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RP-HPLC Analytical Method for Simultaneous Estimation of Related Substances of Glimepiride and Metformin HCl in Combined Dosage Forms

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ABSTRACT: Background: Diabetes mellitus is considered as a dominant public health problem. Metformin inhibits hepatic gluconeogenesis. Glimepiride is a potent first generation sulfonylurea derivative widely used in the treatment of non insulin dependent type-II Diabetes mellitus. **Aim:** The study was aimed to develop a single analytical method for the estimation of the impurity profile of two different drug moieties in a multidrug composition. A simple, rapid, precise and reliable reverse phase HPLC method was developed for the separation and estimation of the impurity profile of two drugs, Glimepiride and Metformin in bulk drug and its pharmaceutical dosage forms. **Method:** The estimation was carried out using the column Inertsil ODS-3V (250 mm × 4.6 mm, 5 µm). The mobile phase consisted of Acetonitrile, Methanol, and buffer consisting of 5 mM Pentane Sodium salt and 20 mM Potassium phosphate at pH 3, adjusted with phosphoric acid. The flow rate was a gradient one and detection was carried out at λ_{max} of 230 nm. All the known impurities of both the API's were well resolved with the Metformin and Glimepiride eluting at 7 and 26 min respectively. **Results:** The method was validated in terms of precision, ruggedness, limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, ruggedness and robustness with all the parameters meeting the pre-determined acceptance criteria as specified in ICH Q2 guidelines on Analytical Method Validation. **Conclusion:** The validated method was successfully applied for the estimation of related substances in combined pharmaceutical dosage form, yielding very good and reproducible results.

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INTRODUCTION:

In the current Indian scenario, most commonly attacking disease to a common man has been found to be diabetes. Recent studies indicate that prevalence of Type-2 diabetes is rapidly increasing in the society. Type-2 diabetes is a progressive disorder with a consistent and steady increase in glycosylated hemoglobin (HbA_1C) overtime associated with enhanced risk of micro- and macrovascular complications and a substantial reduction

in life expectancy. There are three major pathophysiologic abnormalities associated with Type-2 diabetes that are impaired insulin secretion, excessive hepatic glucose output and insulin resistance in skeletal muscles, liver and adipose tissue. These defects have been treated by use of oral insulin secretagogues (Sulphonyl ureas) or Insulin, Biguanides, and Thiazolidinediones respectively [1-4].

Glimepiride is a medium-to-long acting sulphonyl urea antidiabetic drug. It is chemically 1-[[p-[2-(3-Ethyl-4-methyl-2-oxo-3-pyrroline-1 carboxamido) ethyl] phenyl] sulfonyl]-3-(trans-4-methyl cyclohexyl) urea. The primary mechanism of action of glimepiride in lowering blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta cells. Metformin hydrochloride is also an antidiabetic drug in the biguanide class and it is chemically 1,1-dimethyl biguanide monohydrochloride. It decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. The combination of Glimepiride and Metformin sustained release complements each other and provides better glycemic control in the management of Type-2 Diabetes and probably in the prevention of its associated macrovascular and microvascular complications [5-7].

The chemical structures of the drugs are as shown in Fig 1 and 2. Keeping the medical importance in mind, a group of drugs used for treating diabetes, namely Glimepiride and Metformin has been selected for method development and validation [8,9].

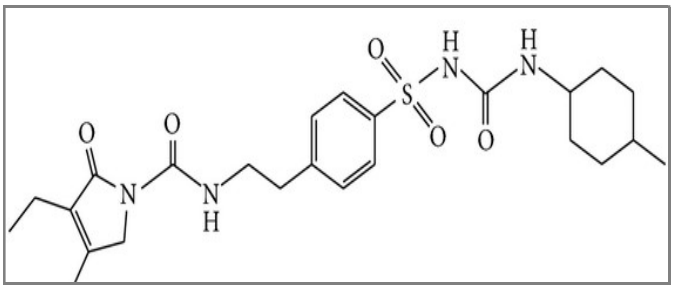

Fig 1. Chemical Structure of Glimepiride.

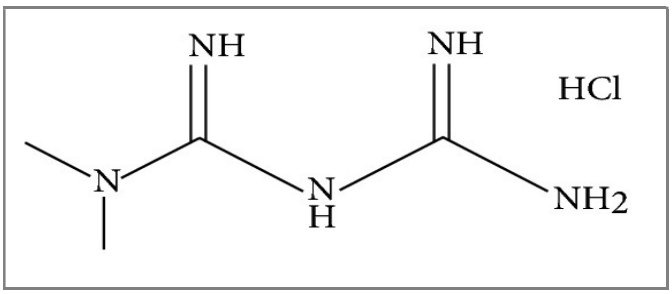

Fig 2. Chemical Structure of Metformin HCl.

For individual estimation of each drug, several methods are available in the literature. There are some methods where even more than 2 drugs are estimated at a time. Very limited work has been done for the simultaneous estimation of all the two drugs Glimepiride and Metformin [10,11].

For contributing such a novel cause, through this article, we have tried our best to develop a fast and user-friendly methodology for the simultaneous estimation of Glimepiride and Metformin, using reverse phase-HPLC method in its combined dosage forms.

MATERIALS AND METHODS:

Chemicals and reagents:

Glimepiride and metformin Hydrochloride Standards used were received from Oman Pharmaceutical Products L.L.C. Combination drug tablets were taken from the commercial batch manufactured at Oman Pharmaceutical Products L.L.C. HPLC-grade Acetonitrile, Methanol, and Orthophosphoric acid were obtained from Merck, Darmstadt, Germany. All other chemical reagents were of analytical grade.

Table 1. The Chromatographic conditions used in estimation of Glimepiride and Metformin.

Parameters	Specified values
Column	Inertsil ODS-3V 250 × 4.6mm, 5µ (Cat # 5020-01802)
Injection	10 µl
Wave length	230 nm
Column	35°C
Elution	Gradient
Run time	35min
Retention time	Metformin: About 7 min Glimepiride: About 26 min

Table 2. The solvent gradient program used in analytical study.

Time (min)	Flow	% Mobile Phase A	% Mobile Phase B	% Mobile Phase C
0.0	0.8	90	8	2
5.0	0.8	90	8	2
15.0	1.6	45	5	50
30.0	1.6	45	5	50
30.1	0.8	90	8	2
35.0	0.8	90	8	2

Preparation of mobile phase (Buffer):

About 0.87 g of pentane sodium salt and 2.74 g of Potassium phosphate were taken in a 1000 ml beaker. To the beaker Milli-Q grade water was added and mixed. The pH was adjusted to 3.0 with orthophosphoric acid.

The buffer solution gave estimated concentration of 5 mM pentane sodium salt and 20 mM potassium phosphate. The solvent A, B and C were buffer, methanol and acetonitrile. The diluent was a buffer: methanol: acetonitrile (90:8:2).

Preparation of Standard stock solution:

Preparation of solution (0.2 mg/ml Metformin and 0.05 mg/ml Glimepiride in Methanol):

About 5 mg of Metformin and 2.5 mg of Glimepiride standard were weighed and transferred into a 50 ml clean and dry volumetric flask. Then, about 20 ml of methanol was added and the mixture was sonicated to dissolve. Finally, the volume was made up to 50 ml with methanol and mixed well.

Standard solutions:

A solution containing 0.002 mg/ml of Metformin and 0.0005 mg/ml Glimepiride was prepared with diluent that is about 2 ml of standard stock was transferred into a 200 ml clean, dry volumetric flask and made up to mark with diluent.

Preparation of placebo stock solution (Glimepiride):

Accurately placebo powder equivalent to 5 Tablets (Weight equivalent to 800 mg) was weighed and transferred into a 100 ml volumetric flask. To the flask, 20 ml water was added and sonicated for 15 min with intermittent swirling. Then, 50 ml acetonitrile was added to the above solution and sonicated for 10 min with intermittent swirling. Finally, the solution was diluted to volume with methanol and mixed well. Then, the solution was centrifuged 10000 RPM for 10 min and used the supernatant solution for Glimepiride related analysis.

Preparation of placebo solution (Metformin):

Further, about 2 ml of the supernatant solution was transferred into a clean and dry 50 ml volumetric flask and then diluted up to the mark with diluent.

Preparation of sample stock solution (Glimepiride):

About 5 intact tablets were weighed and transferred into a 100 ml volumetric flask. Next, 20 ml water was added and sonicated for 15 min with intermittent swirling. Then, 50 ml Acetonitrile was added and sonicated for 10 min with intermittent swirling. Finally, the solution was diluted to volume with methanol and mixed well. Then, the solution centrifuged at 10000 RPM for 10 min and used the supernatant solution for Glimepiride related analysis.

Preparation of sample solution (Metformin):

Further, 2 ml of the supernatant solution was transferred into a clean and dry 50 ml volumetric flask and then diluted up to mark with diluent.

Evaluation of system suitability [12-14]:

- Symmetry factor should be not more than 2.0 for the Metformin and Glimepiride peak from the first injection of standard chromatogram.
- Theoretical plate count should be not less than 2000 for the Metformin and Glimepiride peak from the first injection of standard chromatogram.
- The % RSD for Metformin and Glimepiride peak areas of six injections from standard should be not more than 10.0.

Calculation:

The percentage of degradation products for Metformin and Glimepiride was calculated by using the following equation and disregard unknown impurity below 0.05 %. The percentage of Glimepiride specified impurities (GSI) was calculated as;

$$GSI\ (\%) = \frac{A_{T1}}{A_{S1}} \times \frac{W_{S1}}{50} \times \frac{1}{100} \times \frac{100}{5} \times \frac{1}{L_1} \times \frac{P_1}{RRF}$$

The percentage of Glimepiride unspecified degradation products (GUD) was calculated as;

$$GUD\ (\%) = \frac{A_{T2}}{A_{S1}} \times \frac{W_{S1}}{50} \times \frac{1}{100} \times \frac{100}{5} \times \frac{P_1}{L_1}$$

Where, A_{T1} = Area of degradation product peak in sample preparation, A_{S1} = Average area of Glimepiride peak in standard preparation, W_{S1}= Weight of Glimepiride working standard taken in mg, P_1 = Purity of Glimepiride working standard (on as is basis), L_1= Label claim of Glimepiride in mg and RRF = Relative response factor [15].

The percentage of Metformin specified impurities (MSI) was calculated as;

$$MSI\ (\%) = \frac{A_{T2}}{A_{S2}} \times \frac{W_{S2}}{50} \times \frac{1}{100} \times \frac{100}{5} \times \frac{50}{2} \times \frac{1}{L_2} \times \frac{P_2}{RRF}$$

The percentage of Metformin unspecified degradation products (MUD) was calculated as;

$$MUD\ (\%) = \frac{A_{T2}}{A_{S1}} \times \frac{W_{S2}}{50} \times \frac{1}{100} \times \frac{100}{5} \times \frac{50}{2} \times \frac{P_2}{L_2}$$

Where, A_{T2} = Area of degradation product peak in sample preparation, A_{S2} = Average area of Metformin peak in standard preparation, A_{S1} = Average area of Glimepiride peak in standard preparation, W_{S2}= Weight of Metformin working standard taken in mg, P_2 = Purity of Metformin working standard (on as is basis), L_2=

Label claim of Metformin in mg and RRF = Relative response factor.

RESULTS AND DISCUSSION:

The developed method for determination Related Substances of Metformin and Glimepiride was validated by using the following parameters.

System suitability:

It followed the procedure described in the methodology and established the system suitability before starting the analysis. Standard solutions are mentioned in Table 3 and 4.

Table 3. The System suitability for Metformin Standard.

Injection #	Area	Asymmetry	Theoretical plate
1	7496	1.02	8456
2	8064	0.97	9203
3	7454	1.02	8419
4	7521	1.03	8457
5	7550	1.01	8395
6	7510	1.03	8450
Mean	7599	1.01	8563
SD	229.898	-	-
% RSD	3.0	-	-

Table 4. The System suitability Glimepiride Standard.

Injection #	Area	Asymmetry	Theoretical plate
1	1773	0.98	61559
2	1623	1.00	66891
3	1713	0.94	69325
4	1672	0.99	63808
5	1717	0.94	53379
6	1712	0.98	49081
Mean	1702	0.97	60674
SD	50.254	-	-
% RSD	3.0	-	-

Specificity:

There were no interfering peaks at the retention times of the Metformin and Glimepiride in the presence of excipients. Further, to demonstrate the specificity of the method, the sample had been subjected to acid, base, oxidation, thermal and photolytic degradation. This was evaluated by comparing the purity angle with the purity threshold. The specificity data is mentioned in Fig 3 to 12, for the chromatograms and Table 5 to 9 for the peak purity analysis data.

Table 5. The percentage Interference.

Obser-vation	Placebo prepa-ration	Blank prepa-ration	Impurity prepa-ration
% Inter-ference	No inter-ference	No inter-ference	No inter-ference

Table 6. The Peak purity data.

Details	Purity Angle	Purity Threshold
Metformin standard	2.168	2.698
Glimepiride standard	3.699	17.623
Metformin Sample	0.411	1.005
Glimepiride Sample	0.103	1.075

Table 7. The Peak purity data Spike sample.

Details	RT (min)	RRT	Purity Angle	Purity Threshold
Metformin-Spike sample				
CN	4.086	0.64	0.538	10.234
Melamine	5.428	0.85	2.733	10.176
MS	6.365	1.00	0.390	10.004
MI	11.409	1.79	0.553	10.660
Glimepiride spike Sample				
GI-B	16.296	0.65	1.323	12.611
GI-C	17.288	0.69	4.207	14.284
GS	25.014	1.00	0.109	10.067
GI-D	25.775	1.03	7.738	12.298

GI - Glimepiride impurity, GS – Glimepiride sample, CN – Cynoguanidine, MS - Metformin Sample and MI - Metformin Impurity.

Table 8. Forced degradation study – Metformin.

SN	Sample Area	% Assay	% Degr	Purity Angle	PT
C	43375620	-	-	0.411	1.005
ACD	41299111	95.2	4.8	0.794	1.013
ALD	8306555	19.2	80.8	10.289	12.000
PD	31900027	73.5	26.5	0.345	1.005
TD	43625367	100.6	-0.6	0.593	1.005
PLD	43591903	100.5	-0.5	0.520	1.005
AKD	4259096	57.1	42.9	1.000	-

SN – Sample name, C – Control, ACD - Acid degradation (1N HCl/1 h), ALD - Alkali degradation (1N NaOH/1 h), PD - Peroxide degradation (30 % w/v H_2O_2/1 h), TD - Thermal Degradation (105 °C/1 Day), PLD - Photolytic Degradation (1.2 Million Lux in h), AKD - Alkali degradation (1N NaOH/15 min), Degr. – Degradation and PT – Purity Threshold.

Table 9. Forced degradation study – Glimepiride.

Sample Name	Sample Area	% Assay	% Degradation	Purity Angle	Purity Threshold
Control	1934076	-	-	0.103	1.075
ACD (1N HCl/1 h)	1482207	76.6	23.4	0.123	1.069
ALD (1N NaOH/1h)	1024747	53.0	47.0	0.098	1.122
PD (30 % w/v H_2O_2/1 h)	1278950	66.1	33.9	0.268	1.124
TD (105°C/1Day)	1731867	89.5	10.5	0.368	1.085
PD (1.2 Million Lux in h)	1959735	101.3	-1.3	0.099	1.063
AKD (1N NaOH/15 min)	227110	71.8	28.2	1.000	-

ACD - Acid degradation, ALD - Alkali degradation, PD - Peroxide degradation, TD - Thermal Degradation, PLD - Photolytic Degradation and AKD - Alkali degradation.

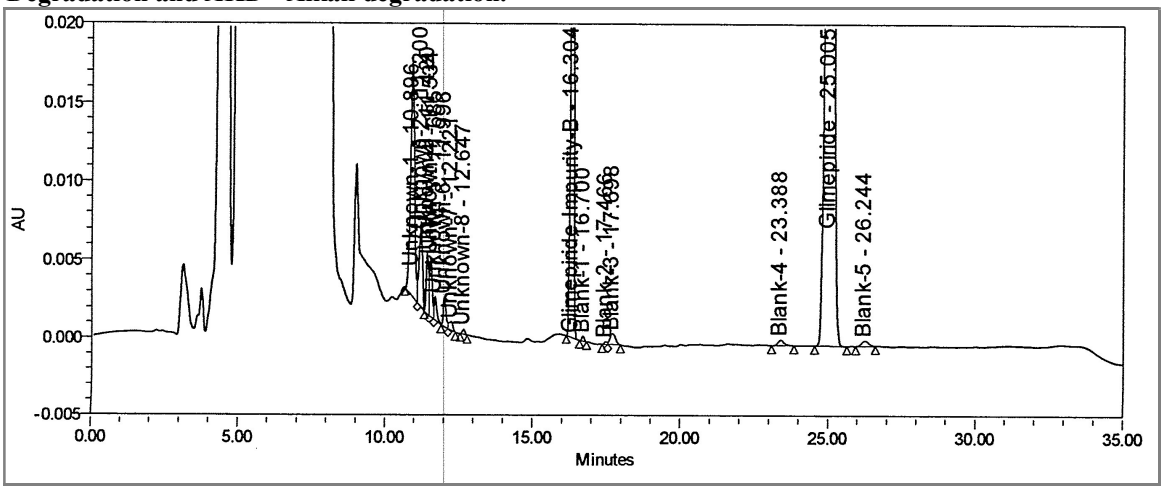

Fig 3. Reference chromatogram of Acid degradation (Glimepiride).

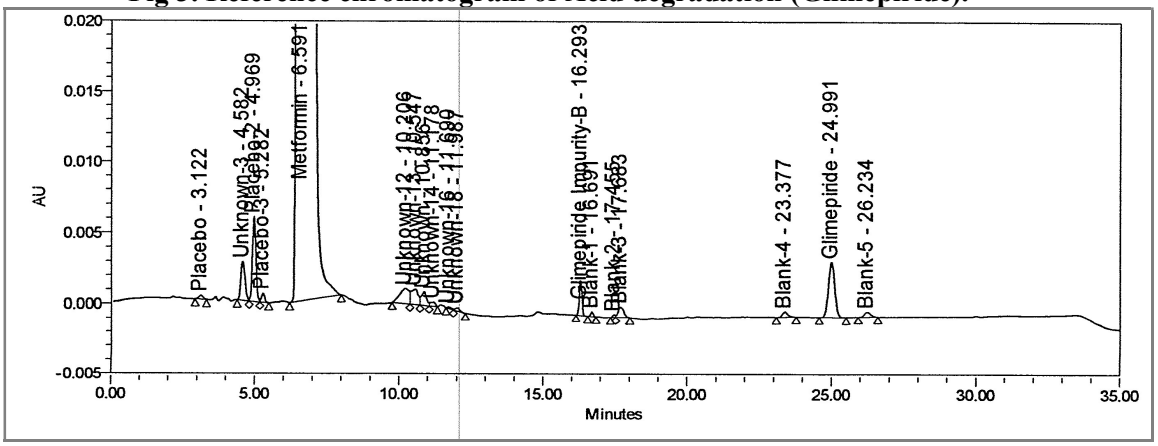

Fig 4. Reference chromatogram of Acid degradation (Metformin)

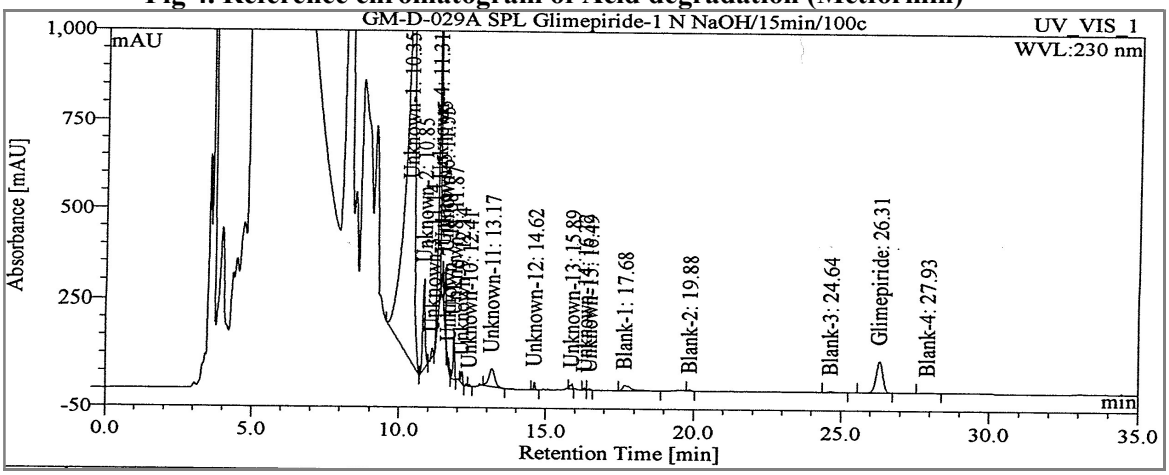

Fig 5. Reference chromatogram of Base degradation (Glimepiride).

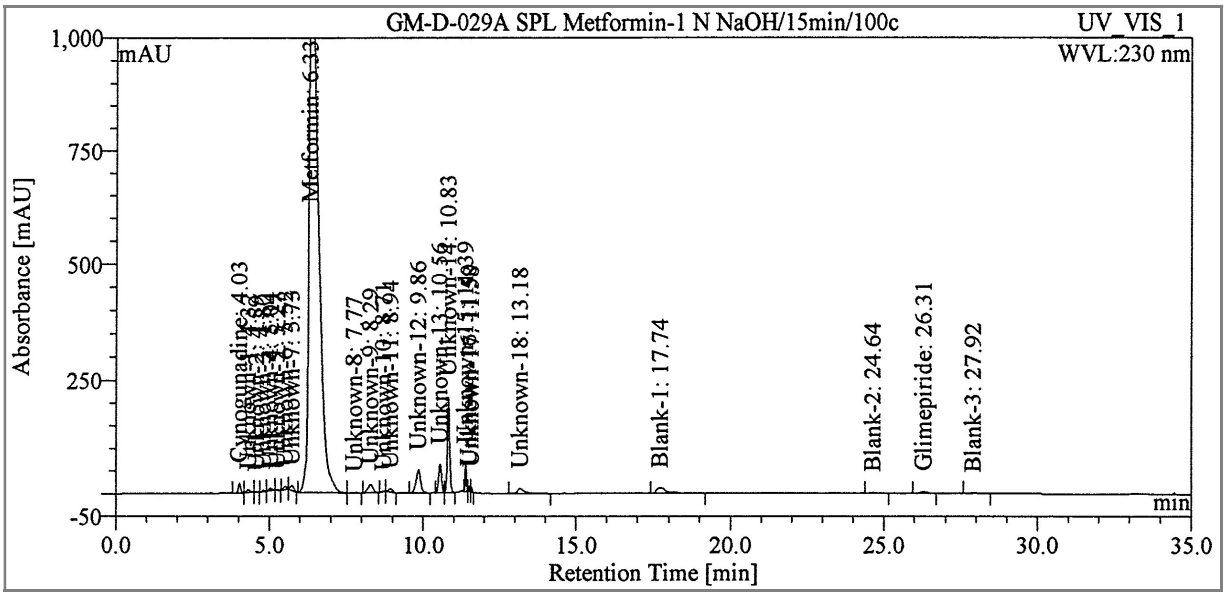

Fig 6. Reference chromatogram of Base degradation (Metformin).

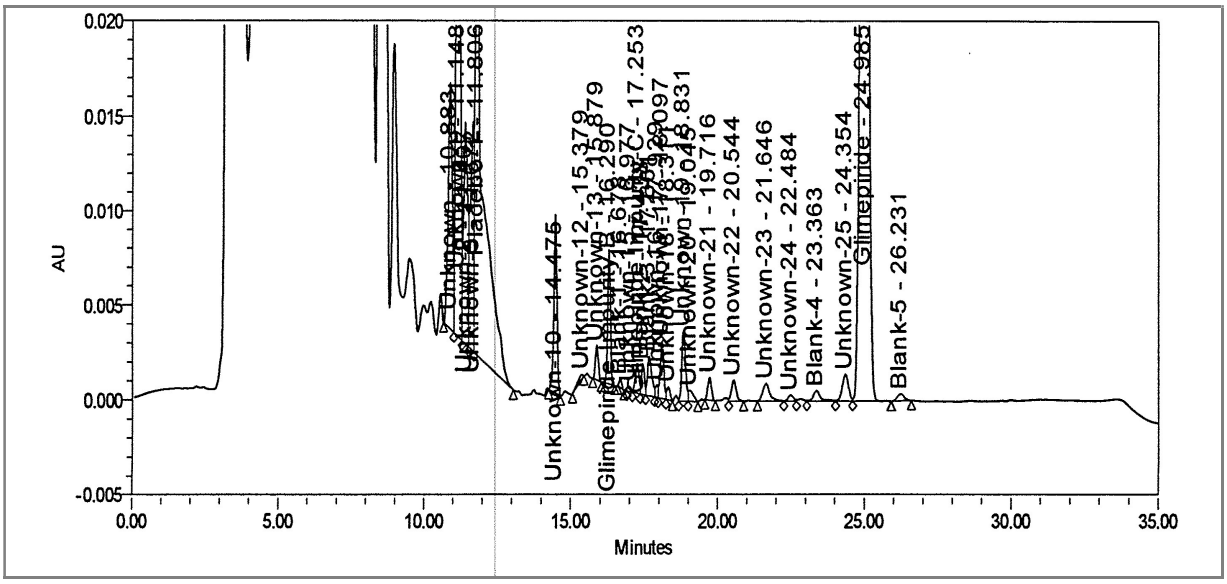

Fig 7. Reference chromatogram of peroxide degradation (Glimepiride).

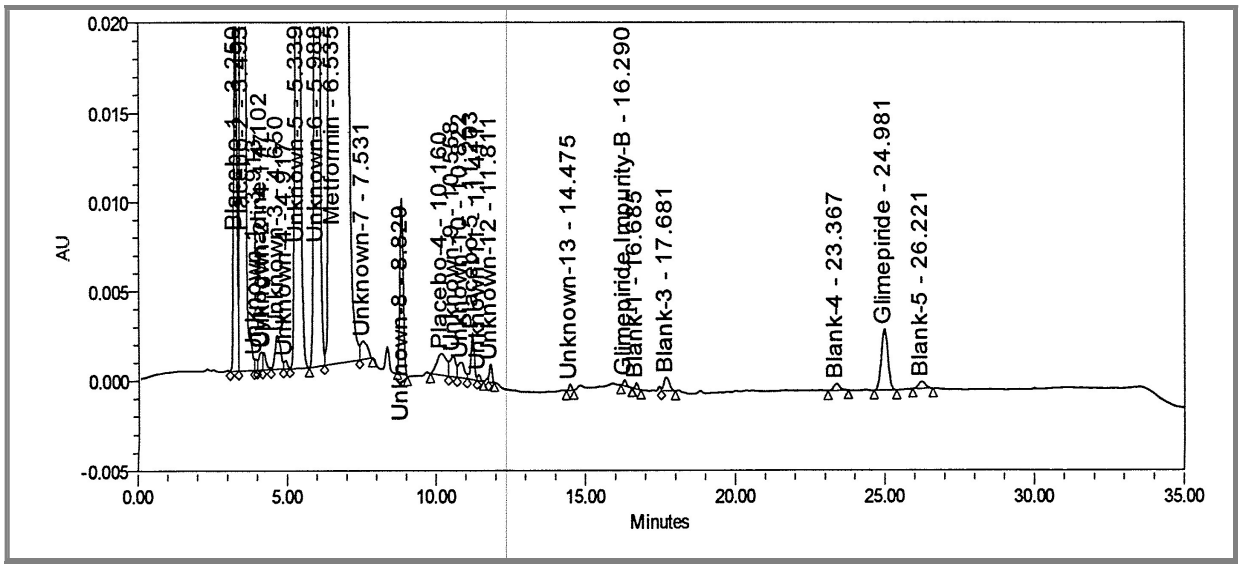

Fig 8. Reference chromatogram of peroxide degradation (Metformin).

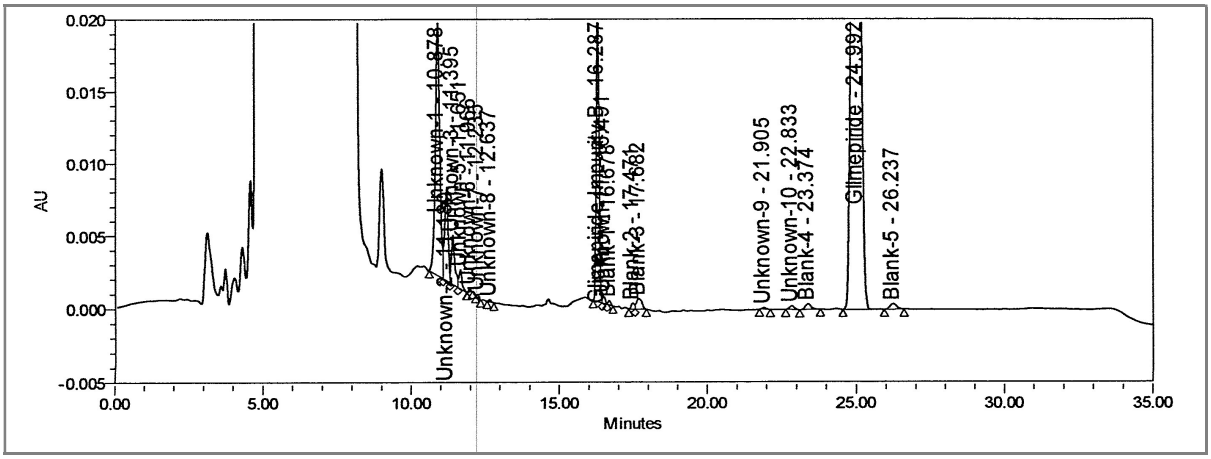

Fig 9. Reference chromatogram of Thermal degradation (Glimepiride).

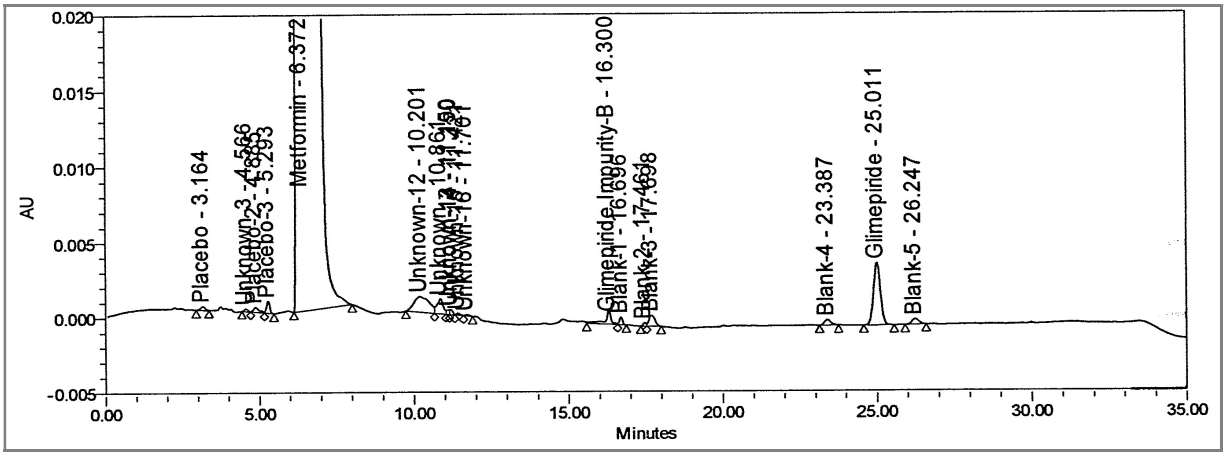

Fig 10. Reference chromatogram of Thermal degradation (Metformin).

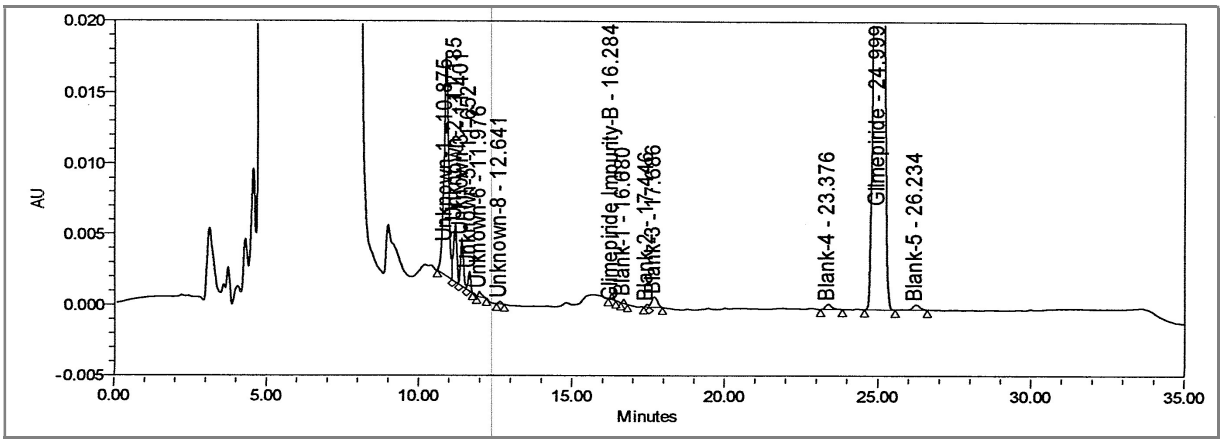

Fig 11. Reference chromatogram of UV degradation (Glimepiride).

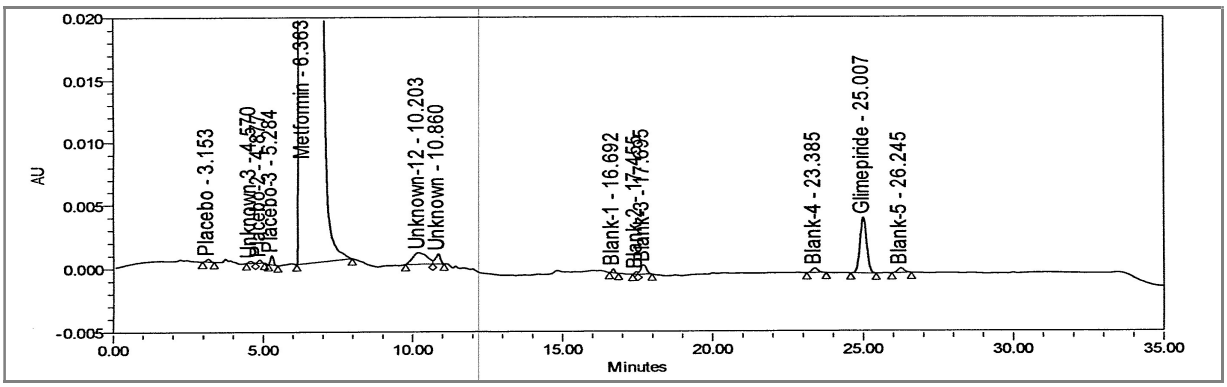

Fig 12. Reference chromatogram of UV degradation (Metformin).

Precision (Unspike sample):

Precision was determined by preparing the standard and sample as per the methodology. The sample was prepared in six replicates and injected into the chromatograph. Calculated the % specified and unspecified impurity for each preparation. Further, deduced % RSD for % specified and % unspecified impurity. The data obtained for the six sample preparations have been presented in Table 10 and 11.

Table 10. Method Precision Study (Metformin Un-spike sample).

Sample Number	Cyno-guanidine	% single max. unknown impurity	% Total impurities
1	0.001	0.013	NA
2	0.001	0.014	NA
3	0.001	0.015	NA
4	0.001	0.014	NA
5	0.001	0.013	NA
6	0.001	0.015	NA
Mean	BDL	BDL	NA
SD	-	-	-
% RSD	-	-	-

Table 11. Method Precision Study (Glimepiride-un-spiked sample).

Sample Number	GI-B	GI-C	% single max. unknown impurity	% Total impurities
1	0.101	0.02	0.031	0.101
2	0.108	0.02	0.031	0.108
3	0.104	0.02	0.035	0.104
4	0.109	0.02	0.041	0.109
5	0.108	0.02	0.037	0.108
6	0.111	0.02	0.034	0.111
Mean	0.107	BDL	BDL	0.107
SD	0.004	-	-	0.004
% RSD	3.7	-	-	3.7

GI - Glimepiride Impurity.

Precision (Spike sample):

Spike precision was determined by preparing the standard and sample as per the methodology. Prepared sample in six replicates as per the proposed method by spiking Cynoguanidine, Glimepiride Impurity-B and Glimepiride impurity-C at the specification level (0.2 and 0.5 % with respect to sample concentration) and injected into the chromatograph. Calculated the % specified impurity for each preparation. Further, deduced % RSD for % specified impurity calculated for the six replicate preparations. The data obtained for six replicate standard injections and the six sample preparations have been presented in Table 12 and 13.

Table 12. Method Spike Precision Study (Metformin Spike sample).

Sample Number	Cyno-guanidine	% single max. unknown impurity	% Total impurities
1	0.194	0.026	0.194
2	0.198	0.014	0.198
3	0.193	0.014	0.193
4	0.194	0.014	0.194
5	0.199	0.014	0.199
6	0.194	0.014	0.194
Mean	0.195	BDL	0.195
SD	0.003	-	0.003
%	1.5	-	1.5

Table 13. Method Spike Precision Study (Glimepiride Spike Sample).

Sample Number	GI-B	GI-C	% single max. unknown impurity	% Total impurities
1	0.515	0.511	ND	1.026
2	0.503	0.521	ND	1.024
3	0.512	0.514	ND	1.026
4	0.519	0.515	ND	1.034
5	0.500	0.512	ND	1.012
6	0.506	0.527	ND	1.033
Mean	0.509	0.517	-	1.026
SD	0.007	0.006	-	0.008
% RSD	1.4	1.2	-	0.8

GI - Glimepiride Impurity.

Table 14. Intermediate Precision Study (Metformin un spike Sample).

Sample Number	Cyno-guanidine	% single max. unknown impurity	% Total impurities
1	ND	0.007	NA
2	ND	0.007	NA
3	ND	0.008	NA
4	ND	0.008	NA
5	ND	0.007	NA
6	ND	0.007	NA
Mean	-	BDL	NA
SD	-	-	-
% RSD	-	-	-

Ruggedness (Unspike sample):

Ruggedness of method was demonstrated by preparing the standard and sample as per the methodology by a different analyst on a different day, using a different column lot and using a different HPLC system. The

sample was prepared in 6 replicates and injected into the chromatograph. Calculated the % specified and unspecified impurity for each preparation. The deduced % RSD for % specified and unspecified impurity. The data obtained for the 6 sample preparations given in Table 14 to 16.

Table 15. Precision & Intermediate comparison Metformin (Un spike).

Sample ID#	Cynoguanidine		highest Unknown	
	SET-I	SET-II	SET-I	SET-II
1	0.001	ND	0.013	0.007
2	0.001	ND	0.014	0.007
3	0.001	ND	0.015	0.008
4	0.001	ND	0.014	0.008
5	0.001	ND	0.013	0.007
6	0.001	ND	0.015	0.007
Mean	BDL	NA	BDL	BDL
SD	-	-	-	-
% RSD	-	-	-	-
Overall	-		-	
Overall	-		-	
Overall	-		-	

Table 16. Intermediate Precision Study (Glimepiride Un Spike sample).

SN	GI-B	GI-C	% single max. unknown impurity	% Total impurities
1	0.118	0.060	ND	0.178
2	0.121	0.074	ND	0.193
3	0.123	0.067	ND	0.190
4	0.120	0.072	ND	0.192
5	0.116	0.070	ND	0.186
6	0.114	0.067	ND	0.181
Mean	0.119	0.068	-	0.187
SD	0.003	0.005	-	0.006
% RSD	2.5	7.4	-	3.2

SN - Sample Number, GI - Glimepiride Impurity.

Ruggedness (Spiked):

Ruggedness of method was demonstrated by preparing the standard and sample as per the methodology by a different analyst on a different day, using a different column lot and using a different HPLC system. Prepared samples in six replicates as per the proposed method by spiking Cynoguanidine, Glimepiride Impurity-B and Glimepiride impurity-C at the specification level (0.2 and 0.5 % with respect to sample concentration) and injected into the chromatograph. Calculated the % specified impurity for each preparation. Further, deduced % RSD for % specified impurity calculated for the six replicate preparations.

Table 17. Precision and Intermediate comparison-Glimepiride (Un spike).

Sample ID#	GI-B		GI-C		HU	
	SET-I	SET-II	SET-I	SET-II	SET-I	SET-II
1	0.101	0.118	0.022	0.060	0.031	ND
2	0.108	0.121	0.021	0.074	0.031	ND
3	0.104	0.123	0.022	0.067	0.035	ND
4	0.109	0.120	0.024	0.072	0.041	ND
5	0.108	0.116	0.025	0.070	0.037	ND
6	0.111	0.114	0.028	0.067	0.034	ND
Mean	0.107	0.119	BDL	BDL	BDL	NA
SD	0.004	0.003	-	-	-	-
% RSD	3.7	2.5	-	-	-	-
Overall Mean	0.113		-		-	
Overall SD	0.007		-		-	
Overall % RSD	6.2		-		-	

GI - Glimepiride Impurity and HU - Highest Unknown.

Table 18. Intermediate Precision Study (Metformin-spiked sample).

Sample Number	Cyno-guanidine	% single max. unknown impurity	% Total impurities
1	0.174	0.010	0.174
2	0.175	0.010	0.175
3	0.176	0.010	0.176
4	0.177	0.009	0.177
5	0.176	0.010	0.176
6	0.176	0.011	0.176
Mean	0.176	BQL	0.176
SD	0.001	-	0.001
% RSD	0.6	-	0.6

Table 19. Intermediate Precision Study (Glimepiride-spiked sample).

SN	GI-B	GI-C	% single max. UI	% TI
1	0.551	0.512	ND	1.078
2	0.541	0.521	ND	1.062
3	0.539	0.525	ND	1.063
4	0.536	0.519	ND	1.055
5	0.542	0.526	ND	1.068
6	0.531	0.520	ND	1.051
Mean	0.540	0.523	-	1.063
SD	0.007	0.003	-	0.01
% RSD	1.2	0.6	-	0.9

SN – Sample Number, GI - Glimepiride Impurity, UI – Unknown impurity and TI – Total Impurity.

The data obtained for six replicate standard injections and the six sample preparations have been presented in Table 17 to 20.

Table 20. Precision and Intermediate comparison (spike sample).

Sample ID#	Cyno-guanidine		GI-B		GI-C	
	SET-I	SET-II	SET-I	SET-II	SET-I	SET-II
1	0.194	0.174	0.515	0.551	0.511	0.527
2	0.198	0.175	0.503	0.541	0.521	0.521
3	0.193	0.176	0.512	0.538	0.514	0.525
4	0.194	0.177	0.519	0.536	0.515	0.519
5	0.199	0.176	0.500	0.542	0.512	0.526
6	0.194	0.176	0.506	0.531	0.527	0.52
Mean	0.195	0.176	0.509	0.540	0.517	0.523
SD	0.003	0.001	0.007	0.007	0.006	0.003
% RSD	1.5	0.6	1.4	1.2	1.2	0.6
Overall Mean	0.185		0.525		0.520	
Overall SD	0.010		0.017		0.006	
Overall % RSD	5.4		3.2		1.2	

GI - Glimepiride Impurity.

Linearity and range:

Standard solutions containing Cynoguanidine, Metformin, Glimepiride, Glimepiride Impurity-B and Glimepiride impurity-C were prepared. Linearity was determined by duplicate injections of six different concentrations (LOQ, 50, 80, 100, 120 and 150 % of the target concentration). The average peak areas were plotted against concentrations. Then linearity was evaluated using the calibration curve to calculate coefficient of correlation, slope and intercept. In general, a value of correlation coefficient (r2) > 0.99 is considered as the evidence of an acceptable fit for the data to the regression line.

The results obtained are shown in Table 21 to 25 and show that the current method was linear for the five analytes in the range specified above with correlation coefficients better than 0.99. The plots have been shown in Fig 13 to 17.

Table 21. Linearity of Cynoguanidine.

Level No.		Conc. (µg/ml)	Mean area
1	LOQ	0.0503	267
2	50 %	1.0067	5454
3	80 %	1.6107	8852
4	100 %	2.0133	10872
5	120 %	2.4160	13139
6	150 %	3.0200	16226
Slope			5383.202
Intercept			67.524
CC			0.9999
R^2			0.999

Table 22. Linearity of Metformin.

Level No.		Conc. (µg/ml)	Mean area
1	LOQ	0.0503	402
2	50 %	0.5032	3738
3	80 %	0.8052	6152
4	100 %	1.0065	7740
5	120 %	1.2078	9022
6	150 %	1.5097	11148
Slope			7293.743
Intercept			177.016
CC			0.9985
R^2			0.997

Table 23. Linearity of Glimepiride Impurity-B.

Level No.		Concentration (µg/ml)	Mean area
1	LOQ	0.0496	218
2	50 %	0.2482	1109
3	80 %	0.3970	1853
4	100 %	0.4963	2238
5	120 %	0.5956	2787
6	150 %	0.7445	3431
Slope			4605.647
Intercept			-10.302
CC			0.9998
R^2			0.999

Table 24. Linearity of Glimepiride Impurity-C.

Level No.		Conc. (µg/ml)	Mean area
1	LOQ	0.0233	173
2	50 %	0.2331	929
3	80 %	0.3730	1483
4	100 %	0.4662	1851
5	120 %	0.5594	2250
6	150 %	0.6993	2562
Slope			3695.467
Intercept			102.460
CC			0.9981
R^2			0.996

Table 25. Linearity of Glimepiride.

Level No.		Concentration (µg/ml)	Mean area
1	LOQ	0.0750	251
2	50 %	0.2498	868
3	80 %	0.3997	1433
4	100 %	0.4997	1740
5	120 %	0.5996	2101
6	150 %	0.7495	2562
Slope			3480.762
Intercept			14.159
CC			0.9991
R^2			0.998

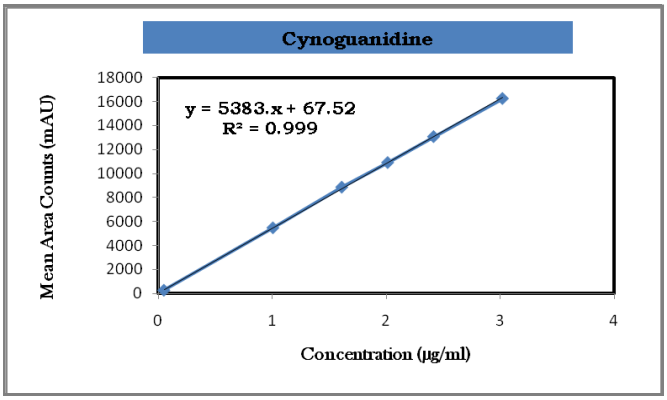

Fig 13. Linearity of Cynoguanidine.

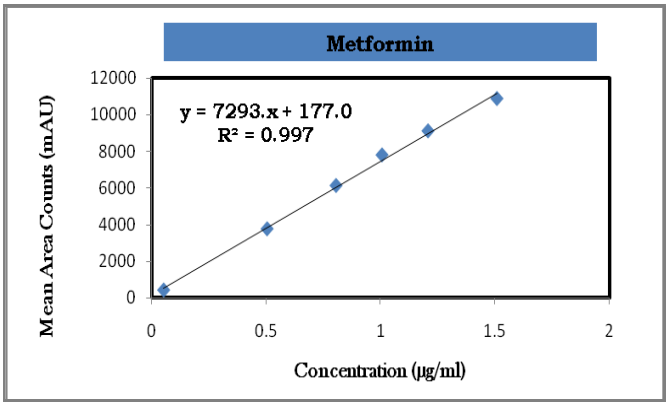

Fig 14. Linearity of Metformin.

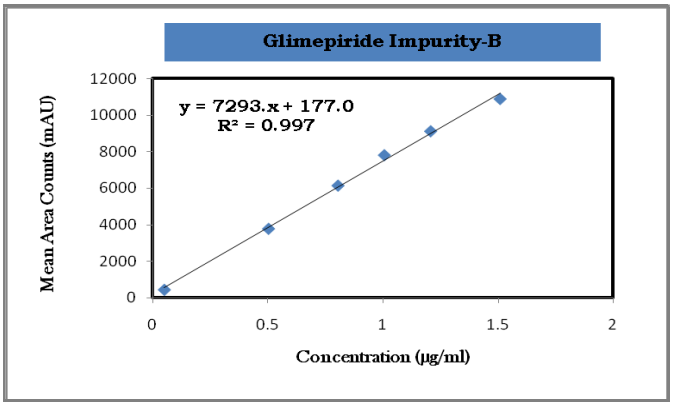

Fig 15. Linearity of Glimepiride Impurity-B.

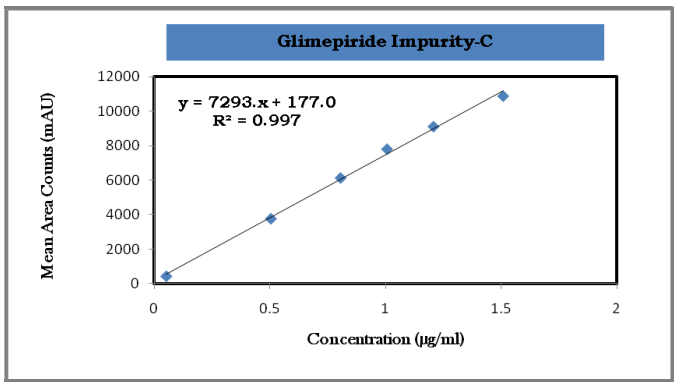

Fig 16. Linearity of Glimepiride Impurity-C.

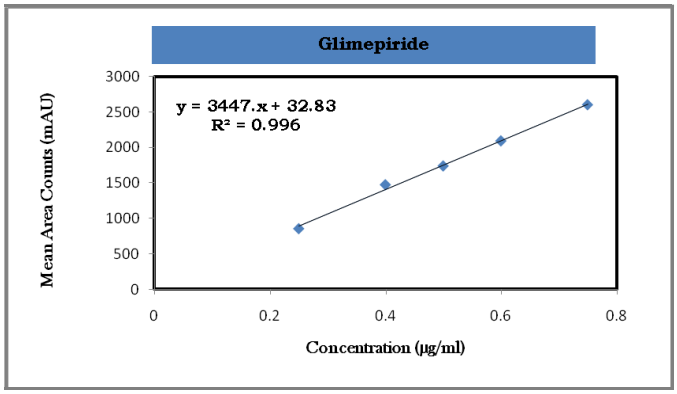

Fig 17. Linearity of Glimepiride.

LOD and LOQ:

Injected the LOQ and LOD solutions at the predicted concentration 6 times and 3 times each respectively. The data of LOD and LOQ are presented in the Table 26 to 30 along with the range data.

Accuracy:

Accuracy study was conducted by spiking the known amount of Cynoguainadine, Glimepiride Impurity-B and Glimepiride Impurity-C in the sample. Accuracy study was conducted in triplicate at four different levels (LOQ, 50, 100 and 150 %) of target concentration of Cynoguainadine, Glimepiride Impurity-B, and Glimepiride Impurity-C. The samples were analyzed as per methodology and % recovery at each spiked level was calculated. The data are presented in Table 31 to 33.

CONCLUSION:

This intended study can be concluded as the proposed method is economical, simple, ultra-fast, sensitive and reliable and is found to be accurate, precise, specific, stability indicating, rugged. All these parameters considered for verification meet the predefined acceptance criteria. So, the method is proposed for the quantitative estimation of related substances of Metformin and Glimepiride in Metformin HCl and Glimepiride Film coated tablets 500/2 mg for intended purpose.

Table 26. Cynoguanidine

Inj #	LOQ		LOD		Range
	Area	S/N	Area	S/N	Area
Conc. (µg/ml)	Conc.: 0.05 µg/ml		Conc.: 0.025 µg/ml		Conc.: 3 µg/ml
Conc. (%)	0.005 %		0.0025 %		150 %
1	267	27.90	144	3.90	16349
2	291	33.80	148	4.10	16300
3	277	31.50	150	4.70	16396
4	278	33.20	-	-	16268
5	260	28.60	-	-	16383
6	271	21.80	-	-	16290
Mean	274	29.47	147	4.23	16331
SD	10.658	-	-	-	52.657
% RSD	3.9	-	-	-	0.32

Table 27. Metformin.

Inj #	LOQ		LOD		Range
	Area	S/N	Area	S/N	Area
Conc. (µg/ml)	Conc.: 0.05 µg/ml		Conc.: 0.025 µg/ml		Conc.: 1.5 µg/ml
Conc. (%)	0.005%		0.0025%		150%
1	402	30.00	296	3.70	11439
2	459	26.00	375	4.50	11327
3	454	24.10	323	4.90	11447
4	473	26.00	-	-	11519
5	483	23.40	-	-	11439
6	461	16.40	-	-	11478
Mean	455	24.32	331	4.37	11442
SD	28.176	-	-	-	64.018
% RSD	6.2	-	-	-	0.56

Table 28. Glimepiride Impurity-B.

Inj #	LOQ		LOD		Range
	Area	S/N	Area	S/N	Area
Conc. (µg/ml)	Conc.: 0.05 µg/ml		Conc.: 0.025 µg/ml		Conc.: 0.75 µg/ml
Conc. (%)	0.05 %		0.025 %		150 %
1	247	31.20	108	3.80	3426
2	209	32.90	96	3.60	3373
3	213	30.70	107	4.40	3392
4	219	33.70	-	-	3351
5	213	28.50	-	-	3426
6	204	20.90	-	-	3343
Mean	218	29.65	104	3.93	3385
SD	15.281	-	-	-	35.986
% RSD	7.0	-	-	-	1.06

Table 29. Glimepiride Impurity-C.

Inj #	LOQ		LOD		Range
	Area	S/N	Area	S/N	Area
Conc. (µg/ml)	Conc.: 0.025 µg/ml		Conc.: 0.0125 µg/ml		Conc.:0.75 µg/ml
Conc. (%)	0.025 %		0.0125 %		150 %
1	173	19.10	94	2.50	2685
2	180	23.90	92	2.80	2668
3	171	21.70	79	2.70	2658
4	180	24.20	-	-	2655
5	178	20.60	-	-	2668
6	180	15.60	-	-	2659
Mean	177	20.85	88	2.67	2666
SD	4.000	-	-	-	10.968
% RSD	2.3	-	-	-	0.41

Table 30. Glimepiride.

Inj #	LOQ		LOD		Range
	Area	S/N	Area	S/N	Area
Conc., (µg/ml)	Conc.: 0.075 µg/ml		Conc.: 0.0375 µg/ml		Conc.: 0.75 µg/ml
Conc. (%)	0.075 %		0.0375 %		150 %
1	251	8.60	115	1.10	2557
2	277	10.60	90	1.00	2670
3	254	9.90	120	1.30	2633
4	290	11.30	-	-	2602
5	280	10.00	-	-	2491
6	273	6.30	-	-	2599
Mean	271	9.45	108	1.13	2592
SD	15.303	-	-	-	62.193
% RSD	5.6	-	-	-	2.40

Table 31. Accuracy for Cynoguanidine.

Sl. No.	Level	Sample	Amount recovered (µg/ml)	Amount added (µg/ml)	%Recovery	% RSD in each level	
1	LOQ-1	1	0.0446	0.0503	88.7	Avg:	91.8
2	LOQ-2	2	0.0491	0.0503	97.6	SD:	5.002
3	LOQ-3	3	0.0449	0.0503	89.3	%RSD:	5.4
4	50%-1	1	1.0173	1.0067	101.1	Avg:	99.6
5	50%-2	2	0.9941	1.0067	98.7	SD:	1.256
6	50%-3	3	0.9970	1.0067	99.0	%RSD:	1.3
7	100%-1	1	1.9623	2.0133	97.5	Avg:	98.0
8	100%-2	2	1.9994	2.0133	99.3	SD:	1.137
9	100%-3	3	1.9576	2.0133	97.2	%RSD:	1.2
10	150%-1	1	2.8928	3.0200	95.8	Avg:	95.6
11	150%-2	2	2.8937	3.0200	95.8	SD:	0.407
12	150%-3	3	2.8720	3.0200	95.1	%RSD:	0.4

Table 32. Accuracy for Glimepiride Impurity-B.

Sl. No.	Level	Sample	Amount recovered	Amount added	%Recovery	% RSD in each level	
1	LOQ-1	1	0.0498	0.0496	100.4	Avg:	97.5
2	LOQ-2	2	0.0467	0.0496	94.2	SD:	3.151
3	LOQ-3	3	0.0486	0.0496	98.0	%RSD:	3.2
4	50%-1	1	0.2722	0.2478	109.8	Avg:	110.5
5	50%-2	2	0.2748	0.2478	110.9	SD:	0.584
6	50%-3	3	0.2746	0.2478	110.8	%RSD:	0.5
7	100%-1	1	0.5117	0.4956	103.2	Avg:	102.2
8	100%-2	2	0.4997	0.4956	100.8	SD:	1.263
9	100%-3	3	0.5088	0.4956	102.7	%RSD:	1.2
10	150%-1	1	0.7677	0.7434	103.3	Avg:	103.5
11	150%-2	2	0.7774	0.7434	104.6	SD:	0.959
12	150%-3	3	0.7635	0.7434	102.7	%RSD:	0.9

Table 33. Accuracy for Glimepiride Impurity-C.

Sl. No.	Level	Sample	Amount recovered	Amount added	%Recovery	% RSD in each level	
1	LOQ-1	1	0.0300	0.0259	115.8	Avg:	109.9
2	LOQ-2	2	0.0303	0.0259	117.0	SD:	11.272
3	LOQ-3	3	0.0251	0.0259	96.9	%RSD:	10.3
4	50%-1	1	0.2990	0.2594	115.3	Avg:	115.2
5	50%-2	2	0.2993	0.2594	115.4	SD:	0.156
6	50%-3	3	0.2985	0.2594	115.1	%RSD:	0.1
7	100%-1	1	0.5080	0.5189	97.9	Avg:	98.7
8	100%-2	2	0.5177	0.5189	99.8	SD:	0.950
9	100%-3	3	0.5113	0.5189	98.5	%RSD:	1.0
10	150%-1	1	0.7847	0.7783	100.8	Avg:	102.1
11	150%-2	2	0.7969	0.7783	102.4	SD:	1.105
12	150%-3	3	0.8013	0.7783	103.0	%RSD:	1.1

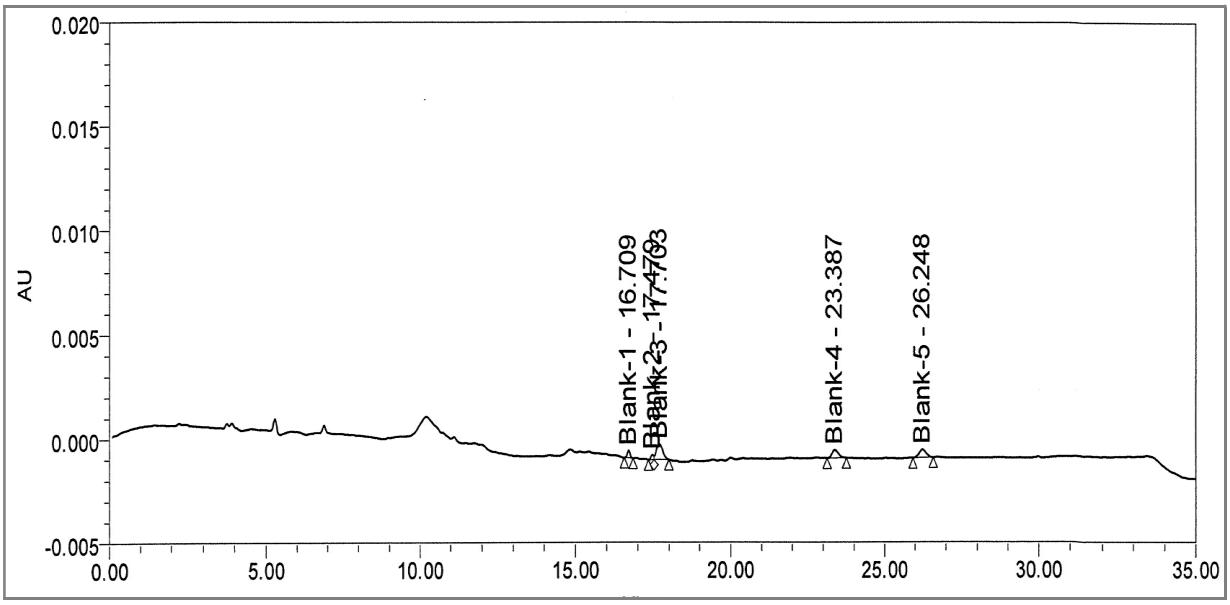

Fig 18. Reference chromatogram of Blank.

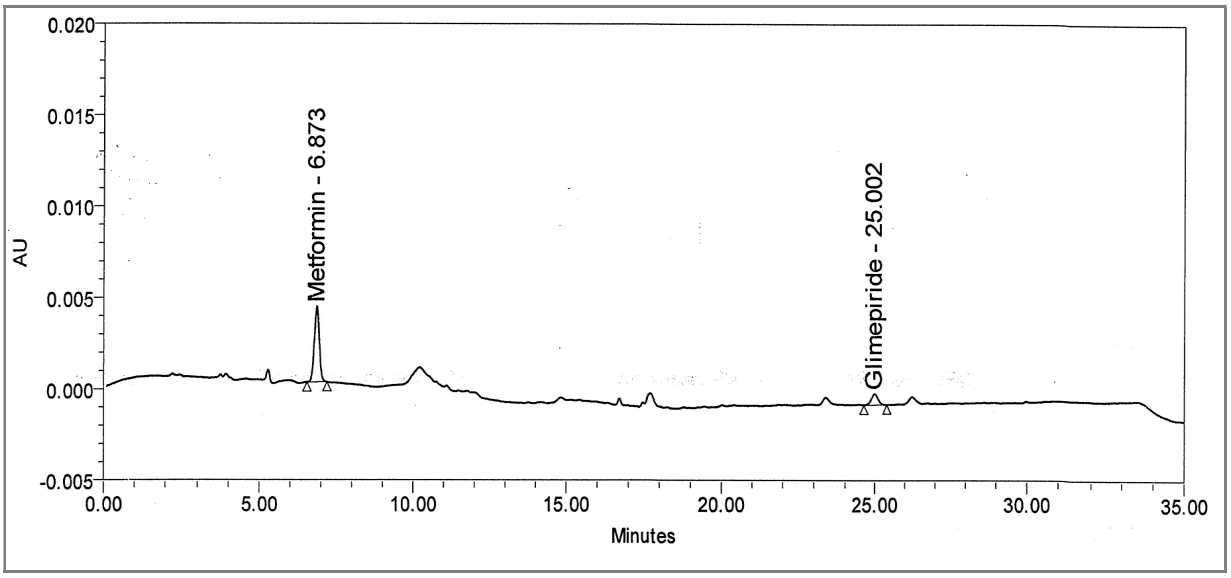

Fig 19. Reference chromatogram of Standard Solution.

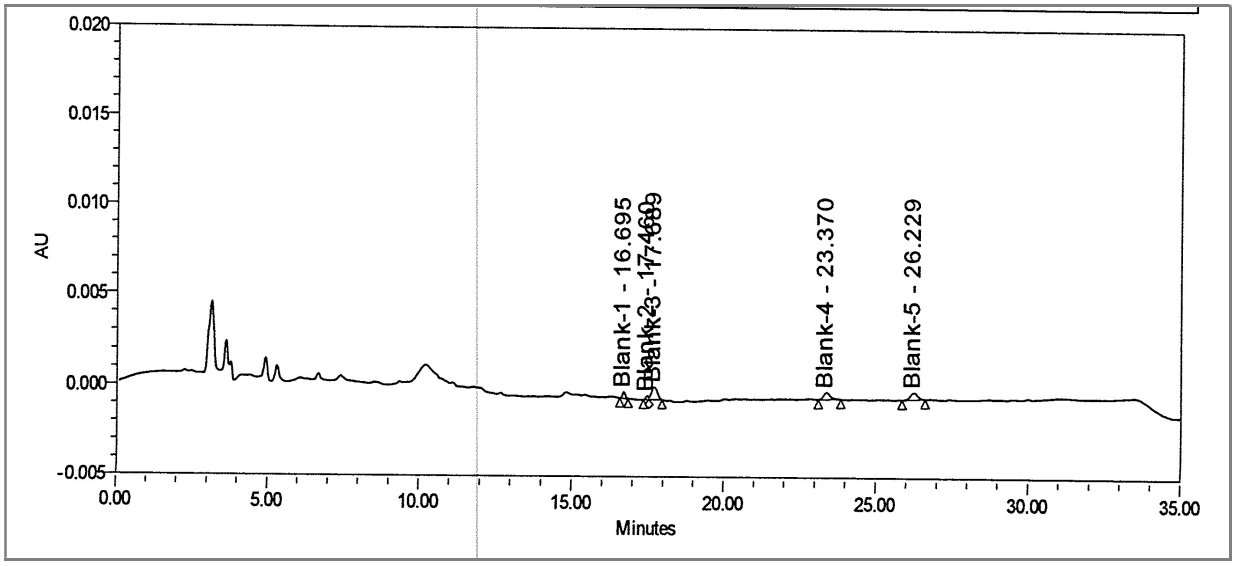

Fig 20. Reference chromatogram of Placebo Solution (Glimepiride)

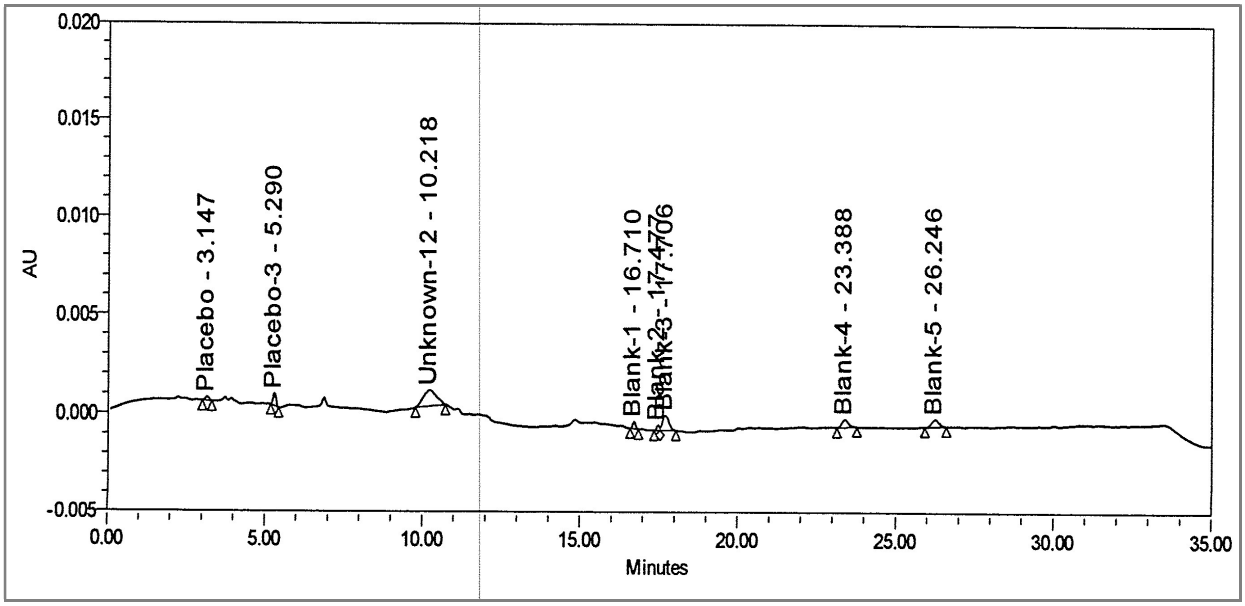

Fig 21. Reference chromatogram of Placebo Solution (Metformin).

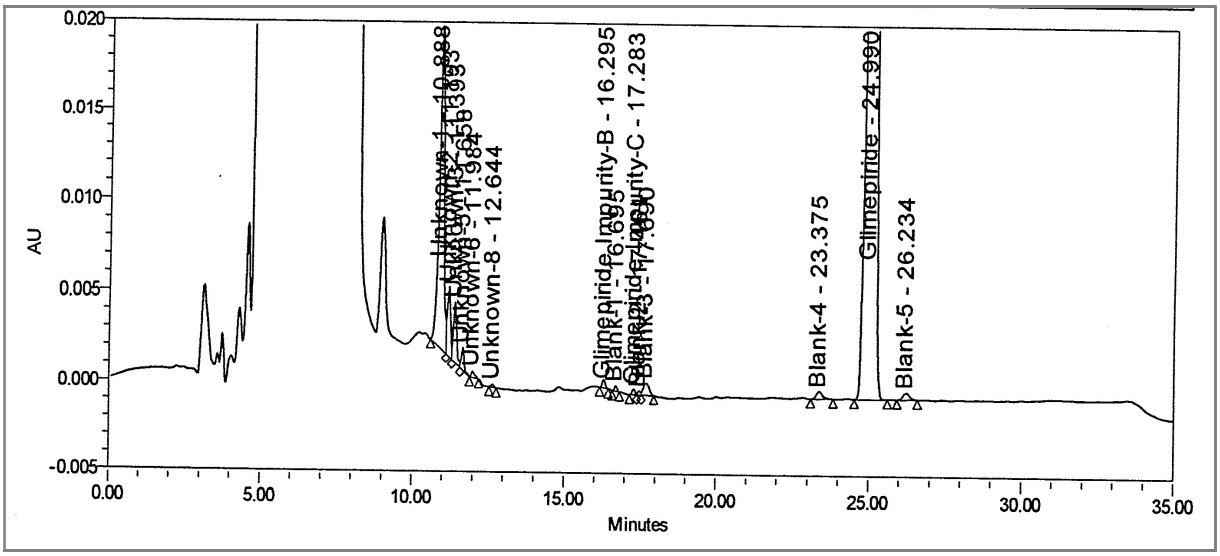

Fig 22. Reference chromatogram of as such Sample Solution (Glimepiride).

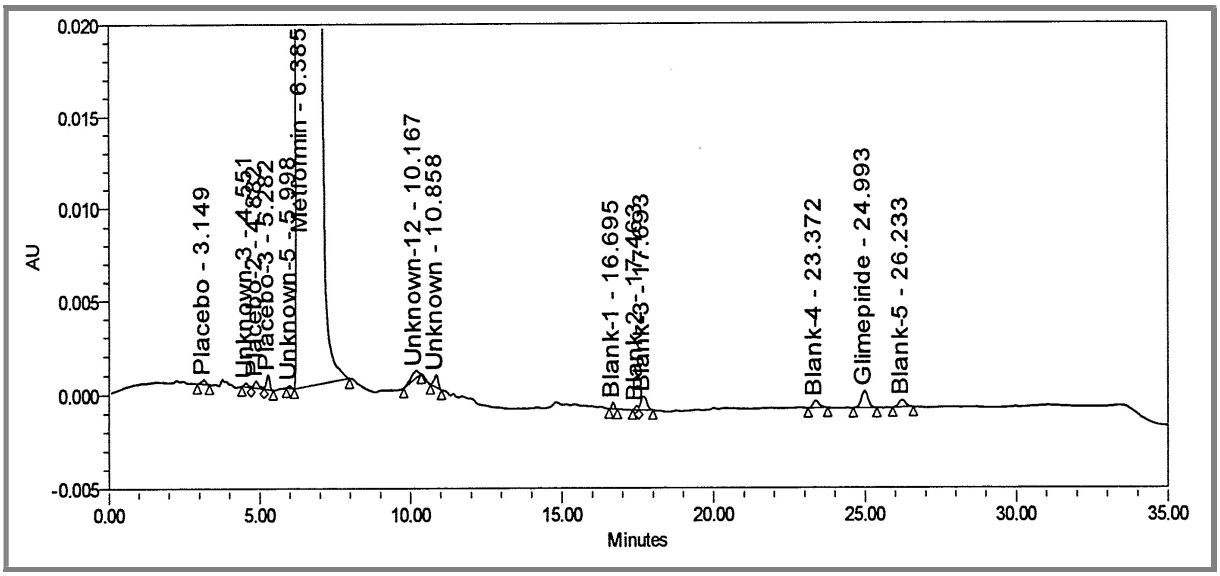

Fig 23. Reference chromatogram of as such Sample Solution (Metformin).

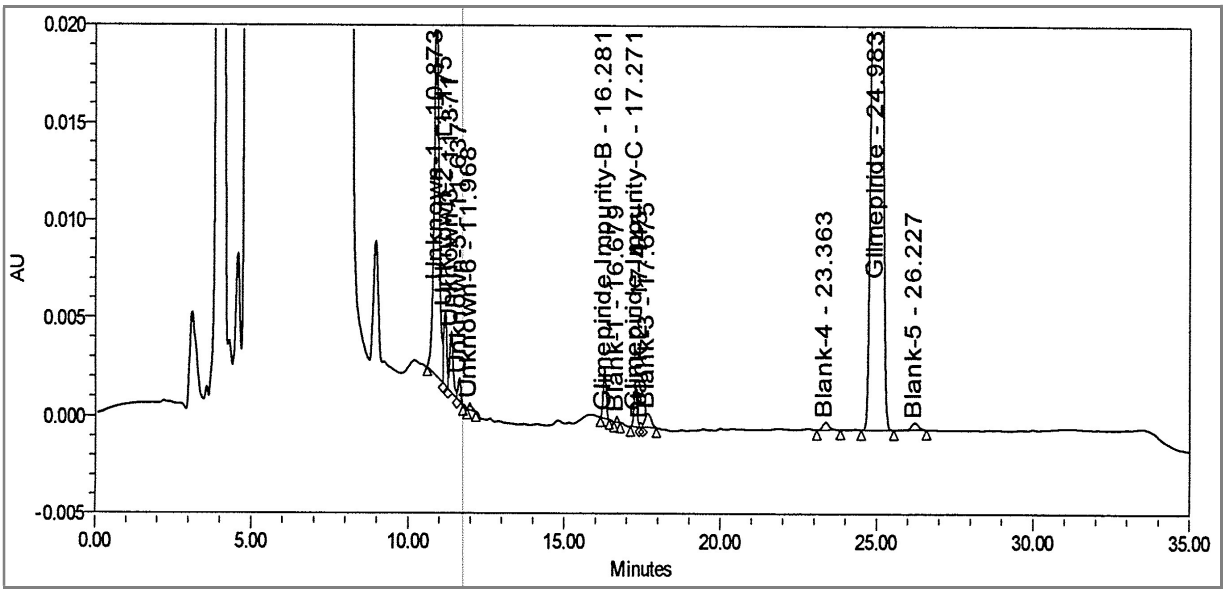

Fig 24. Reference chromatogram of Spike Sample Solution (Glimepiride).

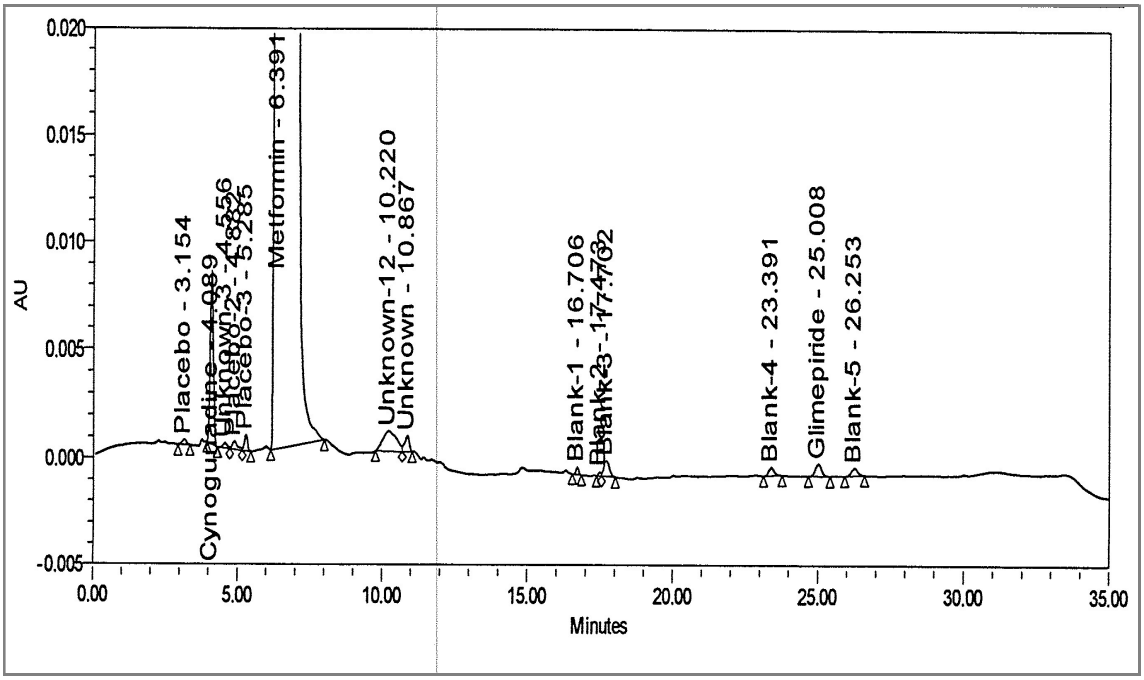

Fig 25. Reference chromatogram of Spike Sample Solution (Metformin).

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