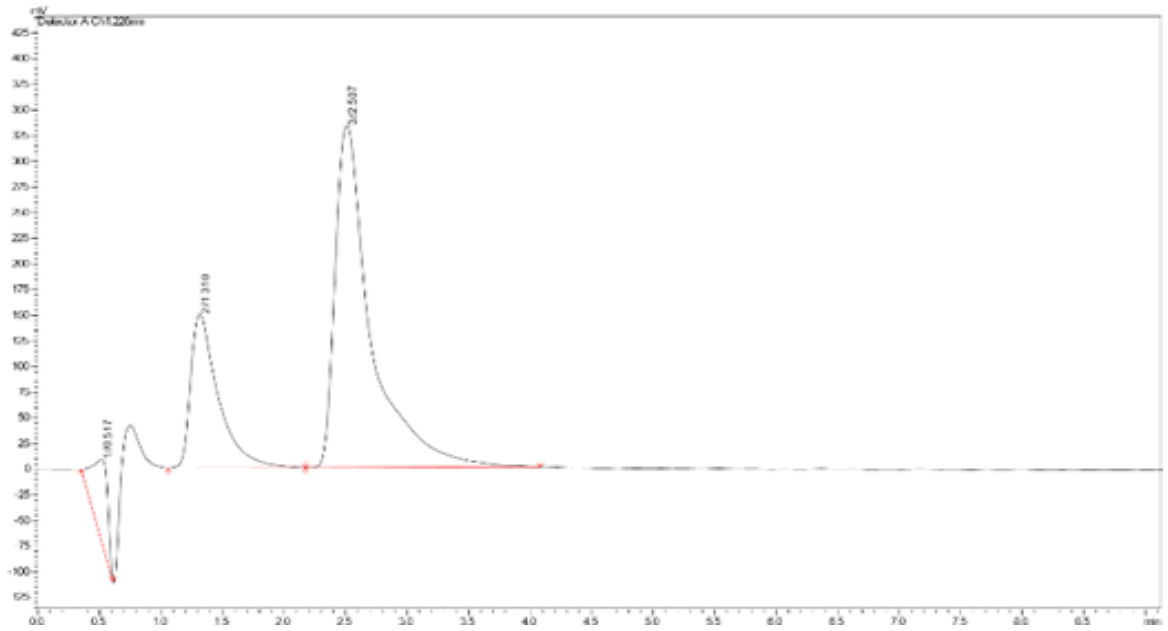

Figure 7 Accuracy data of SXP (1.31 min) and MET(2.50) at 120%

4. Discussion

The proposed reverse phase chromatographic (RP-HPLC) analytical method for the simultaneous estimation of saxagliptin (SXP) and metformin (MET) in both bulk and tablet formulation have complied the ICH and US-FDA guidelines. As per the ICH and USFDA guidelines, the developed method has complied the linearity range (calibration data), accuracy/drug recovery studies (%), repeatability, precision studies (intraday and interday/intermediate), and robustness. Moreover, as per the ICH guidelines, the system suitability test performed for simultaneous estimation of saxagliptin and metformin has achieved all guidelines; including, tailing factor (T), separation factors (α), theoretical plates (N), capacity factor (k'), resolution (R) and RSD (%). In addition, the stability indicating studies or force degradation studies were also performed for both these drugs. As concluded, both drugs were seen stable in thermal, oxidation and acid induced hydrolysis.

5. Conclusion

Above all results we, may conclude that, this developed and validated method for investigation by reverse phase chromatography can be used for routine analysis of simultaneous estimation of either saxagliptin or metformin or both from the mixture of marketed formulation.

Compliance with ethical standards

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Disclosure of conflict of interest

All authors have no conflict of interest.

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