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New Validated Stability Indicating RP-HPLC Method for Simultaneous Estimation of Metformin and Alogliptin in Human Plasma

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Abstract

A validated new stability indicating RP-HPLC method for the quantitative determination of metformin and alogliptin in human plasma was developed as per US-FDA guidelines. The drug was spiked in the plasma and extracted with mobile phase by precipitation method. The extracted analyte was injected into X-Terra C18 (4.6 × 150 mm, 3.5 µm, Make: ACE) or equivalent, maintained at 25°C temperature and effluent was monitored at 235 nm. The mobile phase was consisted of sodium dihydrogen ortho phosphate [pH 4.0]:acetonitrile [HPLC Grade] (70:30 v/v). The flow rate was maintained at 1.0 mL/min. The calibration curve for metformin and alogliptin was linear from 300.0 to 700.0 µg/mL (r^2=0.997) and 7.5 to 17.5 µg/mL (r^2=0.998) respectively. The inter-day and intra-day precision was found to be within limits. The Lower limit of quantification (LLOQ) for metformin and alogliptin were 5.936 and 1.983 µg/mL respectively. The average % recovery for metformin and alogliptin were 100.17 and 99.40-99.55% respectively and reproducibility was found to be satisfactory. This RP-HPLC method is suitable for determining the concentration of metformin and alogliptin in human plasma and it can applied for routine analysis for determination of the metformin and alogliptin from dosage form during pharmacokinetic study.

Keywords: Metformin; Alogliptin; RP-HPLC; ICH-guidelines; Validation; Human Plasma; US-FDA guidelines

Introduction

Diabetes is a chronic condition associated with abnormally high levels of sugar (glucose) in the blood. The production of insulin by the pancreas lowers blood glucose level. Due to the absence or insufficient production of insulin causes diabetes. There are two types of diabetes which are referred to as type 1 and type 2 [1]. Formerly names for these conditions were insulin-dependent and non-insulin-dependent diabetes, or juvenile onset and adult onset diabetes. The symptoms of diabetes include increased urine output, thirst, hunger, and fatigue. Diabetes is diagnosed by blood sugar (glucose) testing. The number of individuals affected by diabetes is continuing to increase worldwide; the need for effective management assumes ever greater urgency [2]. Newer classes of medications, particularly those which work via the incretin pathway, achieve glucose lowering and minimizing risks associated with more traditional therapies. Ideally, combination therapies should be well tolerated, convenient to take, have few contraindications, have a low risk of hypoglycemia and weight gain, and be reasonably effective over both the short and long term such as the combination of metformin (MF) and the dipeptidyl peptidase-4 (DPP-4) inhibitor alogliptin (ALG). The chemical structure of metformin and alogliptin were represented in Figures 1 and 2 respectively. Alogliptin [3-5] is a selective, orally-bioavailable inhibitor of enzymatic activity of dipeptidyl peptidase-4 (DPP-4). DPP-4 inhibitors represent a new therapeutic approach to the treatment of type 2 diabetes that functions to stimulate glucose dependent insulin release and reduce glucagons levels. This is done through inhibition of the inactivation of in cretins, particularly glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP). Alogliptin inhibits dipeptidyl peptidase 4 (DPP-4), which normally degrades the incretin glucose-dependent insulin tropic polypeptide (GIP) and glucagon like peptide 1 (GLP-1), thereby improving glycemic control [3-5]. Metformin hydrochloride (MTF) [6-9] ($C_4H_{11}N_5$.HCl) is 1:1 dimethylbiguanidine monohydrochloride is an anti-diabetic drug from the biguanide class of oral Hypoglycaemic agents, given orally in the treatment of non-insulin-dependent diabetes mellitus. The main action of Metformin HCl is in increasing glucose transport across the cell membrane in skeletal muscle. Several analytical methods based on UV [9,10], Spectroflourimetry [11,12], Reverse Phase-HPLC [13-23], LC-MS/MS [24-27], HPTLC [28] were reported for the determination of metformin either in alone or in combination with other drugs. Although literature survey reveals that no methods were reported for metformin (MTF) and alogliptin (ALG) in combination form.

Materials and Methods

Chemicals and reagents

The reference sample of metformin and alogliptin were supplied by M/s Pharma Train, Hyderabad**.** HPLC grade water (prepared by using 0.45 Millipore Milli-Q) was procured from Standard Reagents, Hyderabad. HPLC grade acetonitrile was purchased from Merck, Mumbai. The chemicals used for preparation of buffer include sodium dihydrogen ortho phosphate (Finar Chemicals, Ahmedabad), and orthophosphoric acid (Standard Reagents, Hyderabad). 0.45 µm membrane filters (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) were used for filtration of various solvents and solutions intended for injection into the column [29-32].

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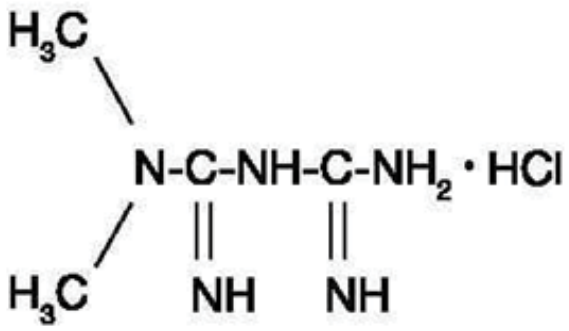

Figure 1: Chemical structure of Metformin HCl.

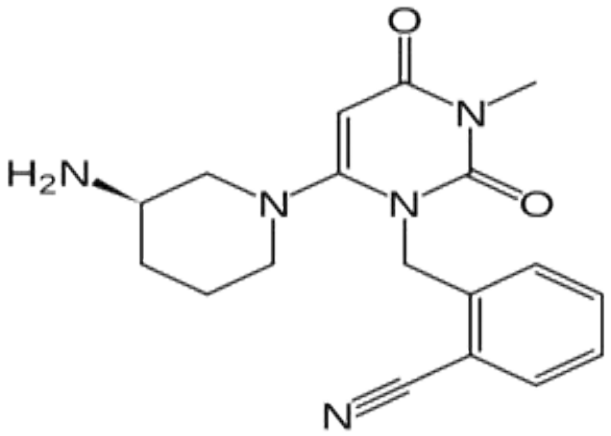

Figure 2: Chemical structure of Alogliptin.

Instrumentation

A Waters Alliance 2695 separation module equipped with a 2487 UV detector was employed throughout this study. Column that was employed in the method was X-Terra C_{18} (4.6 × 150 mm, 3.5 µm, Make: ACE). The samples were injected with an automatic injector. The 20 µL volume of sample was injected. The input and output operations of the chromatographic system were monitored by Waters Empower software. The flow rate selected was 1.0 mL per min. The detection was done at 235 nm. The temperature and run time was monitored at 25°C and 8.0 min respectively. The ultra violet spectra of the drugs used for the investigation were taken on a Lab India UV 3000 spectrophotometer for finding out their λ_{max} values. Solubility of the compounds was enhanced by sonication on an ultra sonicator. (Model: Power Sonic 510, Hwashin Technology). All the weighings in the experiments were done with an Afcoset electronic balance. The HermLe microlitre centrifuge Z100 (model no 292 P01) was used for the centrifugation process and Remi equipments (model no- CM101DX) Cyclomixer was used.

Glassware

All the volumetric glassware used in the study was of Grade A quality Borosil.

Preparation of sodium phosphate buffer (pH 4.0)

The buffer solution was prepared by dissolving 2.5 g of sodium dihydrogen ortho phosphate in 900 mL of HPLC grade water in a 1000 mL clean and dry flask. The mixture was stirred well until complete dissolution of the salt. The volume was made upto the mark with water. The pH was adjusted to 4.0 with 1% ortho phosphoric acid.

Preparation of mobile phase

The mobile phase was prepared by mixing 700 mL phosphate buffer (pH 4.0) and 300 mL of acetonitrile in a 1000 mL clean and dry flask. The resultant mobile phase was filtered through a 0.45 µm membrane filter (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) under vacuum. The resultant mobile phase was degassed in an ultra sonicator for 5 min.

Preparation of diluent

The diluent was prepared by mixing phosphate buffer (pH 4.0) and acetonitrile (HPLC grade) in the ratio of 70:30 v/v. This solution was used to dilute the drug solutions in the study.

Preparation of standard solution of metformin and alogliptin

10 mg metformin was weighed accurately and transferred into a 10 mL clean and dry volumetric flask. Initially, the drug was mixed with 7 mL of diluent. The solution was sonicated for 15 min for complete dissolution of the drug. The final volume was made up to the mark with the same solvent. Similarly, about 10 mg alogliptin was weighed accurately and transferred into a 100 mL clean and dry volumetric flask. Initially, the drug was mixed with 70 mL of diluent. The solution was sonicated for 15 min for complete dissolution of the drug. The final volume was made up to the mark with the same solvent. From the above prepared stock solutions 5.0 mL of metformin and 1.25 mL of alogliptin were pipetted out into a 10 mL clean and dry volumetric flask and it was diluted up to the mark with diluent. This mixed stock solution contains 500.0 µg/mL of metformin and 12.5 µg/mL of alogliptin.

Spiking of metformin and alogliptin into plasma and their extraction from plasma (By precipitation method)

From the above prepared mixed stock solution (500.0 µg/mL of metformin and 12.5 µg/mL of alogliptin), 0.5 mL was pipetted out and spiked into 0.5 mL of plasma in a polypropylene tube (Torson's). Then the tube was cyclo mixed for 5 min. Then 1.0 mL of acetonitrile was added to the tube and centrifuged for 20 min at 3000 rpm. Further the supernatant liquids were collected in another Eppendorf tube and 20 µL supernatant was injected into the analytical column.

Validation Development

Selectivity

An aqueous mixture of metformin and alogliptin (500.0 µg/mL of metformin and 12.5 µg/mL of alogliptin) was prepared and injected into the column and the retention times were checked and any interference at the retention times was checked by comparing the response in the blank. No interference was observed at the retention times for metformin and alogliptin extracted from plasma [33-38]. The method was found to be precise and specific. A typical chromatogram of metformin and alogliptin in plasma is shown in Figure 3.

Sensitivity

To determine the sensitivity in terms of LLOQ, 'Lower Limit of Quantification' where the response of LLOQ must be at least five times greater than the response of interference in blank matrix at the retention time of the analyte(s). The LLOQ obtained by the proposed

method were 5.936 and 1.983 µg/mL for metformin and alogliptin respectively.

Precision

To check the intra and inter-day variations of the method, solutions containing 500.0 µg/mL of metformin and 12.5 µg/mL of alogliptin were subjected to the proposed HPLC method of analysis and results obtained were noted. The precision of the proposed method i.e., the intra and inter-day variations in the peak areas of the drugs solutions in plasma were calculated in terms of percent relative standard deviation (RSD) and the results are represented in Tables 1, 2, 3 and 4. A statistical evaluation revealed that the percentage relative standard deviation of the drugs at linearity level for 6 injections was less than 2.0. Typical chromatogram of metformin and alogliptin in plasma for intra and inter-day precision are shown in Figures 4 and 5.

Accuracy

To determine the accuracy of the proposed method, recovery studies were carried out by analyzing (8.0, 10.0, 12.0 mg of metformin and alogliptin) of pure drugs. The solutions were suitably diluted at linearity level (500.0 µg/mL of metformin and 12.5 µg/mL of alogliptin). Then each dilution was injected thrice (n=3). The percent recoveries of the drugs were determined. The results are shown in Tables 5 and 6.

Linearity

In order to find out the linearity range of the proposed HPLC method in plasma, curves were constructed by plotting peak areas obtained for the analyte against their concentrations. A good linear relationship (r^2=0.997) was observed between the concentrations of metformin and alogliptin and their corresponding peak areas. The relevant regression equations were y=1192.x-38173 for metformin (r^2=0.997) and y=2802x-1113 for alogliptin (r^2=0.998) (where y is the peak area and x is the concentration of metformin and alogliptin (µg/mL)). The slope, intercept and the correlation coefficient of the plots are shown in Tables 7 and 8. The linearity ranges for metformin and alogliptin and their corresponding graphs are shown in Figures 6 and 7.

Injection	Retention Time	Area
Injection-1	2.734	553001
Injection-2	2.731	559476
Injection-3	2.738	553994
Injection-4	2.738	552953
Injection-5	2.731	553012
Injection-6	2.732	553032
Average	2.734	554245
Standard Deviation	0.003	2593.64
%RSD	0.12	0.47

Table 1: Intra-day precision of the proposed method for Metformin in plasma.

Injection	Retention Time	Area
Injection-1	4.451	34316
Injection-2	4.452	34431
Injection-3	4.454	34085
Injection-4	4.459	34350
Injection-5	4.452	34814
Injection-6	4.458	34360
Average	4.454	34392.7
Standard Deviation	0.003	237.7
%RSD	0.076	0.7

Table 2: Intra-day precision of the proposed method for Alogliptin in plasma.

Days	Retention Time	Area
Day-1*	2.734	563116
Day -2*	2.736	563076
Day -3*	2.735	561049
Average	2.735	562414
Standard Deviation	0.001	1182
%RSD	0.04	0.21

Table 3: Inter-day precision of the proposed method for Metformin (on three consecutive days n=6) in plasma.

Days	Retention Time	Area
Day-1*	4.451	33218
Day-2*	4.456	33876
Day-3*	4.451	33790
Average	4.452	33628
Standard Deviation	0.003	358
%RSD	0.06	1.1

Table 4: Inter-day precision of the proposed method for Alogliptin (on three consecutive days n=6) in plasma. *Average of Six injections.

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered (mg)	SD	% RSD
80%	100.05	100.17	8.0	0.032	0.4
	99.88		7.99		
	100.59		8.05		
100%	100.05	100.17	10.01	0.036	0.36
	99.88		9.99		
	100.59		10.06		
120%	100.05	100.17	12.01	0.042	0.35
	99.88		11.99		
	100.59		12.07		

Table 5: Accuracy data of the proposed method for Metformin in plasma.

Conc. Level	% Recovery	Avg. % Recovery	Amount Recovered (mg)	SD	% RSD
80%	98.48	99.55	7.88	0.119	1.50
	101.23		8.1		
	98.94		7.91		
100%	98.48	99.55	9.85	0.146	1.46
	101.23		10.12		
	98.94		9.89		
120%	98.48	99.40	11.82	0.178	1.49
	101.23		12.15		
	98.48		11.87		

Table 6: Accuracy data of the proposed method for Alogliptin in plasma.

Concentration (µg/mL)	Area	Statistical Analysis
300	308855	Slope=1192 Intercept= -38173 C. C=0.997
400	445785	
500	564758	
600	684752	
700	785468	

Table 7: Linearity range of Metformin in plasma.

Concentration (µg/mL)	Area	Statistical Analysis
7.5	19414	Slope=2802 Intercept= -1113 C. C=0.998
10	27219	
12.5	34464	
15	40829	
17.5	47634	

Table 8: Linearity range of Alogliptin in plasma.

S. No.	Standard Sample	Freeze and Thaw Stability Sample	Short Term Stability Sample	Long Term Stability Sample
1.	201585	185470	195862	187452
2.	204758	189562	194258	187569
3.	206984	188475	196541	187414
Mean	204442	187835.7	195554	187478
SD	2713	2120	1172	81
%RSD	1.33	1.13	0.60	0.04
Assay		91.88	95.65	91.70

Table 9: The Stability data for Metformin in plasma.

S. No.	Standard Sample	Freeze and Thaw Stability Sample	Short Term Stability Sample	Long Term Stability Sample
1.	13985	12014	12956	12014
2.	13586	12036	12947	12036
3.	13632	12159	12933	12159
Mean	13734	12069.67	12945	12070
SD	218	78	12	78
%RSD	1.59	0.65	0.09	0.65
Assay		87.88	94.26	87.88

Table 10: The Stability result for Alogliptin in plasma.

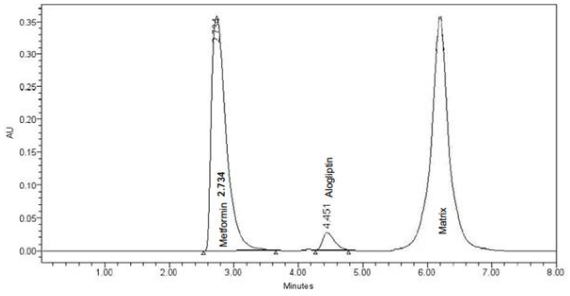

Figure 3: A typical chromatogram of Metformin and Alogliptin standard drugs in plasma.

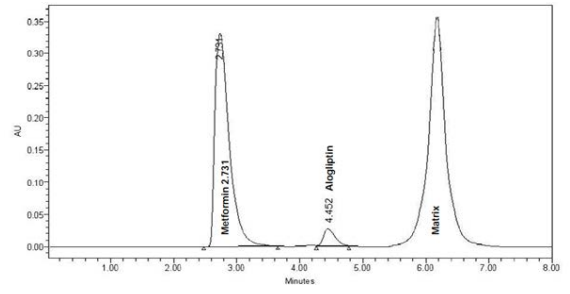

Figure 4: Typical chromatogram of metformin and alogliptin in plasma for intra-day precision.

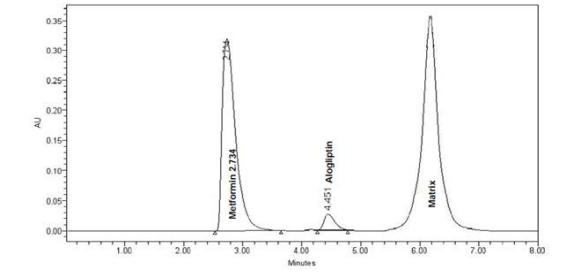

Figure 5: Typical chromatogram of metformin and alogliptin in plasma for inter-day precision.

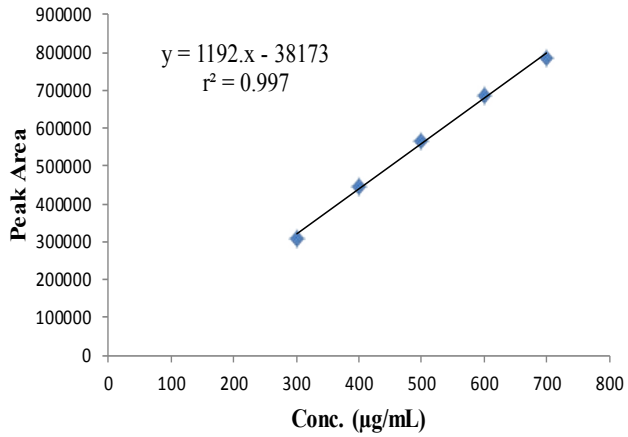

Figure 6: Calibration curve for Metformin in plasma.

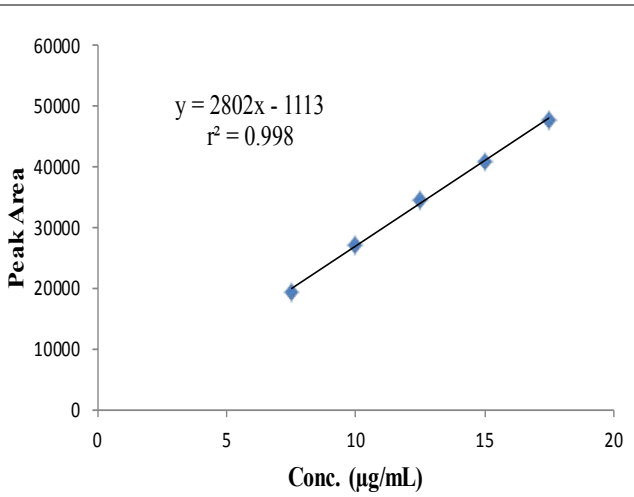

Figure 7: Calibration curve for Alogliptin in plasma.

Stability

All stability determinations used a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix [39]. The stock solutions of the analyte for stability evaluation were prepared in an appropriate solvent at known concentrations. To test the stability of the drug extract, it was subjected to

α) Freeze and thaw stability at -20°C ± 2°C,

β) Short term stability for period of 24 hours stored at room temperature,

χ) Long term stability for period of 15 days stored at 4°C.

Similar to the preparation of the standard preparation, the above samples were spiked into the plasma and extracted and collected in vial and injected into HPLC system. All the stability samples compared against the standard stock solution assessed for stability. The results are presented in Tables 9 and 10 (the figures in the table are in peak area units). Typical chromatograms for standard samples, freeze and thaw stability samples, short term stability samples and long term stability samples were represented in Figures 8, 9, 10 and 11.

Results and Discussion

To optimize the mobile phase, various proportions of sodium phosphate buffer (pH 4.0) with acetonitrile (HPLC Grade) were tested. The use of sodium phosphate buffer (pH 4.0) and acetonitrile (HPLC Grade) in the ratio of 70:30 v/v resulted in peak with good shapes and resolution. A flow rate of 1.0 mL /min was found to be optimum in the 0.4-1.5 mL/min range resulting in short retention time, baseline stability and minimum noise.

The LLOQ obtained for metformin and alogliptin by the proposed method in plasma was 5.936 and 1.983 µg/mL respectively. The retention times obtained for metformin and alogliptin in plasma were 2.734 and 4.451 respectively. Quantitative linearity of drugs in plasma was obeyed in the concentration range of 300-700 µg/mL for metformin and 7.5-17.5 µg/mL for alogliptin respectively. The relevant regression equations were y=1192.x-38173 for metformin (r^2=0.997) and y=2802x-1113 for alogliptin (r^2=0.998) (where y is the peak area and x is the concentration of metformin and alogliptin (µg/mL)). The intra-day and inter-day drugs variations in plasma by the proposed method showed percentage relative standard deviation were less than 2%, indicating that the method is precise. The corresponding mean recoveries of the drugs in plasma were 98.40-100.17%. This reveals that the method is quite accurate. The percentage relative standard deviation obtained for the drugs spiked in plasma for stability studies were less than 2%.

Conclusion

The proposed HPLC method was found to be simple, precise, accurate and sensitive for the simultaneous determination of metformin and alogliptin. The method was validated as per ICH guidelines and all the parameters met within the acceptance criteria. Applicability of this method for simultaneous estimation of metformin and alogliptin in plasma was confirmed.

Acknowledgements

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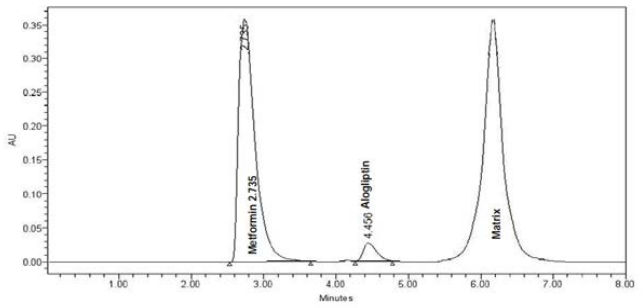

Figure 8: Typical chromatogram for standard samples in plasma for stability studies.

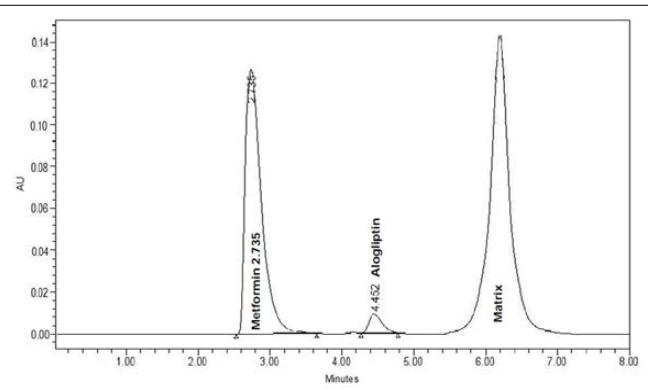

Figure 9: Typical chromatogram for freeze thaw samples in plasma for stability studies.

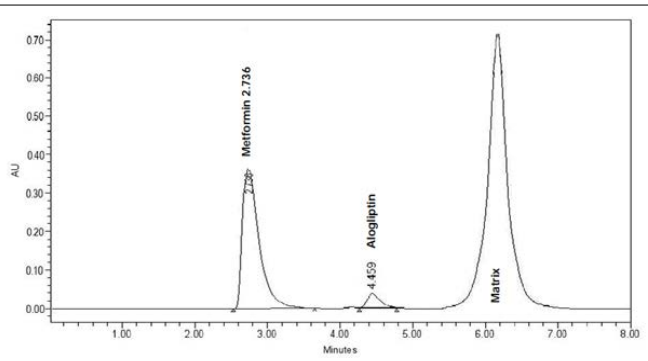

Figure 10: Typical chromatogram for short term samples in plasma for stability studies.

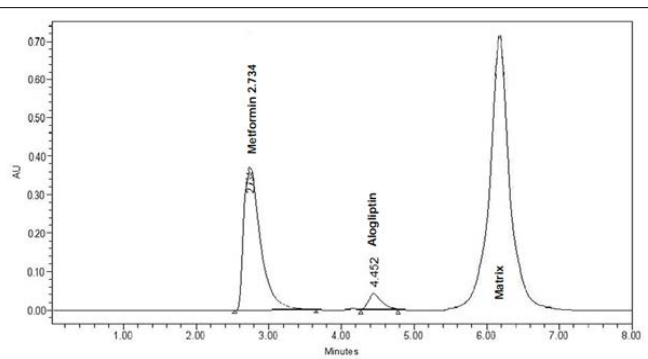

Figure 11: Typical chromatogram for long term samples in plasma for stability studies.

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