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"VALIDATION OF STABILITY INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS DETERMINATION OF ASSAY OF GLYBURIDE AND METFORMIN DRUGS IN THE PHARMACEUTICALS TABLET FORMULATIONS USING ATOVAQUONE AS A COMMON INTERNAL STANDARD"

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ABSTRACT

Glyburide is an oral antihyperglycemic agent used for the treatment of non-insulin-dependent diabetes mellitus (NIDDM). Metformin is a biguanide antihyperglycemic agent used for treating non-insulin-dependent diabetes mellitus (NIDDM).Validation of stability indicating Simple, Specific, Precise, Accurate, Linear, Rugged, Robust High Performance Liquid Chromatographic method of analysis for simultaneous determination of assay of Glyburide and Metformin drugs in the pharmaceuticals Tablet formulations using Atovaquone as a common internal standard was performed. The assay was accomplished using a mixture of Ammonium Dihydrogen phosphate in water and methanol in the volume ratio of 30:70 v/v as mobile phase on an OYSTER ODS3, 150 mm x 4.6mm, 5µ as chromatographic column at a flow rate 1.000 mLmin-1 and with a uv detector at a wavelength 230nm. The temperature of auto injector and column oven was 10^0C and 30^0C receptively. The Injection volume of HPLC system kept as 30 µL. linearity of the analytical method was evaluated at concentration range of 0.5937 µg/ml to 15.0057 µg/ml for Glyburide and 100.0644 µg/ml to 3000.0089 µg/ml for Metformin respectively with Correlation coefficient (r) value more than 0.999.The LOD and LOQ was 0.1242 µg/ml and 0.3764 µg/mL for Glyburide and 21.4220 µg/ml and 64.9153 µg/mL for Metformin respectively. The retention time found to be 2.96 min for Glyburide, 7.14 min for Metformin and 4.24 min for internal standard. Specificity, Method Precision, System Precision, Ruggedness, Robustness, Recovery, Stability of analytical solution, Filter paper selection study, Stress testing(Force Degradation) at various conditions were performed as per the ICH (Q2) recommendations. All the results were found with in acceptance criteria.

KEYWORDS: Glyburide, Metformin Hydrochloride, Atovaquone, High Performance Liquid Chromatographic, Force degradation studies, Assay.

INTRODUCTION

Glyburide is an oral antihyperglycemic agent belongs to the sulfonylurea class of insulin secretagogues used for the treatment of non-insulin-dependent diabetes mellitus (NIDDM), as it stimulates β cells of the pancreas to release insulin. Sulfonylureas not only increase basal insulin secretion, meal-stimulated insulin but also increase peripheral glucose utilization, decrease hepatic gluconeogenesis and may increase the number and sensitivity of insulin receptors. Sulfonylureas are associated with weight gain, though less so than insulin. Due to their mechanism of action, individuals require consistent food intake to decrease hypoglycemia risk as sulfonylureas may cause hypoglycemia. The risk of hypoglycemia is increased in elderly, debilitated and malnourished individuals. Fasting plasma glucose, postprandial blood glucose and glycosolated hemoglobin (HbA1c) levels (reflective of the last 8-10 weeks of glucose control) is decrease by Glyburide. Glyburide appears to be completely metabolized, likely in the liver. Although its metabolites exert a small hypoglycemic effect, their contribution to glyburide's hypoglycemic effect is thought to be clinically unimportant. Glyburide metabolites are excreted in urine and feces in approximately equal proportions. The chemical name is 1-[[p-[2-(5-chloro-o-anisamido) ethyl]phenyl]sulfonyl]-3-cyclohexylurea.The molecular formula for Glyburide is $C_{23}H_{28}ClN_3O_5S$. The molecular weight of Glyburide is 494.01. Glyburide is white to off-white crystalline compound. Glyburide is soluble in Methanol, Ethanol,

DMSO, Chloroform and very slightly soluble in water. The pKa of Glyburide is 4.32.[1-7]

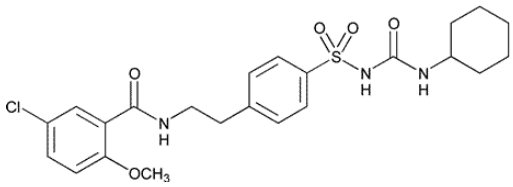


Figure 1: Chemical structure of Glyburide.

Metformin is a biguanide antihyperglycemic agent used for treating non-insulin-dependent diabetes mellitus (NIDDM). It improves glycemic control by decreasing hepatic glucose production, decreasing glucose absorption and increasing insulin-mediated glucose uptake. Metformin improves glucose tolerance in patients with type 2 diabetes, lowering both basal and postprandial plasma glucose. Its pharmacologic mechanisms of action are different from other classes of oral antihyperglycemic agents. Metformin decreases hepatic glucose production, decreases intestinal absorption of glucose, and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. Unlike sulfonylureas, metformin does not produce hypoglycemia in either patients with type 2 diabetes or normal subjects.

The chemical name is 3-(diaminomethylidene)-1,1-dimethylguanidine hydrochloride. The molecular formula is $C_4H_{11}N_5.HCl$. The molecular weight of Metformin Hydrochloride is 165.625. The molecular weight of Metformin is 129.167. Metformin Hydrochloride is white to off-white powder and soluble in organic solvents such as methanol and is practically insoluble in acetone, ether and chloroform. The pKa of Metformin is 12.4.[1-7]

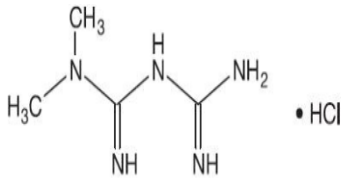


Figure 2: Chemical structure of Metformin Hydrochloride.

Atovaquone is a synthetic hydroxynaphthoquinone class of drug that has antimicrobial activity and is being used in antimalarial protocols. The chemical name is 2-(trans-4-(P-Chlorophenyl)cyclohexyl)-3-hydroxy-1,4-naphthoquinone. The molecular formula is $C_{22}H_{19}ClO_3$. The molecular weight of Atovaquone is 366.84. Atovaquone is white to powder and has a pKa of 8.23. Atovaquone is practically insoluble in water and soluble in organic solvents such as DMSO, Chloroform, Methanol, Ethanol.[1-7]

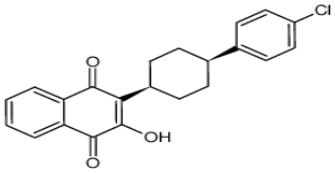


Figure 3: Chemical structure of Atovaquone.

While Reviewing Literature for analytical method of analysis it was observed that many methods have been reported for determination of Glyburide and metformin in combination and individually[8-17] but none of the reported HPLC methods have not been validated using internal standard to compensate any processing related and method related variability. Most of the published method is not performed stability-indicating studies (Acid, Alkali, Peroxide, Thermal, Photolytic, Humidity degradation,) which is mandatory as per the ICH(Q2) recommendations.

The main objective of the work is to develop and validate stability indicating HPLC method of analysis which is Simple, Specific, Precise, Accurate, Linear, Rugged, Robust etc. for simultaneous determination of assay of Glyburide and Metformin drugs in the pharmaceutical Tablet formulations using Atovaquone as an common internal standard.

MATERIAL AND METHODS

Instrumentation

Shimadzu Prominence HPLC system equipped with dual pump, SIL-HTc auto-sampler with cooler, column oven, variable wavelength UV detector and a data acquisition system (Lab Solution Software) were used for the simultaneous determination of assay of Glyburide and Metformin drugs in the pharmaceutical Tablet formulations using Atovaquone as a common internal standard.

Reagents and Materials

The reagents used during analysis include Methanol [HPLC Grade], Water [Milli-Q /HPLC Grade], Ammonium dihydrogen phosphate [ARGrade], Orthophosphoric acid [ARGrade], Cocentrated Hydrochloric acid (37%) [ARGrade], Glyburide standard; Metformin Hydrochloride standard and Atovaquone Hydrochloride were used obtained from Wockhardt Pharmaceutical limited and Mylan laboratories limited. Fixed dose combination tablets containing 2.5 mg Glyburide and 500 mg Metformin Hydrochloride of Teva Pharmaceuticals Ltd. was purchased from Local medical, Aurangabad (Maharashtra). Ammonium phosphate Buffer, Rinsing solution, Mobile Phase was prepared by dissolving required volume and quantity of reagents and chemicals. Methanol with 0.02 % of Concentrated Hydrochloric acid of total volume was used as diluent for dissolving tablet for Formulation and analytical standard.

Analytical solutions

Stock solutions having concentrations approximately, 102.3581 µg/mL of Glyburide in diluent, 3848.6323 µg/mL of Metformin in diluent and 1666.6687 µg/mL of Atovaquone in diluent were prepared and solutions were filtered through 0.45µm nylon membrane filter with discarding first 2 mL of the filtrate before use. The solution of Atovaquone was used as internal standard dilution solution during various experiments performed in an analytical method validation and assay calculations of pharmaceutical formulation.

Standard solutions having concentrations approximately, 7.50 µg/ml of Glyburide, 1500.00 µg/mL of Metformin and 100.000 µg/mL of Atovaquone were prepared in mobile phase and use as a reference solution for related activities and system suitability. Filter the solution through 0.45µm nylon membrane filter with discarding first 2 mL of the filtrate before use.

Sample solution having concentrations 7.50 µg/ml of Glyburide, 1500.00 µg/mL of Metformin and 100.000 µg/mL of Atovaquone was prepared in mobile phase by dissolving a quantity of powder equivalent to Strength of 2.5 mg of Glyburide and 500 mg Metformin and use as a sample solution for related activities. Filter the solution through 0.45µm nylon membrane filter with discarding first 2 mL of the filtrate before use.

RESULT AND DISCUSSION

Method development

Primarily, numerous trials for optimization of method was performed using different mobile phases composition , different ratios of organic to buffer ,different organic solvents , different buffer with different pH, different stationary phases, different internal standards and variable chromatographic settings in an effort to achieve the finest peak resolution and separation between Glyburide, Metformin and internal standard.

The finalized chromatographic method used for simultaneous estimation of estimation of Glyburide and Metformin was as follows.

Mobile phase: Methanol and Ammonium Dihydrogen phosphate in water (70:30v/v)

Rinsing Solution: Methanol: Mill-Q water (70:30v/v)

Chromatographic Column: OYSTER ODS3, 150 mm x 4.6mm, 5µ

Wavelength:	230 nm
Column Oven Temperature:	30 ^{0}C
Sample cooler Temperature:	10 ^{0}C
Flow rate:	1.000 ml per minute
Injection Volume:	30 µl
Run Time:	10 minute
Retention Time (minute):	Glyburide-2.96
Atovaquone-4.24	
Metformin R.T.	-7.14

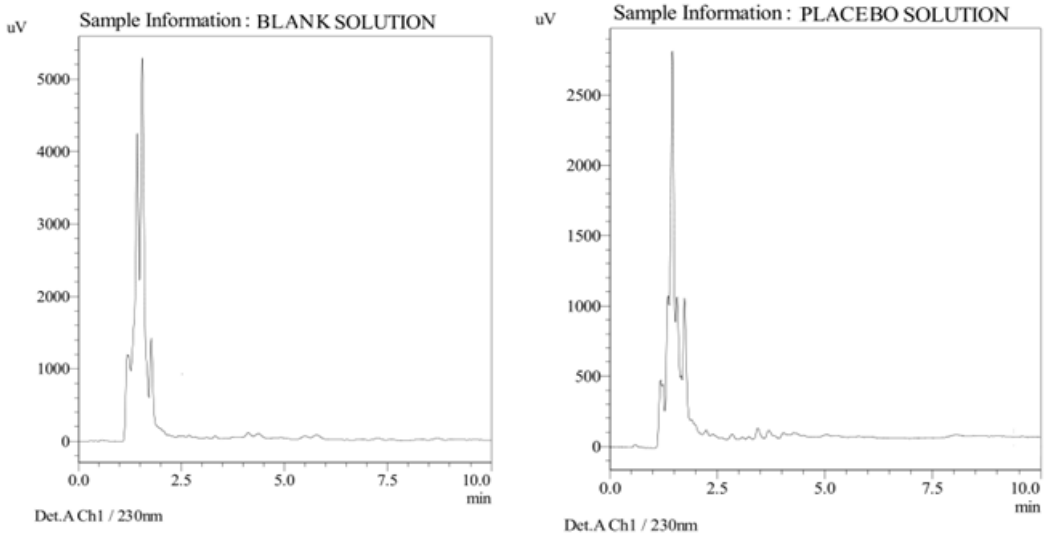
Analytical method validation

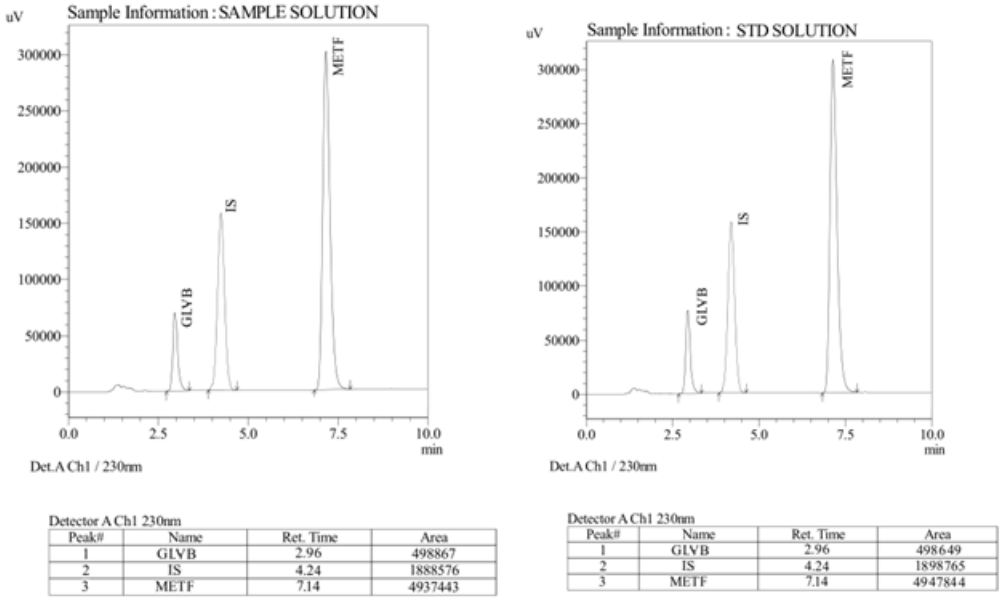
The Analytical method was optimized and validated in accordance with the current ICH guidelines and recommendations by means of a vision to accomplish Simple, Specific, Precise, Accurate, Linear, Rugged, Robust method.[18-21]

Specificity

For the evaluation of specificity; Blank solution, placebo solutions, sample solution, standard solution in triplicate were injected into HPLC system. No interference was observed from blank solution and placebo at the retention time of chromatographic peak of Glyburide, Metformin and internal standard. Peak purity was passes (purity angle was less than purity threshold) for Glyburide and Metformin and % assay difference with respect to method precision was found 0.10% for Glyburide and 0.10% for metformin.

The typical chromatograms of various samples under optimized HPLC conditions was depicted in Figure 4.





Detector A Ch1 230nm

Peak#	Name	Ret. Time	Area
1	GLVB	2.96	498867
2	IS	4.24	1888576
3	METF	7.14	4937443

Detector A Ch1 230nm

Peak#	Name	Ret. Time	Area
1	GLVB	2.96	498649
2	IS	4.24	1898765
3	METF	7.14	4947844

Figure 4: Typical chromatograms of Blank solution, Placebo solution, Sample solution & standard Solution.

System Precision

Six replicates injections of standard solution were injected in to the HPLC system and the chromatograms and area ratio of Glyburide to the Atovaquone and Metformin to the Atovaquone are recorded. % RSD for area ratio of Glyburide to the Atovaquone and Metformin to the Atovaquone of six replicate injections of standard solution was found 0.28% and 0.13% respectively implies that system is précises as tabulated in Table No.1.

Table no. 1: System Precision.

Injection No.	Area ratio (Glyburide to Atovaquone)	Area ratio (Metformin to Atovaquone)
1	0.2632	2.6077
2	0.2640	2.6045
3	0.2626	2.6058
4	0.2629	2.6028
5	0.2637	2.6022
6	0.2646	2.5983
Mean	**0.26350**	**2.60355**
Standard Deviation	**0.000743**	**0.003260**
% R.S.D.	**0.28**	**0.13**

Method Precision

For the evaluation of Method precision of the analytical method, six samples from homogenous mixture of single batch were prepared as per the test procedure of methodology and analyzed on HPLC system .%RSD for % assay of Glyburide and Metformin of six samples found was 0.14% and0.13% as tabulated in Table No.2.

Table No.2: Method Precision.

Serial No.	% Assay of Glyburide	% Assay of Metformin
1	99.2	99.8
2	99.3	99.7
3	99.4	99.5
4	99.6	99.6
5	99.5	99.8
6	99.4	99.8
Mean	**99.4**	**99.7**
Standard Deviation	**0.14**	**0.13**
% R.S.D.	**0.14**	**0.13**

Method Ruggedness

The ruggedness was evaluated through analysis of six samples from a homogenous mixture of single batch by different analyst by using different column, different system and different day. %RSD for % assay of ruggedness found was 0.10 % for Glyburide and 0.10 % for Metformin and Overall %RSD found was 0.14 % for Glyburide and 0.11 % for Metformin as tabulated in Table No.3.

Table No.3: Method Ruggedness.

Sr. No.	Glyburide		Metformin	
	% Assay of Glyburide	% Assay of Glyburide	% Assay of Metformin	% Assay of Metformin
	Method precision	Ruggedness	Method precision	Ruggedness
1	99.2	99.1	99.8	99.9
2	99.3	99.3	99.7	99.8
3	99.4	99.3	99.5	99.7
4	99.6	99.4	99.6	99.8
5	99.5	99.3	99.8	99.7
6	99.4	99.2	99.8	99.6
Mean	**99.4**	**99.3**	**99.7**	**99.8**
S.D.	**0.14**	**0.10**	**0.13**	**0.10**
% R.S.D.	**0.14**	**0.10**	**0.13**	**0.10**
Overall Mean	**99.4**		**99.7**	
Overall S.D.	**0.14**		**0.11**	
Overall R.S.D.	**0.14**		**0.11**	

Accuracy (Recovery)

Accuracy of the analytical method was evaluated at a known concentration of Glyburide and Metformin at about 50%, 100% and 150% of test concentration of sample solution and 50% (1X Blend) and 150% (3x Blend) was calculated. % accuracy at individual level and overall average of % Recovery at all level for both Glyburide and Metformin was found 99% to 100% as tabulated in Table No.4.

Table No.4: Accuracy (Recovery).

Spike level in %	Glyburide				Metformin			
	%Recovery	Mean	SD	% RSD	% Recovery	Mean	SD	% RSD
50% (Assay)	99.2	**99.3**	**0.21**	**0.21**	99.2	**99.7**	**0.45**	**0.45**
	99.5				99.7			
	99.1				100.1			
100% (Assay)	98.7	**99.0**	**0.26**	**0.26**	99.4	**99.4**	**0.20**	**0.20**
	99.1				99.2			
	99.2				99.6			
150% (Assay)	99.4	**99.5**	**0.26**	**0.26**	99.7	**99.7**	**0.35**	**0.35**
	99.8				100.1			
	99.3				99.4			
50% (1X Blend)	99.3	**99.0**	**0.12**	**0.12**	99.2	**99.2**	**0.10**	**0.10**
	99.1				99.3			
	98.9				99.1			
150% (3X Blend)	99.1	**99.6**	**0.10**	**0.10**	100.0	**99.9**	**0.06**	**0.06**
	99.7				99.9			
	99.6				99.9			
Overall Mean	**99.3**				**99.6**			
Overall SD	**0.30**				**0.35**			
Overall %RSD	**0.30**				**0.35**			

Linearity

For the evolution of the linearity of the analytical method, a mixture of standard solution of Glyburide and Metformin in a concentration range of 0.5937 µg/ml to 15.0057 µg/ml for Glyburide and 100.0644 µg/ml to 3000.0089 µg/ml for Metformin respectively were prepared as per the test procedure of methodology and analyzed on the HPLC system.

Correlation coefficient (r) value for Glyburide and Metformin using a regression equation with a 1/ (concentration^2) of weighting factor was calculated.

Correlation coefficient (r) value was found 0.99942 for Glyburide and 0.99990 for Metformin. Lower limit of Detection (LOD) and Lower limit of Quantification (LOQ) was calculated using following formulas.

Limit of detection (LOD) =3.3 X S.D. of Y intercept / Slope of the calibration curve.
Limit of Quantification (LOQ) =10 X S.D. of Y intercept / Slope of the calibration curve.

The LOD and LOQ for Glyburide were 0.1242 µg/ml and 0.3764 µg/ml.
The LOD and LOQ for metformin were 21.4220 µg/ml and 64.9153 µg/ml.
The linearity plot for Glyburide and Metformin was depicted in Figure 5.
Results of linearity was tabulated in Table No.5

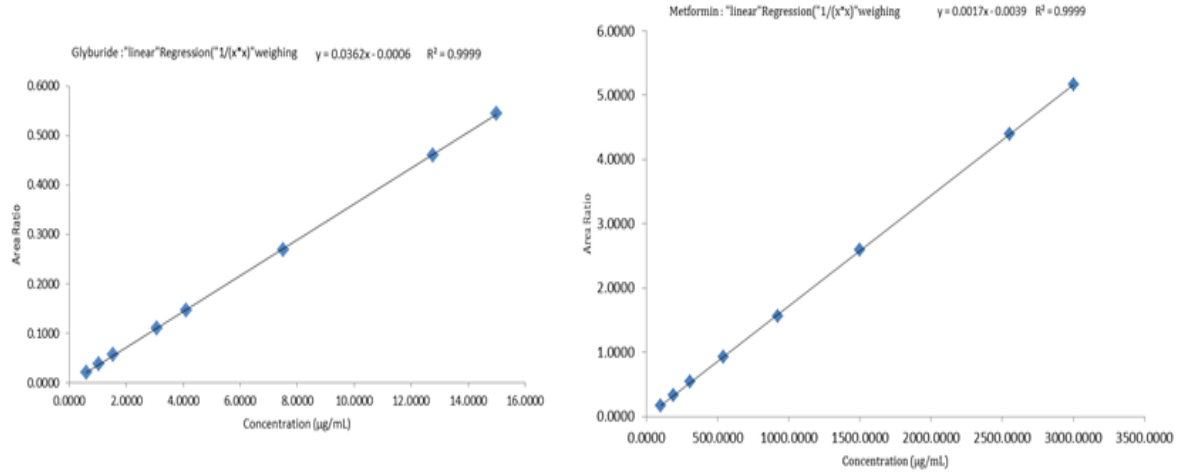


Figure 5: Linearity plot for Glyburide and Metformin.

Table No.5: Results of linearity.

Serial No.	Glyburide		Metformin	
	Concentration in µg/mL	Area Ratio	Concentration in µg/mL	Area Ratio
1	0.5937	0.0208	100.0644	0.1668
2	1.0236	0.0374	192.4316	0.3346
3	1.5354	0.0564	307.8906	0.5361
4	3.0707	0.1104	539.9631	0.9249
5	4.0943	0.1464	923.6717	1.5646
6	7.5028	0.2697	1500.1969	2.5879
7	12.7538	0.4602	2550.1037	4.3931
8	15.0057	0.5447	3000.0089	5.1652
Slope	**0.0362**		**0.0017**	
Intercept	**-0.0006**		**-0.0039**	
CC (r)	**0.9999**		**0.9999**	

The results of the linearity confirmed that an excellent correlation was exists between area ratio and concentration of both drugs within the concentration range.

Stability in analytical solution
For the evolution of stability in analytical solution; standard solution and sample solution was prepared freshly injected on the HPLC system at initially and different time intervals up to 46 hours and 44 hours respectively and the results of standard solution and sample solution were recorded. Absolute % difference and similarity factor were calculated.

For sample solution; absolute % difference between the initial result and results obtained at different time intervals was found 0.20 % for Glyburide and 0.20% for Metformin.

For standard solution; similarity factor between the initial result and results obtained at different time intervals was found 99.1 for Glyburide and 99.6 for Metformin.

The sample solution and standard solution are stable up to 44 hours and 46 hours respectively on bench top at room temperature.

Filter paper study
Filter paper study was performed to measure the analysis impact of filter paper used during various experiments of analytical method validation. For the evolution of the filter paper study of the analytical method, standard

solution was prepared as per test procedure of methodology and distributed the standard solution in two different portions. One portion centrifuged at 4000 rpm for 5 minutes and second portion was filter through 0.45-µm nylon membrane filter with discarding first 2mL of the filtrate and all the samples were analyzed on HPLC system.
Similarity factor between as such standard solution and filtered standard solution was found 99.7 for Glyburide and 100.4 for Metformin.

% absolute difference between average % assay of centrifuged sample solution and filtered sample solution was found 0.10% for Glyburide and 0.20% for Metformin.

Form the results it was concluded that the 0.45-µm nylon membrane filter with discarding first 2mL of the filtrate is suitable for the determination of the Assay Glyburide and Metformin in tablet formulation.

Forced degradation study
Forced degradation study was performed by treating sample tablet of 2.5MG/500MG strength containing 500 mg Metformin Hydrochloride and 2.5 mg Glyburide under acidic, basic, peroxide , thermal, photolytic and humidity conditions but somewhat degradation of the Glyburide observed under peroxide stress condition and slightly acidic degradation detected for metformin as tabulated in Table No.6**.**

Table No.6: Results of Force degradation.

Degradation Condition	% Degradation	
	Glyburide	Metformin
Acid Treated	1.2	5.2
Alkali Treated	1.3	2.3
Peroxide Treated	3.9	3.0
Thermal Treated	3.0	0.3
Photolytic Treated	4.3	0.1
Humidity Treated	0.4	0.2

Method robustness
Robustness of the analytical method was evaluated by accomplishment of analysis under marginally changed in the chromatographic method of analysis such as change in detection wavelength, change in flow rate, change in composition of the mobile phase and change in column oven temperature and change in pH. The assay results were compared with the assay result of method precision i.e. with finalized chromatographic conditions. The analytical method used is robust for change in flow rate, change in column oven temperature, change in pH, change in wavelength and change organic component of mobile phase.

The result was tabulated in Table No.7 and in Table No.8.

Table No.7: Result of Method robustness for Glyburide.

Sr. No.	Method precision	Minus Flow	Plus Flow	Minus Temp	Plus Temp	Minus (Methanol)	Plus (Methanol)	Minus pH	Plus pH	Minus nm	Plus nm
1	99.4	99.2	99.4	99.2	99.5	99.2	99.4	99.3	99.4	99.3	99.4
2	99.6	99.4	99.5	99.1	99.2	99.5	99.2	99.2	99.3	99.2	99.3
3	99.2	99.4	99.1	99.2	99.3	99.3	99.5	99.4	99.3	99.4	99.3
4	99.3	Not Applicable - - - - - - - - -									
5	99.5										
6	99.4										
Overall mean		99.4	99.4	99.3	99.4	99.4	99.4	99.4	99.4	99.4	99.4
Overall SD		0.13	0.16	0.16	0.14	0.14	0.14	0.13	0.12	0.13	0.12
Overall % RSD		0.13	0.16	0.16	0.14	0.14	0.14	0.13	0.12	0.13	0.12

Table No.8 for Result of Method robustness Metformin.

Sr. No.	Method precision	Minus Flow	Plus Flow	Minus Temp	Plus Temp	Minus (Methanol)	Plus (Methanol)	Minus pH	Plus pH	Minus nm	Plus nm
1	99.8	99.3	99.7	99.5	99.6	99.3	99.6	99.2	99.3	99.7	99.3
2	99.7	99.6	99.5	99.7	99.5	99.7	99.6	99.1	99.5	99.5	99.5
3	99.5	99.5	99.4	99.3	99.3	99.4	99.8	99.7	99.8	99.4	99.5
4	99.6	Not Applicable - - - - - - - - -									
5	99.8										
6	99.8										
Overall mean		**99.6**	**99.6**	**99.6**	**99.6**	**99.6**	**99.7**	**99.6**	**99.6**	**99.6**	**99.6**
Overall SD		**0.17**	**0.15**	**0.17**	**0.17**	**0.19**	**0.12**	**0.26**	**0.18**	**0.15**	**0.18**
Overall % RSD		**0.17**	**0.15**	**0.17**	**0.17**	**0.19**	**0.12**	**0.26**	**0.18**	**0.15**	**0.18**

Range

From the analytical procedure data of precision, accuracy and linearity, the range of the analytical method used for simultaneous determination of assay of Glyburide and Metformin drugs in the pharmaceutical Tablet formulations using Atovaquone as a common internal standard was tabulated in Table No.9.

Table No.9: Result of Range.

Name of Analyte (s)	Concentration (µg/mL)
Glyburide	0.5937 µg/ml to 15.0057 µg/ml
Metformin	100.0644 µg/ml to 3000.0089 µg/ml

Analysis of Marketed Products

The potency test of marketed tablet products were performed after the complete validation of the method for simultaneous determination of assay of Glyburide and Metformin drugs in the pharmaceutical Tablet formulations using Atovaquone as a common internal standard were performed by the proposed validated method.

The potency of tested brands was found to be within the limit of 98.00-102.00%.The results are tabulated in Table No.10.

Table No.10 Result of potency of Marketed Products

Sr.No	Brand name code	Glyburide			Metformin		
		Label Claimed (mg)	Amount found (mg)	Potency (%)	Label Claimed (mg)	Amount found (mg)	Potency (%)
1	Glyb and Met **A**	2.5	2.51	100.4	500	503	100.6
2	Glyb and Met **B**	1.25	1.26	100.8	250	248	99.2

CONCLUSIONS

This is the first reported High Performance Liquid Chromatographic method developed used for simultaneous determination of assay of Glyburide and Metformin drugs in the pharmaceuticals Tablet formulations using Atovaquone as a common internal standard was stability indicating as recommended by ICH guidelines and validated for Specificity, System precision, Method precision, Ruggedness, Robustness, Accuracy etc. The present analytical method has a widespread linear concentration range augmenting its applicability to different strength of Glyburide and Metformin tablet formulations. The chromatographic method may also be applied for simultaneous estimation of analytes in plasma, serum, urine after using appropriate sample extraction technique. Thus the method is Simpler, Accurate and Economical as compare to the previous methods.

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