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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF METFORMIN AND EVOGLIPTIN IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A Simple, Accurate, Precise method was developed for the simultaneous estimation of Evogliptin and Metformin in pharmaceutical dosage form. Chromatogram was run through ACCLAIMED mix mode HILIC-1; 5µ, 150 X 4.6mm. ID. Mobile Phase containing Ammonium acetate and acetonitrile (70:30 v/v) as a mobile phase was pumped through column at a flow rate of -1mL.min^{-1}. Room temperature was maintained. Optimized wavelength of Metformin and Evogliptin was 205 nm. Retention time of Evogliptin and Metformin were found to be 4.93 min and 8.42 min. % RSD of the Evogliptin and Metformin were found to be 0.43 and 0.92 respectively. LOD, LOQ values are obtained from equation of Evogliptin and Metformin were 1.34, 0.75 & 4.46, 2.50 respectively. linear regression observed for EVO and MET. As resulted, they were y = 73859x + 44465 and y = 64111x + 19284, respectively.

KEYWORDS: Method Development, Validation, Metformin, Evogliptin, RP-HPLC.

INTRODUCTION

Metformin is an effective biguanide class of oral antihyper glycemic agent and chemically known as 3-(diaminomethylidene)-1,1- dimethyl guanidine; hydrochloride. Metformin hydrochloride has been considered as the first line treatment to control blood glucose level of non-insulin-dependent diabetes mellitus (type II).

Evogliptin tartrate is oral DPP-4 inhibitors and chemically known as (3R)-4-[(3R)-3-amino- 4-(2,4,5-trifluorophenyl) butanoyl]-3-[(2-methylpropan-2-yl) oxymethyl] piperazin-2-one;(2R,3R)-2,3 dihydroxybutanedioic acid and it's used to improve glycaemic control mainly via stimulation of glucose-mediated incretin secretion, resulting in increased insulin secretion and decreased glucagon release with lower risk for hypoglycaemia.

This combination was developed to improve medication adherence for type 2 diabetes mellitus. This review focuses on the recent developments in analytical techniques for estimation of Metformin Hydrochloride & Evogliptin Tartrate, and there was no any method reported for this combination. However, UV, HPLC, Stability indicating RP-HPLC, and HPTLC methods have been reported for Metformin Hydrochloride individual and along with other drugs and for Evogliptin Tartrate only one UV Spectrophotometric method has been reported.[1]

Quality assurance applies for both the drug substance (API) and medical product, and includes current Good Manufacturing Practices (cGMP), as well as any necessary analytical testing and stability studies. It is both state of mind, and an understanding the regulation and guidance's relating to the development and validation manufacturer of medicinal product are of a standard that assures the patient's expectations of safety and efficacy.[1]

Quality can be defined as the character, which define the grade of excellence. A good quality drug is something, which will meet the established product specifications can be safely bought and confidently used for the purpose for which it is intended.[2]

Quality is an effective system of integrating improvement efforts of various groups of the organization so as to provide product at level which allow customer satisfaction. Quality, a source of competitive advantage should remain a official mark of company products. High quality is not an added value; it is an essential basic requirement for pharmaceutical products.[3,4]

The word quality is normally referred to "conformance to specification" or a "degree of excellence". The international organization (ISO) defines quality as the totality of features and characteristics of a product or a service that bear on its ability to satisfy stated or implied needs. In pharmaceutical industry, the quality is a measure of high degree of managerial, scientific and technical sophistication.[5]

A poor quality medicine is one that does not meet specification. The use of poor quality product may have undesirable clinical and economical effect, as well as affect the credibility of the health delivery system. Clinical effect, as well as affect the credibility of the health delivery system. Clinical effect can include prolonged illness or death or adverse reaction. On the economic side, limited financial resources may be wasted on poor quality, packaging, transportation, storage conditions and other factor and these influences may be cumulative.[6]

1.2 Analytical Method Development

Methods are developed for new products when no official methods are available. Alternate method for existing (non-pharmacopoeia) products are to reduce the cost and time. for better precision and ruggedness. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available. The goal of the HPLC-method is to try & separate, quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and any degradants.

- **Basic criteria for new method development of drug analysis**

✓ The drug or drug combination may not be official in any pharmacopoeias.
✓ A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
✓ Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
✓ Analytical methods for the quantitation of the drug in biological fluids may not be available.

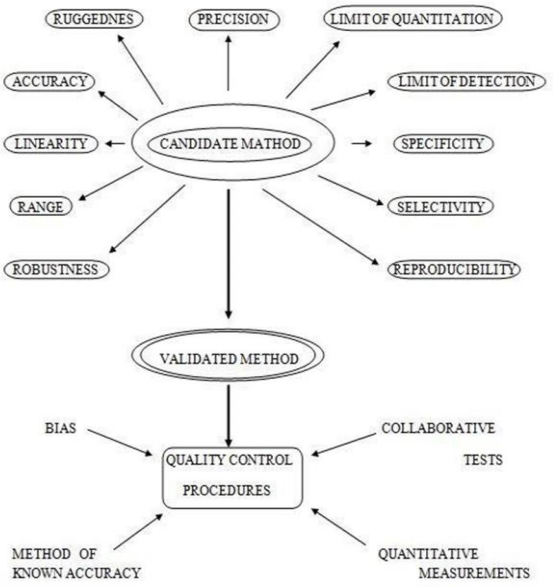

Fig. 1.1: Flow chart for method development strategy.

MATERIAL AND INSTRUMENT

MATERIALS

The drug used for present investigation was obtained from Alkem Laboratories LTD. as a gift sample.

Details of pure drug

Table No 1.1: Details of API.

Drug	Supplied by	Quantity	Purity (Assay)
Evogliptin	Alkem Laboratories LTD	10mg	99.9% w/w
Metformin	Alkem Laboratories LTD	10mg	99.05% w/w

Marketed preparation

Table no 1.2: Details of Marketed Preparation.

Brand name	Mfg. by	Content	Quantity
VALERA M 500	Alkem Laboratories LTD. As a gift sample.	Evogliptin	5mg
		Metformin	500mg

EXPERIMENTAL WORK

Instrumentation

The high-performance liquid chromatography (HPLC) of analytical technology Ltd. in built with binary pump, UV detector (Analytical technology Ltd.), 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. Water Symmetry®, 3.5 µm; 150 x 4.6 mm ID., HPLC column purchased from (Newcastle-UK) was used throughout the analysis. Digital weighing balance (PGB 100) purchased from Mettler-Toledo (USA), Wenser ultra-sonicator Labman®

purchased from Ultra Chrome Ltd, India. Digital pH meter from Mettler-Toledo was purchased from (Mumbai-India). 50µmicro syringe was purchased from Hamilton USA.0.20µ and0.45µ nylon membrane filters were purchased from Phenomenex ®Mumbai, India.

Standard stock solutions

Standard stocks solutions of EVO and MET (1mgml-1) were prepared separately by dissolving 10 mg of the drug in using a 20 ml volumetric flask and completing the final volume adjusted with ammonium acetate-acetonitrile (70:30 v/v) based on the solubility of drugs in particular eluents. Furthermore, freshly prepared sample solution was sonicator for 10 minutes and later filtered through 0.20µ nylon filters. Required serial dilution was made for evaluating the validation studies.

Working stock solutions

Working stock solution of EVO (375µg mL-1) was prepared by serial dilution of 37.5 ml of its stock solution in a 100 mL volumetric flask by completing to volume with the mobile phase. Working solution of MET (125µg mL-1) was prepared by serial dilution of 12.5 ml of its stock solution in a 100mLvolumetric flask by completing to volume with the mobile phase.

Marketed Sample preparation

Exactly 20 tablets of VALERA-500 containing 5 mg of EVO and 500 mg of MET were weighed separately, powdered and mixed in a mortar. An accurately weighed 10 mg amount of the finely powdered VALERA-500 tablets were transferred into 100 mL volumetric flask and the volume was adjusted with 10mL of ammonium acetate-acetonitrile (70:30 v/v) and sonicated until completely dissolved. The solutions were filtered with 0.2 µ nylon filters, followed by serial dilutions to the required concentrations using the same mobile phase for experiment with standard addition technique.

Linearity/Calibration studies

Accurately measured aliquots of stock solutions ranging between 3.9 – 62.5 µg, of EVO and MET combination were made transferring 10 mg of each combination of EVO and MET into 25 ml volumetric flasks. It was then mixed with 10 ml of AA-ACN to prepare 1000 ppm. Serial dilutions of the samples were made by adjusting the volume to make 5 dilutions between 3.9 - 62.5 µg ml^{-1} with same mobile phase, and then 20 µL were injected into the HPLC instrument. A calibration curve (linearity graph) was plotted by calculating peak area against concentration.

Precision of the proposed method

Triplicates of similar concentration so the mixture of EVO and MET (500µg. mL^{-1}) were analyzed nine times, within the same day, using the procedure. Also the triplicates of similar concentrations of the mixture of EVO and MET (500µg.mL^{-1}) were analyzed on three successive days. Using the same procedure mentioned in section.

Robustness for the chromatographic method

The flow rate of the mobile phase was deliberately changed from 1 ml to ±0.2 mL min^{-1} to make 1.2ml min^{-1} and 0.8mL $min^{-1}$and the results were evaluated to understand the separation behavior. Similarly, the variation of organic modifier used as acetonitrile was changed by from 30 to ±2% to make 32% and 28% to monitor the peak area and retention time. Finally, the effect of wavelength was monitored by making deliberate variation from205 by±2nm to make 203nmand 207 nm to understand the peak shape and area.

RESULTS AND DISCUSSION

- **Determination of λmax of Evogliptin and metformin**

The standard stock solution of evogliptin and metformin was prepared as describe in experimental section.

The λmax was determined on UV- visible spectrophotometer (model UV- 1800) in the range of 200-400 nm using methanol as a blank. The solution of mixture exhibited maxima at about 205nm.

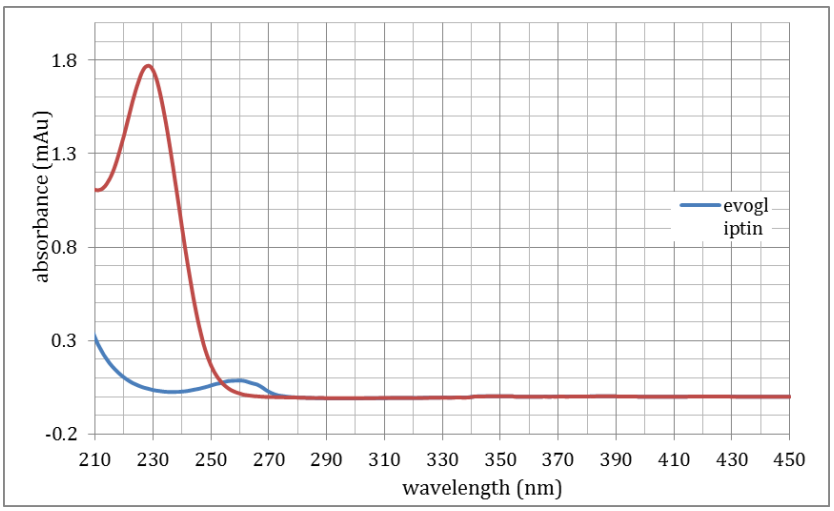

Fig no. 1.2: Overlain spectra of EVO and MET.

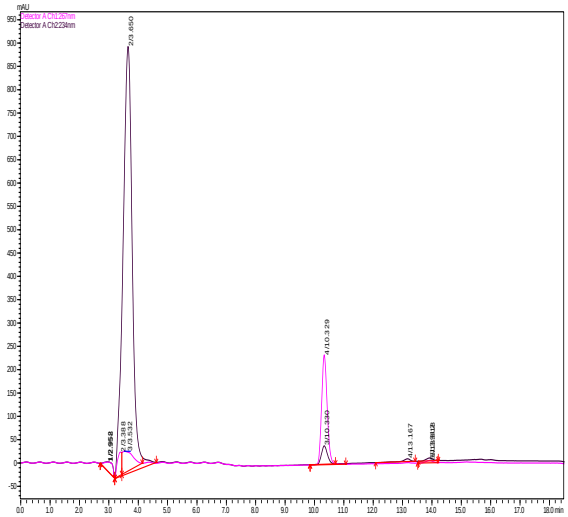

Figure 1.3: Trial reports of MTF and EVO by RP-HPLC.

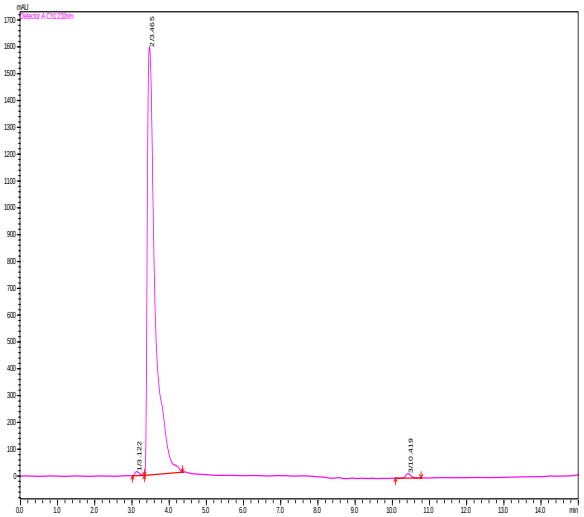

Figure 1.4: Trial reports of MTF and EVO by RP-HPLC.

Several articles have been published on simultaneous estimation of metformin and Evogliptin using C18 column with different dimensions. However, in all published articles the metformin was eluted with the t_0 value. Hence, as per the ICH guidelines, this separation pattern cannot be considered as retained. ICH guideline has recommended that first eluting compound should have more than 0.5 capacity factor. Nevertheless, in all attempted separation carried out in C18 column, metformin was eluted with lower capacity factor. Most importantly, all reported articles have not mentioned the capacity factor in their reported articles which should be mandatory as per the ICH guidelines.

Therefore, considering above limitations of C18 column towards simultaneous estimation of metformin and Evogliptin; Acclaimed mix mode HILIC-1 column (5µ, 150 x 5.6 mm. ID) was selected and all relevant results were displayed in Figures (1.2 & 1.4). In addition, the sensitivity of Evogliptin was found quite low even in lower UV wavelength at 205 nm. Hence it affects to archives stable base line while selecting the gradient elution mode towards simultaneous estimation of metformin and Evogliptin. This obstacle was most predominantly observed in separation carried out C18 column. Altogether, this simultaneous estimation of both selected drugs carried out in Acclaimed mix mode chromatography, proved effectively the separation of Evogliptin and metformin with acceptable resolution (R) and capacity factor (k) with significant improved UV sensitivity at 205 nm wavelength.

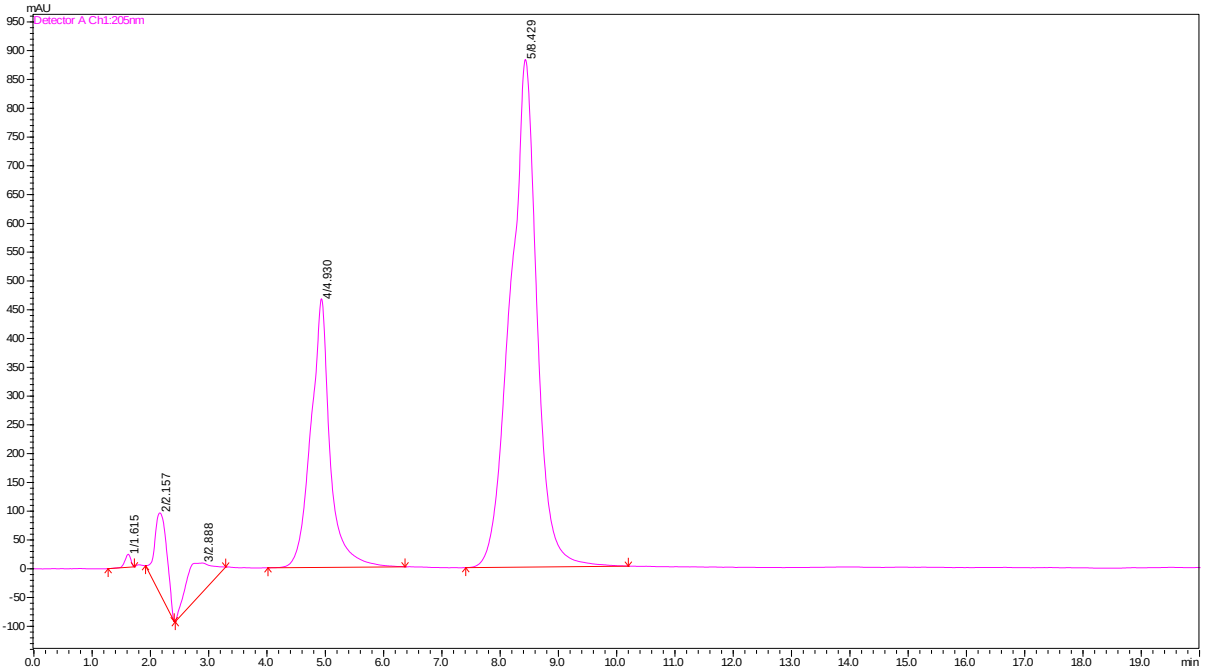

Figure 1.5: Method development of simultaneous analysis of metformin and evogliptin.

After several attempt most often, the metformin was eluted with the void volume. Therefore, further research work was carried out in Acclaimed mix-mode HILIC-1 column (5µ, 150 X 4.6 mm. ID) with isocratic elution technique, consisting 25 mm ammonium acetate-acetonitrile (70:30 v/v) explicit the best results' detection was monitored at 205nm for both selected MTF and EVO as both compounds exhibit optimum absorption at this selected wavelength. The flow rate was adjusted to 1 $mL.min^{-1}$to achieve better resolution, peak symmetry and capacity factor.

System suitability tests for EVO and MET

System suitability studies

The proposed RP-HPLC method for the simultaneous quantification of EVO and MET was validated as per the ICH guidelines and therefore including system suitability studies, other variables such as linearity, accuracy, precision (intra/ intermediate), robustness and specificity studies were tested, evaluated, and displayed in table respectively. The tailing factor (*T*) values <2 represented that the peak width is under the acceptance criteria as per the ICH guideline since both symmetric and asymmetric factors were found of equal magnitude. The separation factor (α) and resolution (Rs) for both EVO and MET were found significantly higher than the minimum requirement as per the ICH guidelines. As demonstrated, the proposed HPLC method signifies a high degree of reproducibility for the simultaneous quantification of EVO and MET. For EVO, this proposed method expressed average retention time (*t*R) of 4.93 minutes with mean k' of 1.21 whereas the tR and k' for MET was 8.42 minutes and 2.79. System suitability test reveals the factors such as, theoretical plate (N), capacity factor (k'), resolution (R), separation factor (α), tailing factor (*T*), Mean±SD and RSD% which should in acceptable range for at least 6 successive injections of same analytes. Table No. 8.3; represents the system suitability for EVO and MET.

Table No. 1.3. System suitability studies.

System Suitability Parameters	Evogliptin	Metformin
Retention time(tR)	4.93 min.	8.42 min.
No.Of Theoretical plates(N)	2115	2493
Tailing Factor(T)	1.05	0.93
Capacity Factor(k')	1.21	2.79
Resolution(R)	---	6.21
Separation factor(α)	4.07	2.29
Intra-Day Precision (%RSD)	0.29 – 0.52	0.47 -1.16
Inter-Day Precision (%RSD)	0.43 – 1.25	0.88 -1.93
Linearity range	3.9 – 62.5 $\mu g.ml^{-1}$	3.9 – 62.5 $\mu g.ml^{-1}$

Linearity and range

The linearity of any HPLC Method represents its ability to explicit the results that should proportional to the concentration of studied analytes within a selected range. Therefore, over the tested range of 3.9-62.5 µg.ml−1 for EVO and 3.9-62.5 µg.ml−1 for MET, significantly, higher proportionality was observed between the concentration against peak area with linear regression observed for EVO and MET were y = 51521x - 188395and y = 91966X-79565, respectively. Moreover, the regression coefficients (R^2) were 0.996 and 1 for both evogliptin and metformin, respectively; which itself represented a high degree of linearity.

Table No. 1.4: Linearity data of Evogliptin.

Name of Drug Evogliptin			
S. No.	**Concentration (µg.mL^{-1})**	**Area**	**Average (Mean)**
1	500	8037947	8037947
2	250	4015541	4015541
3	125	2022212	2022212
4	62.50	1024711	1024711
5	31.25	522355	522355
Regression Equation		y = 16028x + 19097	
Correlation coefficient (R^2)		1	
Std. error of intercept		4877.248397	
Std. Dev. Of intercept		10905.85896	
LOQ		3.04 µg/ml	
LOD		0.91 µg/ml	

Table No. 1.5: Linearity data of Evogliptin.

Name of Drug Metformin			
S. No.	**Concentration (µg.mL^{-1})**	**Area**	**Average (Mean)**
1	100	9275218	9275218
2	50	4644200	4644200
3	25	2400945	2400945
4	12.5	1192777	1192777
5	6.25	596388	596388
Regression Equation		y = 92327x + 44250	
Correlation coefficient (R^2)		0.9999	
Std. error of intercept		22376.51288	
Std. Dev. Of intercept		50035.4039	
LOQ		5.42 µg/ml	
LOD		1.62 µg/ml	

Accuracy

Percentage recoveries of three different concentrations; 80%, 100% and 120% (injected thrice) to determine the MTF and EVO were calculated to determine the drug recovery (%) and variation in RSD% and results obtained were reported in Table. Applying the calibration curve, the Y-intercept and the slope of the graph were used to determine the % drug recovery, attributed to the developed method for the simultaneous quantification of selected drugs.

As resulted, the achieved drug recovery of both MTF and EVO were in the range of 100.4-100.7 and 100-105, respectively whereas the RSD calculated for both drugs were well below the 2%. As recommended by international conferences of Harmonization guidelines the drug recovery should be within the range of 90-110% and the RSD in percentage should be less than 2%. Hence, the calculated drug recovery for MTF and EVO signifies the drug recovery were in the acceptance limit given by ICH guidelines.

Table No. 1.6: Accuracy data of MET.

Conc. (%)	Sr. No	Drug. added	Amt. rec.	% recovery	Peak Area (500 ppm)	Mean Rec %	% RSD
80%	1	400	400.1	44.46	31338357	100.09	0.10
	2	400	400.12	44.46	31339924		
	3	400	400.81	44.53	31393969		
100%	1	500	500.25	50.03	39182737	100.13	0.07
	2	500	500.88	50.09	39232083		
	3	500	500.77	50.09	39223467		
120%	1	600	600.17	54.56	47009102	100.07	0.02
	2	600	600.24	54.57	47014585		
	3	600	600.43	54.58	47029467		

Table No. 1.7: Accuracy data of MET.

Conc. (%)	S. N.	Drug. added	Amt. rec.	% recovery	Peak Area (50 ppm)	Mean Rec %	% RSD
80%	1	4	4.11	102.75	629149	104	1.05
	2	4	4.18	104.50	639864		
	3	4	4.19	104.75	641395		
100%	1	5	5.09	101.80	779165	102	0.82
	2	5	5.11	102.20	782227		
	3	5	5.03	100.60	769980		
120%	1	6	6.13	102.17	938366	102	0.52
	2	6	6.18	103.00	946020		
	3	6	6.12	102.00	936835		

Repeatability

Implementing the procedure mentioned under the experimental section, the homologous mixture of both EVO 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 8.4).

Table 8.4: Repeatability data of EVO and MET.

Sr. No.	Drug Name: Evogliptin	Drug Name; Metformin
	Peak Area; Conc. 375 ppm	Peak Area; Conc. 125 ppm
1	27711844	10563458
2	27889184	10891336
3	28614552	10656305
4	27429374	10639080
5	27935553	10611007
6	27280776	10423112
Mean	**27810213**	**10630716**
STD. DEV.	**469877.87**	**152747.53**
RSD (%)	**1.69**	**1.44**

Precision

The precision of HPLC method reflects its closeness to the agreement among the series of repetitive results, derived after multiple sampling of the same homogenous mixture of selected drugs under the given conditions (Nadella *et al*., 2018). Both intra- and inter-day variability for precision studies, this method is significantly precise over the tested range of 375 µg /ml for EVO and 125 µg/ml for MET.

Moreover, the peak area of the studied samples was also correlated with selected concentration; where the % RSDs were <2%. The RSDs were observed in the range of 1.25%-1.72% for EVO and 1.11%–1.96% for MET of the intra-day studies (Table 3); whereas the % RSDs were observed in the range of 1.25%-1.70% for EVO and 1.00%–1.58% for MET in the inter-day studies that reflects an acceptable precision with minimum variations of the proposed method.

Intraday precision

Implementing the procedure mentioned under section , the homologous mixture of both EVO and MET of three replicates of three different concentrations; 500 ppm, 375ppm and 125 ppm were tested and evaluated within the same day (intra-day precision). The %RSD

Interday (intermediate) precision

Implementing the procedure mentioned under section, the homologous mixture of both EVO and MET of three replicates of three different concentrations; 500 ppm, 375ppm and 125 ppm were tested and evaluated in three successive days (interday/intermediate precision). The %RSD was calculated and found less than 2%was calculated and found less than 2%.

Robustness for the chromatographic method

Robustness of HPLC Method represents its ability to remain unaffected by small but deliberate variations in separation parameters to ascertain its reliability during routine analysis. In this method, robustness was established by making deliberate changes in flow rate (1.0 ± 0.2 ml/minutes), organic modifier (70% ± 2% ml), and temperature (28°C ± 2°C). Therefore, increased the flow rate by +0.2 ml/minutes, reduced the tR values to 4.13 and 7.01 mins of EVO and MET, respectively, whereas reduced the flow rate (−0.2 ml/minutes), extended the tR values to 5.97 and 10.42 minutes of similar drugs; although the variation was almost 27%. However, altering the concentration of acetonitrile as mobile phase by 30% ± 2% as well as altering the temperature by 28°C ± 2°C has not made any significant changes in the retention pattern of MTF but as observed it affect the retention of EVO. Perhaps these differences might incur owing to selection of smaller dimension of acclaimed mix mode column. Although, this difference does not exceed more than 10%.

Thus, increasing the flow rate, organic modifier, and temperature, both EVO and MET were appeared earlier whereas decreasing them, their elution order were elongated. Importantly, excluding the theoretical plates (N); other variables like capacity factor (k'), resolution (Rs)and peak tailing (*Tf*) of selected EVO and MET were almost unchanged which clearly signified that the proposed HPLC Method obliged all minimum requirements led by the ICH guidelines.

Limit of quantification (LOQ) and Limit of detection (LOD)

LOD and LOQ were calculated based on the standard deviation of the response and the slope of the regression equation. As observed, the LOD and LOQ of EVO were 1.34 and 4.46 µg/ml, whereas for MET they were 0.75 and 2.50 µg/ml µg/ml, respectively.

SUMMARY AND CONCLUSION

1. The present study deals with development and validation of stability indicating HPLC method for Evogliptin and Metformin. The stability indicating assay method was established for the analysis of Evogliptin and Metformin in the presence of its degradation products by selecting suitable detecting wavelength and mobile phase.
2. The method provides selective quantification of Evogliptin and Metformin. This developed RP-HPLC method for estimation of Evogliptin and Metformin is accurate, precise and robustness.
3. The method has been found to be better because of its less retention time, gradient mode and use of economical readily available mobile phase, readily available column, UV detector and better resolution of peaks.

4. The run time is relatively short, which will enable rapid qualification many samples in routine and quality-controlled analysis of various formulations containing Evogliptin and Metformin. All these factors make this method suitable for qualification of Evogliptin and Metformin in bulk drugs and in the pharmaceutical dosage forms without any interference.
5. The method was completely validated showing satisfactory data for all the method validation parameters tested. Hence this method can be introduced into routine use for determination of Evogliptin and Metformin.

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