



**DEVELOPMENT OF STABILITY INDICATING ANALYTICAL METHOD FOR  
SIMULTANEOUS ESTIMATION OF EZETIMIBE AND GLIMEPIRIDE IN BULK AND  
PHARMACEUTICAL DOSAGE FORM**

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**ABSTRACT**

A simple, accurate, precise and selective stability indicating High performance thin layer chromatography method was developed and validated for the simultaneous quantitative analysis of Ezetimibe and Glimepiride in combined pharmaceutical dosage form. Degradation studies were carried out by subjecting both the drugs to different stress conditions as recommended by the International Conference on Harmonization (ICH). Stability indicating high performance thin layer chromatographic method for ezetimibe and glimepiride estimation was developed by using the degraded samples. The acceptable resolution of two drugs from their degradation products was achieved with the use of precoated silica gel 60 F<sub>254</sub> aluminium plates as stationary phase and Toluene: Methanol: Acetone: Triethylamine (7: 2: 1: 0.2, v/v/v) as optimum mobile phase. Densitometric detection was carried out at 246 nm. The retention factors were found to be  $0.28 \pm 0.03$  for ezetimibe and  $0.60 \pm 0.001$  for glimepiride. The developed method was validated for linearity, precision, accuracy, specificity and robustness. The results demonstrated that the described method was suitable for quantitative analysis of ezetimibe and glimepiride in the presence of their degradation products formed under a variety of stress conditions. The proposed method will be applicable to the determination of stability of ezetimibe and glimepiride drugs and can be successfully used in quality control of bulk manufacturing and pharmaceutical preparations.

**KEYWORDS:** Glimepiride, Ezetimibe, Stability studies, Method validation.

**INTRODUCTION**

Chemical stability of molecules is one of the vital factors that influence the safety and efficiency of the drug product. Food and Drug Administration (FDA) and the International Conference on Harmonization (ICH) affirm the necessity of stability testing data to recognize how the quality of a drug substance and drug products changes with time under the influence of various environmental factors. The information on the stability of drug substance helps in selecting proper formulation and package as well as providing proper storage conditions and shelf life, which is necessary for regulatory documentation. The forced or stress degradation of drug products and drug substances occurs under severe conditions such as acidic medium, alkaline medium, oxidation with hydrogen peroxide and photochemical conditions to give complete idea about the stability of drug products. The ICH guideline states that stress testing is proposed to recognize the likely degradation products which can further help in determination of the intrinsic stability of the molecule

and establishing degradation pathways, and to validate the stability indicating procedures used here.<sup>[1]</sup>

Ezetimibe (EZE), chemically, (3R, 4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl] - 4-(4-hydroxyphenyl) azetidin-2-one is anti-hyperlipidemic drug used to lower the plasma cholesterol.<sup>[2]</sup> It is not official in any pharmacopoeia. Glimepiride (GLIM) is antidiabetic drug with IUPAC name 3-ethyl-4-methyl-N-{2-[4-((4-methylcyclohexyl) carbamoyl) amino] sulfonyl} phenyl ethyl} -2-oxo-2,5-dihydro-1H-pyrrole-1-carboxamide used with diet to lower blood glucose by increasing the secretion of insulin from pancreas and increasing the sensitivity of peripheral tissues to insulin.<sup>[3]</sup> It is official in Indian pharmacopoeia<sup>[4]</sup>, British pharmacopoeia<sup>[5]</sup>, United States Pharmacopoeia<sup>[6]</sup> and European pharmacopoeia.<sup>[7]</sup> The combination of these two drugs is used for treatment of hypercholesterolemia.

Extensive review of literature on studies of the quantitative analysis of EZE and GLIM showed number

of analytical methods for their estimation. RP-HPLC method was usually used for the estimation of EZE as single and in combination with other drugs.<sup>[8,15]</sup> Stability indicating RP-HPLC<sup>[16,18]</sup>, UPLC<sup>[19,20]</sup>, HPTLC<sup>[21,22]</sup> method were also used to determine EZE in combination with other drugs in pharmaceutical dosage forms. Chemometry<sup>[23]</sup>, HPLC<sup>[24,26]</sup>, HPTLC<sup>[27,28]</sup> were also used for the estimation of GLIM in combination with other drugs in serum and pharmaceutical preparations.

However there was stability indicating HPLC<sup>[29]</sup> and Spectrophotometry<sup>[30]</sup> method reported for simultaneous estimation of EZE and GLIM in combined dosage form. No reports were found in the literature for stability studies of EZE and GLIM in pharmaceutical dosage form by High performance thin layer chromatography method. The aim of the present study was to develop an accurate, precise, specific, reproducible and stable indicating HPTLC method for the estimation of EZE and GLIM in the presence of their degradation products.

## MATERIALS AND METHODS

### Materials

EZE and GLIM reference standards were provided as gift samples by Dr. Reddy's Laboratories and Ranbaxy Pharmaceuticals Ltd., Gurgaon (Maharashtra, India). Tablet formulation EZIWA labeled to contain 1 mg of GLIM and 10 mg of EZE (Kaytross Health Care Private Ltd., India) was procured from a local pharmacy. Toluene, Methanol, Acetone and Triethylamine (all AR grade) were obtained from Sisco Research Laboratories (Mumbai, India).

### Instrumentation and optimized chromatographic conditions

Chromatographic studies were carried out on CAMAG HPTLC system equipped with Camag Linomat V sample applicator, Hamilton syringe (100  $\mu$ L), Camag TLC Scanner-3 with winCATS software version 1.4.2 and Camag twin- trough chamber (20 $\times$ 20 cm), Silica gel 60 F<sub>254</sub> TLC plates (20 $\times$ 20 cm, layer thickness 0.2 mm, E. Merck, Germany) were used for the present work.

The chromatographic resolution was achieved by linear ascending development in twin trough glass chamber (CAMAG, Muttenz, Switzerland) using Toluene: Methanol: Acetone: Triethylamine (7: 2: 1: 0.2, v/v/v/v) as mobile phase. The chamber was saturated with mobile phase vapor for 15 min. The development distance was 9 cm and the development time approximately 15 min. The slit dimensions 6 mm  $\times$  0.30 mm and scanning speed of 20 mm sec<sup>-1</sup> was employed. After chromatographic development, plates were dried and densitometric estimation was done on CAMAG thin layer chromatography scanner-3 at 246 nm for all developments operated by winCATS software version 1.4.2.

### Selection of detection wavelength

Stock solution of both drugs was prepared in 10 mL of methanol and UV spectra were taken and overlain. Both drugs showed maximum absorbance at 246 nm and hence was selected as wavelength for the detection.

### Preparation of standard stock solutions

Accurately weighed 5 mg of GLIM was dissolved in 50 mL of methanol to get concentration of 100 ng  $\mu$ L<sup>-1</sup>. From the above solution, 5 mL was further diluted to 10 mL with methanol to get solution of final concentration 50 ng  $\mu$ L<sup>-1</sup>. Stock solution of EZE was prepared by dissolving 10 mg of drug in 10 mL methanol which was diluted further to acquire final concentration 500 ng  $\mu$ L<sup>-1</sup>.

### Development of optimum mobile phase

Different solvent systems containing various ratios of toluene, methanol, acetic acid, chloroform, triethylamine and ethyl acetate were examined (data not shown) were examined to separate and resolve spot of GLIM and EZE from its impurities and other excipients present in formulation. Finally, the mobile phase comprising of Toluene: Methanol: Acetone: Triethylamine (7: 2: 1: 0.2, v/v/v/v) was selected as optimal for obtaining well defined and resolved peaks for both drugs.

### Tablet formulation analysis

For tablet formulation analysis, twenty tablets (EZIWA labeled to contain 1 mg of GLIM and 10 mg of EZE) were weighed accurately and finely powdered. A quantity of powder equivalent to 0.5 mg of GLIM (5 mg EZE) was weighed and transferred to a 10 mL volumetric flask containing approximately 7 mL of methanol. The contents were sonicated for 10 min, and volume was made with the methanol. The resulting solution was filtered through Whatman filter paper No. 41 and 1 mL of filtrate was further diluted to 10 mL with methanol. Two micro-liter volume of this solution was applied to a TLC plate to furnish final concentration of 100 ng band<sup>-1</sup> for GLIM and 1000 ng band<sup>-1</sup> for EZE. After chromatographic development the peak areas of the bands were measured at 246 nm and the amount of each drug present in sample was estimated from the respective calibration curves. Procedure was repeated six times for the analysis of homogenous sample.

### Forced degradation study

The stability studies were examined by subjecting the bulk drugs to the physical stress and stability was accessed. The hydrolytic studies were carried out by refluxing the stock solution (100 ng  $\mu$ L<sup>-1</sup> of GLIM and 1000 ng  $\mu$ L<sup>-1</sup> of EZE) with 2 N HCl at 60°C for 1 h and 2 N NaOH at 60°C for 1 h, respectively. The stressed samples of acid and alkali were neutralized with NaOH and HCl, respectively to furnish the final concentration of 50 ng band<sup>-1</sup> for GLIM and 500 ng band<sup>-1</sup> for EZE. Neutral hydrolysis study was performed by refluxing both the drugs with water 60°C for 1 h. The oxidative degradation was carried out in 20% H<sub>2</sub>O<sub>2</sub> at 60°C for 2 h and sample was diluted with methanol. Thermal stress

degradation was performed by keeping both drugs individually in oven at 105°C for period of 6 h. Photolytic degradation studies were carried out by exposure of drugs individually to UV light up to 200 watt h square meter<sup>-1</sup> for 7 d. Thermal and photolytic samples were diluted with methanol to get concentration of 50 ng band<sup>-1</sup> for GLIM and 500 ng band<sup>-1</sup> for EZE. The basic concentration of drugs used for stability study was 200 ng band<sup>-1</sup> for GLIM and 2000 ng band<sup>-1</sup> for EZE.

## RESULTS AND DISCUSSION

### Method optimization

The aim of present research work was to develop stability indicating HPLTC method which would be

capable to give the satisfactory resolution between GLIM, EZE and its degradation products. The separation was achieved by linear ascending development in 10 cm × 10 cm twin trough glass chamber using Toluene: Methanol: Acetone: Triethylamine (7: 2: 1: 0.2, v/v/v/v) as mobile phase. Densitometric detection was performed at 246 nm. The retention factors (R<sub>f</sub>) were found to be 0.28 ± 0.03 for EZE and 0.60 ± 0.001 for GLIM. (Figure 1).

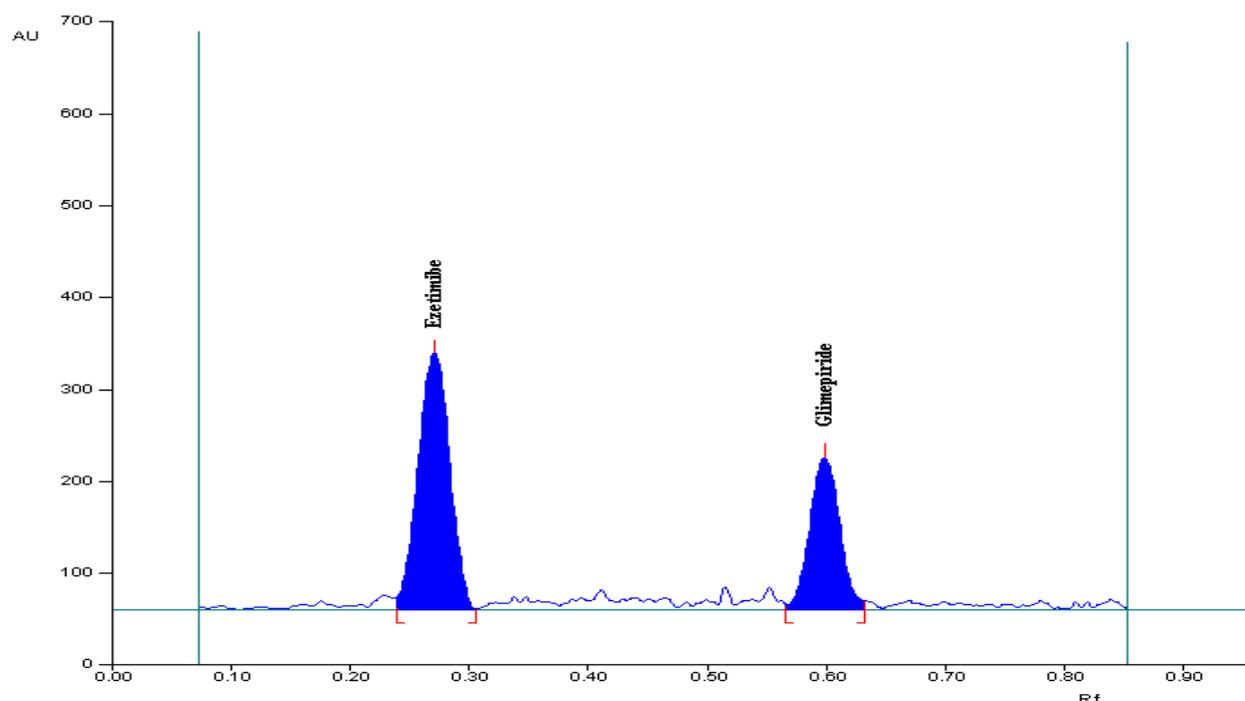


Figure 1: Representative densitogram of mixed standard solution of EZE (2000 ng band<sup>-1</sup>, R<sub>f</sub> = 0.28 ± 0.03) and GLIM (200 ng band<sup>-1</sup>, R<sub>f</sub> = 0.60 ± 0.001).

### Forced degradation study

The stress degradation results indicated the susceptibility of both drugs to acid and base catalysed hydrolysis, oxidation, thermal stress as well as photolysis and stability under neutral hydrolytic condition. Figures 2 and 3 show the densitograms of acid and alkali hydrolytic degradation, while Figures 4-6 show the densitograms of oxidative degradation, thermal degradation and photolytic degradation, respectively. Marked degradation in the densitograms was observed but the degraded products were well resolved from the drugs indicating specificity of the method. The findings of degradation studies are represented in Table 1.

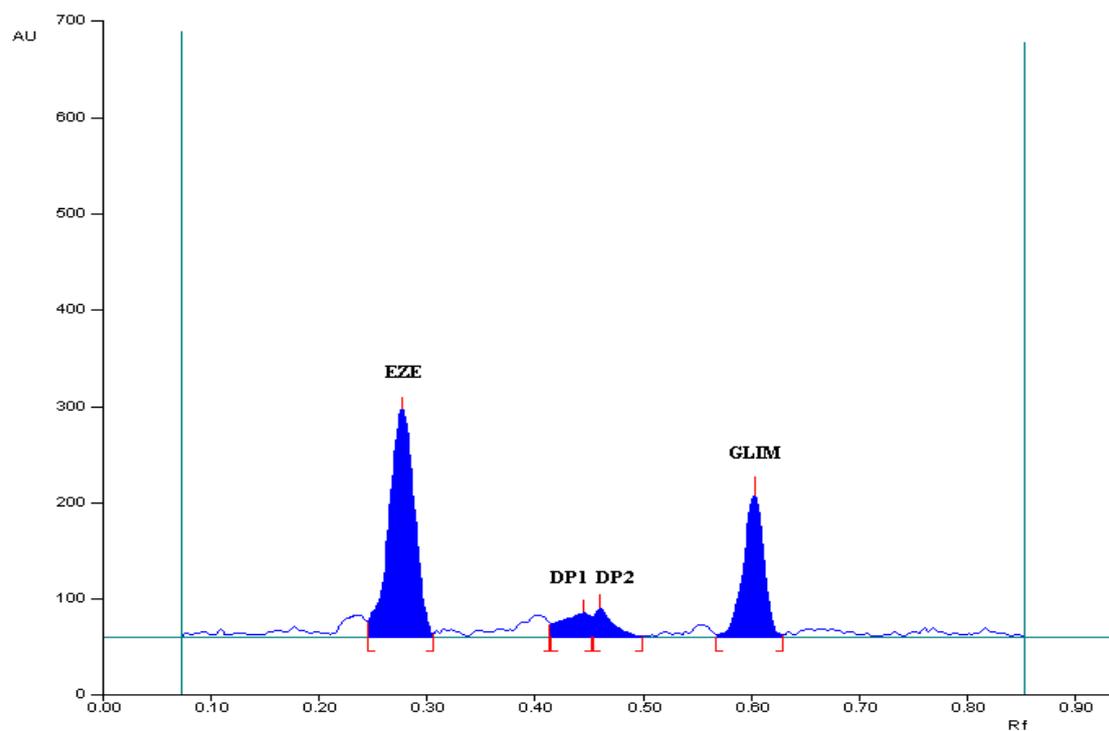


Figure 2: HPTLC densitogram of EZE with degradation products (DP1, R<sub>f</sub> = 0.44 and DP2, R<sub>f</sub> = 0.48) and GLIM after acid hydrolysis with 2 N HCl at 60°C for 1 h.

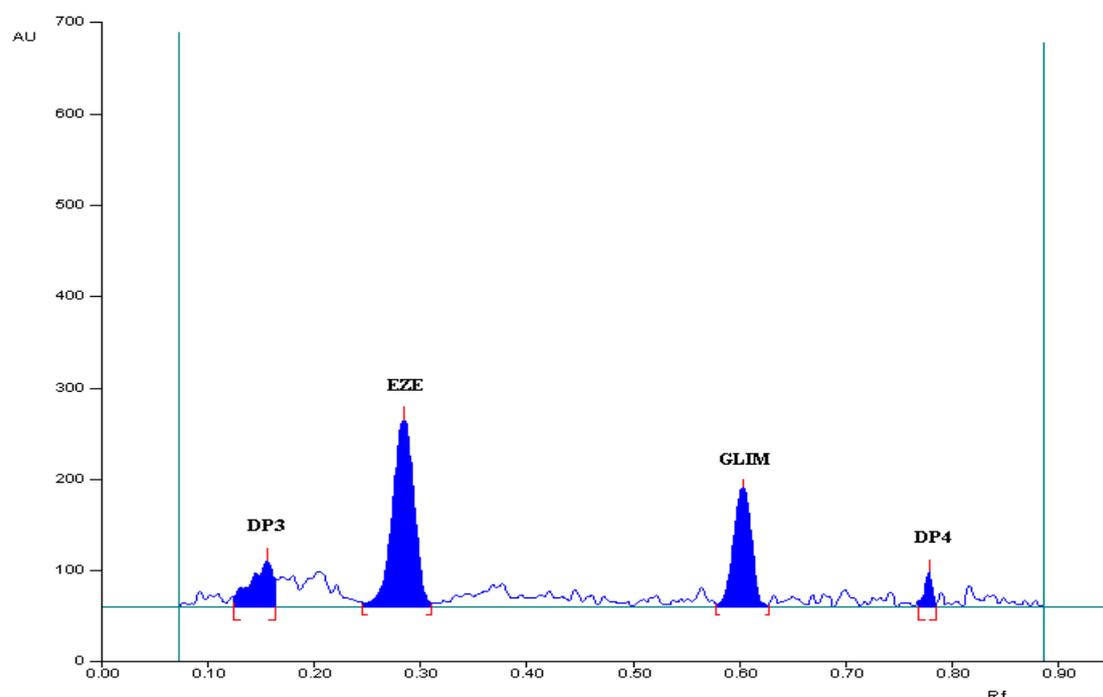


Figure 3: Densitogram of EZE with degradation product (DP3, R<sub>f</sub> = 0.15) and GLIM (DP4, R<sub>f</sub> = 0.78) after base hydrolysis with 2 N NaOH at 60°C for 1 h.

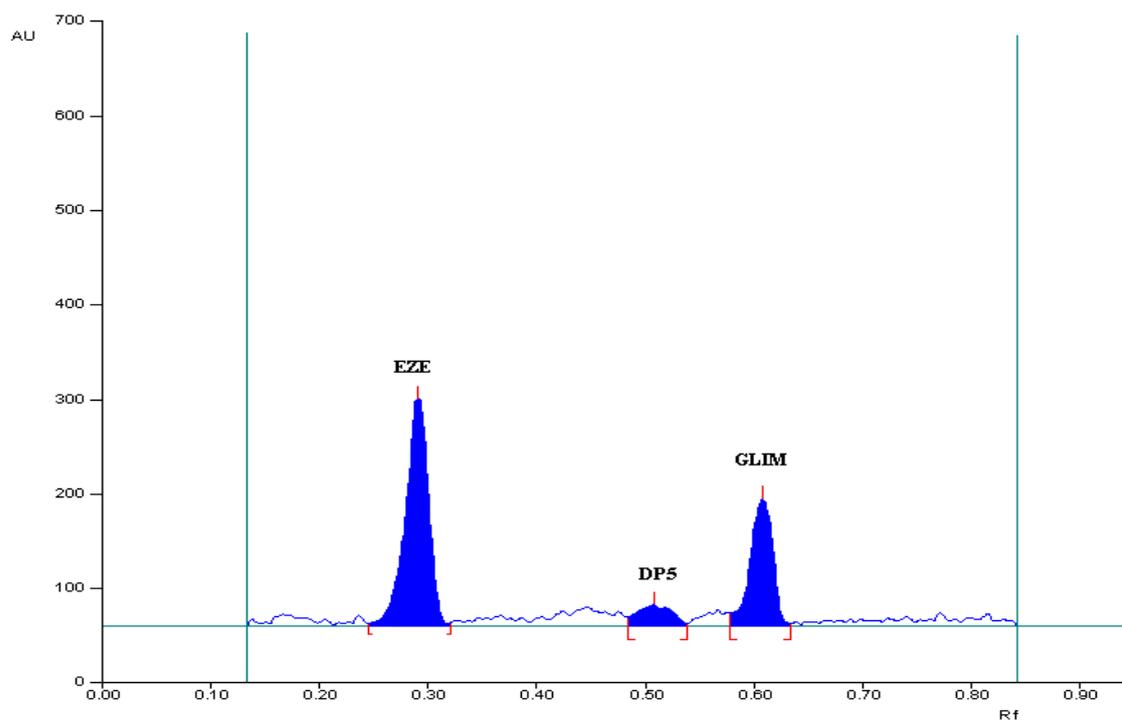


Figure 4: Densitogram of EZE and GLIM with degradation product (DP5, Rf = 0.52) after treatment with 20 % H<sub>2</sub>O<sub>2</sub> at 60°C for 2 h.

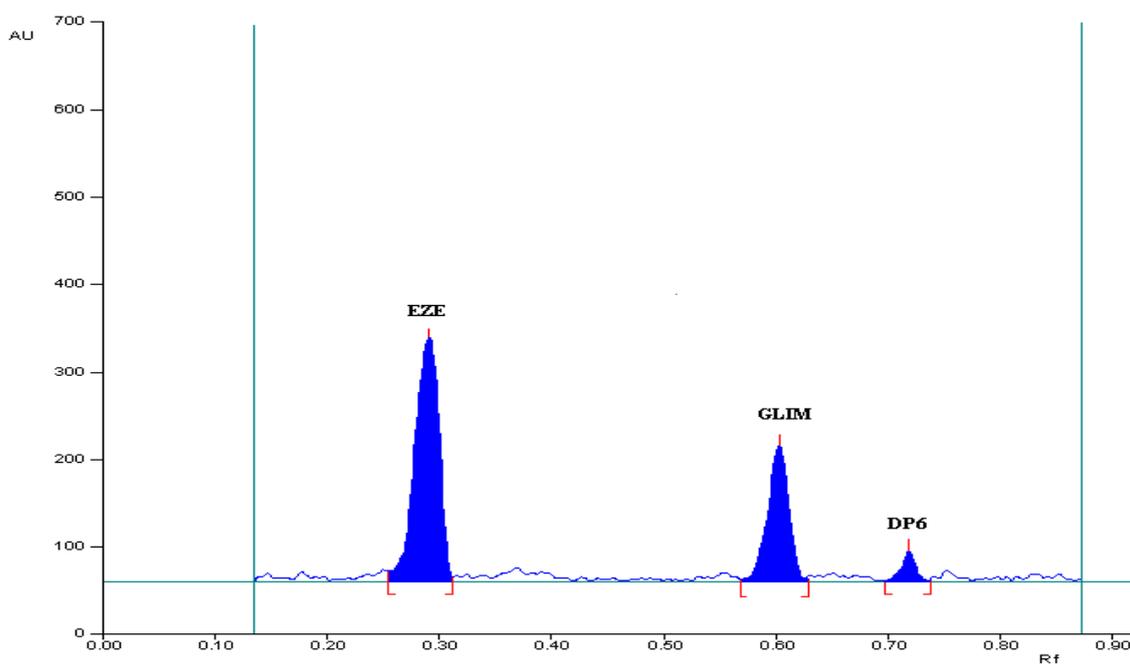


Figure 5: Densitogram of EZE and GLIM with degradation product (DP6, Rf = 0.72) after dry heat at 105°C for 6 h.

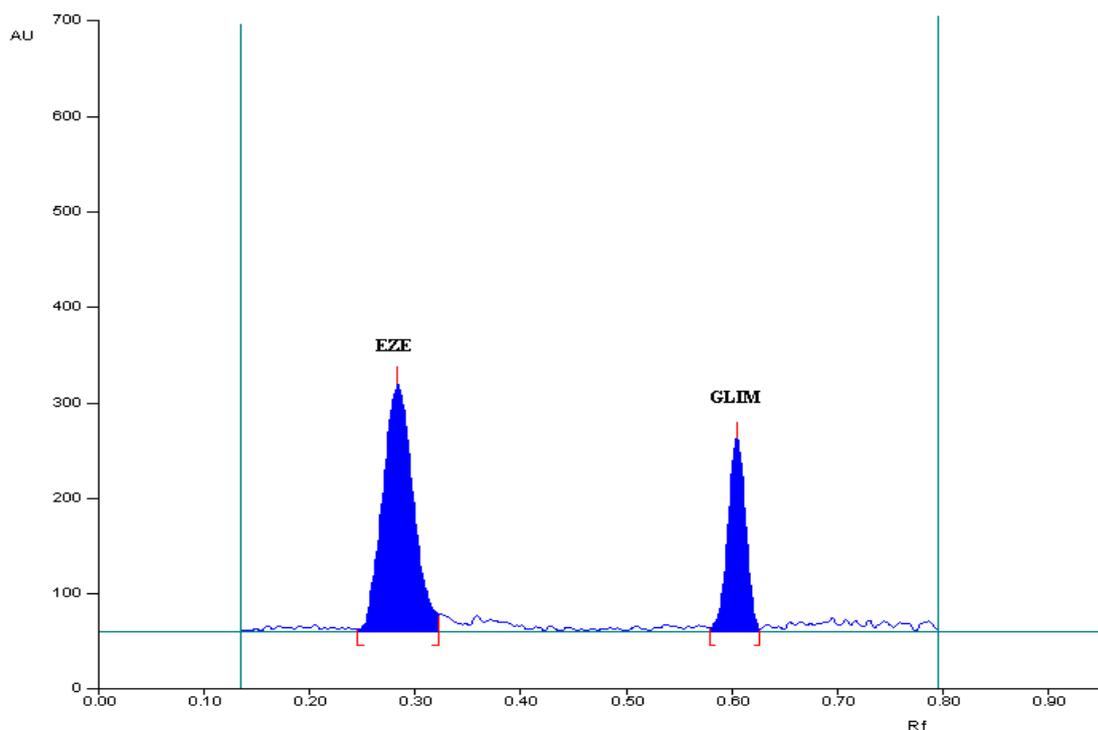


Figure 6: Densitogram EZE and GLIM after exposure to UV light.

Table 1: Results of forced degradation studies.

Stress conditions/ duration	% Assay		% Degradation	
	GLIM	EZE	GLIM	EZE
Acid / 2 N HCl/ Refluxed at 60°C for 1 h	88.50	86.01	11.50	13.98
Alkali / 2 N NaOH/ Refluxed at 60°C for 1 h	87.71	83.89	12.28	16.10
Oxidation / 20 % H <sub>2</sub> O <sub>2</sub> / Refluxed at 60°C for 2 h	86.39	90.54	13.61	09.45
Neutral /Water/ Refluxed at 60°C for 1 h	100.43	99.90	---	---
Photolysis: UV light 200 watt h square meter <sup>-1</sup>	93.41	100.25	10.60	---
Dry heat/ 105°C/ 6 h	89.39	91.80	06.59	08.20

#### Analytical method validation

The developed method was validated with respect to linearity, accuracy, precision, robustness, limit of quantization (LOQ), limit of detection (LOD) to ensure the reliability of results of analysis as per International Conference on Harmonisation (ICH) guidelines for Validation of Analytical Procedures: Text and Methodology Q2 (R1)<sup>[31]</sup> and Stability testing of new drug substances and products, Q1A (R2) (ICH 2005; 2003).<sup>[32]</sup> The linearity of was determined by application of aliquots of 1, 2, 3, 4, 5 and 6  $\mu\text{L}$  of standard solutions of GLIM (50  $\text{ng } \mu\text{L}^{-1}$ ) and EZE (500  $\text{ng } \mu\text{L}^{-1}$ ) separately on TLC plate. The plate was developed and scanned under above established chromatographic conditions. Each standard in six replicates ( $n = 6$ ) was analyzed and peak areas were recorded. The linearity was observed in the range of 50-300  $\text{ng } \text{band}^{-1}$  for GLIM and 500-3000  $\text{ng } \text{band}^{-1}$  for EZE with correlation coefficients 0.999 for both drugs. The Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as signal-to-noise ratio of 3:1 and 10:1. LOD values were found to be 9.37  $\text{ng } \text{band}^{-1}$  and 128.25  $\text{ng } \text{band}^{-1}$  for GLIM and EZE, respectively whereas LOQ values were 28.40  $\text{ng } \text{band}^{-1}$

and 388.66  $\text{ng } \text{band}^{-1}$ . The repeatability of separation was accessed by intra day and inter day precision studies with concentrations of 150, 200 and 250  $\text{ng } \text{band}^{-1}$  for GLIM and 1500, 2000 and 2500  $\text{ng } \text{band}^{-1}$  for EZE. The % RSD values were not more than 2 indicating the precision of developed method. Recovery studies were carried out by addition of standard drug solutions to pre-analyzed sample solution at three different levels 50, 100 and 150%. At each level of the amount, three determinations were carried out. The method was found to be accurate and precise, as indicated by recovery studies as recoveries were close to 100% and % RSD not more than 2 (Table 2). Robustness of the method ( $n = 3$ ) was examined at a concentration level of 300  $\text{ng } \text{band}^{-1}$  for GLIM and 3000  $\text{ng } \text{band}^{-1}$  for EZE under the influence of small, deliberate variations of the analytical parameters. Parameters varied were mobile phase composition ( $\pm 2\%$  methanol), chamber saturation time ( $\pm 10$  min). The areas of peaks of interest remained unaffected by small changes of the operational parameters and % RSD was within the limit ( $< 2\%$ ) indicating the robustness of the developed method.

**Table 2: Recovery Studies of GLIM and EZE.**

Drug	Sample concentration (ng band <sup>-1</sup> )	Added concentration (ng band <sup>-1</sup> )	Total concentration found (ng band <sup>-1</sup> )	% Recovery	% R.S.D.*
GLIM	100	50	149.76	99.84	1.61
	100	100	201.73	100.86	1.57
	100	150	249.71	99.88	0.79
EZE	1000	500	1493.82	99.58	0.77
	1000	1000	1992.50	99.62	0.80
	1000	1500	2501.54	100.05	0.84

\*Average of three determinations.

## CONCLUSIONS

The stability indicating HPTLC method has been developed and validated for the simultaneous determination of GLIM and EZE as bulk drugs and in tablet dosage form. The developed method is simple, precise, accurate, and reproducible and can be used for quantitative analysis of GLIM and EZE in pharmaceutical dosage form as well as for routine analysis in quality control laboratories. The proposed method would be suitable for analysis of GLIM and EZE without any interference from the excipients and can be successfully used to estimate the amount of drugs in the formulations by easily available low cost materials.

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