Analytical Method Development and Validation for the Simultaneous Analysis of Anti-Diabetic Drugs Metformin and Evogliptin in Pure and Marketed Formulations by Uv Spectroscopy and Rp-Hplc

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***Abstract*—Analytical Method Developments and Validations for the Quantitative Estimation of Metformin and Evogliptin in bulk form and Combined Pharmaceutical Dosage Forms by UV Spectroscopy and RP-HPLC methods. In UV method the maximum absorption of Metformin and Evogliptin was found to be 248nm and 265nm respectively. The UV Method was validated as per the ICH Guidelines. In HPLC method, the chromatographic conditions were successfully developed for the separation of Metformin and Evogliptin by using Altima C18 (4.6mm×150mm, 5.0 µm), flow rate was 1.0 ml/min, and mobile phase ratio was Methanol: TEA Buffer pH 4.5: Acetonitrile in the ratio of 50:25:25% v/v/v, detection wavelength was 258nm. The retention times of Metformin and Evogliptin were found to be 2.102mins and 3.537mins respectively. The % purity of Metformin and Evogliptin was found to be 99.6%. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study of Metformin and Evogliptin was found in concentration range of 5µg/ml-25µg/ml and 12.5µg/ml -62.5µg/ml and correlation coefficient (r2) was found to be 0.9993 and 0.9998. The precision study was precise, robust, and repeatable. Hence the suggested UV and RP-HPLC method can be used for routine analysis of Metformin and Evogliptin in bulk and pharmaceutical dosage form for both methods regarding following ICH guidelines.**

***Index Terms*—Metformin and Evogliptin, UV, HPLC, Method Development, Validation, Accuracy.**

I. INTRODUCTION

Metformin is an agent belonging to the biguanide class of anti-diabetics with antihyperglycemic activity. Metformin is associated with a very low incidence of lactic acidosis[1]. This agent helps reduce LDL cholesterol and triglyceride levels, and is not associated with weight gain, and prevents the cardiovascular complications of diabetes. Metformin is not metabolized and is excreted unchanged by the kidneys[2]. Metformin is indicated as an adjunct to diet and exercise to increase glycemic control in adults and pediatric patients 10 years of age and older diagnosed with type 2 diabetes mellitus[3]. The IUPAC Name of Metformin is 3-(diamino methylidene)-1, 1-dimethylguanidine. The Chemical Structure of Metformin is shown in following figure-1.

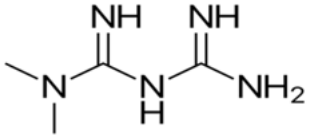

Fig-1: Chemical Structure of Metformin

Evogliptin has been used in trials studying the treatment and screening of Osteoporosis, Renal Impairment, Type 2 Diabetes Mellitus. Evogliptin is an antidiabetic medication[4]. It works by increasing the release of insulin from the pancreas and decreasing the hormones that raise blood sugar levels[5]. This reduces the fasting and postmeal sugar levels[6]. The IUPAC name of Evogliptin is (3R)-4-[(3R)-3-amino-4-(2, 4, 5-trifluoro phenyl) butanoyl]-3-[(2-methyl propan-2-

yl) oxy methyl] piperazin-2-one. The Chemical Structure of Evogliptin is shown in following figure-2.

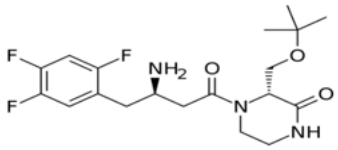

Fig-2: Chemical Structure of Evogliptin

II. MATERIALS AND METHODS

UV Experiment:

Instruments Used:

Table-1: List of Instruments

Sr. No.	Name of Instrument	Instrument Model	Name of Manufacturer
1	UV-Visible double beam spectrophotometer	UV 1800	Elico India
2	HPLC	1575	Hitachi LaChrome
3	Ultra Sonicator	-	Entrech electronics limited
4	Melting point apparatus	-	Lab India
5	Digital pH Meter	-	Lab India
6	Vacuum filtration kit	-	Lab India

Chemicals / Reagents Used

Table-2: List of Chemicals

S. No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.99%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen phosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9	L.R.	Sd fine-Chem ltd; Mumbai
6.	orthophosphoric acid	99.9	L.R.	Sd fine-Chem ltd; Mumbai

Method Development and Its Validation for Simultaneous Estimation of Metformin & Evogliptin by UV Spectroscopy:

Selection of Wavelength:

The λ_{max} of the two ingredients i.e. Metformin & Evogliptin, were found to be 248 nm and 265 nm respectively in methanol as solvent system[7].

Preparation of Standard Solution of Metformin

10 mg of Metformin was weighed accurately and transferred into 100 ml volumetric flask. About 10 ml of HPLC grade methanol was added and sonicated to dissolve. The volume was made up to the mark with same solvent. The final solution contained about 100 µg/ml of Metformin.

Preparation of Standard Solution of Evogliptin

10 mg of Evogliptin was weighed accurately and transferred into 100 ml volumetric flask. About 10 ml of HPLC grade methanol was added and sonicated to dissolve. The volume was made up to the mark with same solvent. The final solution contained about 100 µg/ml of Evogliptin[8].

HPLC Experiment:

Instruments Used:

Table-3: Instruments Used

S. No.	Instruments and Glasswares	Model
1	HPLC	WATERS Alliance 2695 separation module, software: Empower 2, 996 PDA detector.
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital Ultra Sonicator	Labman

Chemicals Used:

Table-4: Chemicals Used

S. No.	Chemical	Brand Names
1	Metformin	Synpharma Research Lab
2	Evogliptin	Synpharma Research Lab
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

HPLC Method Development:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Metformin and Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol. Further pipette 0.1ml of the above Metformin and 0.375ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines[25,30].

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water and Water: Acetonitrile and Methanol: TEA Buffer: ACN with varying proportions. Finally, the mobile phase was optimized to Methanol: TEA Buffer: ACN in proportion 50:25:25 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, Symmetry and Zodiac column. Altima C18 (4.6×150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow at 225nm[9].

Preparation of Triethylamine (TEA) buffer (pH-4.5):

Dissolve 1.5ml of Triethyl amine in 250 ml HPLC water and adjust the p^H 4.5. Filter and sonicate the solution by Vaccum filtration and ultrasonication.

Preparation of Mobile Phase:

Accurately measured 400 ml (40%) of Methanol, 200 ml of Triethylamine buffer (20%) and 400 ml of Acetonitrile (40%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration[10].

Diluent Preparation:

The Mobile phase was used as the diluent.

Method Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1ml of the above Metformin and 0.375ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The resolution, tailing factor and theoretical plates for the five replicate injections was found to be within the specified limits[11].

Specificity:

Preparation of Standard Solution:

Accurately weigh and transfer 10mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1ml of the above Metformin and 0.375ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution:

Take average weight of one Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Metformin and Evogliptin sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.1ml of the above Metformin and 0.375ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:
Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula[12]:
%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity:
Accurately weigh and transfer 10 mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Preparation of Level – I (5 ppm of Metformin & 12.5ppm of Evogliptin): Pipette out 0.05ml of Metformin and 0.125ml of Evogliptin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.
Preparation of Level – II (10 ppm of Metformin& 25ppm of Evogliptin): Pipette out 0.1ml of Metformin and 0.25ml of Evogliptin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.
Preparation of Level – III (15 ppm of Metformin& 37.5ppm of Evogliptin): Pipette out 0.15 ml of Metformin and 0.375ml of Evogliptin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent[13].
Preparation of Level – IV (20 ppm of Metformin& 50ppm of Evogliptin): Pipette out 0.2 ml of Metformin and 0.5ml of Evogliptin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.
Preparation of Level – V (25 ppm of Metformin& 62.5ppm of Evogliptin): Pipette out 0.25ml of Metformin and 0.625ml of Evogliptin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.
Procedure: Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient[14].
Precision
Repeatability
Preparation of Metformin and Evogliptin Product Solution for Precision:
Accurately weigh and transfer 10 mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1ml of the above Metformin and 0.375ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
Procedure: The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.
Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions[15-17].

Procedure:
Day 1: The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.
Day 2: The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.
Accuracy:
For Preparation of 50% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.075ml of the above Metformin and 0.187ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent[18].
For Preparation of 100% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.15ml of the above Metformin and 0.375ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
For Preparation of 150% Standard Stock Solution:

Accurately weigh and transfer 10 mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.225ml of Metformin and 0.56ml of Evogliptin from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

Inject the Three replicate injections of individual concentrations (50%,100%,150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Metformin and Evogliptin and calculate the individual recovery and mean recovery values[19].

Robustness:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For Preparation of Standard Solution: Accurately weigh and transfer 10 mg of Metformin and 10mg of Evogliptin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.15ml of the above Metformin and 0.375ml of the Evogliptin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of Flow Conditions: The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded[20].

Effect of Variation of Mobile Phase Organic Composition: The sample was analyzed by variation of mobile phase i.e. Methanol: TEA Buffer: Acetonitrile was taken in the ratio and 40: 40:20, 60:10:30 instead of (50:25:25), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

III. RESULTS AND DISCUSSION

UV Experiment:

Selection of Wavelength:

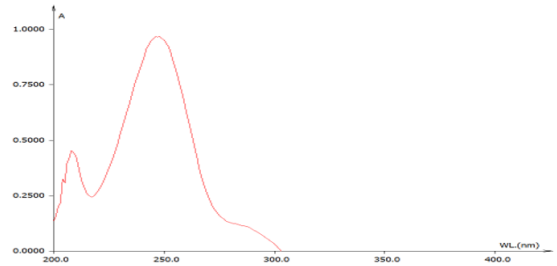

Fig-3: UV Spectrum for Metformin (248nm)

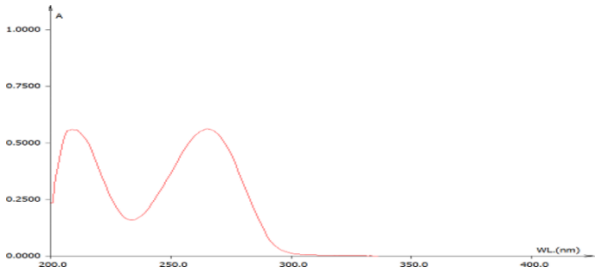

Fig-4:- UV spectrum for Evogliptin (265nm)

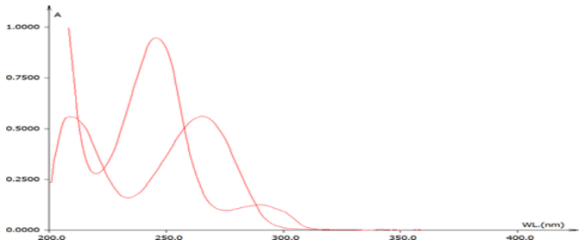

Fig-5: Isobestic Point Metformin & Evogliptin (258nm)

Validation of the Developed Method According to I.C.H Guidelines

Following parameters were taken into consideration for validation of proposed methods:

1. Linearity

Preparation of Calibration Curve for Metformin & Evogliptin

Standard solutions of Metformin in the concentration range of 2 µg/ml to 80 µg/ml were obtained by transferring (0.2,1,2,3,4,5,6,7,8 ml) of Metformin stock solution (100 ppm) to the series of 10 ml volumetric flasks and standard solutions of Evogliptin in the concentration range of 5 µg/ml to 70 µg/ml were obtained by transferring (0.5,1,2,3,4,5,6,7 ml) of Evogliptin stock solution (100 ppm) to the series of 10 ml volumetric flasks. The volumes in each volumetric flask were made up with the solvent system and mixed.

The Absorbances of the solutions were measured at 248 nm and 265 nm against the solvent system as blank and calibration curves were plotted. The Lambert-Beer's Law is linear in concentration range of 2 to 80 µg/ml at 248 nm and 2 to 80 µg/ml at 265 nm for Metformin. The Lambert-Beer's Law is linear in concentration range of 5 to 70 µg/ml at 248 nm and 5 to 70 µg/ml at 265 nm for Metformin. The results are shown in Table no. 7 and 8.

Table-7: Absorbances of Metformin

Conc. in ppm	Absorbance at 248nm
2	0.083
10	0.326
20	0.628
30	0.934
40	1.251
50	1.546
60	1.839
70	2.135
80	2.459

Table-8: Absorbances of Evogliptin

Conc. in ppm	Absorbance at 265nm
5	0.161
10	0.302
20	0.581
30	0.887
40	1.176
50	1.471
60	1.749
70	2.041

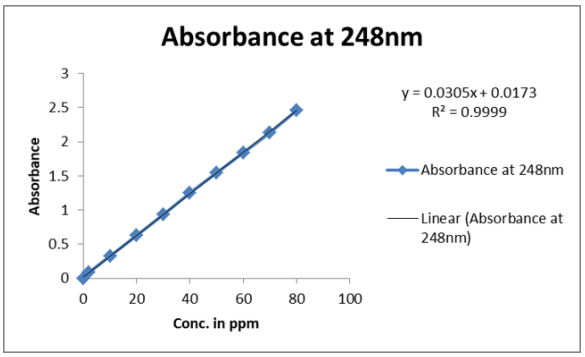

Fig-8: Calibration Curve for Metformin at 248nm

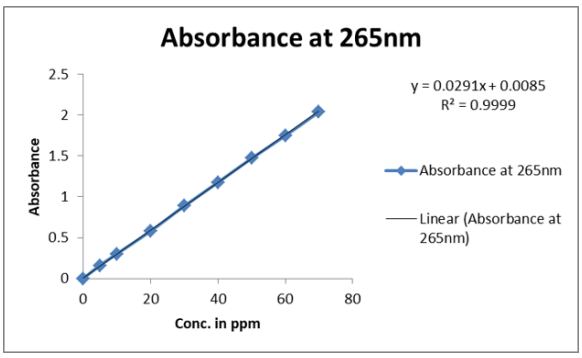

Fig-9: Calibration Curve for Evogliptin at 265nm

Result & Discussion: Linearity range was found to be 2-80 µg/ml for Metformin at 248 nm. The correlation coefficient was found to be 0.9999, which adhere good linearity between above range. The slope was found to be 0.0305 and intercept was found to be 0.0173 which was close to zero intercept.

For Evogliptin at 265nm linearity range was found to be 5-70µg/ml. The correlation coefficient was found to be 0.9999, which adhere good linearity between above range. The slope was found to be 0.0291 and intercept was found to be 0.0085 which was close to zero intercept.

2. Range

Range of an analytical method is the interval between the upper and lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. It includes working range, linearity range and target range and 100% concentration or test concentration.

3. Accuracy

The results of analysis, obtained in three group containing three replicate experiments with API and different tablet dosage forms, had good agreement with the labelled amount of the drug[23].

Method: In nine different 10 ml volumetric flasks, 1 ml of the pre-analyzed tablet solution (100 µg/ml) was taken and added 1, 2, 3 ml of standard solution of bulk (API) mixture (100µg/ml) and the volume was made up to 10 ml with methanol. The results are shown in Table no.9.

Table-9: Data of Recovery Studies

Mix.	WL. (nm)	Conc. of Tablet Sol^n (ppm)	Std. Added (ppm)	Abs.	Amt. Found (mg)	% Recovery	Mean Recovery ± S.D.	% RSD
1	248	2.079	1	0.569	4.066	99.35	MET	EVO
	265	9.996	10	0.678	19.987	99.91		
	248	2.079	1	0.568	4.050	98.55		0.757
	265	9.996	10	0.677	19.964	99.68	99.383 ± 0.752	
	248	2.079	1	0.570	4.081	100.10	MET	EVO
	265	9.996	10	0.679	20.01	100.140	99.782 ± 0.216	0.216
2	248	2.079	2	0.851	6.082	100.075		
	265	9.996	20	1.014	29.892	99.480		
	248	2.079	2	0.853	6.068	99.725		
	265	9.996	20	1.017	30.001	100.025		
	248	2.079	2	0.852	6.097	100.450		
	265	9.996	20	1.015	29.915	99.595		
3	248	2.079	3	1.131	7.977	98.300		
	265	9.996	30	1.350	39.879	99.610		
	248	2.079	3	1.134	8.024	99.083		
	265	9.996	30	1.353	39.947	99.836		
	248	2.079	3	1.133	8.008	98.817		
	265	9.996	30	1.352	39.924	99.760		

Result & Discussion: The results obtained for the accuracy study (recovery method) from three sample studies (n = 3) for each level indicated that the mean of the % recovery was 99.383 % and 99.782 % and R.S.D was 0.757% and 0.216 % for Metformin & Evogliptin respectively in synthetic mixture (MET 1 µg/ml: EVO 10 µg/ml). Here the mean % recovery is in between 98-102 % thus showing that the analytical technique has a good recovery study.

IV. PRECISION

Repeatability was assessed using: Six-time repetition of target concentration 100 % that is (10µg/ml). Intermediate precision can be assessed by intra-day and inter day analysis[24].

4.1 Repeatability

Method: In the study of the repeatability precision which was conducted on the solution which has the concentration value 100 % of the target concentration (n = 6). The results are shown in Table-10.

Table-10: Data of Repeatability of Absorbances

S. No.	Conc. µg/ml	Wavelength (nm)	Abs.	Mean ± S.D.	% RSD
1	(MET 7.5 + EVO 37.5) µg/ml	248	1.067	MET	EVO
		265	1.272	7.547 ± 0.033	0.437
2		248	1.065		
		265	1.212		
3		248	1.066		
		265	1.272		
4		248	1.068	MET	EVO
		265	1.274	37.553 ± 0.036	0.096
5		248	1.067		
		265	1.273		
6		248	1.064		
		265	1.270		

Result & Discussion: Repeatability study showed a R.S.D of 0.437 % for Metformin and 0.096 % for Evogliptin. Thus, it is concluded that the analytical technique has a good repeatability precision as R.S.D for both drugs were less than 2 %.

V. LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

The slope S may be estimated from the calibration curve of the Analyte.

Result & Discussion: The LOD was found to be 0.2682 µg/ml and 0.2542 µg/ml and LOQ was found to be 0.7698 µg/ml and 0.6365 µg/ml for Metformin and Evogliptin respectively which represents that sensitivity of the method is high[25].

Estimation of Metformin and Evogliptin in Pharmaceutical dosage form

Twenty tablets/Capsules were taken and the I.P. method was followed to determine the average weight.

Method: Above weighed tablets/Capsules were finally powdered and triturated well. A quantity of powder equivalent to 120 mg of drugs were transferred to 100 ml volumetric flask, and mixed with 70 ml of methanol and solution was sonicated for 10 minutes there after volume was made up to 100 ml with same solvent system. The solution was filtered through Whatman filter paper. Then 10 ml of the above filtrated solution was diluted to 100 ml with the solvent system. From this stock solution (1ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system. The Absorbances of these solutions were measured at 248 nm and at 265 nm using methanol as blank[26].

Result & Discussion: The amount of drug in tablet was found to be 4.658 mg/tab for Evogliptin and 499.059 mg/tab for Metformin respectively.

HPLC Experiment:

Analytical Method Development:

Optimised Chromatographic Conditions:

Mobile phase: Methanol: TEA Buffer pH 4.5: Acetonitrile (50:25:25% v/v/v)

Column: Altima C18 (4.6mm×150mm, 5.0 µm)

Flow rate: 1.0 ml/min

Wavelength: 225 nm

Column temp: 40ºC

Injection Volume: 10 µl

Run time: 7 minutes

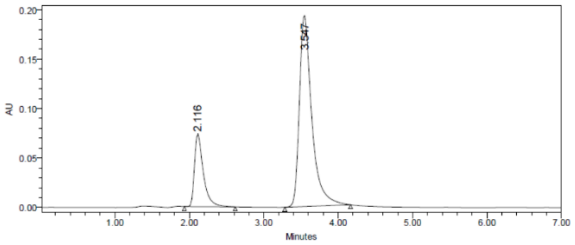

Fig-10: Optimized Chromatographic Condition

Analytical Method Validation:

System Suitability:

Table-11: Results of System Suitability for Metformin

S.No.	Name	Rt	Area	Height	USP Plate Count	USP Tailing
1	Metformin	2.117	608452	71498	5643	1.9
2	Metformin	2.118	606820	126412	5432	1.6
3	Metformin	2.116	608452	126471	5123	1.6
4	Metformin	2.109	595267	129859	5207	1.7
5	Metformin	2.102	596608	124691	5481	1.6
Mean			603119.8			
Std. Dev			6607.31			
% RSD			1.09			

Table-12: Results of System Suitability for Evogliptin

S.No.	Name	Rt	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Evogliptin	3.547	2234724	188631	5043	1.2	2.07
2	Evogliptin	3.539	2240080	2614821	5432	1.4	2.05
3	Evogliptin	3.547	2234724	2321451	5987	1.5	2.0
4	Evogliptin	3.565	2204466	2324710	5845	1.6	2.01
5	Evogliptin	3.537	2209574	2531247	5371	1.6	2.01
Mean			2224714				
Std. Dev			16399.05				
% RSD			0.73				

Table-13: System Suitability Results for Metformin and Evogliptin

S.No.	Parameter	Limit	Result
1	Asymmetry	T ≤ 2	Metformin = 0.12, Evogliptin = 0.19
2	Theoretical plate	N > 2000	Metformin = 5586.0, Evogliptin = 5371.0
3	Tailing Factor	(Tf) < 2	Metformin = 0.96, Evogliptin = 1.22
4	Resolution	Rs > 2	10.68
5	Capacity Factor (K)	< 1	0.25
6	Retention Time (Rt)	--	Metformin = 2.116, Evogliptin = 3.547

Specificity

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components. Analytical method was tested for specificity to measure accurately quantitates Metformin and Evogliptin in drug product[27].

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Metformin and Evogliptin in pharmaceutical dosage form was found to be 99.6%.

Linearity

Chromatographic Data for Linearity Study:

Table-14: Linearity Data of Metformin:

Concentration µg/ml	Average Peak Area
5	205035
10	381239
15	561128
20	740162
25	909922

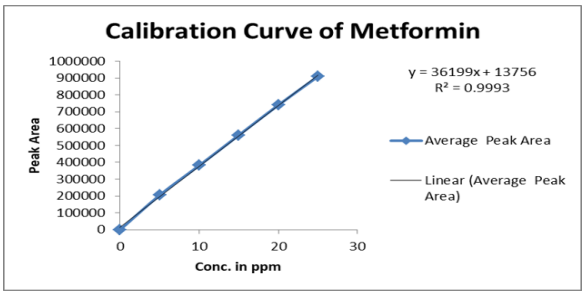

Fig-11: Calibration Graph for Metformin

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Metformin is a straight line[28].

Y = mx + c

Slope (m) = 36199

Intercept (c) = 13756

Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 13756. These values meet the validation criteria.

Table-15: Linearity Data of Evogliptin

Concentration µg/ml	Average Peak Area
12.5	527881
25	1087881
37.5	1658941
50	2232457
62.5	2771811

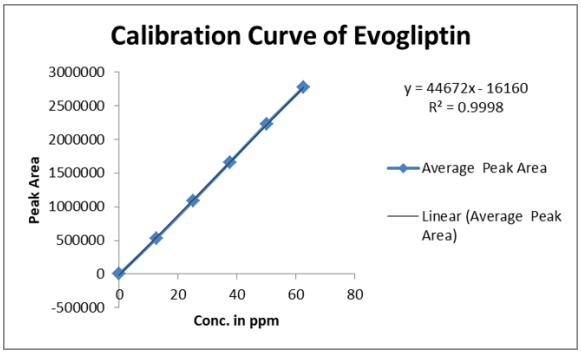

Fig-12: Calibration Graph for Evogliptin

Linearity Plot: The plot of Concentration (x) versus the Average Peak Area (y) data of Evogliptin is a straight line.

Y = mx + c

Slope (m) = 44672

Intercept (c) = 16160

Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 16160. These values meet the validation criteria[29].

Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions[27].

Repeatability: Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD[30].

Table-16: Results of Repeatability for Metformin:

S. No.	Name	Rt	Area	Height	USP Plate Count	USP Tailing
1	Metformin	2.108	602223	128898	2586	1.6
2	Metformin	2.105	607748	129233	2947	1.4
3	Metformin	2.113	607302	127409	2468	1.6
4	Metformin	2.109	608674	127047	2146	1.9
5	Metformin	2.109	607376	129859	2307	1.7
Mean			606665			
Std. Dev			2542.3			
% RSD			0.42			

Table-17: Results of Method Precision for Evogliptin:

S. No.	Name	Rt	Area	Height	USP Plate Count	USP Tailing
1	Evogliptin	3.552	2220333	2231111	1.6	2371
2	Evogliptin	3.550	2221573	2674210	1.6	2841
3	Evogliptin	3.564	2215483	2231261	1.5	2816
4	Evogliptin	3.564	2217379	2421301	1.5	2872
5	Evogliptin	3.565	2211255	2324710	1.6	2845
Mean			2217205		1.6	2841
Std. Dev			4100.8			
% RSD			0.18			

Intermediate Precision[31]:

Day 1:

Table-18: Results of Intermediate Precision for Metformin

S.No.	Name	Rt	Area	Height	USP Plate Count	USP Tailing
1	Metformin	2.108	596608	128898	2547	1.6
2	Metformin	2.105	598959	129233	2944	1.4
3	Metformin	2.113	595728	127409	2361	1.6
4	Metformin	2.109	594485	127047	2546	1.9
5	Metformin	2.109	595267	129859	2207	1.7
6	Metformin	2.102	596608	124691	2481	1.6

Mean			596209			
Std. Dev			1718.7			
% RSD			0.29			

Table-19: Results of Intermediate Precision for Evogliptin

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Evogliptin	3.552	2207732	2231134	8371	1.5	2.04
2	Evogliptin	3.550	2202266	2674210	6841	1.6	2.03
3	Evogliptin	3.564	2209375	2247461	7816	1.6	2.01
4	Evogliptin	3.564	2204037	2454301	8872	1.6	2.05
5	Evogliptin	3.565	2204466	2324710	4845	1.6	2.02
6	Evogliptin	3.537	2209574	2531247	8371	1.6	2.03
Mean			2205575				
Std. Dev			2899.8				
% RSD			0.13				

Day 2:

Table-20: Results of Intermediate Precision Day 2 for Metformin

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Metformin	2.102	602155	127998	5586	1.5
2	Metformin	2.105	603662	134844	5636	1.6
3	Metformin	2.112	603931	161103	5432	1.6
4	Metformin	2.113	607302	127409	5468	1.6
5	Metformin	2.109	608674	127047	5146	1.9
6	Metformin	2.109	607376	129859	5307	1.7
Mean			605516.7			
Std. Dev			2602.622			
% RSD			0.42			

Table-21: Results of Intermediate Precision for Evogliptin

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Evogliptin	3.537	2241579	2263528	2371	1.6	7.98
2	Evogliptin	3.552	2236409	2224418	2414	1.6	6.4
3	Evogliptin	3.560	2239093	2233725	2384	1.6	8.9
4	Evogliptin	3.564	2215483	2231261	2816	1.5	8.3
5	Evogliptin	3.564	2217379	2421301	2872	1.5	7.5
6	Evogliptin	3.565	2211255	2324710	2845	1.6	5.3
Mean			2226866				
Std. Dev			13567.02				
% RSD			0.60				

Accuracy: Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated[32].

Table-22: The Accuracy Results for Metformin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	287774	7.5	7.56	100.8	99.6%
100%	551495	15	14.8	98.6	
150%	825175	22.5	22.4	99.5	

Table-23: The Accuracy Results for Evogliptin

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1104782	18.75	18.73	100%	100%
100%	2105321	37.5	37.4	99.9%	
150%	3211306	56.25	56.21	100%	

Limit of Detection: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value[33].

LOD= 3.3 × σ / s

Were

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Metformin: = 0.2µg/ml

Evogliptin: = 0.9µg/ml

Limit of Quantitation: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined[34].

LOQ = 10×σ/S

Were

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Metformin: = 0.8µg/ml

Evogliptin: = 2.7µg/ml

Robustness: The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Metformin and Evogliptin. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Metformin and Evogliptin were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count[35].

Table-24: Results for Robustness for Metformin:

Parameter used for Sample Analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	607323	2.102	5586	1.7
Less Flow rate of 0.9 mL/min	674735	2.330	5231	1.7
More Flow rate of 1.1 mL/min	1408920	1.950	5234	1.7
Less organic phase	606093	2.290	5643	1.4
More organic phase	603559	1.998	5298	1.5

Table-25: Results for Robustness for Evogliptin:

Parameter used for Sample Analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	558777	3.537	5371	1.6
Less Flow rate of 0.9 mL/min	2505636	3.885	5324	1.7
More Flow rate of 1.1 mL/min	1408920	3.263	5098	1.7
Less organic phase	2239255	4.435	5239	1.2
More organic phase	2300346	3.009	5647	1.0

Stability Studies: The specificity of the method can be demonstrated by applying stress conditions using acid, alkaline, peroxide, thermal, UV, water degradations. The sample was exposed to these conditions the main peak of the drug was studied for peak purity that indicating the method effectively separated the degradation products from the pure active ingredient[36-39].

Table-26: Results of Forced Degradation Studies of Metformin

S. No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	607323	0	100%	100%
2	Acidic	560905.303	7.643	92.357	100%
3	Basic	587803.638	3.214	96.786	100%
4	Oxidative	601037.206	1.035	98.965	100%
5	Thermal	574989.123	5.324	94.676	100%
6	Photolytic	580400.371	4.433	95.567	100%

Table-27: Results of Forced Degradation Studies of Evogliptin

S.No.	Stress Condition	Peak Area	% of Degraded Amount	% of Active Amount	Total % of Amount
1	Standard	2231111	0	100%	100%
2	Acidic	2089167.718	6.362	93.638	100%
3	Basic	2166118.736	2.913	97.087	100%

4	Oxidative	2166319.536	2.904	97.096	100%
5	Thermal	2120827.183	4.943	95.057	100%
6	Photolytic	2158733.759	3.244	96.756	100%

VI. SUMMARY AND CONCLUSION

Summary of UV Experiment:

The standard solutions of Metformin and Evogliptin in Methanol (10µg/ml) subjected to a scan individually at the series of wavelengths of 200 nm to 400 nm. Absorption maximum of Metformin and Evogliptin was found to be at 248 nm and 265nm respectively. Therefore, 248nm and 265nm were selected as λ_{max} of Metformin and Evogliptin for the present study. The calibration curve of Metformin and Evogliptin was found to be linear in the range of 2-80 µg/ml at 248 nm and 5 µg/ml to 70 µg/ml at 265nm respectively. The % recovery was carried out at 3 levels, 80%, 100% and 120% of Metformin and Evogliptin standard concentration. Three samples were prepared for each recovery level. The developed method was subjected to do the various method validation parameters such as specificity, accuracy, precision, linearity and range, limit of detection and limit of quantification, robustness and ruggedness etc.

Summary of HPLC:

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for simultaneous analysis of Metformin and Evogliptin different chromatographic conditions were applied & the results observed are presented in previous chapters. In case of RP-HPLC various columns are available, but here Altima C18 (4.6mm×150mm, 5.0 µm) column was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Metformin and Evogliptin it is evident that most of the HPLC work can be accomplished in the wavelength range of 240-300 nm conveniently. Further, a flow rate of 1.0 ml/min & an injection volume of 20 µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay & stability which can help in the simultaneous analysis of Metformin and Evogliptin in different formulations.

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