IJBPAS, April, 2025, 14(4): 1969-1983

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR EVOGLIPTIN AND METFORMIN IN BULK AND TABLETS

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Received 15th Nov. 2023; Revised 19th Dec. 2023; Accepted 8th June 2024; Available online 1st April 2025

https://doi.org/10.31032/IJBPAS/2025/14.4.8857

ABSTRACT

A combination of Metformin and Evogliptin is used to treat type 2 diabetes mellitus. A selective, accurate and precise RPHPLC method was developed and validated for simultaneous estimation of these drugs in bulk and tablet dosage forms. The drugs were resolved on a C18 (150mm × 4.6mm, 5µ) column using Acetonitrile: water (75:25 v/v) as the mobile phase. The detection wavelength was 226 nm. The retention times obtained for metformin and evogliptin were 1.187& 2.273 min respectively. The linearity ranges were 250-1250 & 2.5-12.5 µg/ml respectively with Regression coefficients of 0.9974 & 0.9994. The % RSD of precision studies was found to be less than 2%. The Accuracy of the proposed method was determined by recovery studies and the mean recovery was found to be in between 98-102%. The method was also applicable for quantitative analyses of the marketed tablet formulations and in studying stability of the drugs under acidic, alkaline, oxidation, thermal and UV conditions.

Keywords: RP-HPLC; Method Development; Validation; Metformin; Evogliptin

1 INTRODUCTION:

Diabetes mellitus (DM) is a severe disease marked by high blood glucose levels (hyperglycemia), which is caused by insulin secretion, action, or either. T2DM is caused by a combination of two basic aspects: insufficient insulin synthesis by pancreatic cells and the failure of insulin-sensitive tissues to adapt to insulin **[1-3]**. Type 2 diabetes (T2D), a disease characterized by insulin resistance and poor blood glucose control **[4, 5]**.

Metformin is a biguanide that is used as the first-line oral treatment for T2DM in people of all ages, **[6, 7]** Metformin lowers blood glucose levels through enhancing insulin sensitivity in the liver and peripheral tissues without changing insulin output **[8-13]**. This biguanide derivate is the most often given prescription to regulate glucose metabolism in type 2 Diabetic patients **[14-19]**. Evogliptin is a β-amino amide derivative. Dong-A ST which is a South Korean pharmaceutical company has developed Evogliptin. In South Korea, Evogliptin is approved for use. Evogliptin is derived from the words "Evolution" and "Gliptin," and it refers to an advanced type of gliptin that has the highest points of known DPP-IV inhibitors **[20]**. Evogliptin is a dipeptidyl peptidase IV inhibitor that is both competitive and reversible. This enzyme slows the breakdown of GLP-1(Glucagon like peptide) and GIPs. GLP- 1 & GIP stimulate the release of insulin while inhibiting the release of glucagon from beta cell of pancreas **[21]**.

Figure 1: Structure of Metformin

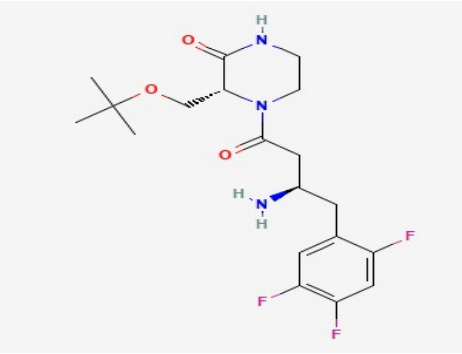

Figure 2: Structure of Evogliptin

2 MATERIALS AND METHOD:

2.1 Instrumentation

Model 1260 infinity II HPLC system was used for liquid chromatography method development and validation (Agilent technologies), equipped with pump (model 1260 Quat pump VL), 1260 manual injection, and ODS C18 column (150mm,4.6mm,5µ) and the detector consisted of 1260 DAD WR at 226nm. Open lab software was used for data processing and evaluation.

2.2 Chemicals and Reagent

API of Evogliptin were provided as gift sample from Alkem Laboratory limited, and API of Metformin were provided as gift sample from Wockhardt (Aurangabad). Methanol, Water, Acetonitrile (HPLC grade) and Hydrochloric Acid, Hydrogen Peroxide and Sodium Hydroxide (AR grade) were purchased from Merck. Commercial tablets (Valera M 500: 500mg metformin + 5mg of Evogliptin) were purchased from local Pharmacy store.

2.3 Preparation of mobile phase

Acetonitrile and water was used as a mobile phase in the ratio (75:25).

2.4 Preparation of Diluents

For the dilutions methanol and water was used as a diluents in the ratio (50:50).

2.5 Preparation of standard stock solution

Accurately weighed 500mg of metformin (API) and 5mg of Evogliptin (API) and transferred into clean and dried 100ml volumetric flask separately. Add diluents to both of these flasks, in the ratio (50:50). Sonicated for 15 min and finally made up to the mark with diluents. The resultant concentrations are 5000µg/ml of MET and 50µg/ml of EVO.

2.6 Preparation of standard working solution

Pipette 1ml from each stock solution and transferred into clean and dried 10ml volumetric flask and finally make up to the mark with diluents. The resultant concentrations are 500µg/ml of MET and 5µg/ml of EVO.

2.7 Preparation of sample stock solution

10 tablets are randomly selected, weighed and the average weight of each tablet is calculated, all tablets were triturated into porcelain mortar. the weight equivalent to 1 tablet was transferred into 100ml volumetric flask, add 50ml diluents, sonicated for 25 minutes and finally make up to the mark with diluents. All the content was passed through 0.45µ filter paper. The resultant concentration 5000µg/ml of MET and 50µg/ml of EVO.

2.8 Preparation of sample working solution

Pipette 1ml of filtered sample stock solution, transfer it into 10ml volumetric flask and

make up to the mark with diluents. The resultant concentrations were 500µg/ml of MET and 5µg/ml EVO.

3 Optimized chromatographic condition:

The separation of Metformin and Evogliptin was achieved on a C_{18} Column (150mm,4.6mm,5µ) and eluting with a mobile phase consisting of a 75:25v/v mixture of Acetonitrile and Water at a flow rate of 1.0ml/min. The analysis were monitored at 226nm. The injection volume was 20µl. The total run time for elution of compound was 10 min.

Column	**C_{18} (150mm × 4.6mm, 5µ)**
Mobile phase	**Acetonitrile: Water (75:25)**
Wavelength	**226 nm**
Flow rate	**1.0 ml/min**
Injection volume	**20 µl**
Run time	**10 min**

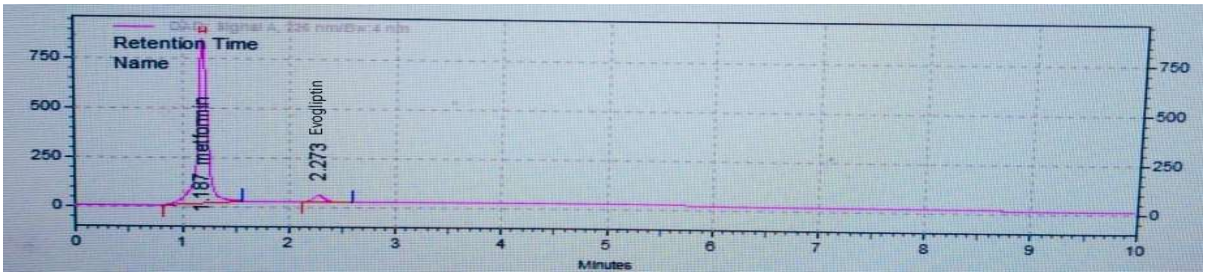

Figure 3: Optimized chromatogram of Metformin and Evogliptin

4 Method validation:

"The process of validating an analytical technique includes establishing, through laboratory investigations, that the method's performance characteristics fulfil the requirements for the intended analytical application."

The processes were verified in accordance with the International Conference on Harmonization (ICH) requirements for analytical procedure validation.

The parameter assessed were

- Accuracy
- Precision
- Specificity
- Linearity
- Range
- Robustness
- Limit of detection
- Limit of quantitation

4.1 System suitability

System suitability test is commonly applied to validate resolution, column efficiency, and repeatability of a chromatographic system to ensure its capability for a specific analysis. The parameters used in the system suitability tests (SST) report are as follows: Number of theoretical plates or Efficiency, tailing factor,

asymmetry factor, retention time. The acceptable limit of Retention time is ≥2, Tailing / asymmetric factor is not more than 2, Theoretical plates is ≥2000.

Table 1: System suitability parameter

Parameter	Metformin	Evogliptin
Tailing factor	1.05	1.03
Peak asymmetry	1.10	1.07
Retention time	1.187	2.273
Theoretical plates	7744	4793
Area	7016142	242817

4.2 Specificity

Specificity of the pharmaceutical analysis is the ability to measure accurately and specifically the concentration of API, without interference from other active ingredients, diluents, mobile phase. Specificity was evaluated by comparing the chromatograms of mobile phase blank solution, standard solution, and sample solution. For this purpose, 20µl solution of mobile phase blank, standard solution and sample solution were injected into HPLC system separately, and the chromatogram results are shown in **Figure 4, 5, and 6**.

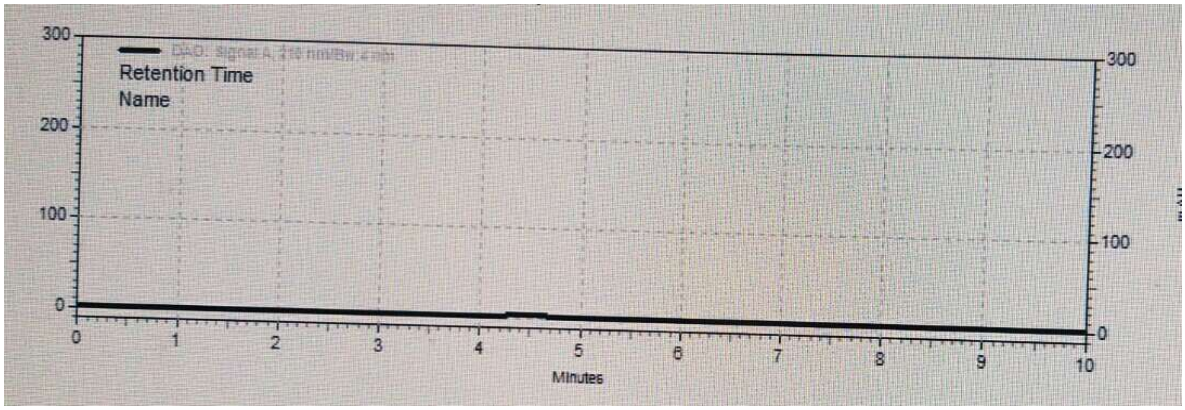

Figure 4: Chromatogram of blank

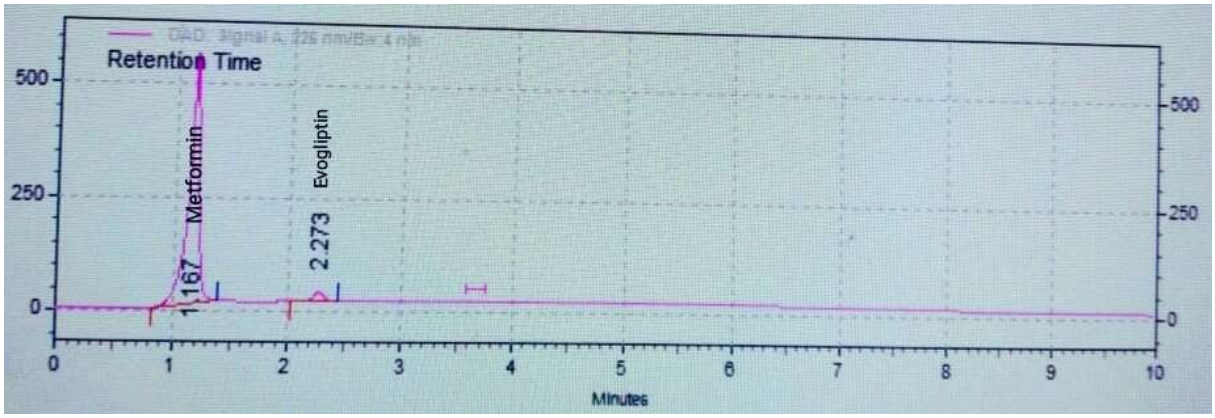

Figure 5: Chromatogram of sample solution

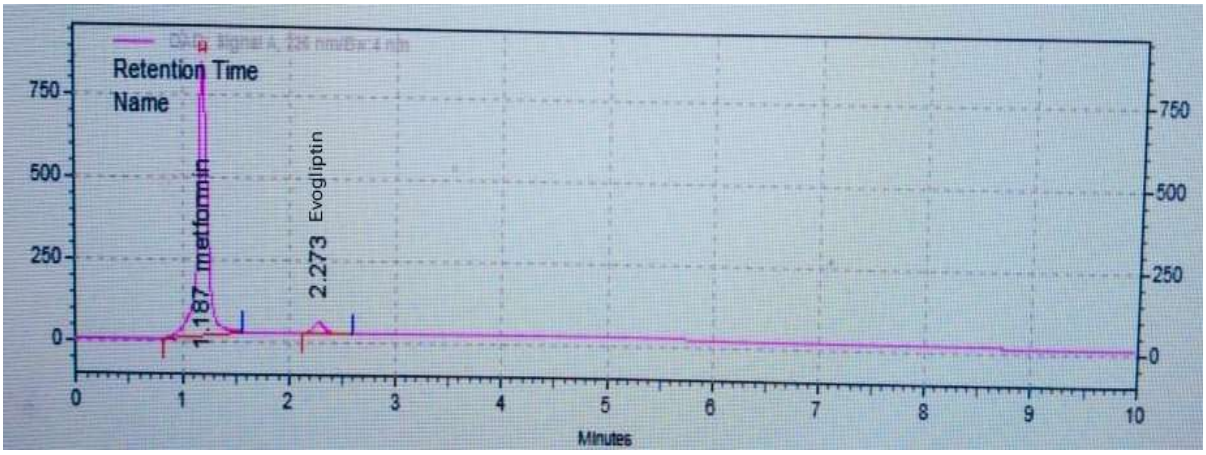

Figure 6: Chromatogram of Standard solution

4.3 Accuracy

The accuracy of the method was evaluated by standard addition method. The known amount of the reference standard was added to the known amount of standard solution at three different levels. The solution were analyzed for mean recovery and %RSD. The studies were performed for both MET and EVO at three different levels 50%, 100% and 150% solution. The 10µl was injected into HPLC and %recovery and %RSD were noted as shown in **Table 2 and Table 3**.

Table 2: Accuracy results for Metformin

Conc. %level	Actual Sample	Sample added	Area	Amount found (µg/ml)	%recovery	Avg.% recovery
50%	500	250	10134231	736.18	98.15	98.88
	500	250	10203641	741.42	98.85	
	500	250	10282310	747.36	99.64	
100%	500	500	13608596	998.57	99.85	100.59
	500	500	13702987	1005.70	100.57	
	500	500	13808523	1013.67	101.36	
150%	500	750	16690398	1231.32	98.50	99.24
	500	750	16800396	1239.62	99.17	
	500	750	16948786	1250.83	100.06	

% level	Mean	SD	%RSD
50%	10206728	74087.7	0.73%
100%	13706702	100015.26	0.73%
150%	16813194	129668.4	0.77%

Table 3: Accuracy results for Evogliptin

Conc. %level	Actual sample	Sample added	Area	Amount recovered	% Recovery	Avg. % recovery
50%	5	2.5	391025	7.40	98.67	99.08
	5	2.5	390654	7.39	98.59	
	5	2.5	396845	7.50	100.0	
100%	5	5	534985	9.86	98.69	99.18
	5	5	538964	9.93	99.37	
	5	5	539647	9.94	99.49	
150%	5	7.5	690856	12.54	100.33	100.50
	5	7.5	695610	12.62	100.98	
	5	7.5	690010	12.52	100.21	

%Level	Mean	SD	%RSD
50%	392842	3472.23	0.88%
100%	537866	2517.70	0.47%
150%	692159	3018.72	0.44%

4.4 Precision

Precision is the degree of closeness of agreement between the series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions. It is expressed in terms of standard deviation (SD) or relative SD (RSD). Precision may be a measure of either the degree of repeatability or the reproducibility of the analytical method.

Repeatability was determined by injecting a sample five times under same operating condition. Inter day and intraday precision: Intra-day precision was determined by injecting sample at same day but some interval of time. Inter-day precision was determined by injecting a sample solution for three consecutive days.

Table 4: Intra-day precision of MET

Sr no	Concentration µg/ml	Area			Mean	SD	%RSD
		10 AM	1PM	4PM			
1	250 µg/ml	3504892	3564123	3500654	3523223	35483.76	1.01%
2	500 µg/ml	7016142	7002369	7056139	7024884	27930.47	0.40%
3	750 µg/ml	10185940	10012456	10036598	10078332	93970.35	0.93%

Table 5: Intra-day precision of EVO

Sr no	Concentration µg/ml	Area			Mean	SD	%RSD
		10 AM	1PM	4PM			
1	2.5 µg/ml	112034	111265	114562	112621	1724.93	1.53%
2	5 µg/ml	241706	240863	245691	242753	27930.47	0.40%
3	7.5 µg/ml	393456	391052	392207	392239	1202.30	0.31%

Table 6: Inter day precision of MET

Sr no	Concentration µg/ml	Area			Mean	SD	%RSD
		Day 1	Day 2	Day 3			
1	250 µg/ml	3526410	3502146	3510896	3513151	12288.12	0.35%
2	500 µg/ml	7085641	7023458	7048693	7052598	31274.81	0.44%
3	750 µg/ml	1054896	1054789	1065897	1058528	6382.54	0.60%

Table 7: Inter-day precision of EVO

Sr no	Concentration µg/ml	Area			Area	SD	%RSD
		Day 1	Day 2	Day 3			
1	2.5 µg/ml	112035	115687	114523	114082	1865.57	1.64%
2	5 µg/ml	241056	245367	247645	244690	3346.36	1.37%
3	7.5 µg/ml	395214	390214	394256	393228	2653.78	0.67%

4.5 Linearity and Range

Linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in samples within a given range. Range is the interval between the upper and lower levels of analyte. Linearity was performed by diluting standard stock solution to give final concentration in the range 250-1250µg/ml of MET & 2.5-12.5µg/ml of EVO for 20µl of concentration injected & calibration curve was constructed by plotting the peak area versus drug concentration.

Table 8: Linear regression data for the calibration curves

Sr no	Metformin		Evogliptin	
	Concentration	Area	Concentration	Area
1	250	3503456	2.5	111562
2	500	7006989	5	242817
3	750	10564231	7.5	397687
4	1000	13905398	10	539909
5	1250	16605238	12.5	692054

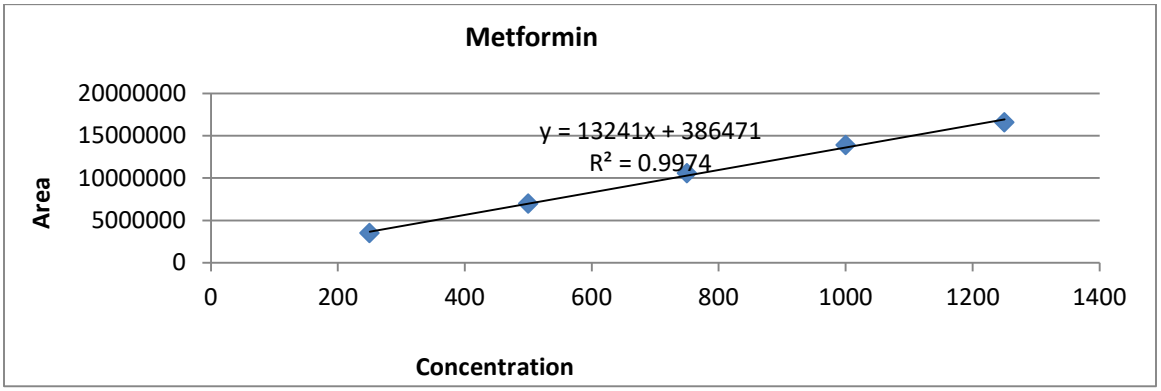

Figure 7: linearity graph of MET

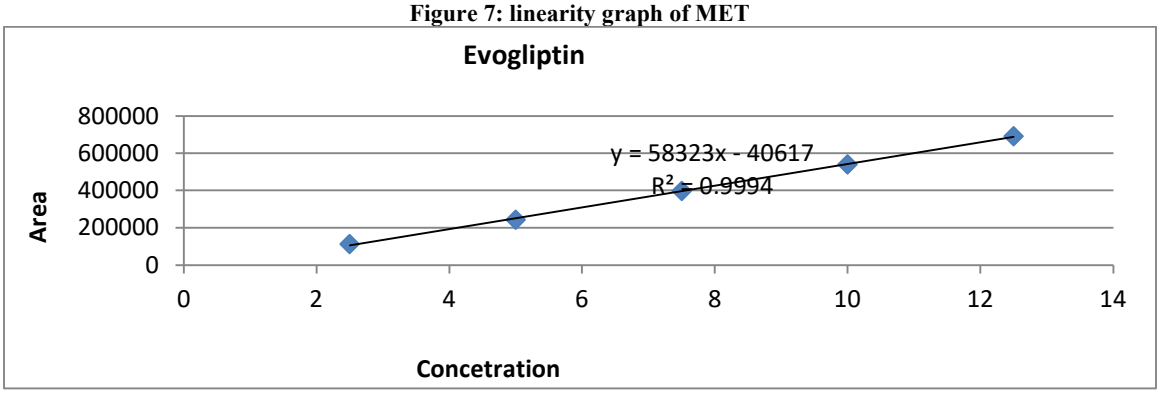

Figure 8: Linearity graph of EVO

4.6 Robustness

It is the ability of analytical procedure to remain unaffected by the small but deliberate variations in the method parameter like pH, mobile phase, temperature, and composition sample.

Robustness was evaluated by making minor changes in the method parameter like

- Change in flow rate (±0.2ml/min),
- Change in mobile phase ratio (±2),
- Change in wavelength (±1).

Table 9: Robustness values of Metformin

Sr no.	Area at flow rate (+0.2ml/min)	Area at flow rate (-0.2ml/min)	Area at mobile phase (+2)	Area at mobile phase (-2)	Area at wavelength (+1)	Area at wavelength (-1)
1	7010453	7054238	7052388	7086234	7080036	7009654
2	7082564	7084586	7086423	7186485	7005409	7099896
3	7013916	7099130	7089645	7089645	7098328	7198995
Mean	7035645	7079318	7076152	7120788	7061258	7102849
SD	40670.4	22904.9	20643.1	56920.8	49223.5	94705.01
%RSD	0.58%	0.32%	0.29%	0.80%	0.70%	1.33%

Table 10: Robustness values of Evogliptin

Sr no.	Area at flow rate (+0.2ml/min)	Area at flow rate (-0.2ml/min)	Area at mobile phase (+2)	Area at mobile phas e(-2)	Area at wavelength (+1)	Area at wavelength (-1)
1	240632	245078	240856	240786	240010	240462
2	245637	245876	245876	241086	241008	248960
3	245861	240879	240123	248573	240634	241058
Mean	244044	243945	242285	243482	240550	243494
SD	2956.42	2684.47	3131.41	4411.77	504.19	4743.64
%RSD	1.21%	1.10%	1.29%	1.81%	0.21%	1.95%

4.7 LOD and LOQ

The detection limit or LOD is the lowest amount of analyte in a sample that can be detected. The detection limit or LOQ is the lowest amount of analyte in a sample that can be quantitatively determined. The results of LOD and LOQ shown in **Table 11**.

Table 11: LOD results of MET and EVO

Drugs	LOD Value	LOQ Value
Metformin	0.37 µg/ml	1.03µg/ml
Evogliptin	1.89 µg/ml	3.4µg/ml

4.8 Assay of Marketed Tablet

An assay is an investigative (analytical) procedure for qualitatively assessing or quantitatively measuring the presence, amount or functional activity of a target entity (the analyte). The assay performed by the marketed product (VALERA M 500) 500 mg of MET & 5mg of EVO.

Table 12: Assay of sample (tablet dosage form)

Drug	Label claim(mg)	% Drug Content
Metformin	500	99.88
Evogliptin	5	100.08

4.9 Stability study

The International Conference on Harmonization (ICH) guideline entitled stability testing of new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the Metformin and Evogliptin using the proposed method.

4.9.1 Preparation of Stock Solution for Degradation Studies

10 tablets are randomly selected, weighed and the average weight of each tablet is calculated, all tablets were triturated into porcelain mortar. the weight equivalent to 1 tablet was transferred into 100ml volumetric flask, add

50ml diluents, sonicated for 25 minutes and finally make up to the mark with diluents. All the content was passed through 0.45µ filter paper. The resultant concentration 5000µg/ml of MET and 50µg/ml of EVO.

4.9.2 Hydrolytic Degradation under Acidic Condition

Pipette 1.5 ml of above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then the volumetric flask was kept at 60°C for 6 hours and then neutralized with 0.1 N NaOH and make up to 10ml with diluent. Filtered the solution with 0.22 microns syringe filters and placed in vials.

4.9.3 Hydrolytic Degradation under Alkaline Condition

Pipetted 1.5ml of above solution into a 10ml volumetric and 3ml of 0.1N NaOH was added in 10ml of volumetric flask. Then the volumetric flask was kept at 60°C for 6 hours and then the solution neutralized with 0.1N HCl and made up to 10ml with diluent. The solution was filtered with 0.22 microns syringe filters and placed in vials.

4.9.4 Thermal Induced Degradation

Metformin and Dapagliflozin sample was taken in Petridish and kept in Hot air oven at 110°C for 24 hours. Then the sample was taken and diluted with diluents and injected into HPLC and analysed.

4.9.5 Oxidative Degradation

Pipetted 1.5ml of above stock solution into a 10ml volumetric flask and 1ml of 3% w/v of hydrogen peroxide was added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. The solution was filtered with 0.45 microns syringe filters and placed in vials.

4.9.6 Photo Degradation

Pipetted 1.5 ml of above stock solution into a 10ml volumetric flask and exposed it to sunlight for 24hrs and the volume was made up to the mark with diluent. The solution was filtered with 0.45 microns syringe filters and placed in vials. The stressed samples were respectively analyzed for Metformin and Evogliptin and the results are shown in **Table 13 & 14**.

Table 13: Stability study of MET

Condition	Area	%assay	%Net degradation
Acidic	**6301247**	**89.18**	**10.18**
Alkaline	**6517572**	**92.9**	**7.10**
Oxidative	**6901254**	**98.37**	**1.63**
Thermal	**6733045**	**95.97**	**4.03**
Photo	**6603541**	**94.12**	**5.88**

Table 14: Stability study of EVO

Condition	Area	%assay	%Net degradation
Acidic	**205478**	**84.67**	**15.33**
Alkaline	**215630**	**88.81**	**11.19**
Oxidative	**235841**	**97.13**	**2.87**
Thermal	**228641**	**94.17**	**5.83**
Photo	**230212**	**94.81**	**5.19**

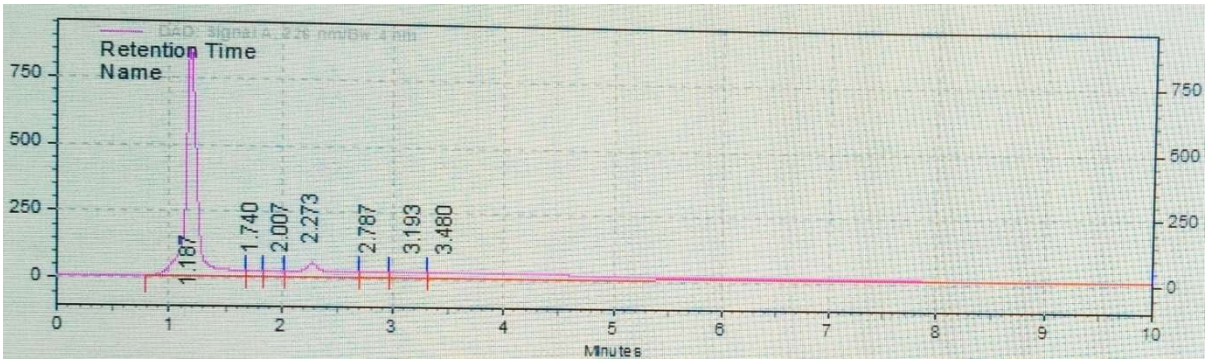

Figure 9: Chromatogram of Hydrolytic Degradation under Alkaline Condition

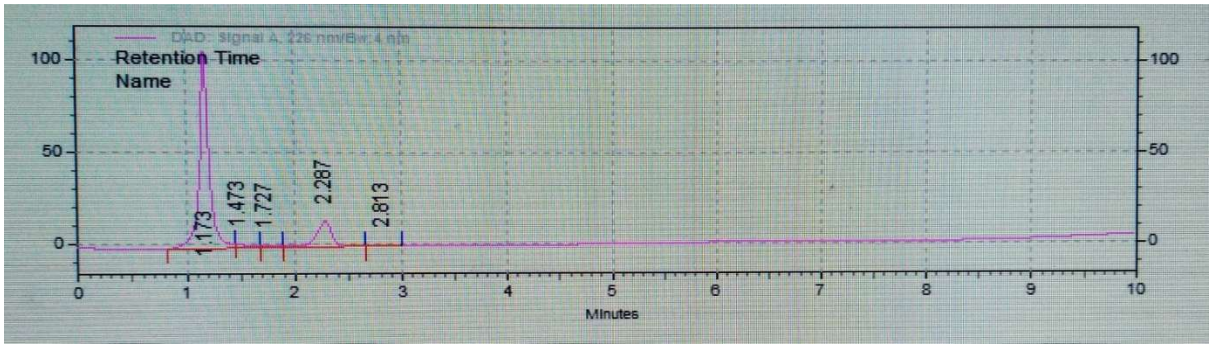

Figure 10: Chromatogram of hydrolytic degradation under Acidic condition

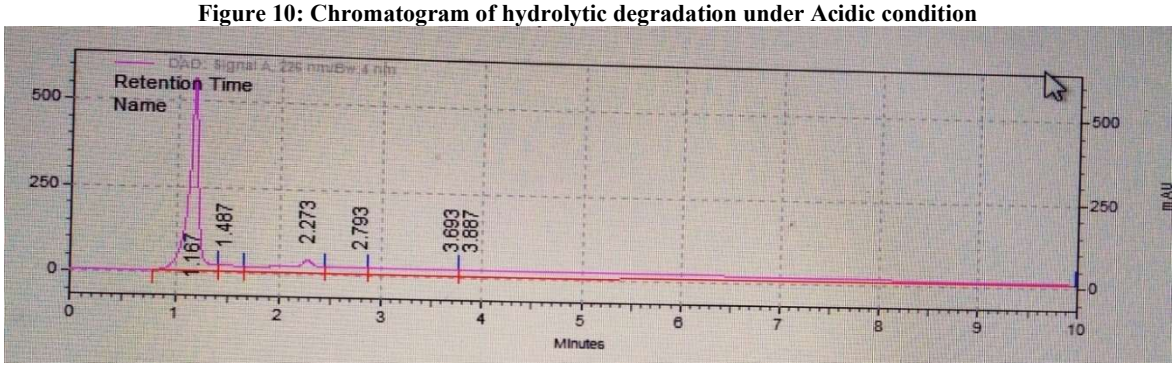

Figure 11: Chromatogram of hydrolytic degradation under oxidative condition

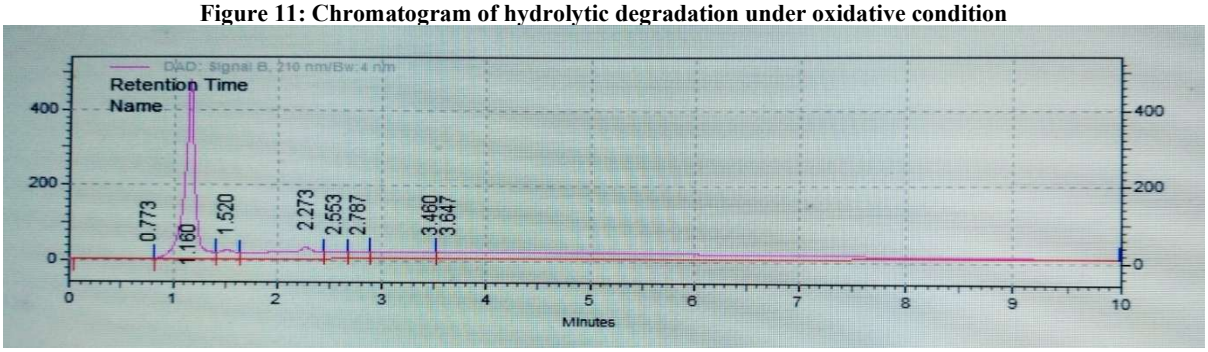

Figure 12: Chromatogram of hydrolytic degradation under thermal condition

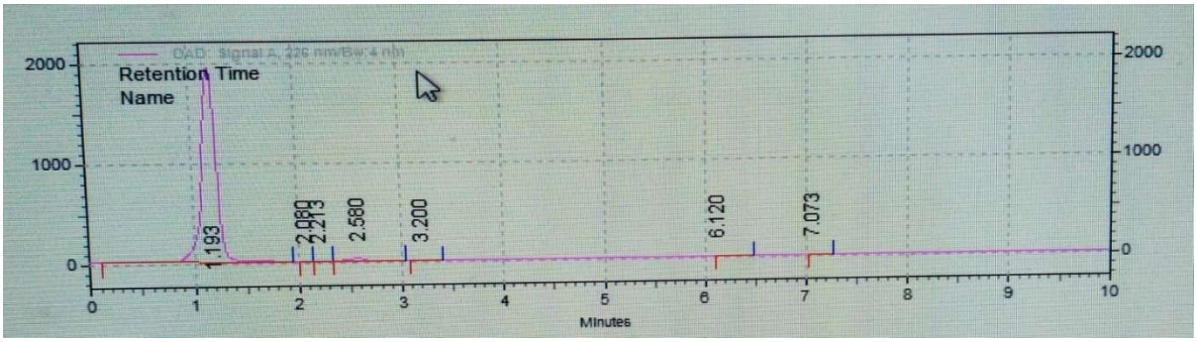

Figure 13: Chromatogram of hydrolytic degradation under photo degradation

5 RESULTS AND DISCUSSION:

The proposed method was simple, precise and accurate for the simultaneous determination of metformin and evogliptin in combined tablet dosage form. The drugs were resolved on a C18 (150mm × 4.6mm, 5µ) column using Acetonitrile & water 75:25v/v as mobile phase, flow rate of 1ml/min and detention wavelength was 226nm. The retention time for metformin and evogliptin were found to be 1.187 and 2.273 min respectively.

The developed method was validated for accuracy, precision, linearity, robustness, LOD and LOQ. The linearity of the method was determined by Regression analysis. A linear relationship was evaluated in the concentration range of 250-1250µg/ml of MET & 2.5-12.5µg/ml of EVO with correlation coefficient of 0.9974 & 9994 for MET & EVO respectively. The system suitability studies and method precision were carried and %RSD were found to be less than 2%. The accuracy of the method was determined by recovery studies and mean recovery was observed to be 98-102%. The LOD and LOQ were found to be 0.37µg/mL & 1.03µg/mL for metformin and 1.89 µg/mL & 3.4µg/mL for evogliptin. The robustness of the method was studies by deliberate changes in the flow rate, mobile phase composition and wavelength. The %RSD were found to be not more than 2% and results indicates that the slight variations on the chromatographic conditions have negligible effect and conformed that the method was highly robust. The proposed method was successfully applied to the assay of commercial formulation and showed 99.88% and 100.08% of metformin and evogliptin respectively.

The specificity of the developed method was evaluated by applying different stress conditions like acid, base, oxidation, thermal, photolytic to metformin and evogliptin in combined dosage form. The result obtained indicates that the purity angle was always less than the purity threshold and it indicates the proposed method was stable.

6 CONCLUSION:

The developed method was simple, precise, accurate and reliable for the simultaneous estimation of metformin and evogliptin in combined dosage form and envisages the stability behavior of both the drugs as per ICH guidelines. The %RSD of all results is less than 2% that shows high degree. Hence, the proposed method was simple, easy, cost-effective and can be used for routine analysis of metformin and evogliptin combined dosage form.

Funding

This research received no external funding.

Acknowledgments

The authors acknowledge the Rajarshi Shahu College of Pharmacy, Buldhana 443001 Maharashtra, India for the present work.

Conflicts of Interest

The authors declare that they have no competing interests.

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