Analytical Method Development and Validation of Stability Indicating RP-HPLC Method For the Simultaneous Estimation of Pitavastatin Calcium and Ezetimibe in Pharmaceutical Dosage Form

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***Abstract*—Hyperlipidemia is a major clinical concern strongly associated with the development of coronary heart disease. Research has shown that combining statins with ezetimibe improves therapeutic outcomes in the management of this condition. This study aims to develop a simple, accurate, and sensitive stability-indicating method for the simultaneous estimation of Pitavastatin and Ezetimibe in pharmaceutical dosage forms. Chromatographic separation was achieved using an Inert Sustain C18 column (250 mm × 4.6 mm, 5 µm particle size) with a mobile phase comprising buffer and acetonitrile (30:70 v/v). Detection was carried out at 235 nm with a flow rate of 1 mL/min. Method validation was performed according to ICH Q2 (R2) guidelines. The method exhibited linearity over concentration ranges of 20–60 µg/mL for Pitavastatin and 50–150 µg/mL for Ezetimibe, yielding correlation coefficients (R^2) of 0.9996 and 0.9992, respectively. The developed RP-HPLC method demonstrated excellent accuracy, precision, and reproducibility, with no observed interference from excipients. Additionally, the stability-indicating capability was confirmed, showing specificity without interference from placebo or degradation products. Thus, the method is suitable for routine quality control and stability analysis of Pitavastatin and Ezetimibe in pharmaceutical formulations.**

***Index Terms*—Ezetimibe, Method Development and Validation, Pitavastatin, RP-HPLC**

I. INTRODUCTION

Hyperlipidemia (high cholesterol) is an excess of lipids or fats in your blood. This can increase your risk of heart attack and stroke because blood can't flow through your arteries easily. Adding exercise and healthy foods can lower your cholesterol. Some people need medication as well. Managing your cholesterol is a long-term effort.(1) People who need medicine to treat their high cholesterol usually take statins. Statins are a type of medication that decreases how much bad cholesterol is circulating in your blood. (2)

Pitavastatin calcium is a novel statin used to lower cholesterol and induce plaque regression while elevating HDL-cholesterol levels. Chemically, it is the calcium salt of (E,3R,5S)-7-[2-cyclopropyl-4-(4-fluorophenyl) quinolin-3-yl]-3,5-dihydroxyhept-6-enoate pentahydrate ($C_5H_{24}FNO_4$; MW 440.49 g/mol). Pitavastatin exhibits high protein binding (>99%) primarily to albumin and alpha-1 acid glycoprotein, with a mean volume of distribution of 148 L. It functions as a competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, thereby reducing hepatic cholesterol and upregulating LDL receptors to enhance clearance of circulating LDL-C. The calcium salt formulation has moderate solubility (~0.5 mg/mL in DMF: PBS, pH 7.2) and primarily exerts its effects in the liver. (3)

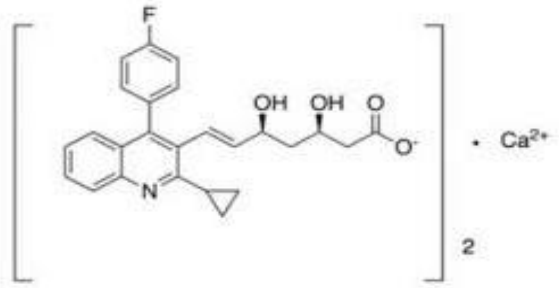

FIGURE NO.1- STRUCTURE OF PITAVASTATIN

Ezetimibe is a cholesterol-lowering medication that selectively inhibits the absorption of cholesterol and phytosterols in the small intestine without affecting the uptake of fat-soluble vitamins and other nutrients. Chemically, it is (3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl) azetidin-2-one ($C_{24}H_{21}F_2NO_3$; MW 409.4 g/mol). Ezetimibe is a white crystalline powder that is freely to very soluble in ethanol, methanol, and acetone but practically insoluble in water. It belongs to the class of selective cholesterol-absorption inhibitors and is widely used to manage hypercholesterolemia. (4)

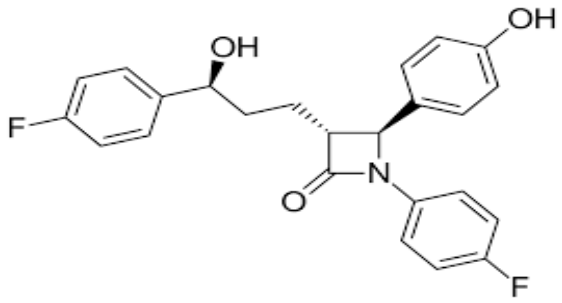

FIGURE NO.2- STRUCTURE OF EZETIMIBE

A literature review revealed that while a few UV spectrophotometric and HPLC methods have been reported for the estimation of PITA and EZET individually and in combination with other drugs, no method has been reported for their simultaneous estimation in a combined dosage form. The developed method was validated as per ICH guidelines. Stress testing under various conditions such as pH (acid/base), temperature, light, oxidation, etc. was also carried out. The present work objective is to develop novel, accurate and precise method for simultaneous estimation.

II. MATERIAL AND METHODS

CHEMICAL REAGENTS

All chemicals and solvents used in this study were of analytical or HPLC grade. Methanol and acetonitrile were obtained from Rankem (HPLC grade, Pvt. Ltd.), while potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and HPLC-grade water were purchased from Merck Life Science Pvt. Ltd. All reagents were used as received without further purification.

INSTRUMENTS

The instruments used in this study included a Shimadzu UV-1800 double-beam UV–VIS spectrophotometer with UV Probe software, and a Shimadzu HPLC system (LC 2010 CHT) equipped with a 100 µL fixed-loop injector, UV detector, and LC Solution software. Other laboratory equipment comprised a Sartorius analytical balance (accuracy 0.001 g), Agilent Cary 630 FT-IR spectrometer with MicroLab Expert software. All instruments were used according to the manufacturer's instructions.

PREPARATION OF MOBILE PHASE

Buffer: Buffer was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml of water.

DILUENT

Pitavastatin (40 µg/mL) and Ezetimibe (100 µg/mL) were prepared in a mixed standard solution using methanol as the diluent. Complete solubility and compatibility with the chromatographic conditions were given by methanol.

III. STOCK SOLUTION PREPARATION

A. PITA (400 µG/ML)

About 21 mg of Pitavastatin calcium (equivalent to 20 mg of pitavastatin) working standard was accurately weighed and transferred into 50 ml volumetric flask. To this, 1 ml of 0.1 N HCL was added to solubilize pitavastatin calcium. Then 20 ml of diluent was added and dissolved by sonication for 10 minutes. The solution was diluted up to the mark with diluent and used as a stock solution.

B. EZET (1000 µG/ML)

About 50 mg of Ezetimibe working standard was accurately weighed and transferred into 50 ml volumetric flask. To this, 30 ml of diluent was added and dissolved by sonication. The solution was diluted up to the mark with diluent and used as a stock solution.

WORKING STANDARD SOLUTION PREPARATION

A. PITA (40 µG/ML)

To prepare the working standard solution, 1 mL of the stock solution was pipetted into a 10 mL volumetric flask and diluted to the mark with methanol.

B. EZET (100 µG/ML)

To prepare the working standard solution, 1 mL of the stock solution was pipetted into a 10 mL volumetric flask and diluted to the mark with methanol.

C. MIXED STANDARD SOLUTION (40 µG/ML PITA + 100 µG/ML EZET)

To prepare the mixed working standard solution, 1 mL each of the PITA stock solution and the EZET stock solution was pipetted into a 10 mL volumetric flask, and the volume was adjusted to the mark with methanol.

SAMPLE PREPARATION

Finely powder the sample of Pitavastatin Calcium (PITA) using a mortar and pestle to ensure uniformity. Repeat the same for Ezetimibe (EZET).

IV. METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

The aim of the present study was to develop and validate a stability-indicating RP-HPLC method for the simultaneous estimation of Pitavastatin Calcium and Ezetimibe in a pharmaceutical dosage form. During method development, key chromatographic parameters such as the mobile phase composition, flow rate, detection wavelength, column type, and column temperature were carefully optimized to improve the performance and reliability of the chromatographic system. Multiple analytical columns, including the InertSustain C18 (250 mm × 4.6 mm, 5 µm), were investigated for their efficiency. System suitability parameters—such as retention time, number of theoretical plates, tailing factor, and resolution—were evaluated to identify the most appropriate column, with the InertSustain C18 being selected as optimal. Various combinations of methanol, acetonitrile, and buffer in different ratios were tested to achieve the best possible separation of the analytes.

Chromatographic Mode	Reversed Phase
Mobile Phase	Buffer: ACN (30:70 % v/v)
Column	Inert Sustain C18 (250 mm x 4.6 mm x 5 µ)
Flow Rate	1.0 ml/min
Detection Wavelength	235 nm
Injection Volume	30 µL
Column Tempreture	25°C
Run Time	10 min
Mode of Elution	Isocratic
Diluent	Mixed Standard PITA (40 µg/ml) and EZET (100 µg/ml)

V. VALIDATION OF RP-HPLC METHOD

“Validation is the process of providing documented proof that a certain method will regularly result in a product that fulfills the desired standard and quality criteria.” (5)

A method’s performance parameter and limitation, as well as the variable that may have an impact on these qualities and to what extent are all determined through the process of method validation. Method compatibility for the use that it is intended for is also verified. The validation procedure makes sure that analytical techniques are carefully examined and approved for usage or rejected if they don't adhere to the required criteria.

The proposed RP-HPLC method was validated in accordance with the ICH Q2 (R2) guidelines. The validation parameters evaluated included system suitability, precision, accuracy, specificity, linearity, range, limit of detection (LOD), limit of quantification (LOQ), robustness, and stability of the analyte in the analytical solution. Each parameter was assessed to ensure the method's reliability, reproducibility, and suitability for the simultaneous estimation of Pitavastatin Calcium and Ezetimibe in a pharmaceutical dosage form.

VI. RESULT & DISCUSSION

SELECTION OF WAVELENGTH

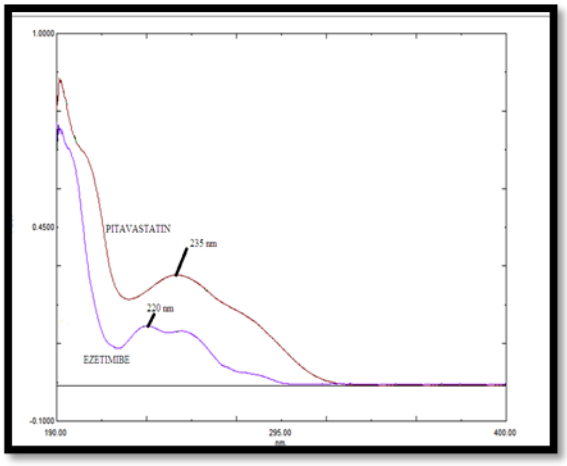

FIGURE NO.3- OVERLAIN UV SPECTRA OF PITA AND EZET

SYSTEM SUITABILITY STUDY

Prior to method validation, system suitability tests were carried out to ensure the performance of the chromatographic system. The results met the predefined acceptance criteria: the retention times for PITA and EZET were consistent, the theoretical plate count exceeded 2000, the tailing factor was less than 2, and the resolution between the peaks was within acceptable limits. > 2 and % RSD of peak areas of six injections were not more than 2%.

TABLE NO. 1- SYSTEM SUITABILITY STUDY

Sr. NO	System Suitability Parameters	PITA		EZET	
		Mean (n = 6)	% RSD	Mean (n = 6)	% RSD
1	Theoretical Plate	2542	1.12	2872	1.45
2	Retention Time	4.538	1.56	6.232	0.67
3	Tailing Factor	1.27	0.89	1.25	0.90
4	Resolution	0.0	0.34	4.31	0.0

SPECIFICITY

The specificity of the developed method was evaluated to confirm that there was no interference from other compounds or excipients in the analysis. This was demonstrated by the chromatograms shown in Figures 4 to 6.

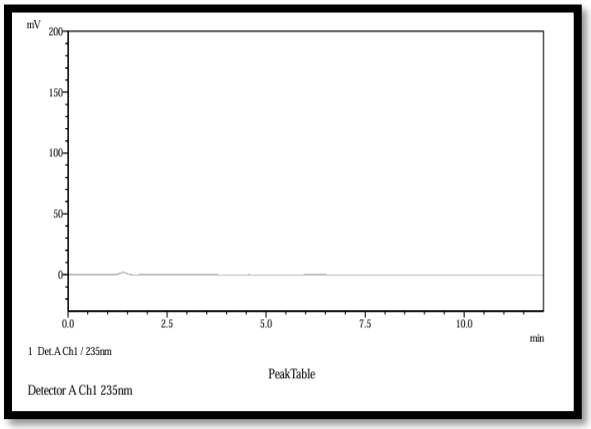

FIGURE NO. 4- CHROMATOGRAM OF DILUENT

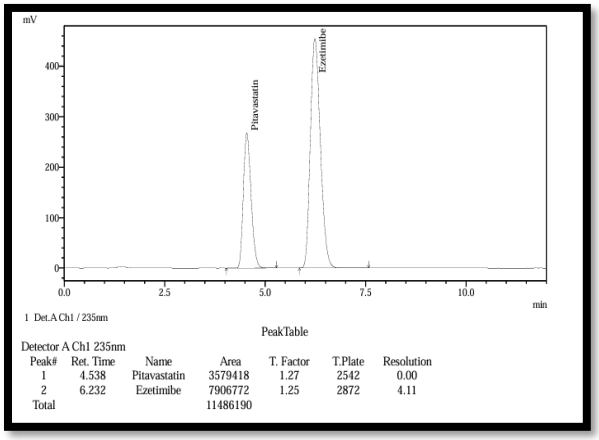

FIGURE NO.5- OVERLAY SPECTRA OF STANDARD OF PITA AND EZET

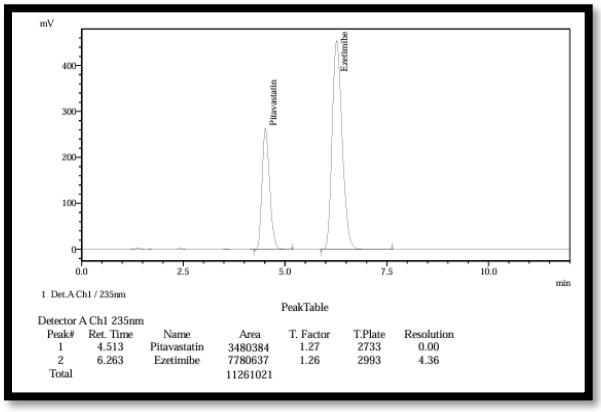

FIGURE NO.6- OVERLAY SPECTRA OF SAMPLE OF PITA AND EZET

LINEARITY AND RANGE

Linearity was evaluated by determining five standard solutions of PITA and EZET in triplicate. The method was linear over the concentration range 20-60 µg/ml with Corelation co-efficient value R^2 = 0.9996 for PITA and the concentration range 50 – 150 µg/ml with correlation co-efficient value R^2 = 0.9992 for EZET. The regression line equation for PITA and EZET were found to be y = 88849x + 71074 and y = 80708x + 315302 respectively. The results of Linearity for PITA and EZET was shown in Table 2 and 3 respectively. Calibration Curve of PITA and EZET was Shown in Figure 7 and 8 respectively. Overlay of Linearity shown in Figure 9.

TABLE NO. 2- LINEARITY DATA FOR PITA

Sr. No.	Concentration (µg/ml)	Peak Area
1	20.00	1842397
2	30.00	2769242
3	40.00	3583648
4	50.00	4520924
5	60.00	5409021

TABLE NO. 3- LINEARITY DATA FOR EZET

Sr. No.	Concentration (µg/ml)	Peak Area
1	50.00	4306669
2	75.00	6404811
3	100.00	8353058
4	125.00	10537133
5	150.00	12329065

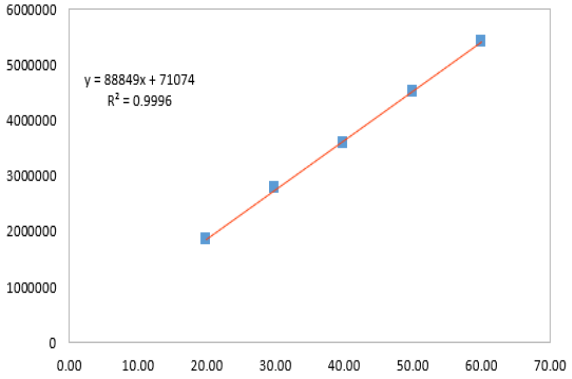

FIGURE NO.7- CALIBRATION CURVE OF PITA

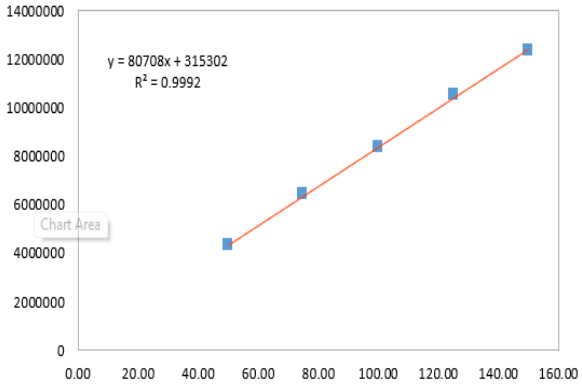

FIGURE NO.8- CALIBRATION CURVE OF EZET

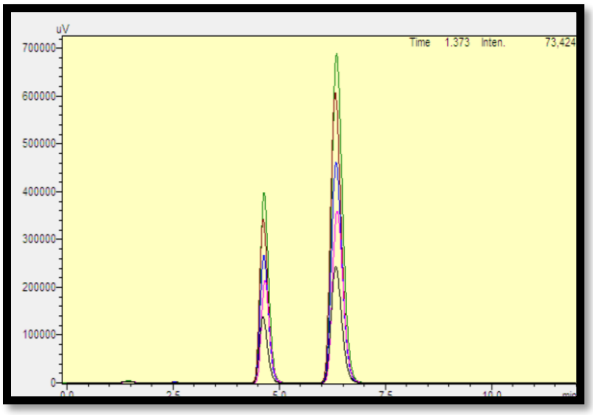

FIGURE NO. 9- OVERLAY LINEARITY CHROMATOGRAM OF PITA AND EZET

PRECISION

REPEATABILITY

The repeatability data was shown in Table 4. The % RSD was found to be 1.54 % for PITA and 0.72 % for EZET.

TABLE NO. 4- REPEATABILITY DATA OF PITA AND EZET

Conc of PITA	Peak area	Conc of EZET	Peak area
40 µg/ml	3579418	100 µg/ml	7906772
	3449198		7759552
	3448189		7768515
	3449938		7781528
	3448676		7772975
	3445455		7767866
Mean (n = 6)	3470146	Mean (n = 6)	7792868
SD	53554.21	SD	56261.13
% RSD	1.54	% RSD	0.72

INTRADAY PRECISION

Same day precision was evaluated by injecting six solutions (n = 6) of sample preparations prepared from Sample solution (PITA 40 µg/ml and EZET 100 µg/ml) as per the proposed method.

TABLE NO. 5- INTRA DAY PRECISION DATA OF PITA AND EZET

Conc of PITA	Peak area	Conc of EZET	Peak area
40 µg/ml	3425760	100 µg/ml	7768987
	3521035		7993565
	3529821		8036544
	3494876		7917863
	3636709		8156553
	3532691		8051598
Mean (n = 6)	3492205.333	Mean (n = 6)	7933032
SD	68292.345	SD	132458.90
% RSD	1.95	% RSD	1.66

INTER DAY PRECISION

To evaluate inter day precision, solutions containing PITA at concentrations of 20, 40, and 60 µg/mL, and EZET at 50, 100, and 150 µg/mL were analyzed over three consecutive days. The inter day precision data are presented in Table 6.

TABLE NO. 6- INTER DAY PRECISION DATA OF PITA AND EZET

Drug	Concentration (µg/ml)	Mean peak area ± SD (n = 3)	% RSD
PITA	20	1755304 ± 11548.1219	0.66
	40	3426994 ± 26978.63	0.79
	60	5278287 ± 49611.04	0.94
EZET	50	4122160 ± 27366.1438	0.66
	100	7844652 ± 62494.54	0.80
	150	11892463 ± 145548.43	1.22

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

Using slope and Y-intercept, the determined values of LOD and LOQ were evaluated.

All the results of LOD and LOQ was shown in Table 7.

TABLE NO. 7- LIMIT OF DETECTION AND LIMIT OF QUANTITATION DATA FOR PITA AND EZET

Parameters	LOD (µg/ml)	LOQ (µg/ml)
PITA	0.21	0.64
EZET	0.60	1.82

Accuracy (n=3)

The accuracy of the methodology was evaluated by determining the recoveries of PITA and EZET using the standard addition method. The accuracy data for PITA and EZET are presented in Tables 8 and 9. The results demonstrate that the method provides accurate measurements for both PITA and EZET.

TABLE NO. 8- ACCURACY DATA OF PITA

Name of drug	Conc Level (%)	Added (ml)	Amount Added (mg)	Amount Recovered (mg)	% Recovery	%Mean Recovery	% RSD
PITA	50 %	0.500	0.200	0.204	102.2	101.7	0.67
	50 %	0.500	0.200	0.202	101.1		
	50 %	0.500	0.200	0.204	101.9		
	100%	1.000	0.400	0.407	101.6	101.8	0.34
	100%	1.000	0.400	0.409	102.2		
	100%	1.000	0.400	0.406	101.6		
	150 %	1.500	0.600	0.611	101.8	101.9	0.14
	150 %	1.500	0.600	0.612	101.9		
	150 %	1.500	0.600	0.612	101.9		

TABLE NO. 9- ACCURACY DATA OF EZET

Name of drug	Conc Level (%)	Added (ml)	Amount Added (mg)	Amount Recovered (mg)	% Recovery	%Mean Recovery	% RSD
EZET	50 %	0.500	0.500	0.505	101.0	101.8	0.89
	50 %	0.500	0.500	0.508	101.7		
	50 %	0.500	0.500	0.513	102.7		
	100%	1.000	1.000	0.988	98.8	99.2	0.49
	100%	1.000	1.000	0.991	99.1		
	100%	1.000	1.000	0.996	99.6		
	150 %	1.500	1.500	1.520	101.3	100.4	1.15
	150 %	1.500	1.500	1.510	100.6		
	150 %	1.500	1.500	1.488	99.2		

OBSERVATION:

Accuracy of the method was confirmed by recovery study at three levels (50%, 100% and 150%) of standard addition. The method was found to be accurate as the % recovery ranged from 98.2 – 101.2 %.

ROBUSTNESS

Robustness was assessed by introducing small, deliberate variations in method parameters, including temperature (±5°C), flow rate (±0.2 mL/min), and organic phase composition (±2%). The % RSD for both PITA and EZET remained below 2%, indicating that the method is robust under these modified conditions. The results are presented in Tables 10 and 11.

TABLE NO. 10- ROBUSTNESS DATA OF PITA

Sr. No.	Area atTemp.-5°C	Area at Temp.+5°C	Area at Flow Rate-10%	Area at Flow Rate+10%	Area atOrganic Phase-2%	Area atOrganic Phase+2%
1	3395864	3398827	3727217	3073217	3366755	3422804
2	3393452	3402990	3730555	3078849	3370325	3419042
3	3398425	3407966	3724831	3075682	3370336	3430221
Mean	3395914	3403261	3727534	3075916	3369139	3424022
% RSD	0.14	0.19	0.10	0.12	0.11	0.26
Theoretical Plates	2734	2708	2670	2733	2735	2668
Tailing Factor	1.27	1.27	1.25	1.28	1.27	1.27

TABLE NO. 11- ROBUSTNESS DATA OF EZET

Sr. No.	Area atTemp.-5°C	Area at Temp.+5°C	Area at Flow Rate-10%	Area at Flow Rate+10%	Area atOrganic Phase-2%	Area atOrganic Phase+2%
1	7836996	7839086	8583906	7081657	7811791	7792893
2	7833655	7850776	8595293	7094557	7820869	7785462
3	7843637	7866842	8585288	7089035	7825110	7801868
Mean	7838096	7852235	8588162	7088416	7819257	7793408
% RSD	0.1	0.2	0.1	0.1	0.1	0.1
Theoretical Plates	3089	2915	2858	3029	2930	2940
Tailing Factor	1.25	1.25	1.23	1.27	1.24	1.26

OBSERVATION

The typical variations studied under this parameter were temperature, flowrate and Organic phase. Overall

% RSD was found to be less than 2% for all the variations which indicates that the proposed method is robust.

ASSAY OF MARKETED FORMULATION

Applicability of the proposed method was tested by analyzing the formulation. Results as % Assay is shown in Table 12.

TABLE NO. 12- ASSAY OF FORMULATION

Drug	Label Claim	Amount Found	Assay (%) Mean ± SD (n = 3), % RSD
PITA	4 mg	4.05 mg	101.3 ± 0.143, 0.14
EZET	10 mg	10.2 mg	102 ± 0.784, 0.76

The assay outcomes were comparable to each drug's labelled value in the Formulation. These results indicates that the developed method is specific, accurate, precise, simple, sensitive, robust and rapid

SUMMARY

Summary of Validation parameters of RP-HPLC Method for PITA and EZET were shown in Table 13.

TABLE NO. 13- SUMMARY OF RP-HPLC VALIDATION PARAMETERS OF PITA AMD EZET

Parameters	PITA	EZET
Linearity Range (µg/ml)	20 – 60	50– 150
ssion equation, y = mx + c	y = 88849x + 71074	y = 80708x + 315302
Correlation Coefficient (R^2)	0.9996	0.9992
Detection limit(µg/ml)	0.21	0.60
Quantitation limit(µg/ml)	0.64	1.82
tability (%RSD, n = 6)	1.54	0.72
Intra-day, % RSD	1.95	1.66
Inter-day (n = 3), % RSD	0.66 – 0.79	0.66 – 1.22
acy (%recovery % RSD (n= 3)	7 -101.9, 0.14-0.67	99.2-101.8, 0.49- 1.15
Robustness		
e in Temperature (+5 ℃ and -5 ℃) Change in Flow rate (+1 ml/min and -1 ml/min) Change in Organic Phase (+ 2 % and -2 %)	All are Within the Range	
amount found % label claim ± SD	.05, 102 ± 0.143	10.2, 102 ± 0.784

VII. CONCLUSION

A simple, precise, and accurate stability-indicating RP-HPLC method was developed and validated for the simultaneous estimation of Glimepiride and Sitagliptin Phosphate Monohydrate in a synthetic mixture. Separation was achieved using a mobile phase consisting of Buffer: Methanol (33:67 % v/v) at a detection wavelength of 235 nm. A forced degradation study was performed under various stress conditions, including acidic, basic, oxidative, and thermal environments. The degraded components were well separated from the main drug peaks, with no interference from the active pharmaceutical ingredient (API). The degradation study also revealed that degradation products could be isolated and characterized in sufficient quantities for further analysis. The method demonstrated specificity, and all tested parameters met the acceptance criteria. Therefore, this method is suitable for stability assessment of Glimepiride and Sitagliptin Phosphate in pharmaceutical formulations.

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[4] Drug profile of Ezetimibe https://go.drugbank.com/drugs/DB00973

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