IJPSR (2015), Vol. 6, Issue 3 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 18 July, 2014; received in revised form, 11 October, 2014; accepted, 19 January, 2015; published 01 March, 2015

STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF GLIMEPIRIDE AND EZETIMIBE IN BULK AND TABLET DOSAGE FORM

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Keywords:

Glimepiride, Ezetimibe, RP-HPLC, stability studies, Method development, validation,

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ABSTRACT: A simple, rapid, sensitive, precise, accurate and economic isocratic stability indicating RP-HPLC chromatographic method was developed and validated for the simultaneous estimation of Glimepiride and Ezetimibe in bulk and tablet dosage form. The method was developed through a Hypersil ODS C_{18} (150mm x 4.6 mm, 5µ) column, mobile phase comprised of phosphate in water as buffer P^H adjusted to 4.8 with tri ethylamine, acetonitrile in proportion ratio 30:70v/v and at the flow rate of 1 ml/min. GLM and EZE were eluted at acceptable retention times of 3.328 and 2.322 minutes respectively with good resolution by monitoring UV detection at 237 nm. Calibration plots were linear in the concentration range of 2.5-15 µg/ml for GLM and 25-150 µg/ml for EZE with correlation coefficient(r^2) 0.999 and 0.999 respectively. The total run time is 6 min. The studies were carried out by conducting deliberate degradation of the sample with exposure to stress conditions like acidic (1M HCl), alkaline (1M NaOH), 105^0C heat, oxidizing agents (H_2O_2) and water. This method was validated and met the regulatory requirements for specificity, Linearity, LOD, LOQ, Precision, accuracy and stability for the determination of glimepiride and ezetimibe in bulk and tablet dosage form by RP-HPLC.

INTRODUCTION: Diabetes is a metabolic disorder accompanied by insulin insufficiency and by impaired insulin secretion.The symptoms are characterized by hyper glycaemia, impaired insulin secretion, glucosuria, hyper lipaemia (insulin resistance in skeletal muscles, liver and adipose tissue), negative nitrogen balance, sometimes ketonaemia. Such patients are often obese and generally present in adult life, the incidences rising progressively with β-cell function declines. These defects have been treated by use of oral insulin secretogogues (sulphonyl urea/glinides) or insulin, biguanides, thiazolidinediones and anti-cholesteremic agents [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(3).1066-77

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(3).1066-77

Glimepiride ($C_{24}H_{34}N_4O_5S$) is chemically 3-ethyl-4-methyl-N-(4-[N-((1r,4r)-4-methyl cyclohexyl carbamoyl) sulfamoyl] phenethyl) - 2- oxo - 2, 5-dihydro-1H-pyrrole-1 carboxamide (**Figure 1**) [2], 3rd generation sulfonylurea derivative used in the treatment of type-II diabetes mellitus and also non insulin dependent diabetes mellitus(NIDDM) [3]. The primary mechanism of action of glimepiride in lowering blood glucose (Secretagogue) appears to be dependent on stimulating the release of insulin from functioning pancreatic β-cells and by inducing increased activity of intracellular insulin receptors [4]. It is official in Indian pharmacopoeia (IP) [4], British pharmacopoeia (BP) [5], United States Pharmacopoeia (USP) [6] and European pharmacopoeia (EU) [7] describe liquid chromatographic method for estimation.

Ezetimibe ($C_{24}H_{21}F_2NO_3$) is chemically (3R, 4S)-1-(4-fluorophenyl)-3-[(3S) – 3 - (4-fluorophenyl) – 3 - hydroxypropyl] – 4 - (4-hydroxyphenyl) azetidin-2-one (**Figure 2**) [8], a new anti-hyper lipidemic

agent [9], which inhibits the absorption of cholesterol from intestine, used in the treatment of primary hypercholesterolemia. It inhibits the absorption of biliary and dietary cholesterol from small intestine without affecting absorption of fat soluble vitamins, triglycerides and bile acids. After oral administration, EZE is metabolized into glucuronidein the liver and small intestine, which is also active in prevention of absorption of cholesterol.

It is not official in any pharmacopoeia. Literature survey reveals that UV spectrophotometric method for simultaneous estimation of GLM and EZE in tablet dosage form [10] was found, and detailed survey revealed several methods(RP-HPLC,UV)for determination of GLM in single dosage form and as well as with other combination dosage forms [11-14]. Similarly literature survey also reveals spectrophotometric (UV) and HPLC, HPTLC methods for determination of EZE in single dosage form and with other drugs in combination [15-22]. The combination of these two drugs is not official in any pharmacopoeia.

Literature survey does not reveal any simple chromatographic RP-HPLC method for simultaneous estimation of GLM and EZE in combination dosage forms and also stability studies of both combination tablet dosage form. This work presents a study of acidic, alkaline, neutral, oxidative, and thermal and photo degradation of GLM and EZE. The manuscript describes simple, sensitive, rapid, accurate, precise and cost effective stability indicating isocratic chromatographic method based on method development and validation for simultaneous estimation of GLM and EZE in their bulk and tablet dosage form.

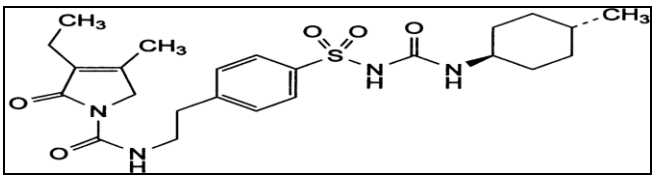

FIGURE 1: GLIMEPIRIDE (Mol wt. 490.616)

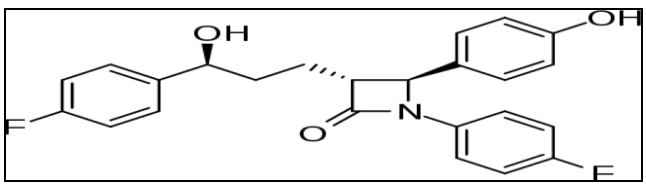

FIGURE 2: EZETIMIBE (Mol wt.490.425)

MATERIALS AND METHODS:

Chemicals and reagents: Glimepiride and Ezetimibe reference standards were provided by Dr. Reddy's Laboratories and Ranbaxy Pharmaceuticals Ltd., Gurgaon India. Tablet formulation of EZIWA (1+10) Manufactured by Kaytross Health Care Private Limited in India were procured from a local pharmacy. Potassium di hydrogen ortho phosphate and ortho phosphoric acid were analytical grade obtained from SD Fine chemicals(Hyderabad, India), methanol and acetonitrile of HPLC grade procured from Rankem and high purity water was prepared by using Milli pore Milli-Q water purification system was used. 0.45µm nylon membrane filters were obtained from spincotech private limited, Hyderabad. Methanol and water in the ratio of 50:50 is used as diluent.

Instrumentation and Chromatographic conditions: HPLC analysis was performed on Waters 2695 Alliance HPLC system connected with PDA Detector 2996.The drug analysis data were acquired and processed using Empower 2 software. HPLC system (Waters) was equipped with auto sampler.

Series: Waters 2695-Alliance

Software: Empower 2

Column: Hypersil ODS C_{18} (150mm x 4.6 mm, i.d. , 5µ)

Mode: Isocratic

Flow rate: 1 ml/ml

Detector: Photo diode array detector

U.V: T 60 PG instrument

Sonicator: Wensar MUC 6L

P^H meter: Mettler Toledo S 220

Weighing balance: Shimadzu AY-200

Preparation of solutions:

Preparation of 0.01N of potassium di hydrogen ortho phosphate (KH_2PO_4) buffer solution (pH 4.8): Accurately weighed 1.36 gm of potassium di hydrogen ortho phosphate in a 1000 ml of

volumetric flask, added about 900ml of milli-Q water and 1ml of tri ethylamine. Then it was degassed in an ultrasonicator for 10 minutes and filtered through 0.45 µ membrane filter and finally made up the volume with water and then the P^H adjusted to 4.8 with dilute ortho phosphoric acid.

Preparation of mobile phase: Mixture of above buffer 300 ml and 700 ml of acetonitrile HPLC grade and degassed in an ultrasonic water bath for 10 minutes. Filter through 0.45 µ filter under vacuum filtration.

Preparation of stock and working solutions: Accurately weighed and transferred 5 mg and 10 mg of GLM and EZM into 50ml and 10 ml of clean dry volumetric flask respectively, add $3/4^{th}$ of diluent and sonicated for 30 minutes and made up to the final volume with diluent to obtain the 100 µg/ml and 1000 µg/ml concentration. From the above stock solutions, 1 ml of solution was transferred into a 10 ml volumetric flask to that the diluent was added up to the mark to get final concentration of 10 µg/ml GLM and 100 µg/ml EZE.

Sample Solution Preparation: Accurately 20 tablets were weighed individually and the average weight was calculated and powdered. The tablet powder equivalent to 10mg EZE and 1 mg GLM transferred into a 100 ml volumetric flask, to that 60ml of diluent was added and sonicated for 30 minutes at controlled temperature to dissolve the powder, further the volume made up with diluent, and filtered through 0.45 µ membrane filter (Stock solution). From this solution 0.5 ml was diluted to 10 ml with diluent to give a concentration of 100 µg/ml and 10 µg/ml solution of EZE and GLM respectively.

Method Validation: The HPLC method was validated in according to ICH guidelines for validation of analytical procedures for different validation parameters. The method was validated for its specificity, linearity, accuracy, precision, robustness, ruggedness, LOD and LOQ.

System suitability: System suitability test was carried out to verify that the analytical system is working properly and can give accurate and precise results. The overall system suitability was evaluated for the system suitability of the proposed method. Data from six injections (10µg/mL) were utilized for calculating parameters like theoretical plates, resolution, tailing factor and %RSD of 6 injections.

Specificity: The specificity studies were carried out by varying specific conditions i.e., placebo study. Specificity of the method was established by demonstrating that there was no interference from the excipients. This was demonstrated by preparing the placebo containing all excipients except the drug and also the sample prepared from the same. The samples were injected individually and chromatogram was obtained.

Linearity: Linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the samples [23]. The linearity of the method was determined by preparing serial dilutions of minimum 5 concentration of working stock solutions in the range of 2.5-15µg/ml for GLM and 25-150µg/ml for EZE. The area of each injection was obtained and the peak area was plotted against actual concentration. The regression coefficient 'r^2', y-intercept and slope of the regression were calculated.

Detection and quantitation limits (Sensitivity): The LOD is defined as the lowest concentration of an analyte in a sample that can be detected but not quantified. The LOQ is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. The LOD and LOQ can be calculated based on the standard deviation of the response and the slope of calibration curve.

$$LOD=3.3\sigma/S$$

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

where

'σ' is the standard deviation of the intercept of the regression lines and
's' is the slope of the calibration curve.

Accuracy: Accuracy was carried out by % recovery studies of GLM and EZE at three different

concentration levels (50%, 100%, 150%). In the proposed method recovery studies were carried out by collecting the sample solution of 20 tablets containing GLM and EZE and analyzed. Percentage of recovery was calculated from the amount added and amount recovered. The percentage recovery was within the acceptance criteria, this indicates the accuracy of the method. (Acceptance criteria: % recovery between 98 to 102).

Precision: Precision of an analytical procedure as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions [23]. It has done by the following methods.

System precision (Reproducibility): System precision of method was carried out by measuring the peak response of GLM and EZE for six replicate injections of standard solution. The retention time and area ratio of six determinations were measured and percent coefficient variation (% RSD) calculated. (Acceptance criteria: % RSD not more than 2%).

Method precision: It can be done by 2 methods.

Repeatability (Intraday precision): Repeatability was carried out by analyzing six replicate injections of assay concentration (10 µg/ml) of standard and sample solutions over a short time interval (within a day) under same conditions. The percentage relative standard deviation (% RSD) was calculated for the resultant peak areas.

Intermediate precision (Inter day precision): Intermediate precision was assessed by analyzing the same standard and sample solutions on different days. % RSD of assay results were calculated. To assess the degree of reproducibility of the method, 10 µg mL^{-1} was analyzed on different day. The assay procedure was repeated six times and the chromatogram was recorded and the %RSD was calculated.

Robustness: The robustness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters such as flow rate, column temperature and mobile phase were varied within a realistic range and the quantitative influence of the variables was determined. It provides an indication of the procedure's reliability during normal usage. It is concluded that the method is robust as it is found that the % RSD is less than 1 concerning % assay despite deliberate variations done concerning flow rate (± 0.1 ml), composition of mobile phase (±10 ml) and temperature (±5 ºC) [23].

Stability studies: Forced degradation studies typically involved the exposure of samples of the drugs to the relevant stress conditions of acid, base, hydrolysis, oxidation, thermal, photo stability. Stability testing was established for estimating the allowed time span between sample collection and sample analysis. It is also important to evaluate an analytical method's ability to measure drug products in the presence of its degradation products [23].

Acid Degradation:
To 1 ml of the stock solution of GLM and EZE, 3 ml of 2N HCl was added in 10 ml volumetric flask and immediately the solution was refluxed for 30 mins at 60 ºC.The solution was diluted to obtain 100 µg/ml and 10 µg/ml. 10µl solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation:
To 1 ml of the stock solution of GLM and EZE, 3 ml of 2N NaOH was added in 10 ml volumetric flask and immediately the solution was refluxed for 30 minutes at 60 ºC. The solution was diluted to obtain 100 µg/ml and 10 µg/ml. 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Oxidative Degradation:
To 1 ml of the stock solution, 3 ml of 20 % H_2O_2 was added in 10 ml volumetric flask and immediately the solution was refluxed for 30 minutes at 60 ºC. The solution was diluted to obtain 100µg/ml and 10 µg/ml. 10µl was injected into the system and the chromatograms were recorded to assess the stability of sample.

Thermal Degradation:
The standard drug solution was placed in an oven at 105 ºC for 6 hour to study dry heat degradation. For HPLC study, the resultant solution was diluted to 100 µg/ml and 10 µg/ml solution and from that 10µl solution was injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies:
The photo chemical stability of the drug was also studied by exposing the sample solution to UV light by keeping the beaker in UV chamber for 7 days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 100 µg/ml and10 µg/ml solutions and filter through 0.45µ membrane filter. From that 10 µl solution was injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation:
Stress testing under neutral conditions was studied by refluxing the drug in water for 6 hours at a temperature of 60 ºC. For HPLC study, the resultant solution was diluted to 100 µg/ml and 10 µg/ml solution and filtered through 0.45 µ membrane filter. From that10 µl was injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

RESULTS AND DISCUSSIONS:
Method Development and Optimization:
Optimization of UV conditions: The method development work was started by taking UV-spectra in the range 200-400 nm of GLM and EZE (10PPM) standard solution. These samples were scanned in the range 200-400 nm using UV-visible spectrophotometer. By observing the UV spectra of standard solutions, the detection wavelength was selected as 237 nm for trails to develop HPLC method. The spectra are shown in **Figure 3**.

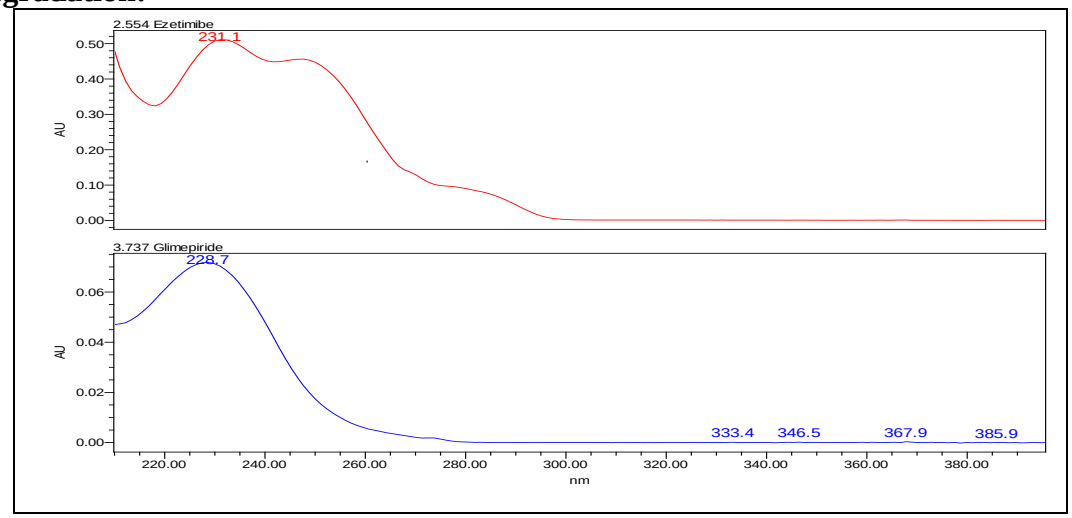

FIGURE 3: UV SPECTRA OF EZETIMIBE AND GLIMEPIRIDE

Method Optimization: Optimization of the method was carried out by performing various trials by change in mobile phase composition, column, flow rate, etc.

Optimized Method: In this work, an isocratic, simple, accurate and sensitive HPLC method suitable for the simultaneous determination of GLM and EZE in pure form and in pharmaceutical formulations using a 150 mm x 4.6 mm, i.d. Hypersil ODS C_{18} 5µ analytical column has been developed. The mobile phase was chosen after several trials to match the optimum stationary/mobile phase. The present method contains mobile phase phosphate buffer (pH-4.8): Acetonitrile (30:70 v/v) which was found to be the most suitable, as the chromatographic peaks obtained were better defined, well resolved and almost free from tailing. The flow rate is 1 ml/min. The average retention times under the conditions described were 3.328 minutes for GLM and 2.322 minutes for EZE. The total run time is 6 minutes with which all the system suitability parameters are ideal for the mixture of standard solutions. **Figure 4** represent chromatogram of mixture of standard solutions, respectively **Table 1**.

TABLE 1: OPTIMIZED CHROMATOGRAPHIC PARAMETERS

Optimized Chromatographic Conditions	
Mode of separation	Isocratic
Mobile phase	ACN:PO_4(70:30)
Column	Hypersil ODS C_{18} (150mm x 4.6 mm i.d., 5µ.)
Column temperature	30°C
Detector wave length	237nm
Run time	6 min
Injection volume	10µl
Flow rate	1.0 ml/min.

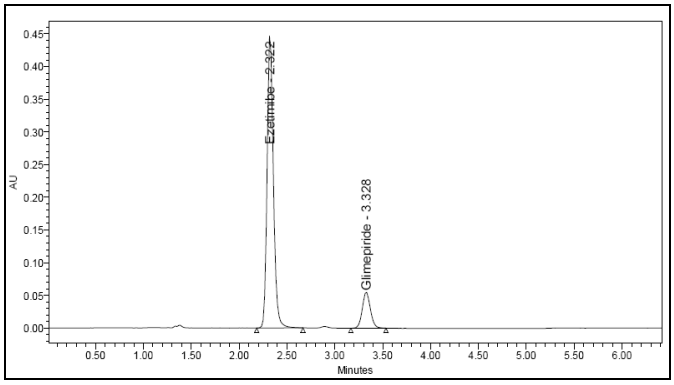

FIGURE 4: CHROMATOGRAM UNDER OPTIMIZED CHROMATOGRAPHIC CONDITIONS (EZE AND GLM)

METHOD VALIDATION:

System suitability: A RP-HPLC method was developed by monitoring the system suitability parameters i.e. tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness. System suitability method acceptance criteria set in each validation run were: tailing factor ≤ 2.0 and theoretical plates > 2000. In all cases, the relative standard deviation (R.S.D) for the analytic peak area for two consecutive injections was < 2.0%. A chromatogram obtained from reference substance solution was presented. System suitability parameters are tabulated in **Table 2**.

TABLE 2: SYSTEM SUITABILITY PARAMETERS IN SOLUTION STATE

Parameters	Glimepiride	Ezetimibe
Tailing factor (T)	1.07	1.17
Number of theoretical plate(n)	7445	6055
Retention time (RT)	3.328	2.322
Linearity range	2.5-15µg/ml	25-150µg/ml
Correlation coefficient(r^2)	0.999	0.999
Slope	34980	38081
Limit of detection(LOD)(µg/ml)	0.05 µg/ml	0.03 µg/ml
Limit of Quantification(LOQ)(µg/ml)	0.15 µg/ml	0.09 µg/ml

Specificity: The specificity studies were carried out by varying specific conditions i.e., placebo study. A study conducted to demonstrate that diluent and placebo were not interfering with the analyte peak in the proposed method. Solutions of sample, placebo and blank were prepared individually and chromatograms were obtained. The blank chromatogram showed no interference peaks at the retention time of GLM and EZE. This indicates that diluent solution used in sample preparation do not interfere in the estimation of GLM and EZE. Similarly the placebo sample chromatogram showed no interference peaks at the retention time of GLM and EZE respectively, which indicates the specificity of the proposed method. The chromatogram of the blank, placebo and sample using proposed method for GLM and EZE are shown in **Figures 5, 6 and 7.**

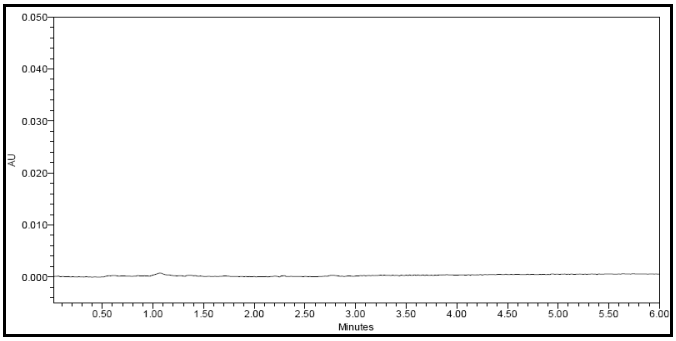

FIGURE 5: CHROMATOGRAM SHOWING NO INTERFERENCE OF BLANK FOR THE GLIMEPIRIDE AND EZETIMIBE

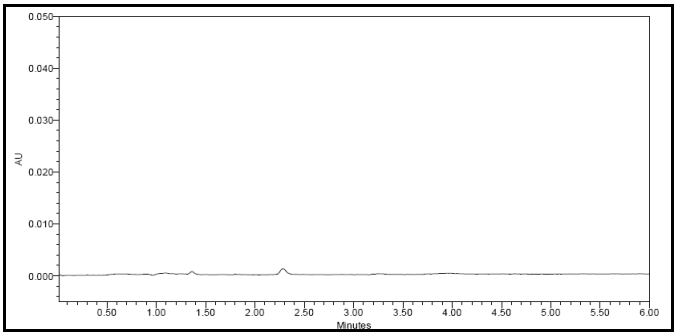

FIGURE 6: CHROMATOGRAM SHOWING NO INTERFERENCE OF PLACEBO FOR THE GLIMEPIRIDE AND EZETIMIBE

S.No	Parameters	Acceptance criteria	Interference
1.	Blank	No interference peak of RT of analyte peak	Passes
2.	Placebo	No interference peak of RT of analyte peak	Passes

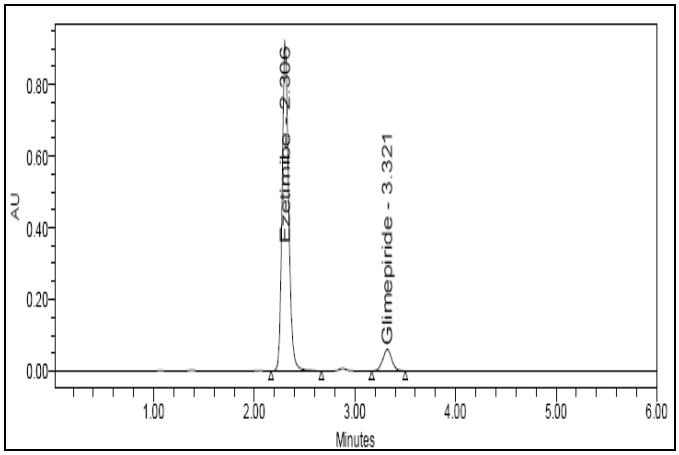

FIGURE 7: CHROMATOGRAM OF SAMPLE SOLUTION OF GLIMEPIRIDE AND EZETIMIBE (SPECIFICITY)

Linearity: The linearity of GLM and EZE was carried out at different concentrations ranging from 2.5-15 µg/ml for GLM and 25-150 µg/ml for EZE and correlation coefficient was found to be 1, which indicates that the concentration had given good linearity as shown in **Figure 8 and 9**. Linearity results are tabulated in Tables 3 and 4.

TABLE 3: LINEARITY RESULTS OF EZETIMIBE AND GLIMEPIRIDE.

S. No.	Glimepiride		Ezetimibe	
	Concentration (µg/mL)	Area	Concentration (µg/mL)	Area
1.	2.5	85798	25	992242
2.	5	174668	50	1815411
3.	7.5	263949	75	2880318
4.	10	355151	100	3834184
5.	12.5	441909	125	4795457
6.	15	518632	150	5677142

Linearity of Glimepiride:

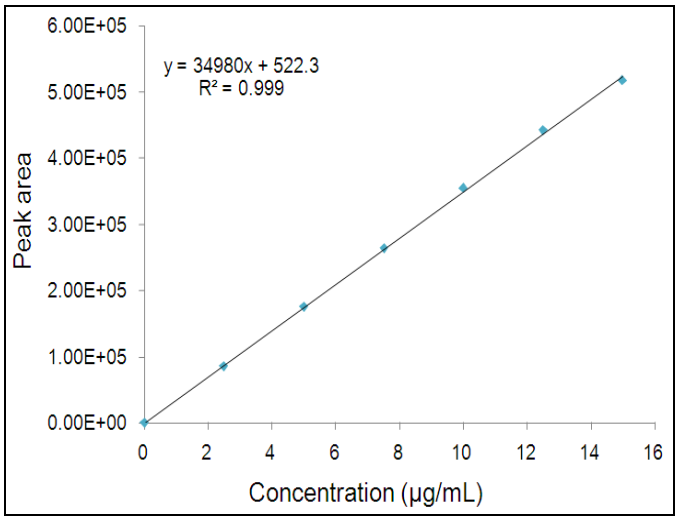

FIGURE 8: LINEARITY CURVE OF GLIMEPIRIDE

Linearity of Ezetimibe:

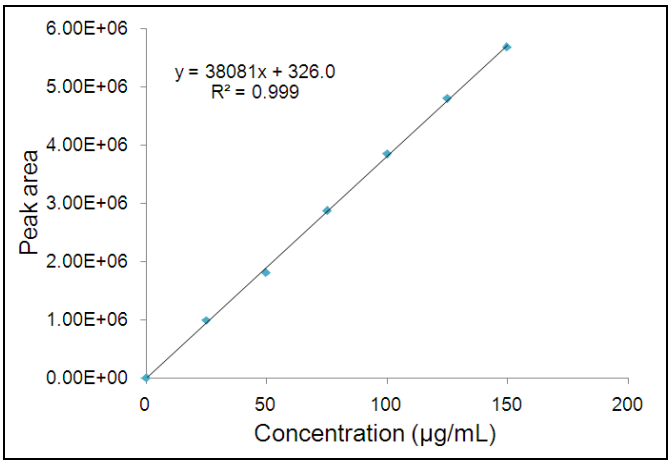

FIGURE 9: LINEARITY CURVE OF EZETIMIBE

TABLE 4:REGRESSION ANALYSIS OF LINEARITY DATA OF GLM AND EZE

Parameter	GLM	EZE
Slope	34980	38081
Intercept	522.3	326.0
Correlation coefficient(r^2)	0.999±0.001	0.999±0.001

Sensitivity:

LOD and LOQ of Glimepiride and Ezetimibe: The values were within the limit which indicates the sensitivity of the method. The results of the LOD and LOQ are tabulated in **Table 5.**

TABLE 5: LODAND LOQ OF GLIMEPIRIDE AND EZETIMIBE

S. No	Drug Name	Standard deviation (S.D)	Slope	LOD	LOQ
1.	GLM	522.3	34980	0.05 µg/ml	0.15 µg/ml
2.	EZE	326.0	38081	0.03 µg/ml	0.09 µg/ml

Accuracy: The accuracy of the method was found at 3 different concentration levels i.e. 50 %, 100 %, 150 % and showed acceptable % recoveries in the range of 99.88 for GLM and 99.83 for EZE. The results are tabulated in **Tables 6 and 7.**

Precision: The precision of the method was determined by system precision, method precision i) repeatability and ii) intermediate precision studies. In this study the system precision RSD values are 0.64 % and 0.16 % were obtained for the standard area of GLM and EZE, respectively. The method precision study for 6 sample preparations

in marketed samples showed a RSD of 1.65 % and 0.57 %. The intermediate precision study was performed on a different day, and the % RSD results were 0.80% and 0.83% for GLM and EZE. Results are tabulated in **Tables 8, 9 and 10** that are in the acceptance limit of less than 2%.

TABLE 6: ACCURACY DATA FOR GLIMEPIRIDE AND EZETIMIBE

	GLM			EZE		
Injection	**50%**	**100%**	**150%**	**50%**	**100%**	**150%**
Inj-1	178669	358616	530299	2081569	4128418	6216820
Inj-2	176314	357216	532193	2084495	4181678	6258934
Inj-3	177186	354478	537590	2079469	4191546	6276747
AVG	99.626	100.18	99.85	99.89	99.976	99.976
S.D	0.669	0.59	0.71	0.12	0.815	0.492
%RSD	0.671	0.59	0.71	0.12	0.81	0.49

TABLE 7: ACCURACY (%RECOVERY) RESULT FOR GLM AND EZE

Drug Name	Level	Amout added(mg)	Amount recovered(mg)	%Recovery	% Mean recovery
1. Glimepiride	50%	2.5 mg	2.49	99.62	99.88
	100%	5 mg	5.009	100.18	
	150%	7.5 mg	7.49	99.84	
2. Ezetimibe	50%	5 mg	4.99	99.86	99.83
	100%	10 mg	9.99	99.9	
	150%	15 mg	14.97	99.8	

TABLE 8: SYSTEM PRECISION RESULTS FOR GLIMEPIRIDE AND EZETIMIBE

S. No	Injections	Area of GLM	Area of EZE
1.	Injection-1	357132	4161481
2.	Injection-2	356745	4160414
3.	Injection-3	358030	4158152
4.	Injection-4	352492	4174603
5.	Injection-5	354381	4165539
	Avg.	355756	4164038
	Standard Deviation	2268.64	6484.382
	%RSD	0.64	0.16

TABLE 9: METHOD PRECISION (REPEATABILITY) FOR GLM AND EZE

S. No	Injections	Area of GLM	%Assay	Area of EZE	%Assay
1.	Injection-1	345056	96.89533	4148192	99.51984
2.	Injection-2	349190	98.0562	4191836	100.5669
3.	Injection-3	349927	98.26316	4182687	100.3474
4.	Injection-4	357114	100.2813	4162464	99.86224
5.	Injection-5	341802	95.98157	4196256	100.673
6.	Injection-6	354932	99.66861	4138937	99.2978
	Avg.	349670	98.191	4170062	100.04
	S.D	5772.221	1.621	23767.27	0.5712
	%RSD	1.65	1.65	0.57	0.57

TABLE 10: INTERMEDIATE (DAY-DAY) PRECISION RESULT FOR GLM AND EZE

S. No	Injections	Area of GLM	Area of EZE
1.	Injection-1	346023	4011151
2.	Injection-2	353754	4004876
3.	Injection-3	351199	3994473
4.	Injection-4	349072	3985576
5.	Injection-5	348828	4070666
	AVG	349775	4013348
	S.D	2886.5	33501.88
	%RSD	0.80	0.83

Robustness: To evaluate the robustness, the developed method was subjected to small deliberate variations in the optimized method parameters like variation of flow rate 1.0 ± 0.1 ml/min (i.e., 0.9 ml/min, 1.0 ml/min and 1.1 ml/min) and mobile phase ratio i.e. 30:70 (± 10 ml) and temperature 30 °C (± 5 °C). The mixed standard solution containing 100 µg/ml EZE and10 µg/ml GLM was injected in triplicate with varied chromatographic conditions and the standard deviation of the retention time of each analyte was calculated. The method was found to be robust as the slight deliberate variations in flow rate, mobile phase and temperature ratio did not lead to changes in retention time of the peak of interest and there was no significant change in chromatographic parameters (% RSD is found to be less than 2% for all the variation). Results are tabulated in **Table 11**.

TABLE 11: RESULTS OF ROBUSTNESS OF GLM AND EZE FOR RP-HPLC

Parameter	Optimized	Used	GLM R_t	GLM Peak area	GLM Mean± %RSD	EZE R_t	EZE Peak area	EZE Mean± %RSD
1.Flow rate (± 0.1ml)	1.0 ml/min	0.9	3.688 3.722	390446 391491	390968.5 ± 0.2	2.568 2.596	4289284 4357510	4323397± 1.1
		1.1	3.318 3.354	354291 357025	355658± 0.5	2.315 2.331	4066615 4106221	4086418± 0.7
2.Mobile phase composition (30:70)	Buffer:ACN(30:70)	40:60	3.167 3.184	354425 356174	355299.5 ± 0.3	2.236 2.240	3908393 3925784	3917089± 0.3
		20:80	3.352 3.564	362817 358055	360436± 0.9	2.439 2.441	4013801 3993747	4003774± 0.4
3.Temperature (± 10 °C)	30 °C	25	3.168 3.303	336111 341552	338831.5 ± 1.1	2.229 2.306	3855737 3896109	3875923± 0.7
		35	3.140 3.215	357244 356579	356912± 0.1	2.219 2.252	3916382 3911346	3913864± 0.1

Analysis of marketed tablet formulation: The validated RP-HPLC method was applied for the quantitation of tablet formulation that was obtained by injected 3 replicates of the sample solutions. The amounts of GLM and EZE estimated were found to 99.79 and 99.84, respectively. The results are tabulated in **Table 12.**

TABLE 12: ANALYSIS OF TABLET FORMULATION

Drug	Labeled amount (mg)	% Assay
GLM	1	99.79
EZE	10	99.84

Stability studies: The stability was initially determined by analyzing both standard and sample solutions during analysis over a period of 24 hr at room temperature. The results show that for solution, the retention time and peak area of GLM (0.8) and EZE (0.8) gives almost similar (% RSD less than 2.0) and there was no degradation within indicated period, so that indicates both solutions were stable for at least 24 hr, which was sufficient to complete the whole analytical process. Further forced degradation studies conducted indicates the stability of proposed method. The peak purity of GLM and EZE was found to satisfactory under different stress conditions as shown in the chromatograms in Fig. 10 (a, b, c, d, e) which indicates that the developed method was stability indicating. The results were tabulated in **Table 13 and 14.**

TABLE 13: FORCED DEGRADATION STUDY RESULTS FOR GLIMEPIRIDE

Stress condition	Area	R_t(min)	% Degradation	Purity Angle	Purity Threshold	USP Tailing
Standard (control)	358552.6	3.312	-	-	-	1.06
Acid degradation	329653	3.310	8.76	0.241	0.473	1.1
Base degradation	332665	3.322	7.78	0.399	0.442	1.1
Peroxide degradation	336739	3.319	6.47	0.255	0.351	1.1
Thermal degradation	338323	3.279	5.97	0.416	0.670	1.1
UV degradation	348016	3.257	3.02	0.437	0.726	1.1
Water degradation	352715	3.250	1.65	0.420	0.662	1.1

TABLE 14: FORCED DEGRADATION STUDY RESULTS FOR EZE

Stress condition	Area	R_t(min)	% Degradation	Purity Angle	Purity Threshold	USP Tailing
Standard (control)	4162943	2.306	-	-	-	1.17
Acid degradation	3851648	2.305	8.08	0.158	0.265	1.2
Base degradation	3881847	2.321	7.24	0.131	0.269	1.2
Peroxide degradation	3929388	2.319	5.94	1.526	2.135	1.1
Thermal degradation	3965596	2.292	4.97	0.135	0.295	1.2
UV degradation	4094652	2.281	1.66	0.168	0.304	1.1
Water degradation	4128192	2.273	0.84	0.154	0.296	1.2

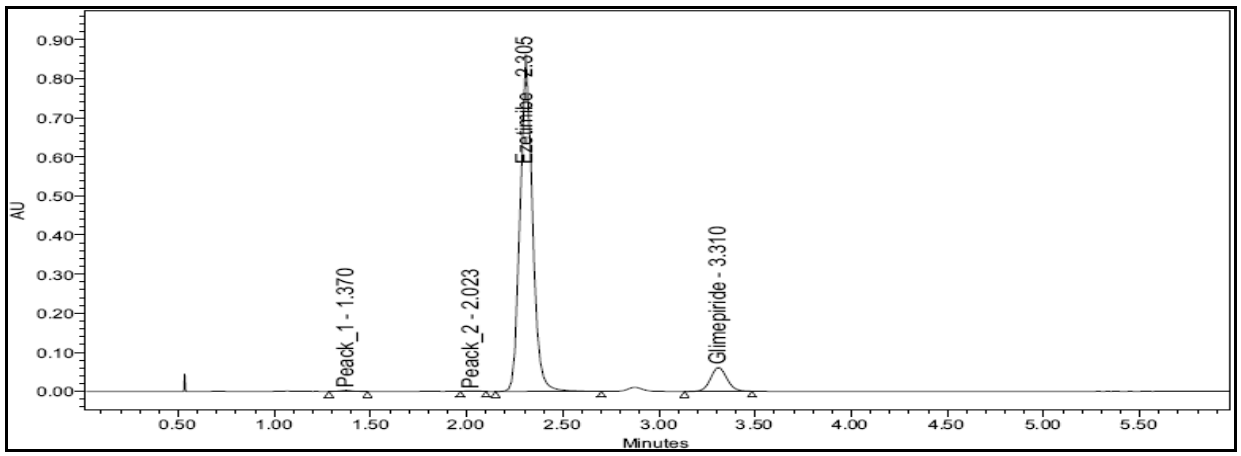

FIGURE 10(a) TYPICAL CHROMATOGRAM OF ACID DEGRADATION PATTERN OF EZE AND GLM

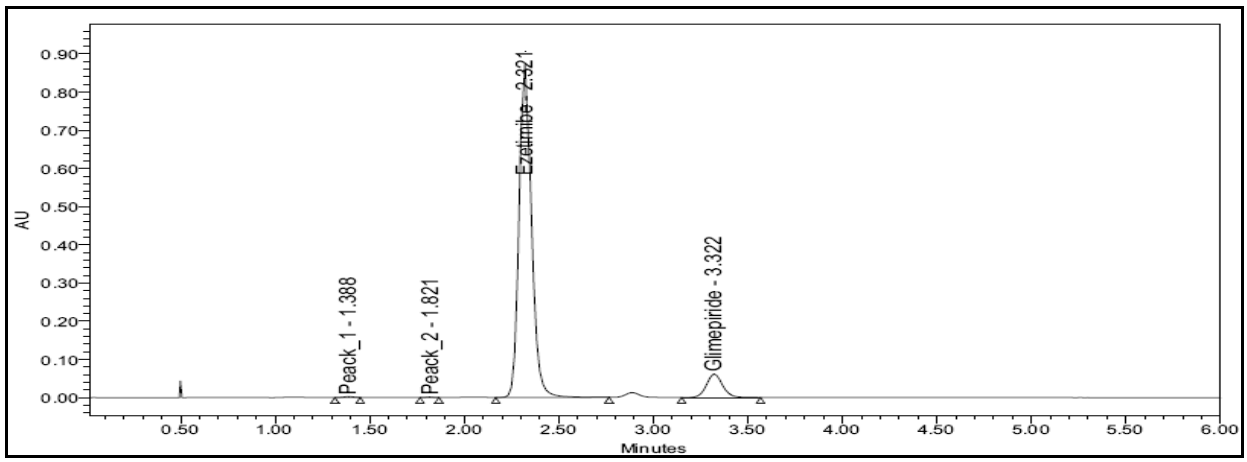

FIGURE 10 (b) TYPICAL CHROMATOGRAM OF BASE DEGRADATION PATTERN OF EZE AND GLM

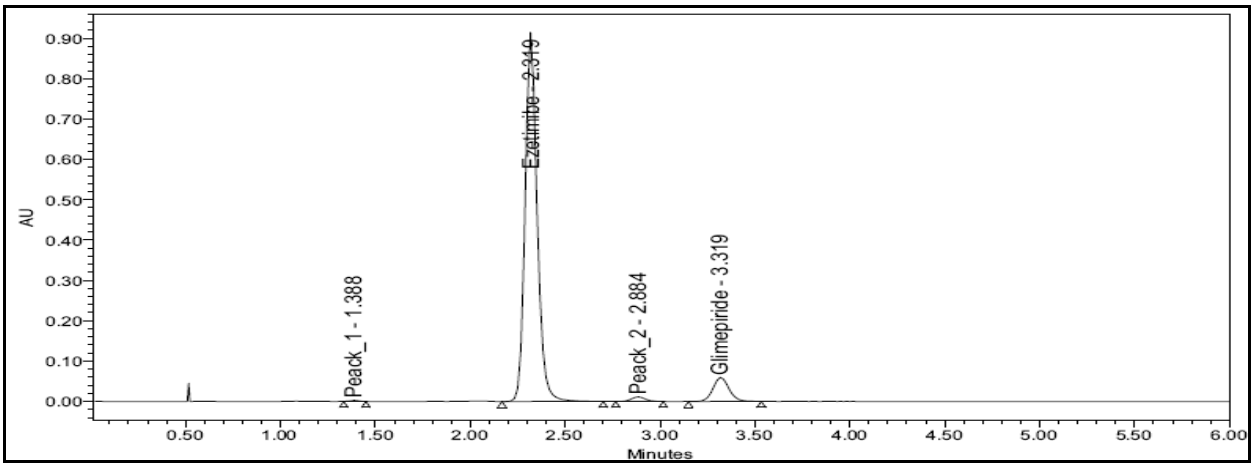

FIGURE 10 (c) TYPICAL CHROMATOGRAM OF OXIDATIVE DEGRADATION (H_2O_2) PATTERN OF EZE AND GLM

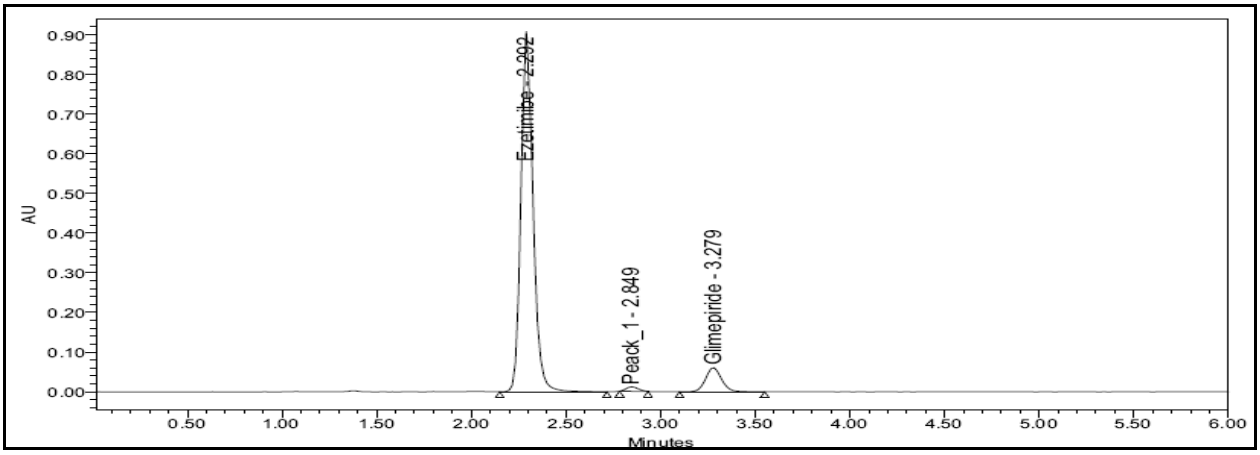

FIGURE 10 (d) TYPICAL CHROMATOGRAM OF THERMAL DEGRADATION PATTERN OF EZE AND GLM

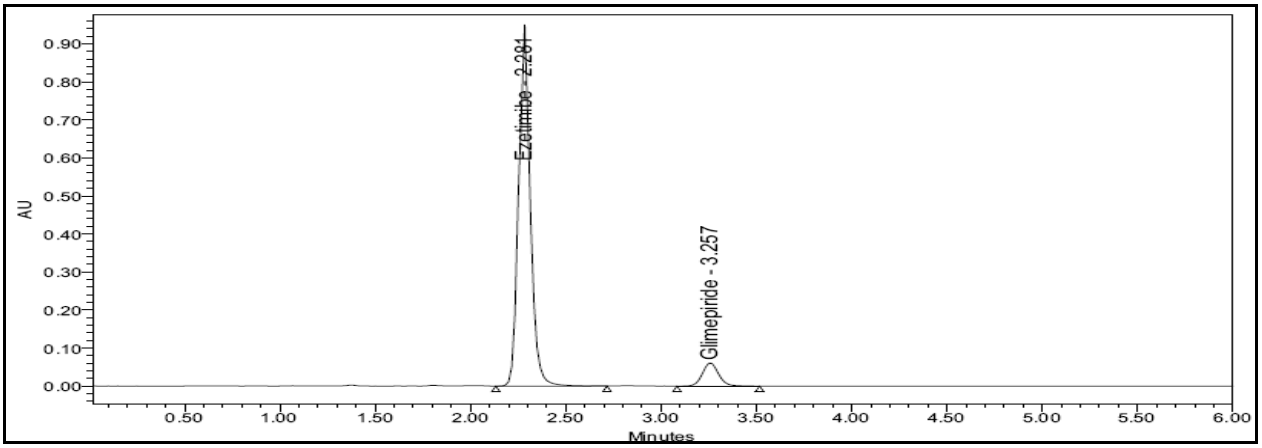

FIGURE 10 (e) TYPICAL CHROMATOGRAM OF UV DEGRADATION PATTERN OF EZE AND GLM

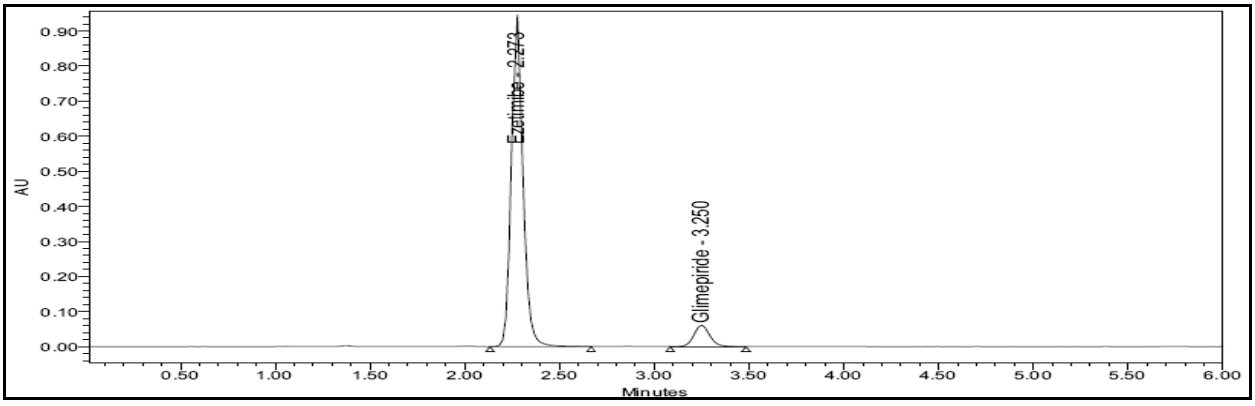

FIGURE 10 (f) TYPICAL CHROMATOGRAM OF DEGRADATION PATTERN OF EZE AND GLM IN WATER

CONCLUSION: The developed and validated method is very simple, rapid, precise, accurate, isocratic and stability-indicating RP-HPLC analytical method. The method was validated for specificity, linearity, accuracy, precision, LOD, LOQ, robustness and system suitability. No interfering peaks were found in chromatogram, indicating that the estimation of drugs is free from inference of excipients. The rapid run time of 6 min and the relatively low flow rate (1 ml/min) allows the analysis of large number of samples with less mobile phase that proves to be cost-effective. Therefore, the developed method can be used for routine analysis for simultaneous estimation and stability indicating studies of GLM and EZE in bulk and pharmaceutical dosage form.

ACKNOWLEDGEMENTS: PP and AS would like to thank the Director, CSIR-Indian Institute of Chemical Technology, Hyderabad for giving us an

opportunity to carry out our dissertation work in the institute. We greatly acknowledge Dr. Reddy's Laboratories, Pvt. Ltd, Hyderabad and Ranbaxy Pharmaceuticals Ltd., Gurgaon, India for providing the gift sample of GLM and EZM.

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How to cite this article:
Pavani P, Srilekha A and Sreedhar B: Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Glimepiride and Ezetimibe in Bulk and Tablet Dosage Form. Int J Pharm Sci Res 2015; 6(3): 1066-77.doi: 10.13040/IJPSR.0975-8232.6 (3).1066-77.