DEVELOPMENT AND VALIDATION OF RP HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF METFORMIN HCL AND SITAGLIPTIN PHOSPHATE IN TABLET DOSAGE FORM

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ABSTRACT

We developed and validated a simple, efficient, and less time-consuming analytical method for simultaneous estimation of metformin hydrochloride and sitagliptin phosphate in tablet dosage form by RP-HPLC. Chromatographic separation was accomplished in the isocratic mode using potassium dihydrogen phosphate and acetonitrile (70:30) as the mobile phase, nucleodur C18 (250mm x 4.6 mm) column as the stationary phase with a flow rate of 0.8 ml/min using an UV detector maintained at 267 nm. The reported periods of retention for metformin and sitagliptin are 2.8 min and 7.5 min respectively. As per ICH guidelines, this procedure has been validated for Specificity, Linearity and Range, Accuracy, System Precision, Method Precision, Intermediate Precision, Robustness, Solution stability and Filter Integrity. Our research focusses on reducing the complexity and duration of time required for analyzing metformin hydrochloride and sitagliptin phosphate tablets. The results obtained were good and found within the limit, proving that the developed method can be used for determination of metformin and sitagliptin tablets.

Keywords: RP-HPLC, Metformin, Sitagliptin, ICH guidelines.

INTRODUCTION

Metformin hydrochloride is an oral hypoglycemic agent that works by decreasing glucose production in the liver and absorption of glucose by the intestines. It is freely soluble in water, slightly soluble in alcohol and is partially insoluble in acetone, methylene chloride, ether, and chloroform [1]. Molecular formula for the compound is C4H11N5.HCl and the

molecular weight is 165.63 g/mol. It is chemically known as *N, N*-dimethyl imido dicarbonimidic diamide hydrochloride, white to off-white crystalline, non-hygroscopic powder. Metformin is prescribed as a first-line therapy in type-2 diabetes. It exerts the glucose-lowering effect via (i) inhibition of gluconeogenesis in the liver, (ii) delaying the action of glucagon, (iii) facilitating the action of insulin, and (iv) delaying glucose absorption from the intestine [2]. Its structural formula is presented in Fig 1.

Sitagliptin phosphate is an orally active Dipeptidyl peptidase 4 (DPP-4) inhibitor that works by regulating the levels of insulin produced after meal. It also enhances glycemic control and shows a positive influence on the growth of *β*-cells in pancreatic islets. It is a white to off-white crystalline, non-hygroscopic powder that is soluble in water and N, N-dimethyl

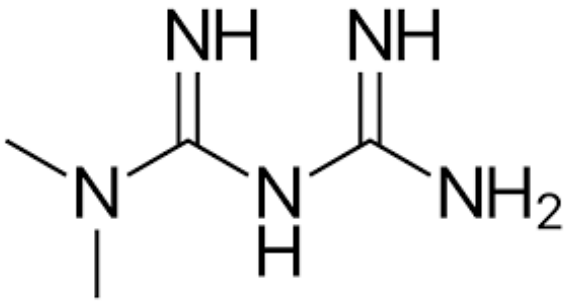
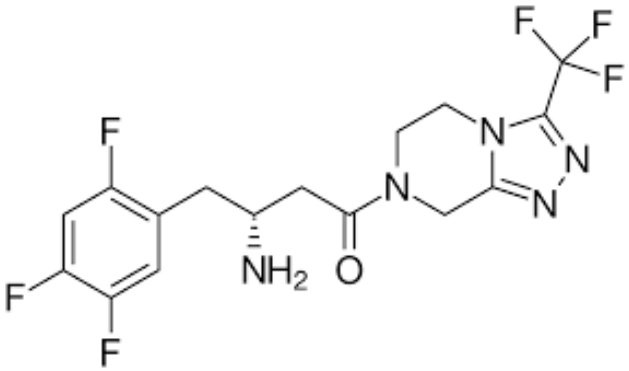


Fig. SEQ Figure * ARABIC 1: Chemical

formamide, slightly soluble in methanol and very slightly soluble in ethanol, acetone, and acetonitrile and insoluble in isopropanol [3]. Empirical formula of the compound is C16H15F6N5O.H3PO4.H2O, molecular weight is 523.32 g/mol. It is chemically known as 7-[(3*R*)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl) butyl]-5,6,7,8-tetrahydro-[3-(trifluoromethyl)-1,2,4-triazolo[4,3-*a*] pyrazine phosphate (1:1)monohydrate. Sitagliptin is used for the treatment of type 2 diabetes. It is effective in lowering HbA1c, fasting as well as postprandial glucose in monotherapy and can be used in combination with other oral antidiabetic agents [2]. Its structural formula is presented in Fig 2



There are several methods developed in the past for estimation that were found to have longer retention times, higher consumption of organic solvents, and poor resolution. Thus, there is a need to develop an analytical technique with less time consumption along with less organic solvent consumption. The present research work aimed to develop and validate a stability indicating HPLC method for simultaneous estimation of the drugs in combined solid oral dosage form by using parameters like specificity, linearity and range, accuracy, system precision, method precision, intermediate precision, robustness, solution stability and filter integrity as per ICH guidelines.

MATERIALS AND METHODS:

Chemicals and Reagents:

Acetonitrile - Analytical Research Grad, Potassium dihydrogen phosphate - Analytical Research Grad**,** Orthophosphoric acid - HPLC Grade, Water - HPLC Grade**,** Metformin Hydrochloride - Working Reference standard**,** Sitagliptin Phosphate - Working Reference standard from Sai Mirra Innopharm in India.

Instruments and Apparatus Required:

High Performance Liquid Chromatography system (Shimadzu i - prominence) with UV detector using the Lab solution software, Sonicator and apparatus such as mortar and pestle, 250 ml & 50 ml volumetric flasks, 100 ml beakers, 5 ml pipettes were used.

Instrumentation and Chromatographic conditions:

The HPLC system (Shimadzu i - prominence) equipped with UV/Visible dual absorbance detector, Nucleodur C18 (250 × 4.6 mm), was used to achieve chromatographic separation. Mobile phase was composed of potassium dihydrogen phosphate buffer pH 4.3 adjusted with orthophosphoric acid and acetonitrile in a 70:30 v/v ratio. The mobile phase was filtered through 0.45 µ membrane filter, degassed and injected onto the column at 0.8 ml/min flow rate. Load volume of the drug solution was 20 µl, and the detection was recorded at 276 nm.

Preparation of Mobile Phase:

Buffer: 2.27 g of Potassium dihydrogen phosphate was added to 1 liter of water. The pH was adjusted to 4.3 with Orthophosphoric acid. The mixture of buffer and Acetonitrile was mixed in the ratio of 70:30 v/v and degassed in the ultrasonic water bath for few minutes. The solution was filtered under vacuums using a 0.45µ filter and used.

Standard Solution Preparation:

Sitagliptin stock solution was prepared by weighing accurately 31.0 mg of Sitagliptin phosphate WS, transferred into a 50 ml volumetric flask, dissolved, and diluted to volume with water. 25.0 mg of Metformin Hydrochloride WS was weighed accurately into a 50 ml

volumetric flask, dissolved in 10 ml mobile phase, 5 ml of the Sitagliptin stock solution was added and made up the volume with mobile phase. This was used as a standard solution.

Sample Solution Preparation:

Sample stock solution: 20 tablets were powdered, and 700 mg of the powdered sample weighed accurately into a 250 ml volumetric flask, added with 50 ml water, and sonicated for 30 minutes and then made up the volume with water.

Sample solution: Pipetted out 5 ml of the above sample stock solution into a 20 ml volumetric flask and diluted with mobile phase up to the volume. Filtered through 0.45µm membrane filter discarding first 5 ml.

Chromatographic Method Development:

Different mobile phases were used to run the standard drugs at various pH levels, along with organic mobile phase modifiers such as acetonitrile, methanol and water. Additional trials were carried out to minimize tailing by altering pH, however these changes caused the peaks to split or the resolution to diminish between the two drugs. It was also attempted to modify the organic phase, however this led to peaks merging or to no changes in the drug's tailing. The peak forms were observed to be symmetrical under the specified chromatographic conditions.

Selection of Wavelength:

The UV Spectrophotometer was used to scan the individual standard solutions of the specified drugs at a concentration of 10 mg/ml in the selected mobile phase. On the basis of the drug's increased response, detection was performed at 267 nm.

Method Validation:

System suitability

Procedure:

Inject 20 µl of the standard preparation in 6 replicates and check the system suitability. If system suitability is found satisfactory, proceed with the injection of sample preparations.

The order of elution will be as follows:

1. Metformin Hydrochloride
2. Sitagliptin phosphate monohydrate

System suitability tests were carried out to ascertain the adequate resolution and repeatability of the developed method. Investigations were done on the following parameters: column efficiency, resolution and relative standard deviation. It was reported that the Column efficiency: Not less than 1500 theoretical plates for both Metformin Hydrochloride and Sitagliptin peaks. Resolution: Not less than 4.0 between Metformin Hydrochloride and Sitagliptin phosphate peaks. Relative standard deviation: NMT 2.0 % for the 6 areas of Metformin Hydrochloride and sitagliptin phosphate peaks from 6 replicate standard injections. The above mentioned parameters were all within acceptable ranges.

Specificity

By comparing the test sample's retention time to that of reference drugs, the peaks of the test drugs were assessed. The retention times of the standard and test samples showed a good correlation. It was noted that the Metformin Hydrochloride and Sitagliptin phosphate peaks were unaffected by the diluent or excipient peaks. Table.1 represents the observations of the specificity samples. The chromatogram of standard and test sample without any interference and are there are no interference of the placebo was observed which is shown in Fig.3 and 4

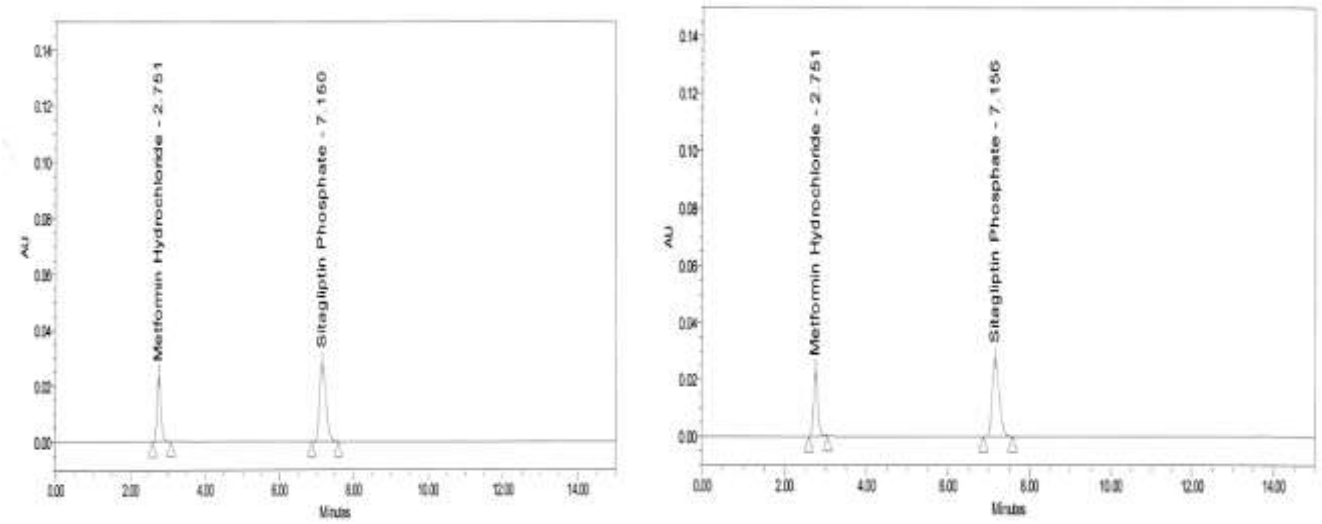


Table 1: Solubility Specificity parameters of Metformin Hydrochloride and Sitagliptin phosphate

Injection No.	**Response of the peak with Retention time**	**Influence of placebo**
1.Blank	No peaks observed	-
2.Placebo	No peaks observed	No influence
3.Standard solution	**Metformin Hydrochloride** Area = 166253, Retention time = 2.751 **Sitagliptin phosphate** Area = 328540, Retention time = 7.150	-
4.Test solution	Two peaks observed at 2.751 minutes with an area 161540 and at 7.156 minutes with an area 323532 which corresponds to Metformin Hydrochloride and Sitagliptin Phosphate respectively	No influence due to placebo

Remarks: There is no interference of the placebo. Hence the method is specific.

Linearity:

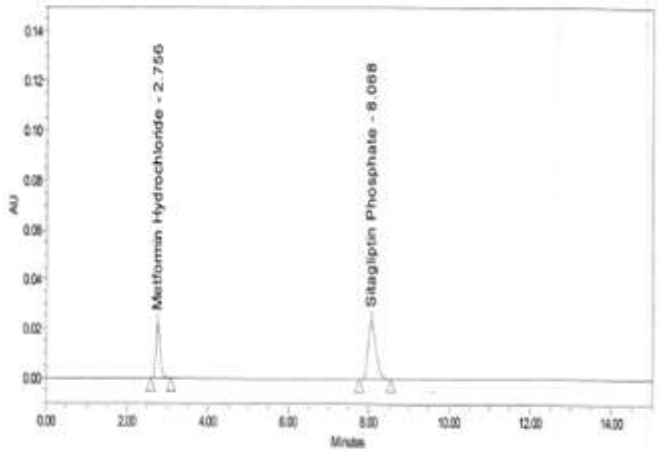
Determined the Linearity of Metformin Hydrochloride and Sitagliptin phosphate by plotting a graph between concentration of the test solution on X-axis and response of the corresponding solutions on Y-axis, from 50 % to the 150 % as shown in Fig 5, 6 and determine the correlation coefficient square & y-intercept which is shown in table 2.

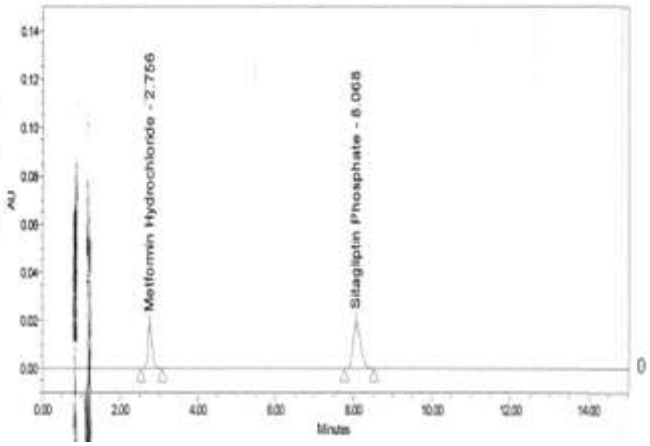
Acceptance Criteria for Linearity: Correlation Coefficient square – NLT 0.995, y-intercept: Not more than ± 2.0 %

Table 2: Linearity of response of Metformin Hydrochloride and Sitagliptin phosphate

Sample ID	Metformin Hydrochloride		Sitagliptin	
	Concentration, in mcg/mL	Area	Concentration, in mcg/mL	Area
50 % of operating concentration	248.80	81616	24.56	157751
80 % of operating concentration	398.08	133723	39.29	256940
100 % of operating concentration	497.59*	160489	49.12*	316497
120 % of operating concentration	597.11	187209	58.94	375469
150 % of operating concentration	746.39	243897	73.68	472672
Correlation coefficient (*r* >0.995)	**0.9957**		**0.9996**	
y- intercept (NMT ±2.0 %)	**+1.90 %**		**+1.11 %**	

The plot between concentration and response is as follows –


Metformin Hydrochloride - 2.756
Sitagliptin Phosphate - 8.068
AU
Minutes


Metformin Hydrochloride - 2.756
Sitagliptin Phosphate - 8.068
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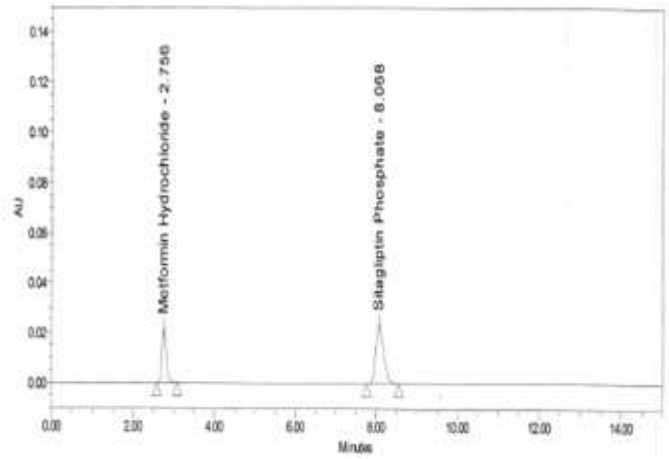
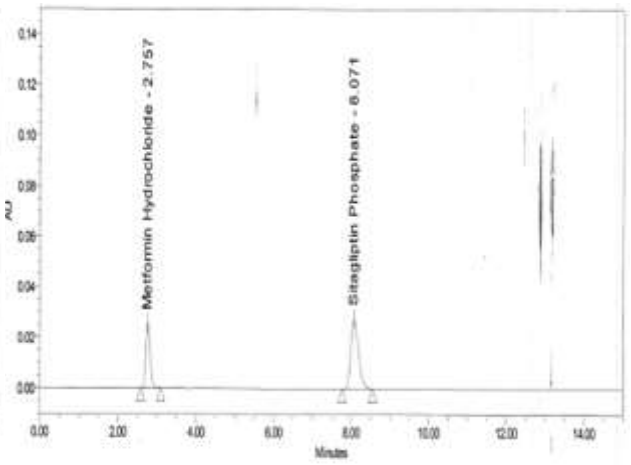
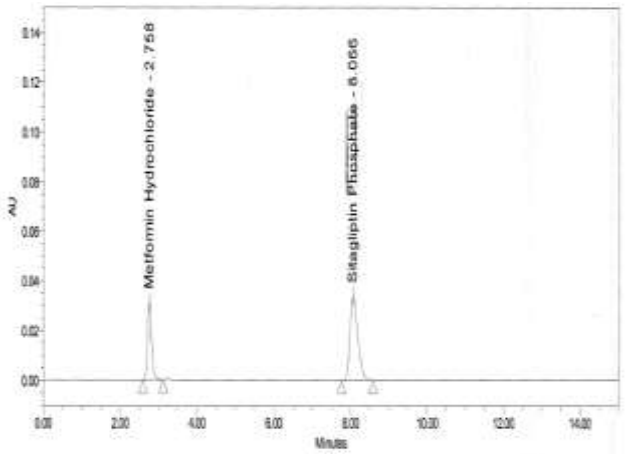

Metformin Hydrochloride - 2.756
Sitagliptin Phosphate - 8.068
AU
Minutes


Metformin Hydrochloride - 2.757
Sitagliptin Phosphate - 8.071
AU
Minutes


Metformin Hydrochloride - 2.758
Sitagliptin Phosphate - 8.066
AU
Minutes

Range

One solution with higher concentration and one with lower concentration (as prepared under Linearity) in 6 replicates each was injected and the peak areas were recorded. Calculate the related standard deviation for the 6 areas which is shown in table 3.

Acceptance Criteria for Range: RSD for 6 areas at two linearity levels - NMT 2.0 %

Table 3 : Range of Least and Highest Concentration Peak Area of 6 replicates

S.No	Metformin Hydrochloride		Sitagliptin phosphate	
	50 % Standard	**150 % Standard**	**50 % Standard**	**150 % Standard**
1	89637	229462	157557	472251
2	89224	229442	156681	471890
3	89369	229703	157112	472235
4	89351	229530	156650	472139
5	89136	229209	156686	471691
6	89660	229007	157239	470370
Average	**89396**	**229392**	**156988**	**471763**
RSD (NMT 2.0 %)	**0.24 %**	**0.11 %**	**0.24 %**	**0.15 %**

Remarks: Correlation Coefficient square, y-intercept and RSD are within the acceptable limit.

Accuracy

The accuracy of the assay method was determined by adding known amounts of Metformin Hydrochloride and Sitagliptin Phosphate to the placebo at 50 %, 100 % and 150 % of actual concentration. The standard preparations and test preparations were injected separately in 6 replicates. The chromatograms were recorded, responses were measured which is shown in table 4 & 5. Percentage recovery was calculated.

Acceptance Criteria for Accuracy: The recovery at various levels is between 98.0 % and 102.0 %, The RSD for Recovery of triplicate samples at various levels is not more than 2.0 %.

Table 4: Recovery Study Values of Metformin Hydrochloride

S. No.	Sample ID		Amount added (mg)	Amount recovered (mg)	Recovery (98.0 % to 102.0 %)	Mean and RSD
1	50	A1	25.5295	25.5365	100.03 %	**Mean = 99.11 %** **RSD** = 0.86. %
2		A2	25.9316	25.6611	98.96 %	
3		A3	26.0321	25.6016	98.35 %	
4	100	A1	46.9382	46.9217	99.96 %	**Mean = 99.64 %** **RSD** = 0.34 %
5		A2	47.1392	46.9812	99.66 %	
6		A3	47.2397	46.9019	99.29 %	
7	150	A1	46.9382	67.4332	98.52 %	**Mean = 98.58 %** **RSD** = 0.07 %
8		A2	47.1392	67.6234	98.65 %	
9		A3	47.2397	67.3777	98.58 %	

Table 5: Recovery Study Values of Sitagliptin Phosphate

S. No.	Sample ID		Amount added (mg)	Amount recovered (mg)	Recovery (98 % to 102 %)	Mean and RSD
1	50	A1	24.7078	24.3680	98.62 %	**Mean = 98.74 %** **RSD** = 0.30 %
2		A2	24.7541	24.3850	98.51 %	
3		A3	24.6152	24.3867	99.07 %	
4	100	A1	49.4156	49.1620	99.49 %	**Mean = 99.68 %** **RSD** = 0.23 %
5		A2	49.5082	49.3128	99.61 %	
6		A3	49.2303	49.1980	99.93 %	
7	150	A1	74.1234	73.9913	99.82 %	**Mean = 99.88 %** **RSD** = 0.14 %
8		A2	74.2623	74.0904	99.77 %	
9		A3	73.8455	73.8762	100.04 %	

Remarks: The recovery and RSD for recovery at each level meets the acceptance criteria.

Precision

i. System Precision: (Table 6)

Acceptance criteria for System Precision:

a) Column efficiency: Not less than 1500 theoretical plates for both Metformin Hydrochloride and Sitagliptin peaks
b) Resolution: Not less than 4.0 between Metformin Hydrochloride and Sitagliptin phosphate peaks.

c) Relative standard deviation: NMT 2.0 % for the 6 areas of Metformin Hydrochloride and sitagliptin phosphate peaks from 6 replicate standard injections.

Table 6: % RSD of area and Average of Column efficiency for the standard solution, Resolution between Metformin Hydrochloride and Sitagliptin Phosphate

Metformin Hydrochloride			Sitagliptin phosphate		Resolution NLT4.0
SET	(RSD NMT 2.0 %)	Number of theoretical plates NLT 1500	(RSD NMT 2.0 %)	Number of theoretical plates NLT 1500	
1	0.19 %	3003	0.13 %	8127	17.20
2	0.28 %	3026	0.11 %	7869	17.13
3	0.16 %	3013	0.17 %	7703	17.24
4	0.13 %	3019	0.12 %	7741	17.46
5	0.10 %	3446	0.06 %	8679	19.27
6	0.14 %	3411	0.44 %	8384	19.12

Remarks: Relative standard deviation for peak response, Number of theoretical plates and Resolution are within acceptable limits.

ii. Method Precision: (Table 7)

Acceptance criteria for Method Precision: The % RSD for the six Assay determinations is NMT 2.0 %

Table 7: Mean value of % of Drug Obtained in Method Precision and % RSD

Metformin Hydrochloride			Sitagliptin
S. No.	Sample ID	Content (% of Label claim)	Content (% of Label claim)
1	Sample-1	494.46 mg (98.89 %)	50.53 mg (101.06 %)
2	Sample-2	500.10 mg (100.02 %)	50.34 mg (100.68 %)
3	Sample-3	491.13 mg (98.23 %)	50.18 mg (100.36 %)
4	Sample-4	499.23 mg (99.85 %)	50.30 mg (100.60 %)
5	Sample-5	498.11 mg (99.62 %)	50.37 mg (100.74 %)
6	Sample-6	497.43 mg (99.49 %)	49.36 mg (98.72 %)
Mean		**496.74 mg (99.35 %)**	**50.18 mg (100.36 %)**
RSD (NMT 2.0 %)		**0.68 %**	**0.83 %**

Remarks: The % RSD for 6 assay values is within acceptable limits.

iii. Intermediate Precision: (Table 8, 9)

Analyst, Instrument and Day variability test was performed, and Overall RSD of Metformin Hydrochloride is 0.93 % (Table 8), Overall RSD of Sitagliptin Phosphate is 0.97 % (Table 9). **Acceptance Criteria for Intermediate Precision**: The % RSD for the six assay determinations shall be NMT 2.0 %, the overall % RSD for the two sets (Intermediate Precision and Precision) is NMT 2.0 %.

Table 8: Mean value, % RSD of Metformin Hydrochloride Obtained in Intermediate Precision and Precision

S.No	Intermediate Precision		Precision	
	Content /tablet	% of label claim	Content /tablet	% of label claim
1	493.29 mg	98.66 %	494.46 mg	98.89 %
2	492.26 mg	98.45 %	500.10 mg	100.02 %
3	499.48 mg	99.90 %	491.13 mg	98.23 %
4	496.85 mg	99.37 %	499.23 mg	99.85 %
5	498.18 mg	99.64 %	498.11 mg	99.62 %
6	491.59 mg	98.32 %	497.43 mg	99.49 %
Average	495.28 mg	99.06 %	**496.74 mg**	**99.35 %**
RSD (NMT 2.0 %)	**0.68 %**	**0.68 % %**	**0.68 %**	**0.68 %**

Table 9: Mean value, % RSD of Sitagliptin Phosphate Obtained in Intermediate Precision and Precision

S.No	Intermediate Precision		Precision	
	Content /tablet	% of label claim	Content /tablet	% of label claim
1	50.09 mg	100.18 %	50.53 mg	101.06 %
2	49.82 mg	99.64 %	50.34 mg	100.68 %
3	50.36 mg	100.72 %	50.18 mg	100.36 %
4	50 .36 mg	100.72 %	50.30 mg	100.60 %
5	50.04 mg	100.08 %	50.37 mg	100.74 %
6	50.22 mg	100.44 %	49.36 mg	98.72 %
Average	**381.31mg**	**101.68 %**	**50.18 mg**	**100.36 %**
RSD (NMT 2.0 %)	**1.10 %**	**1.105 %**	**0.83 %**	**0.83 %**

Remarks: RSD between the assay values and overall RSD between the two sets are within acceptable limits.

Robustness

By making minor, purposeful modifications to the wavelength, flow rate, pH of buffer and mobile phase, the method's robustness was demonstrated as shown in table 10. The samples were injected in 6 replicates and % RSD was calculated which is shown in table 10.

Acceptance Criteria for Robustness: Should pass the system suitability under each variable parameter.

Table 10: variation and its resulted values like % RSD, Theoretical plate and Resolution between Metformin Hydrochloride and Sitagliptin Phosphate

Metformin Hydrochloride				Sitagliptin Phosphate		Resolution NLT 4.0
Parameters	**Variation**	**RSD NMT 2.0 %**	**Theoretical plate NLT 1500**	**RSD NMT 2.0 %**	**Theoretical plate NLT 1500**	
Change in wavelength	265 nm	0.37 %	4056 - 4221	0.26 %	8740 - 8839	18.17-18.37
	269 nm	0.56 %	3529 - 3797	0.31 %	8822 - 8969	17.59-17.91
Flow rate	0.7 mL/min	0.32 %	4129 - 4202	0.18 %	9271 - 9450	18.58-18.67
	0.9 mL/min	0.91 %	4887- 5119	0.69 %	9385 - 9608	23.49-23.92
pH of buffer	pH 4.2	0.32 %	3552 - 3639	0.43 %	8592 - 8663	17.80-18.05
	pH 4.4	1.02 %	3582- 3709	0.83 %	8578 - 8772	17.77-18.02
Mobile phase composition	Buffer: Acetonitrile (58:42)	0.49 %	4721 – 4782	0.36 %	9004 -9157	17.65-17.91
	Buffer: Acetonitrile (62:38)	0.50 %	4484 – 4652	1.06 %	8944 -9114	20.31-20.87

Remarks: Passes the system suitability under each variable parameter.

Filter Integrity

After passing through 0.45µm PVDF, 0.45 µm Nylon and 0.45 µm Teflon filters, the filtered samples were injected. The areas obtained for the filtered samples were compared against the centrifuged sample as shown in table 11 & 12.

Acceptance Criteria for Filter Integrity: The % Deviation in area of the filtered solution from the area obtained in centrifuged solution is NMT 2.0 %.

Table 11: Area and % Deviation in Area of Metformin Hydrochloride Standard and Test Obtained Using Different Filter

S.No	Filtration	Standard Area	% Deviation in area	Test Area	% Deviation in area
1.	Centrifuge	164507	-	163835	-
2.	PVDF	162758	1.06 %	164163	0.21 %
3.	Nylon	167475	1.80 %	163575	0.57 %
4.	Teflon	163846	0.40 %	164248	0.16 %

Table 12: Area and % Deviation in Area of Sitagliptin Phosphate Standard and Test Obtained Using Different Filter

S.No	Filtration	Standard Area	% Deviation in area	Test Area	% Deviation in area
1.	Centrifuge	326100	-	325484	-
2.	PVDF	324319	0.55 %	325307	0.24 %
3.	Nylon	324419	0.52 %	324264	0.56 %
4.	Teflon	325208	0.27 %	325456	0.20 %

Remarks: The areas obtained for the solutions filtered through PVDF, Nylon and Teflon filters are well within specified limits. PVDF, Nylon and Teflon filters are suitable for filtration.

Solution stability

The sample solutions were prepared and their stability was to be tested for the initial hour, 4 hours, 8 hours, 12 hours, 24 hours, 48 hours and 72 hours. The percentage of deviation was also be measured which is shown in table 13, 14.

Acceptance Criteria for Solution Stability: The % Deviation from the initial area at each time is NMT 2.0 %.

Table 13: Solution stability of Metformin Hydrochloride standard and test at different time period.

S.No	Time (Hour)	Standard Area	% Deviation from the initial absorbance	Test Area	% Deviation from the initial absorbance
1.	Initial	161322	-	161747	-
2.	After 2 hours	162597	1.16 %	162929	0.96 %
3.	After 8 hours	162946	0.95 %	163458	0.64 %
4.	After 12 hours	164306	0.12 %	166477	1.20 %
5.	After 24 hours	162833	1.02 %	168680	2.54 %

Table 14: Solution stability of Sitagliptin phosphate standard and test at different time period.

S.No	Time (Hour)	Standard Area	% Deviation from the initial absorbance	Test Area	% Deviation from the initial absorbance
1.	Initial	323107	-	323757	-
2.	After 2 hours	323887	0.68 %	323790	0.71 %
3.	After 8 hours	325032	0.33 %	326325	0.07 %
4.	After 12 hours	324044	0.63 %	328620	0.77 %
5.	After 24 hours	326442	0.10 %	335858	2.99 %

Remarks: The % Deviation from the initial area is within acceptable limits for both the peaks in standard and test solutions even after 12 hours. Hence the solutions are stable up to 12 hours.

RESULTS:

A simple, efficient, and less time-consuming RP-HPLC method for simultaneous estimation of metformin hydrochloride and sitagliptin phosphate in tablet dosage form was developed and validated for Specificity, Linearity and Range, Accuracy, System Precision, Method Precision, Intermediate Precision, Robustness, Solution stability and Filter Integrity. The results obtained were good and found within the limit, proving that the developed method can be used for determination of metformin and sitagliptin tablets.

CONCLUSION:

Most of the methods used in the past for estimating Metformin Hydrochloride and Sitagliptin Phosphate were tedious and time-consuming. Thus, a reversed phase HPLC method was developed and validated. The results of the system suitability and applicability indicated that the proposed method is suitable and applicable for routine laboratory analysis. With less retention time, the approach offers good resolution between the drugs. The proposed technique is linear, exact, accurate, precise, robust, specific, and selective, according to the results of the validation parameters which confirms that the results obtained meet the pre-established acceptance criteria. Therefore, the study results confirm that the developed method is a suitable technique for simultaneous estimation of Metformin Hydrochloride and Sitagliptin phosphate in tablet dosage form.

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CONFLICTS OF INTEREST

There was no conflicts of interest.

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