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Development of liquid chromatographic (LC) method for simultaneous estimation of novel anti diabetic drug Evogliptin and Metformin

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Abstract: Evogliptin and Metformin are antidiabetic medications. Evogliptin works by increasing the release of insulin from the pancreas and decreasing the hormones that raise blood sugar levels. This reduces the fasting and post-meal sugar levels. Metformin works by lowering glucose production in the liver, delaying the absorption of sugar (glucose) from the intestines, and increasing the body's sensitivity to insulin. Accurate and precise high performance liquid chromatographic method has been developed for the estimation of Metformin and Evogliptin. Agilent C_{18} Column (250mm x 4.6mm, 5µm particle size) was used as stationary phase and methanol: 0.11% acetic acid in water (31: 69 % V/V) was used as mobile phase. The method was linear in the concentration range 50-250 µg/mL and 0.5 to 2.5 µg/mL of Metformin and Evogliptin respectively with a correlation coefficient of 0.999. The proposed method was validated with respect to linearity, accuracy, precision, and robustness as per International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use ICH Q2 (R2) guideline. The method was successfully applied for the analysis of Metformin and Evogliptin.

Keywords: Metformin ; evogliptin; design of experiment; validation; accuracy; robustness and liquid chromatography

1. Introduction

Hyperglycemia, or consistently elevated blood sugar levels, is a symptom of a group of metabolic diseases collectively known as diabetes mellitus (DM), or simply diabetes. Multiple medical issues are linked to diabetes. Possible immediate outcomes include metabolic acidosis, hyperosmolar hyperglycemia, and mortality. Serious long-term risks include nerve damage, heart disease, stroke, chronic kidney illness, foot ulcers, visual issues, and cognitive impairment. Metformin hydrochloride is an antidiabetic medication administered orally that is used to treat non-insulin-dependent diabetes. It works by mainly blocking the production of glucose in the liver and the process of glycogenolysis. Biguanides are a class of antidiabetic medications that lower blood sugar, and metformin hydrochloride is one of them [1]. Evogliptin tartrate is a novel, potent dipeptidyl peptidase IV inhibitor used for the treatment of type 2 diabetes. In October 2015, South Korea became the first country to globally approve

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evogliptin for the management of blood glucose in individuals suffering from type 2 diabetes [2]. Evogliptin is used to treat type 2 diabetes mellitus, a chronic metabolic condition characterized by insulin insufficiency and insulin resistance, as a monotherapy, oral antihyperglycemic medicine or in combination with other antidiabetic agents. Evogliptin is a piperazine derivative in clinical trials as a novel DPP-IV inhibitor for the treatment of type 2 diabetes mellitus. Its International Union of Pure and Applied Chemistry (IUPAC) name is 4- [3-amino-4- (2, 4, 5-trifluropenyl) butanoyl] -3 - [2-methyl propane-2-yl) oxymethyl] piperazine-2-one; 2,3-dihydroxybutanedioic acid [3-7]. Evogliptin functions by reducing the hormones that elevate blood sugar levels and stimulating the pancreas' secretion of insulin. As a result, the post-meal and fasting blood sugar levels drop. Metformin functions by decreasing the liver's synthesis of glucose, postponing the intestines' absorption of sugar (glucose), and enhancing the body's sensitivity to insulin. Literature review revealed that Ultra-Violet visible spectroscopy (UV) and High-Performance Liquid Chromatography (HPLC) methods have been reported for the estimation of evogliptin and metformin. The reported UV method is less sensitive and non-specific [8]. The reported HPLC methods require preparation of complex mobile phase and monitoring of liquid chromatographic parameters [9-12] and sensitivity of reported methods are low. The present study involves development of sensitive RP-HPLC method with optimization of chromatographic condition using quality by design approach which helps in development of robust analytical method.

2. Experimental

2.1. Reagents and Chemicals

Active pharmaceutical ingredient of Metformin (99.21% w/w) and Evogliptin (99.34% w/w) were obtained as gift sample from Swapnroop Drugs and Pharmaceuticals. HPLC Grade Methanol, Acetonitrile, Orthophosphoric acid, and Water were purchased from Merck Limited, India. Marketed formulation (Brand name VALERA M containing 5 mg of Evogliptin and 500 mg of Metformin Hydrochloride manufactured by ALKEM Health Science, Mumbai, India) was procured from local pharmacy store.

2.2. HPLC Instrument

The Agilent (1100), Gradient System with autosampler, diode array detector, quaternary pump with Chemstation (10.04) Software was used for analysis. Reverse Phase C_{18} column (4.6mm x 250mm; 5µ) was used as stationary phase.

2.3. Chromatographic Conditions

The Agilent (1100), Gradient System with autosampler, diode array detector, quaternary pump with Chemstation (10.04) Software was used for the study (1100). Reverse phase C_{18} Column (250mm x 4.6mm, 5µm particle size) was used as the stationary phase which was equilibrated with mobile phase Methanol: 0.11% Acetic acid in water (31: 69 % V/V). The flow rate was maintained at 0.8 mL/min, eluent was monitored with diode array detector at 256 nm, the injection volume was 20 µL and total run time kept was of 15 mins.

2.3.1. Preparation of Stock Solution

Metformin was weighed (250 mg) and Evogliptin was weighed accurately (2.5 mg) and transferred in to 100 mL volumetric flask and dissolved in methanol, volume was made up to the mark 100 mL with methanol and sonicated for 15 mins to dissolve it in the solution and yield a stock solution containing 2500 µg/mL of Metformin and 25 µg/mL of Evogliptin. The stock solution were stored at ambient room temperature (25 ± 2°C).

2.4. Validation

The method was validated as per ICH Q2 (R2) [13] guideline for accuracy, precision, linearity, detection limit, quantitation limit and robustness.

2.4.1. Linearity

Appropriate aliquot (0.2, 0.4, 0.6, 0.4, 0.8 and 1.0 mL) was withdrawn from stock solution and transferred to 10 mL of volumetric flask, volume was made up to the mark with mobile phase to obtain 50, 100, 150, 200 and 250 µg/mL of Metformin and 0.5, 1.0, 1.5, 20 and 2.5 µg/mL of Evogliptin solution. Chromatograms were recorded after the solutions were injected. Regression equations were calculated for both the drugs and the calibration curve curve was constructed by plotting average peak area versus concentrations

2.4.2. Accuracy

The accuracy of the method was determined by calculating recovery of Metformin and Evogliptin by the method of standard addition in a pre-analyzed tablet formulation. The standard drugs were spiked at 80%, 100% and 120% of the target concentration i.e. 50 µg/mL and 0.5 µg/mL for Metformin and Evogliptin respectively. The solutions were injected and analyzed. The amount of Metformin and Evogliptin was estimated by measuring peak area.

2.4.3. Precision

The degree of consistency between individual testing results when a technique is done repeatedly to numerous samplings of the homogenous sample is known as the precision of an analytical method. Standard deviation/relative standard deviation are commonly used to express the precision of an analytical process. Additionally, one-way Analysis of variance (ANOVA) was used to compare the data, and the within-day and between-day mean squares were calculated.

2.4.3.1 Intermediate Precision

The intra-day and inter-day precisions were used to assess intermediate precision. The analysis of sample solutions containing 100, 150, and 200 µg/mL of metformin and 1.0, 1.5, and 2.0 µg/mL of evogliptin, covering low, middle, and high concentrations of the calibration curve, was done three times on the same day (n = 3). This allowed for the determination of intra-day precision. Analyzing sample solutions containing the drug at low, medium, and high concentrations over the course of three days (n = 3) allowed for the determination of inter-day precision. The mean and relative standard deviation (% RSD) values were computed using the acquired peak regions.

2.4.4. Robustness

The robustness of the method was confirmed by making small but deliberate changes in the flow rate, wavelength, and composition of the mobile phase. The amount of Methanol in the mobile phase was altered to (+1 mL/ min, -1 mL/ min), and the detection wavelength was modified to (+1nm, -1nm). The impact of the results was then investigated, and study was carried in replicates using 100 µg/mL of Metformin and 1 µg/mL of Evogliptin. The effect of these changes was recorded, and results were examined.

2.4.4.1 Application of Experimental Design for Optimization of Method

Preliminary experiments and screening along with optimization of data analysis was performed using Taguchi Screening method to identify the critical method variables and critical analytical attributes. Chromatographic Column C_{18}, column temperature, mobile phase, concentration of the buffer was investigated at initial level as critical method variables. The identified critical material variables and critical analytical attributes were optimized by DoE based response surface analysis using Box Behnken design (BBD).

2.4.5 Limit of Detection and Limit of Quantification

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. The limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy.

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve

2.5. Analysis of Marketed Formulation

Twenty tablets were weighed and powdered. Powder equivalent to 500 mg of Metformin and 5 mg of Evolgliptin was weighed and transferred to 10 mL volumetric flask. Few ml of methanol (5 mL) was added, and mixture was sonicated for 15 mins to achieve thorough extraction. The solution was filtered in another 10 mL volumetric flask and volume was made up to the mark with methanol. Aliquot (0.4 mL) was taken in 10 mL volumetric flask and volume was made up to the mark with mobile phase. The resultant solution was injected into the optimized chromatographic system and response was measured. The quantity of Metformin and Evogliptin was determined using the regression equation.

3. Results and Discussion

3.1. Selection of Detection of Wavelength

The solution of Metformin and Evogliptin (20 µg/mL) was prepared in methanol and scanned in the range of 400 – 200 in UV visible double beam spectrophotometer. The absorbance spectra gave wavelength maxima at 255 nm, so it was selected as detection wavelength in liquid chromatographic method for the estimation of both the drugs.

3.2. Optimization of Mobile Phase

The objective of the method development was to obtain the sharp and well resolved peak of Metformin and Evogliptin with less asymmetric factor. Different solvents like methanol and water were tried in different proportion to obtain sharp peak **(**Table 1). The mobile phase methanol:0.11 % Acetic Acid (31:69 v/v) gave satisfactory sharp peak (Figure 1). Further optimization of mobile phase was carried out using Quality by design (QbD) approach.

Table 1. Optimization of mobile phase

Trial	Mobile Phase Composition (pH of OPA was 3.0)	Flow Rate (mL/min)	RT (min)	Inference
1	MeOH+0.5%Acetic Acid (80:20)	1.0	1.776	Retention time is near void volume
2	MeOH+0.5%Acetic Acid (70:30)	0.7	1.785	Peaks are near void volume
3	MeOH+0.5%Acetic Acid (70:30)	1.0	1.784	Peaks are near void volume
4	MeOH+0.5%Acetic Acid (50:50)	1.0	1.821	Splitting of Peak is observed in Evogliptin
5	MeOH+0.5%Acetic Acid (50:50)	1.0	1.819	Peaks are near void volume
6	MeOH+0.5%Acetic Acid (50:50)	0.7	2.620	Tailing of Peak is observed
7	MeOH+0.1%Acetic Acid (50:50)	0.7	2.620	Tailing of Peak is observed
8	MeOH+0.1%Acetic Acid (30:70)	0.7	3.184	Tailing of Peak is observed
9	MeOH+0.1%Acetic Acid (40:60)	0.7	3.137	Tailing of Peak is observed
10	MeOH+0.11%Acetic Acid (31:69)	0.8	3.184	Satisfactory Peak is observed

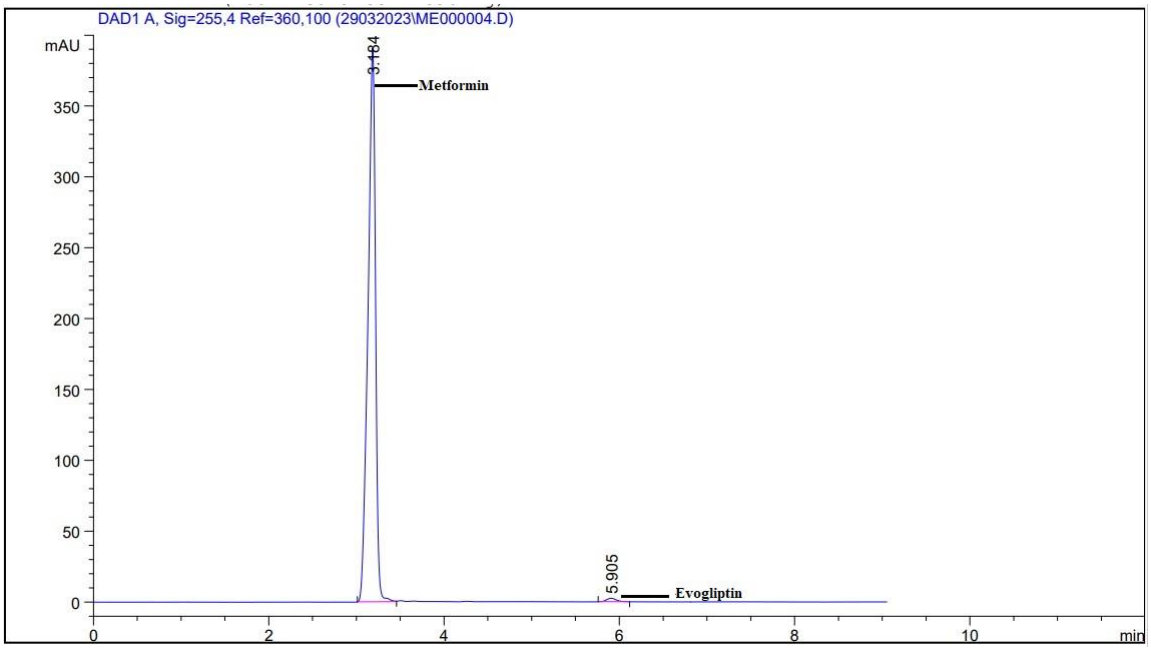

Figure 1. Chromatogram of Metformin and Evogliptin in optimized mobile phase methanol:0.11 % acetic acid (31:69, v/v), Retention time of Metformin was 3.2 and for Evogliptin was 5.9

3.3. Validation

The calibration curve of Metformin and Evogliptin was found to be linear in the range of 50-250 µg/mL and 0.5 to 2.5 µg/mL respectively with a correlation coefficient of 0.999. The regression analysis of calibration curves is reported in the (Table 2). The accuracy of the method was determined by calculating recoveries of Metformin and Evogliptin by method of standard addition. The recovery of the drug Metformin was found to be 101.91±0.76, 101.12±0.24, 98.81± 0.25 and the recovery of the drug Evogliptin was found to be 100.50±0.015, 99.74±0.039 and 99.30±0.08, the high values indicate that the method is accurate. Instrument precision was determined by performing injection repeatability test and the RSD value of Metformin was found to be 0.43 and 0.07 of Evogliptin. The intraday and interday precision studies were carried out and the results are reported in the (Table 3). The low relative standard deviation (RSD) values indicate that the method is precisx1e. The limit of detection of Metformin was found to be 1.377 µg/mL and of Evogliptin was found to be 0.017 µg/mL and the limit of quantification was found to be 4.17 µg/mL and 0.052 µg/ml of Metformin and Evogliptin respectively. By purposefully

altering the experimental settings, a robustness analysis was carried out. Critical parameters that impact the effectiveness of the process include wavelength, flow rate, and the composition of the mobile phase. The flow rate was changed from 0.8 ml/min to 0.9 ml/min, composition of the mobile phase was changed from +1ml/min and -1ml/min and wavelength from 255nm to 256nm. Analysis was performed and recovery of the drug was found to be more than 101% of Metformin and 99% of Evogliptin with less than 1 % standard deviation which proves that method is robust and do not get affected by small but deliberate changes.

Table 2. Regression analysis of calibration curve

Parameters	Results of Metformin	Results of Evogliptin
Linearity (µg/mL)	50-250	0.5-2.5
Correlation coefficient (r^2)	0.999	0.999
Slope of regression equation	23.26	60.61
Average standard deviation of slope	0.45	0.32
Intercept of regression	4.40	2.155

Table 3. Intermediate precision studies (Intra-day and Inter-day)

	Conc (µg/mL)	Intraday Precision			Interday Precision		
		Mean Area*± SD	% Found	% RSD	Mean*± SD	% Found	%RSD
Metformin	100	2296.65±0.71	98.55	0.03	2288.23±9.79	98.19	0.43
	150	3519.18±3.54	100.74	0.10	3519.15±4.91	100.74	0.14
	200	4684.10±2.12	100.60	0.05	4683.69±1.54	100.59	0.03
Evogliptin	1	57.73±0.83	98.81	1.44	57.95±1.07	99.16	1.84
	1.5	85.68±0.76	96.61	0.88	84.99±1.04	95.85	1.22
	2	111.30±1.63	93.59	1.46	111.96±1.17	94.14	1.04

One of the multivariate optimization techniques, Box–Behnken design model was used to optimize chromatographic conditions. The composition of mobile phase, flow rate and wavelength were selected as critical method variables (independent variables) which affect critical analytical attributes i.e. retention time, theoretical plates, peak area, and tailing factor. The Second-order three-level full factorial design experiment was performed by 17 runs at low, intermediate, and high levels of variables coded as −1, 0, and +1 respectively. The measured responses were entered against the respective experimental run (Table 4).

As per the data shown in ANOVA table, the P-value of effect of composition of mobile phase, flow rate and wavelength selected were found to be <0.0500, which indicates that the effect these variables were significant for the development of HPLC method. The optimization of identified critical method variables was carried out by BBD. 17 experimental runs were suggested by design expert software (trial version 13) which were performed in the laboratory and data were entered into the software. The P-value for critical analytical attributes were found to be <0.05, which indicate that the relationship between identified Critical method variables and critical analytical attributes were quadratic. The 3D surface plots of retention time, flow rate, mobile phase composition and wavelength of Metformin and Evogliptin are depicted in (Figure 2 and Figure 3).

Table 4. Layout of actual design of DOE of Metformin and Evogliptin

					Metformin				Evogliptin			
Std	Run	Factor 1 A:Methanol %	Factor 2 B:Flow rate mL/min	Factor 3 C:Wavelength Nm	Response 1 RT min	Response 2 PA	Response 3 TP	Response 4 TF	Response 5 RT 2 min	Response 6 PA 2	Response 7 TP 2	Response 8 TF 2
10	1	31	0.9	255	2.4	2344.61	5499	1.54	4.545	22.9	11446	0.86
4	2	32	0.9	256	2.4	1881.46	5052	1.54	4.398	17.95	11466	0.85
17	3	31	0.8	256	2.8	2105.81	5361	1.51	5.084	23.17	11977	0.83
11	4	31	0.7	257	3.2	1910.89	5529	1.54	5.777	22.77	12841	0.86
15	5	31	0.8	256	2.7	2111.22	5220	1.54	5.077	22.26	12241	0.83
7	6	30	0.8	257	2.8	1654.7	5364	1.59	5.218	20.19	12025	0.85
16	7	31	0.8	256	2.7	2114.83	5198	1.54	5.091	21.44	12621	0.85
13	8	31	0.8	256	2.8	2116.85	5205	1.52	5.089	21.53	12654	0.84
12	9	31	0.9	257	2.4	1480.83	4669	1.58	4.524	18.21	11239	0.83
9	10	31	0.7	255	3.2	3054.81	5380	1.48	5.786	25.47	12881	0.82
2	11	32	0.7	256	3.2	2454.74	5367	1.5	5.639	24.33	12794	0.84
14	12	31	0.8	256	2.7	2022.87	5264	1.59	5.08	20.66	12256	0.85
3	13	30	0.9	256	2.4	1866.81	5347	1.54	4.633	19.28	10995	0.83
1	14	30	0.7	256	3.2	2715.74	5837	1.54	5.976	24.01	12869	0.84
6	15	32	0.8	255	2.7	2696.95	5027	1.53	4.954	22.82	12254	0.85
5	16	30	0.8	255	2.8	2659.25	5236	1.54	5.194	22.06	11913	0.83
8	17	32	0.8	257	2.7	1701.29	4527	1.54	4.986	22.7	12643	0.84

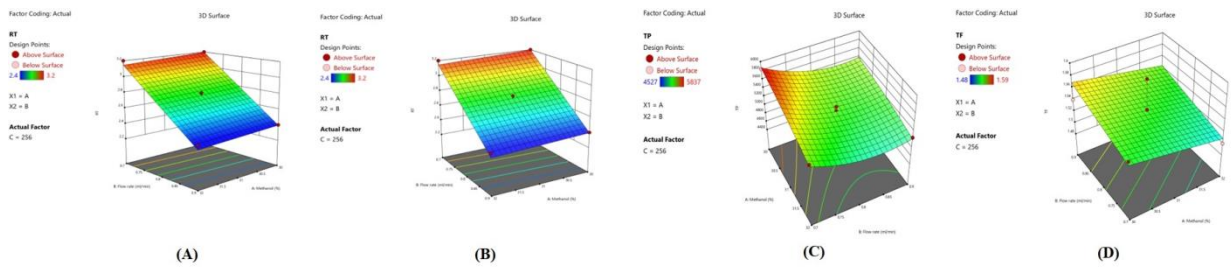

Figure 2. Contour plots against mobile phase and flow rate of Metformin (A) retention time, (B) peak area, (C) theoretical plates, (D) tailing factor

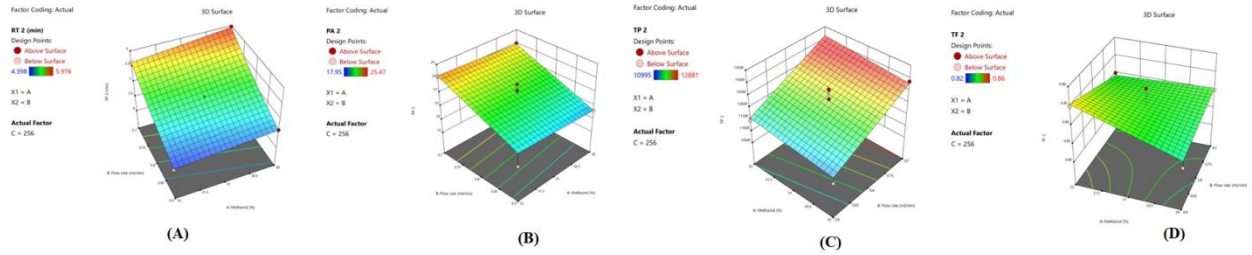

Figure 3. contour plots against mobile phase and flow rate of Evogliptin (A) retention time, (B) peak area, (C) theoretical plates, (D) tailing factor

Based on the result of design experiment, the final chromatographic condition selected for estimation of Metformin and Evogliptin was as below. C_{18} Column (250mm x 4.6mm, 5µm particle size) was selected as stationary phase with a flow rate of 0.8 mL/min. Methanol: 0.11% acetic acid in water was selected as mobile phase with detection wavelength of 256 nm with a total run time of 15 minutes.

3.4. Assay of Marketed Formulation

The developed method had been applied to analyse the dosage of Evogliptin and Metformin in marketed tablet form. It was found that the percentage drug content of Evogliptin was 99.092 ± 0.035 %w/w and Metformin was 100.01±0.04% w/w.

3.5 Uncertainty Assessment

The uncertainty assessment for method was done as per the EURACHEM/CITAC guide and the corresponding literatures [8-12]. Combined uncertainty ($u_{combined}$) and expanded uncertainty ($U_{Expanded}$), calculated from the uncertainty in standard preparation ($u_{standard}$), uncertainty associated with the slope of calibration curve ($u_{calibration}$), uncertainty of recovery ($u_{recovery}$) and uncertainty of repeatability ($u_{repeatability}$) has been using following equation 1.

$$u_{combined} = \sqrt{(u_{standard})^2 + (u_{calibration})^2 + (u_{recovery})^2 + (u_{repeatability})^2} \quad (1)$$

(1)

$U_{standard}$ of analyte was calculated from the %purity provided by the supplier using equation 2.

$$u_{standard} = 100 - \%Purity/\sqrt{3} \quad (2)$$

$U_{calibration}$ was calculated for analyte from the standard error of slope and slope value for the calibration curve using equation 3.

$$u_{calibration} = (\text{Standard Error of Slope} * 100)/\text{ Slope} \quad (3)$$

The mean relative standard deviation (RSD) associated with the recovery studies was considered as $U_{recovery}$ while that of repeatability studies was considered as $U_{repeatability}$ for the analyte. Expanded uncertainty at a 95% confidence interval is calculated by multiplying combined uncertainty with the coverage factor (k = 2) The uncertainty profile for the present method is given in Table 5.

Table 5. Uncertainty Assessment of RP-HPLC method

Uncertainty (U)	Evogliptin	Metformin
$u_{standard}$	0.15	0.22
$u_{calibration}$	1.93	0.53
$u_{recovery}$	0.22	1.43
$u_{repeatability}$	0.01	0.19
$u_{combined}$	1.96	1.60
$U_{expanded}$	3.90	3.20

$U_{Expanded}$: k=2 95 % confidence level; U % values reported

4. Conclusions

HPLC method has been developed using DoE approach for the estimation of Metformin and Evogliptin in combination. The method was validated as per ICH guideline and found to be accurate, precise and sensitive. The method was linear in the concentration range 50-250 µg/mL and 0.5 to 2.5 µg/mL of Metformin and Evogliptin respectively with a correlation coefficient of 0.999. The limit of detection of Metformin was found to be 1.377 µg/mL and of Evogliptin was found to be 0.017 µg/mL and the limit of quantification was found to be 4.17 µg/mL and 0.052 µg/mL for Metformin and Evogliptin respectively. The %RSD obtained for validation parameter indicates the suitability of this method for routine analysis and quantitative analysis. Compared to reported methods developed method is more sensitive and uses simpler mobile phase [8-12]. The method can be used for the routine analysis of bulk drug, formulation, and stability samples.

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