

## Development and Validation of a New Stability Indicating RP-UFLC Method for the Simultaneous Determination of Alogliptin and Metformin Tablets

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### Abstract

Alogliptin is a new hypoglycemic drug and Metformin is anti-diabetic drug. The combination of Alogliptin and Metformin is useful for the treatment of high blood sugar levels caused by type 2 diabetes. A new stability indicating RP-UFLC method has been developed for the simultaneous determination of Alogliptin and Metformin using mobile phase mixture consisting of tetra butyl ammonium hydrogen sulphate and methanol and validated. Shimadzu Model UFLC system with Agilent C<sub>18</sub> column and PDA detector with flow rate 0.6 mL/min with UV detection at 235 nm were the chromatographic conditions for the present study. Metformin shows linearity over the concentration range 0.05-100 mg/ml and the linear regression equation was found to be  $y = 10104x + 5157$  ( $R^2 = 0.999$ ) whereas Alogliptin shows linearity over the concentration range 0.1-25 mg/ml and the linear regression equation was found to be  $y = 75448x + 5118$  ( $R^2 = 0.999$ ). The LOQ and LOD of Metformin were found to be 0.0415 mg/ml and 0.01316 mg/ml respectively. The LOQ and LOD of Alogliptin were found to be 0.0961 mg/ml and 0.0309 mg/ml respectively. Stress degradation studies were performed and the method was validated as per ICH guidelines.

**Keywords:** Alogliptin; Metformin; RP-UFLC; Stability Indicating; Validation; ICH Guidelines

### Introduction

Metformin is anti-diabetic drug approved by FDA in 1994. It is chemically N, N-dimethyl imido-carbonimidic diamide (Figure 1A) and used for the treatment of type 2 diabetes mellitus. It decreases blood glucose levels by decreasing the hepatic glucose production and improving the insulin sensitivity by increasing the peripheral glucose uptake [1]. Alogliptin (AG) (Figure 1B) is a new hypoglycemic drug. It belongs to dipeptidyl-peptidase-4 inhibitor class and stimulates glucose-dependent insulin release [2,3]. It is chemically 2-({6-[(3R)-3-amino piperidin-1-yl]-3-methyl-2,4-dioxo-3,4-dihydro pyrimidin-1(2H)-yl} methyl) benzo nitrile mono benzoate.

Several liquid chromatographic methods such as HPTLC, LC-MS/MS and HPLC methods were developed for the estimation of Metformin and Alogliptin in biological samples as well as tablet dosage forms.

Ashutosh, *et al.* developed a RP-HPLC method for the simultaneous estimation of Metformin and Alogliptin in human plasma [4] using X-Terra C18 column with a mixture of mobile phase consisting of sodium dihydrogen ortho phosphate (pH 4.0): acetonitrile (70:30) with flow rate 1.0 ml/min and detection wavelength 235 nm. Linearity was observed over the concentration range 7.5-17.5 µg/ml and 300-700 µg/ml for Alogliptin and Metformin respectively.

Shereen., *et al.* developed a specific bioanalytical LC-MS/MS method [5] for the estimation of Alogliptin and Metformin in human plasma using Sitagliptin as an internal standard on gradient mode. Mobile phase consisting of acetonitrile and 0.2% formic acid was used with Hypersil Gold column in mass spectrometer in positive ion mode. Linearity was observed over the concentration range 5-400 ng/ml and 25-2000 ng/ml for Alogliptin and Metformin respectively.

Sharma., *et al.* developed a HPTLC method [6] for the simultaneous estimation of Metformin hydrochloride and Alogliptin benzoate in bulk drugs and combined dosage forms using Merck HPTLC aluminium sheets of silica gel 60F254 and acetonitrile: 1% ammonium acetate in methanol (4.5:5.5 v/v) as mobile phase for the chromatographic separation with UV detection at 253 nm. Linearity was observed over the concentration range 100-2500 ng/spot for both Alogliptin and Metformin.

Magdy., *et al.* developed two liquid chromatographic methods [7], HPTLC and HPLC for the determination of Alogliptin and Metformin hydrochloride in presence of metformin impurity, known as Melamin in pure form and in its pharmaceutical formulation. In HPTLC method mobile phase consisting of ethyl acetate: methanol: formic acid (60: 38: 2, v/v) was used with UV detection at 230 nm. In HPLC method, a mixture of 0.1% w/v sodium lauryl sulfate buffer (pH 3.0): methanol (70:30, v/v) was chosen as mobile phase with C<sub>18</sub> column and UV detection at 220 nm.

Runia., *et al.* developed a stability indicating RP-HPLC method [8] for simultaneous estimation of Alogliptin benzoate and Metformin hydrochloride in tablet dosage forms using Hypersil BDS C18 column with a mixture of mobile phase consisting of phosphate buffer and acetonitrile (48:52 % v/v) pH adjusted to 4.8 with ortho phosphoric acid with flow rate 1.0 ml/min and detection wavelength 210 nm with which Alogliptin and Metformin were eluted at 3.78 min and 2.78 mins. Linearity was observed over the concentration range 3.12-18.75 µg/ml for Alogliptin and 125-750 µg/ml of Metformin respectively.

Praveen Kumar., *et al.* developed a RP-HPLC method [9] for the simultaneous estimation of Alogliptin and Metformin hydrochloride tablet dosage forms using Agilent C18 column with a mixture of mobile phase consisting of 0.2% TEA (pH adjusted to 6.0 with ortho phosphoric acid) and methanol (30: 70, v/v ± 0.2%) with flow rate 1.0 ml/min and detection wavelength 254 nm. Linearity was observed over the concentration range 25-150 µg/ml for both Alogliptin and Metformin.

Indraja developed a RP-HPLC method [10] for the simultaneous estimation of Alogliptin and Metformin hydrochloride pharmaceutical formulation by using PDA detector and Agilent C18 column with a mixture of mobile phase consisting of phosphate buffer of pH adjusted to 3.0 with 0.1% OPA and methanol (20:80 v/v) with flow rate 0.7 ml/min and detection wavelength 242 nm. Linearity was observed over the concentration range 10-30 µg/ml for both Alogliptin and Metformin HCl. Alogliptin and Metformin were eluted at 2.900 min and 1.727 mins respectively.

Swathi., *et al.* developed a RP-HPLC method [11] for the simultaneous estimation of Alogliptin and Metformin hydrochloride tablet dosage forms using X Bridge RP C18 column with a mixture of mobile phase consisting of phosphate buffer (pH adjusted to 3.0 with ortho phosphoric acid), methanol and acetonitrile (20: 60: 20) with flow rate 1.0 ml/min and detection wavelength 290 nm. Linearity was observed over the concentration range 1.5-2.5 µg/ml and 60-100 µg/ml for both Alogliptin and Metformin respectively. Alogliptin and Metformin were eluted at 3.346 min and 1.592 mins respectively.

In the present study a new stability indicating RP-UFLC method has been proposed for the simultaneous estimation of Alogliptin and Metformin and the method was validated as per ICH Q2(R1) guidelines.

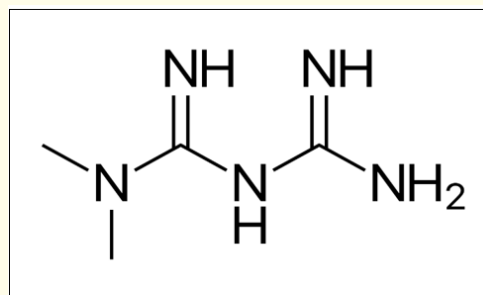


Figure 1A: Structure of Metformin (MF)

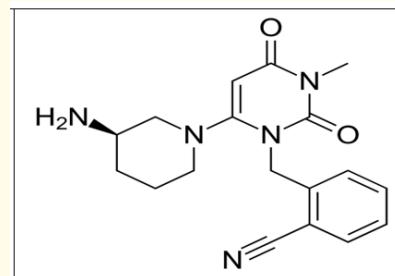


Figure 1B: Structure of Alogliptin (AG)

## Materials and Methods

### Chemicals and reagents

HPLC grade methanol (Merck), Milli-Q water, tetra butyl ammonium hydrogen sulphate (TBHS) (Merck), hydrochloric acid (S D Fine Chemicals), and sodium hydroxide (Qualigens) hydrogen peroxide (30% w/v) (Merck) were procured for the present study. Alogliptin API and Metformin API were obtained from Indoco Remedies Ltd. (India) and Lupin Ltd (India) respectively as gift samples and were used as it is without further purification.

### Preparation of 10 mM Tetra butyl ammonium hydrogen sulphate solution (pH 3.4)

Tetra butyl ammonium hydrogen sulphate ( $C_{16}H_{37}NO_4S$ ) is an ion pairing agent with molecular weight 339.54 grams/mole. 3.3954 grams Tetra butyl ammonium hydrogen sulphate was accurately weighed and transferred to a 1000 ml volumetric flask and dissolved in HPLC grade water to prepare 10 mM solution and the pH of the resulting solution is 3.4. This buffer solution was filtered through 0.42 micron membrane filter and used for the preparation of mobile phase.

### Preparation of Alogliptin and Metformin stock and working standard solutions

25 mg of Alogliptin and Metformin was accurately weighed, transferred and dissolved in 25 mL volumetric flask in methanol and the volume was made up volume (1000 µg/mL) and this stock solution was further diluted with mobile phase to get working standard solution (100 µg/mL) and from which dilutions were made as per requirement.

### Instrumentation and chromatographic conditions

Shimadzu Model UFLC system with Agilent  $C_{18}$  column and PDA detector was used for the chromatographic study. Mobile phase consisting of tetra butyl ammonium hydrogen sulphate in combination with methanol in 40: 60, v/v ratio with flow rate 0.6 mL/min with UV detection at 235nm are the chromatographic conditions and a mixture of tetra butyl ammonium hydrogen sulphate and methanol in 60: 40, v/v ratio was used as diluent. The injection volume was 20 µl and the total run time was 10 minutes.

### Method validation [12]

#### Linearity, precision, accuracy and robustness

A series of solutions consisting of 0.1-25 µg/ml Alogliptin and 0.05-100 µg/ml Metformin were prepared from their stock solutions and 20 µL of each solution was injected in to the UFLC system and the peak area of the chromatogram was noted. Calibration curves were plotted by taking the concentration of Alogliptin and Metformin solutions on the x-axis and the corresponding peak area values on the y-axis.

The intra-day precision of the assay method was evaluated by carrying out 6 independent assays for Alogliptin (0.25 µg/ml) and Metformin (10 µg/ml) and the mean peak area, standard deviation and finally the %RSD was calculated. The inter-day precision study was also performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels and the % RSD was calculated. The accuracy of the assay method was evaluated using standard addition method followed by recovery studies (50, 100 and 150%). The robustness of the assay method was established by introducing very small changes in the optimized UFLC conditions that include detection wavelength, mobile phase composition, pH and flow rate.

#### Assay of alogliptin and metformin tablets

The combination of Alogliptin (12.5 mg) and Metformin (1000 mg) with brand name Vipdomet and Kazano (Takeda Pharmaceuticals India Pvt. Ltd) consisting of Alogliptin (12.5 mg) and Metformin (500 mg) are the existing tablet formulations. 20 Tablets of the available brand from the local pharmacy was procured weighed, crushed in to fine powder and powder equivalent to 100 mg of Metformin and 2.5 mg of Alogliptin were accurately weighed and transferred carefully into a 100 ml volumetric flask and made up to volume with HPLC grade methanol. The contents of the volumetric flask were sonicated for 30 min and filtered. The filtrate was then diluted with mobile phase as per the requirement and 20 µL of this solution was injected into the UFLC system after filtration through 0.45 µm membrane and the peak area was recorded from the respective chromatogram. The percentage purity of each drug was calculated from the calibration curve.

### Stress degradation studies [13]

Stress degradation studies (ICH Q1A (R2)) guidelines were performed to evaluate the specificity of the method as well as the

stability of Metformin and Alogliptin towards acidic hydrolysis, alkaline hydrolysis, thermal degradation and oxidation.

Acidic hydrolysis was performed by heating Alogliptin and Metformin combined solution with 1 mL of 0.1 N HCl solution at 60° for 30 minutes on a water bath. The stressed sample was then cooled, neutralized with 1.0 mL 0.1N sodium hydroxide solution, diluted with mobile phase and then 20 µl of the solution was injected in to the UFLC system.

Alkaline hydrolysis was performed by heating Alogliptin and Metformin combined solution with 1.0 mL 0.1N sodium hydroxide solution at 60° for 30 minutes on a water bath. The stressed sample was then cooled, neutralized with 1.0 mL of 0.1 N HCl solution, diluted with mobile phase and then 20 µl of the resulting solution was injected in to the UFLC system.

Thermal degradation was performed by heating the Alogliptin and Metformin combined solution at 60° for 30 minutes on a water bath and then cooled, diluted with mobile phase and 20 µl of the resulting solution was injected in to the UFLC system.

Oxidative degradation was performed by heating Alogliptin and Metformin combined solution with 1.0 mL 30% hydrogen peroxide solution at 60° for 30 minutes on a water bath. The stressed sample was then cooled, diluted with mobile phase and then 20 µl of the resulting solution was injected in to the UFLC system.

## Results and Discussion

A new stability indicating RP-UFLC method has been developed and validated for the determination of Alogliptin and Metformin combined solution using an ion pairing agent, tetra butyl ammonium hydrogen sulphate in combination with methanol in 40: 60, v/v ratio with flow rate 0.6 mL/min and PDA detection at 235 nm. Some of the parameters of the previously published analytical methods for the estimation of Alogliptin and Metformin combined dosage forms were summarized and compared with the proposed method in table 1. The representative chromatograms obtained for the placebo and that of Alogliptin and Metformin combined solution (API) and that of tablet dosage forms were shown in figure 2.

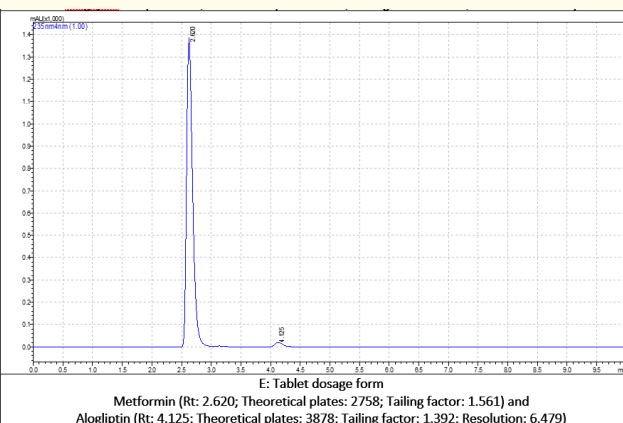
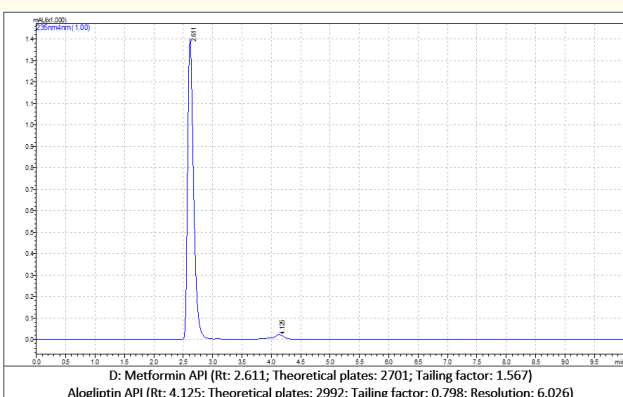
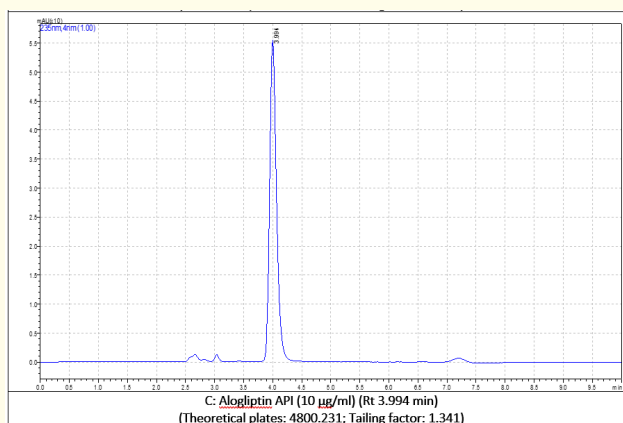
**Table 1:** Review of literature.

Mobile phase/Flow rate/Detection wavelength (% v/v)/(mL/min)/(nm)	Column	Linearity (µg/mL)	Comments	Ref
Sodium dihydrogen ortho phosphate (pH 4.0): Acetonitrile (70:30)/1/235	X Terra C18	7.5-17.5 (AG) 300-700 (MF)	RP-HPLC (Human plasma)	[4]
Acetonitrile: 0.2% Formic acid	Hypersil Gold	0.005-0.4 (AG) 0.025-2.0 (MF)	UHPLC-MS/MS (Human plasma) Sitagliptin (Internal standard)	[5]
Acetonitrile: 1% Ammonium acetate in methanol (4.5: 5.5)/253		100-2500 ng/ spot (AG & MF)	HPTLC	[6]
Ethyl acetate: Methanol: Formic acid (60: 38: 2)/230 0.1% w/v Sodium lauryl sulfate buffer (pH 3.0): Methanol (70:30)/220	- C18	- -	HPTLC  HPLC Melamin Impurity	[7]
Phosphate buffer: Acetonitrile (48: 52) (pH 4.8 adjusted with ortho phosphoric acid) /1/210	Hypersil BDS C18	3.12-18.75 (AG) 125-750 (MF)	RP-HPLC	[8]
0.2% TEA (pH adjusted to 6.0 with ortho phosphoric acid) and methanol (30: 70)/1/254	Agilent C18	25-150 (AG & MF)	RP-HPLC	[9]
Phosphate buffer: Methanol (20: 80) (pH 3.0 adjusted with ortho phosphoric acid) /0.7/242	Agilent C18	10-30 (AG & MF)	RP-HPLC	[10]
Phosphate buffer (pH 3.0 adjusted with ortho phosphoric acid): Methanol: Acetonitrile (20: 60: 20)/1/290	X Bridge RP C18	1.5-2.5 (AG) 60-100 (MF)	RP-HPLC	[11]
Tetra butyl ammonium hydrogen sulphate(pH 3.4): Methanol (40:60)/0.6/235	Agilent C18	0.1-25 (AG) 0.05-100 (MF)	RP-UFLC	Present method

**Method development and optimization**

Metformin solution (10 µg/mL) was initially injected in to the system attached with Agilent C18 column with flow rate 1.0 ml/min and mobile phase tetra butyl ammonium hydrogen sulphate: methanol (45:55) by which Metformin was eluted at 1.546 min. Therefore the flow rate was changed as 0.8 ml/min keeping the mobile phase ratio as it is and the retention time was reported as 1.947 min. Finally the flow rate was modified as 0.6 ml/min with mobile phase (tetra butyl ammonium hydrogen sulphate: methanol) ratio 40:60 and Metformin was eluted at 2.582 min (Figure 2B) with UV detection at 235 nm. Alogliptin solution (10 µg/mL) was injected in to the UFLC system under the same chromatographic conditions and the peak was eluted at 3.994 mins (Figure 2C) with maximum peak area at UV detection at 277 nm.

A combined solution (API) of Alogliptin (10 µg/mL) and Metformin (10 µg/mL) was introduced in to the system with UV detection at 277 nm but the Metformin peak was not observed. As the UFLC system was provided with PDA detector the detection wavelength was chosen as 235 nm and initially the placebo was injected in to the system (Figure 2A) followed by the combined solution where Alogliptin and Metformin were eluted at 4.125 min and 2.611 min (Figure 2D) with the acceptable system suitability parameters (Resolution: 6.026).

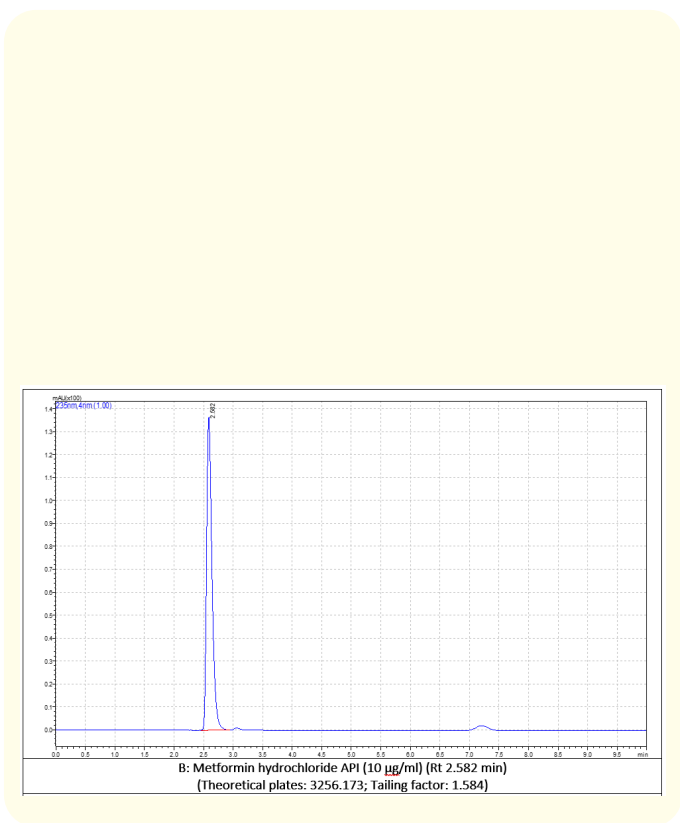


**Figure 2:** Representative chromatograms of Metformin hydrochloride and Alogliptin.

**Method validation**

**Linearity, precision, accuracy and robustness**

Metformin shows linearity over the concentration range 0.05-100 mg/ml and the linear regression equation was found to be  $y = 10104x + 5157$  ( $R^2 = 0.999$ ) (Figure 3A) whereas Alogliptin



shows linearity over the concentration range 0.1-25 mg/ml (Table 2) and the linear regression equation was found to be  $y = 75448x + 5118$  ( $R^2 = 0.999$ ) (Figure 3B). The LOQ and LOD of Metformin were found to be 0.0415 mg/ml and 0.01316 mg/ml respectively. The LOQ and LOD of Alogliptin were found to be 0.0961 mg/ml and 0.0309 mg/ml respectively. The proposed method is precise (Table 3) as the % RSD was less than 2% (Metformin (0.0092) and Alogliptin (0.4874)). The proposed method is accurate (Table 4) as the % RSD was less than 2% (Metformin (0.73-1.07) and Alogliptin (0.52-1.11)) and robust (Table 5) as the percentage RSD (MF: 0.0139-0.4183 and AG: 0.5621-1.0997) was less than 2.

**Table 2:** Linearity of Metformin and Alogliptin.

Metformin (MF)		Alogliptin (AG)	
Conc. (µg/ml)	*Mean peak area	Conc. (µg/ml)	*Mean peak area
0	0	0	0
0.05	5181	0.1	7245
0.1	10142	0.2	14539
0.5	51723	0.5	36183
1	101242	1	72393
2	202391	2	144881
5	506342	2.5	182712
10	1011618	5	365787
20	2093921	10	731024
50	5021781	20	1492215
100	10114993	25	1898936

\*Mean of three replicates.

**Table 4:** Accuracy study of Metformin and Alogliptin.

Conc. (µg/ml)						% Recovery (% RSD)	
Formulation		Pure Drug		Total		MF	AG
MF	AG	MF	AG	MF	AG		
40	1	20	0.5	60	1.5	99.82 (0.73)	99.63 (0.91)
40	1	20	0.5	60	1.5		
40	1	20	0.5	60	1.5		

**Table 3:** Precision study of Metformin and Alogliptin.

Intraday precision				
Conc. (µg/ml)			*Mean peak area	
MF	AG		MF	AG
10	0.25		1011618	18324
10	0.25		1011524	18251
10	0.25		1011729	18288
10	0.25		1011557	18454
10	0.25		1011497	18472
10	0.25		1011692	18339
Mean			1011602.833	18354.667
SD			93.3968	89.4554
% RSD			0.0092	0.4874
Interday precision				
Conc. (µg/ml)			*Mean peak area	
Day	MF	AG	MF	AG
Day 1	10	0.25	1011618	18324
Day 2	10	0.25	1013451	18981
Day 3	10	0.25	1012962	18645
Mean			1012677	18650
SD			949.1528	328.5285
% RSD			0.0937	1.7616

\*Mean of three replicates.

40	1	40	1	80	2	98.93 (0.82)	99.25 (0.52)
40	1	40	1	80	2		
40	1	40	1	80	2		
40	1	60	1.5	100	2.5	99.72 (1.07)	99.49 (1.11)
40	1	60	1.5	100	2.5		
40	1	60	1.5	100	2.5		

\*Mean of three replicates.

**Table 5:** Robustness study of Metformin and Alogliptin.

Parameters	Conditions	*Mean peak Area		*Mean peak area ± SD (% RSD)	
		MF	AG	MF	AG
Flow rate (± 0.1 ml/min)	0.7	1019342	18121	1014465 ± 4243.32 (0.4183)	18323 ± 201.50 (1.0997)
	0.6	1011618	18324		
	0.8	1012435	18524		
Detection wavelength (± 2 nm)	237	1011427	18112	1011582 ± 140.5027 (0.0139)	18215.67 ± 106.077 (0.5823)
	235	1011618	18324		
	233	1011701	18211		

Mobile phase composition (10 mM Tetra butyl ammonium hydrogen sulphate: Methanol) (± 2 v/v)	38:62	1012561	18427	1012402.5 ± 1109.4505 (0.1096)	18324 ± 103 (0.5621)
	40:60	1011618	18324		
	42:58	1013187	18221		
pH	3.3	1012683	18119	1011865 ± 726.6959 (0.0718)	18311.33 ± 186.3232 (1.0175)
	3.4	1011618	18324		
	3.5	1011294	18491		

\*Mean of three replicates.

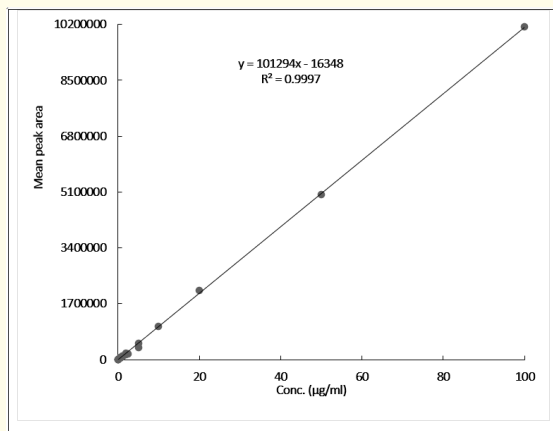


Figure 3A: Calibration curve of Metformin (MF)

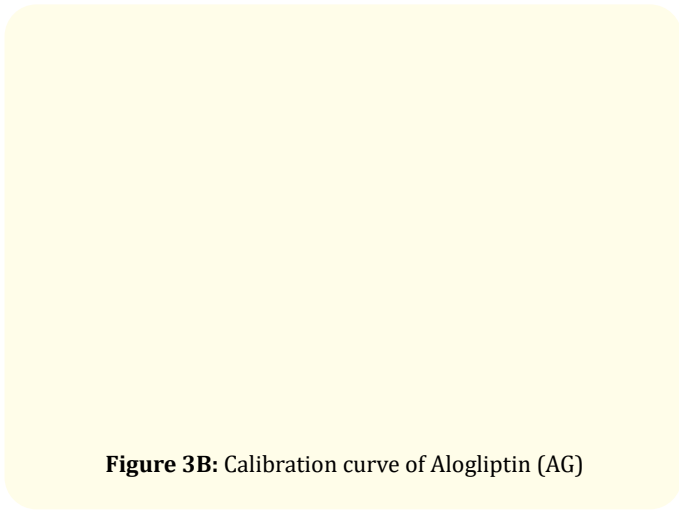


Figure 3B: Calibration curve of Alogliptin (AG)

Assay of commercial formulations

The proposed method was applied for the determination of Metformin and Alogliptinin marketed formulations available i.e. Tablets and the % recovery was 99.84-99.86% (Metformin) and 99.12-99.44% (Alogliptin) and the corresponding chromatogram observed was shown in figure 2E (Table 6).

Table 6: Assay of Metformin and Alogliptin tablets.

Labelled claim (mg)		Amount found* (mg)		Recovery* (%)	
MF	AG	MF	AG	MF	AG
500	12.5	499.21	12.43	99.84	99.44
1000	12.5	998.64	12.39	99.86	99.12

\* Mean of three replicates.

Table 7: Stress degradation study of Alogliptin and Metformin.

Stress condition	Rt (min)		Mean peak area		%Recovery (% Drug degradation)		Theoretical plates		Tailing factor		Resolution
	MF	AG	MF	AG	MF	AG	MF	AG	MF	AG	
Standard	2.611	4.125	10122514	148326	100	100	5700	5321	1.021	1.026	6.026
Acidic hydrolysis 0.1N HCl/60°C/30 min	2.676	4.184	9923800	136764	98.04	92.21	5362	5523	1.085	1.337	8.126
Alkaline hydrolysis 0.1N NaOH/60°C/30 mins	2.648	4.034	8771046	125369	86.65	84.52	5855	5554	1.022	1.316	7.810
Oxidative degradation 3% H <sub>2</sub> O <sub>2</sub> /60°C/30 min	2.602	4.058	10113880	123711	99.92	83.41	28600	2192	1.510	1.340	2.154 3.864
Thermal hydrolysis (60°C/30 min)	2.585	4.011	9765996	143849	96.48	96.98	2934	4757	1.528	1.369	6.733

\* Mean of three replicates

### Stress degradation studies

Stress degradation studies such as acidic hydrolysis, alkaline hydrolysis, thermal treatment and oxidation were performed individually for both Metformin and Alogliptin and later a combined solution of Metformin and Alogliptin was exposed to stress conditions and the resultant solution was injected in to the UFLC system.

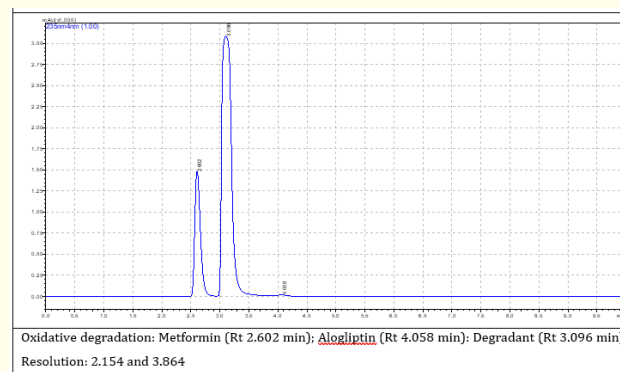
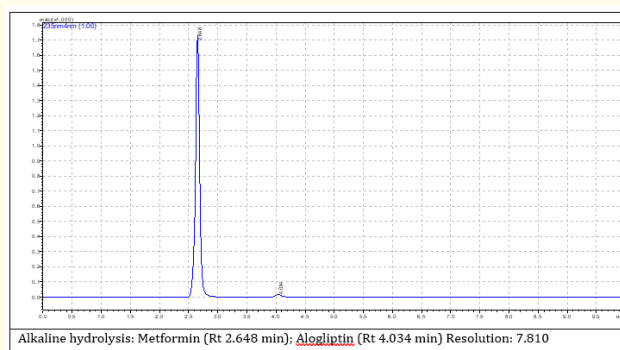
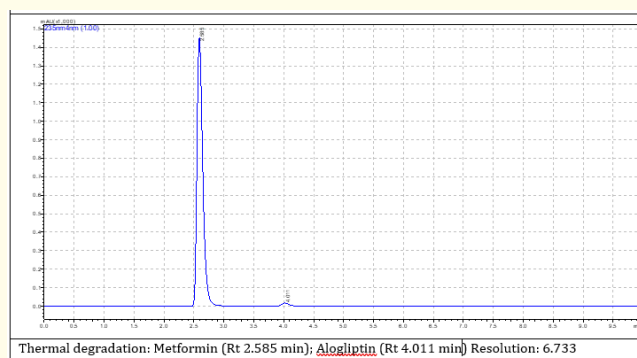
During the acidic hydrolysis Metformin was eluted at 2.676 min and Alogliptin was eluted at 4.184 min. The % recovery was 98.04 and 92.21 for Metformin and Alogliptin respectively with resolution 6.733.

During the alkaline hydrolysis Metformin was eluted at 2.648 min and Alogliptin was eluted at 4.034 min. The % recovery was 86.65 and 84.52 for Metformin and Alogliptin respectively.

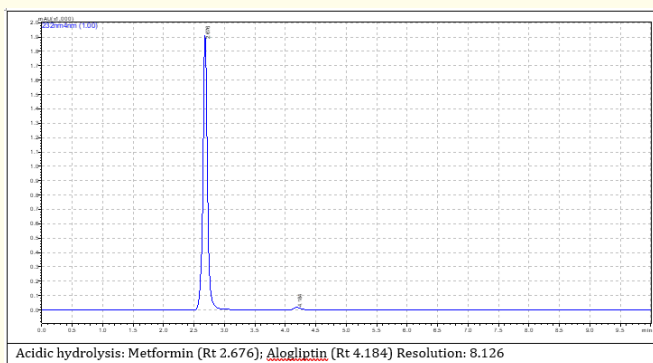
During the Oxidation degradation study Metformin was eluted at 2.602 min and Alogliptin was eluted at 4.058 min. The % recovery was 99.92 and 83.41 for Metformin and Alogliptin respectively. A degradant peak was observed at 3.096 and the resolution values were found to be 2.154 and 3.864 which were greater than 2.

During the thermal degradation study Metformin was eluted at 2.585 min and Alogliptin was eluted at 4.011 min. The % recovery was 96.48 and 96.98 for Metformin and Alogliptin respectively with resolution 6.733.

In all the degradation studies less than 15% was reported and the system suitability parameters such as theoretical plates were greater than 2000, tailing factor was less than 2.0 and the resolution was greater than 2. During the degradation studies it was also observed that none of the degradants were interfering with the degradant peaks and the method was validated as per ICH Q1A (R2) guidelines.



**Figure 4:** Representative chromatograms of Alogliptin and Metformin during stress degradation studies.



### Conclusion

The proposed stability indicating RP-UFLC method is simple, precise, accurate, robust and was validated as per ICH guidelines and can be used for the analysis of Alogliptin and Metformin combined dosage forms. The system suitability parameters such as theoretical plates, tailing factor, resolution etc. are within the acceptable criteria. The method can be applicable for the pharmacokinetics study as well as for the biological sample study.



## Acknowledgement

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