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Simultaneous Determination and Validation of Pitavastatin Calcium and Ezetimibe in Binary Mixture by Liquid Chromatography

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Abstract: A simple and accurate method to determine pitavastatin calcium (PIT) and ezetimibe (EZE), in binary mixture, was developed and validated using liquid chromatography (LC). The LC separation was achieved on a Phenomenex Luna C_{18} column (250 mm, 4.6mm i.d., 5µm), in the isocratic mode using 0.1 % orthophosphoric acid: acetonitrile: triethylamine (19.8: 80: 0.2, v/v/v), pH 3 ± 0.05, at a flow rate of 1.4 mL/min. The retention times were about 6.98 and 2.36 min for PIT and EZE, respectively. Quantification was achieved with Photodiode array (PDA) detector at 235 nm over the concentration range of 0.5-5 µg/mL for each, with mean recoveries of 99.35 ± 0.19 % and 99.51 ± 0.23 % for PIT and EZE, respectively. The method was validated, and was found to be simple, specific, accurate, precise and robust. The method was successfully applied for the determination of PIT and EZE in binary mixture without any interference from common excipients.
Key words: Pitavastatin calcium, Ezetimibe, Binary Mixture, Liquid Chromatography.

INTRODUCTION

Pitavastatin (PIT), (3R, 5S, 6E)-7-[2-cyclopropyl-4-(p-fluorophenyl)-3-quinolyl]-3, 5-dihydroxy-6-heptenoic acid, is a novel, fully synthetic statin, which has a more potent cholesterol-lowering action than other drugs in its class. PIT is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, used as the calcium salt in the treatment of hyperlipidemia[1,2]. Ezetimibe (EZE, 1-(4-fluoro phenyl)-3-[3-(4-fluorophenyl)-3-hydroxy-propyl]-4-(4-hydroxyphenyl)-azetidin-2-one, is a lipid lowering agent which selectively inhibits the intestinal absorption of cholesterol and related phytosterols [3].

Literature survey revealed several analytical methods such as spectrophotometry[4, 5], simple high performance thin layer chromatography (HPTLC) [6, 7], simple and column-switching high performance liquid chromatography (HPLC) with UV and PDA detection [8-12], HPLC with fluorescence detection [13], stability indicating HPLC [14, 15], ultra fast performance chromatography (UPLC) [16] LC-MS/MS [17, 18] and LC-ESI-MS [19] have been reported for the determination of PIT in pharmaceutical dosage forms and or in biological samples. Literature survey revealed several analytical methods such as simple and stability indicating spectrophotometry [20, 21], simple and stability indicating HPTLC [22, 24], simple and column-switching

HPLC with UV detection [25-28], stability indicating HPLC [29-31], LC-MS/MS [32, 33] and LC-ESI-MS [34, 35] have been reported for the determination of EZE in pharmaceutical dosage forms and biological samples.
PIT and EZE combination has additive effects, the concomitant use of the PIT and EZE according to the European Patent has a remarkable blood cholesterol level lowering effect, compared with that of the concomitant use of another HMG-CoA reductase inhibitor and EZE[36]. Also Phase-I clinical trial has been completed, by Schering-Plough, to evaluate the pharmacokinetic interaction and safety of co-administration of PIT and EZE in high cholesterol levels [37]. Hence, the binary mixture is selected.
There is no method reported for the estimation of PIT and EZE in combination forms. Hence, the aim was to develop a selective, sensitive and accurate method which can determine both components simultaneously. The present investigation describes a simple, accurate, sensitive and precise Liquid chromatographic method for simultaneous determination of PIT and EZE in combined form.

EXPERIMENTAL

Reagents and materials

PIT and EZE powder with 99.94 and 99.96 % purity, respectively. LC grade methanol and acetonitrile were from Finar Chemicals Pvt. Ltd. (Ahmedabad, India). LC grade methanol, acetonitrile and triethylamine were from SD Fine Chemicals Pvt. Ltd. (Ahmedabad, India) and orthophosphoric acid was from Spectrochem Pvt. Ltd. (Mumbai, India). The water for LC was prepared by triple glass distillation and filtered through nylon 0.45µm-47mm membrane filter (Millipore, Bedford, MA).

Apparatus and chromatographic conditions

A Shimadzu (Kyoto, Japan) LC system (LC-2010CHT) equipped with autosampler, photodiode array (PDA) detector and Phenomenex (Torrence, CA) Luna C_{18} column (250mm, 4.6mm i.d., 5µm) was used. A Sartorius CP224S (Gottingen, Germany) analytical balance, and an ultra sonic cleaner (Frontline FS 4, Mumbai, India) were used. The LC system was operated isocratically at 25 ± 2 ^{0}C using mobile phase comprised of 0.1 % orthophosphoric acid: acetonitrile: triethylamine (19.8: 80: 0.2, v/v/v), pH 3 ± 0.05, at a flow rate of 1.4 mL/min. The mobile phase was filtered through nylon 0.45µm-47mm membrane filter and was degassed before use. The determination was performed at 235 nm using LC solution (v 1.2; Shimadzu) software. The injection volume was 20 µL and the total run time was 10 min.

Preparation of PIT and EZE Standard solutions

Accurately weighed PIT (10 mg) and EZE (10 mg) standards were transferred to a 50 mL volumetric flask, dissolved in and diluted to the mark with methanol to obtain a standard stock solution (200 µg/mL) for PIT and EZE, each. An aliquot (2.5 mL) of the solution was transferred to a 50 mL volumetric flask, and diluted to the mark with mobile phase to obtain a working standard solution (10 µg/mL) for PIT and EZE, each.

Preparation of Sample solution

The binary mixture of PIT and EZE was prepared in the ratio of 1:5. Accurately weighed PIT (2 mg) and EZE (10 mg) was transferred to a 50 mL volumetric flask and methanol (30 mL) was added. Common excipients, which are used in the tablet formulation, were added in this mixture and the content was sonicated for 15 min. The flask was allowed to stand at room temperature for 5 min, and the volume was diluted to the mark with methanol to obtain the sample stock solution (40 and 200 µg/mL) of PIT and EZE, respectively. The solution was filtered through 0.45µm-47mm membrane filter. Sample stock solution (2.5 mL) was transferred to a 50 mL volumetric flask, and diluted to the mark with mobile phase to obtain working sample solution (2 and 10 µg/mL) for PIT and EZE, respectively. An aliquot (2.5 mL) was transferred to a 10 mL volumetric flask, and diluted to the mark with mobile phase to obtain the sample solution (0.5 and 2.5 µg/mL) for PIT and EZE, respectively.

Method validation

The methods were validated for the following parameters following the International Conference on Harmonization (ICH) guidelines [38].

Specificity

The specificity of the methods was established by comparing the chromatograms and measuring the peak purities of PIT and EZE from standard and sample solutions of the binary mixture. The peak purity spectra of PIT and EZE were recorded using PDA detector.

Linearity (Calibration curve)

Aliquots (0.5, 1, 2, 3, 4 and 5 mL) of mixed working standard solution (equivalent to 0.5, 1, 2, 3, 4 and 5 µg/mL for PIT and EZE, each) were transferred in a series of 10 mL volumetric flasks, and the volume was made up to the mark with mobile phase. An aliquot (20 µL) of each solution was injected under the operating chromatographic conditions as described above. Responses were recorded. Calibration curves were

constructed by plotting the peak areas versus the concentrations, and the regression equations were calculated. Each response was average of three determinations.

Accuracy (% Recovery)

The accuracy of the methods was determined by calculating recoveries of PIT and EZE by the standard addition method. Known amount of standard solutions of PIT (0, 0.5, 1.5 and 2.5 µg/mL) and EZE (0, 0.5, 1.5 and 2.5 µg/mL) were added to a prequantified sample solutions of PIT and EZE (0.5 and 2.5 µg/mL). Each solution was injected in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves.

Precision

The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentrations of PIT (1, 3 and 5 µg/mL) and EZE (1, 3 and 5 µg/mL). The results are reported in terms of relative standard deviation.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the PIT and EZE were calculated using the standard deviation of responses and slopes using signal-to-noise ratio.

Robustness

The robustness was studied by analysing the same samples of PIT and EZE by deliberate variation in the method parameters. The change in the responses of PIT and EZE were noted. Robustness of the method was studied by changing the extraction time of PIT and EZE from binary mixture by ± 2 min, composition of mobile phase by ± 2 % of organic solvent, flow rate by ± 0.2 mL/min.

System-Suitability Test

System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were asymmetry of the chromatographic peak, peak resolution and repeatability, as RSD of peak area for replicate injections. The precision of the instruments was checked by repeatedly injecting (n = 6) solution of PIT and EZE (2 µg/mL, each).

Determination of PIT and EZE in binary mixture

The responses of sample solutions were measured at 235 nm for quantitation of PIT and EZE by the proposed method. The amount of PIT and EZE present in the sample solutions were determined by fitting the responses into the regression equations of the calibration curve for PIT and EZE, respectively.

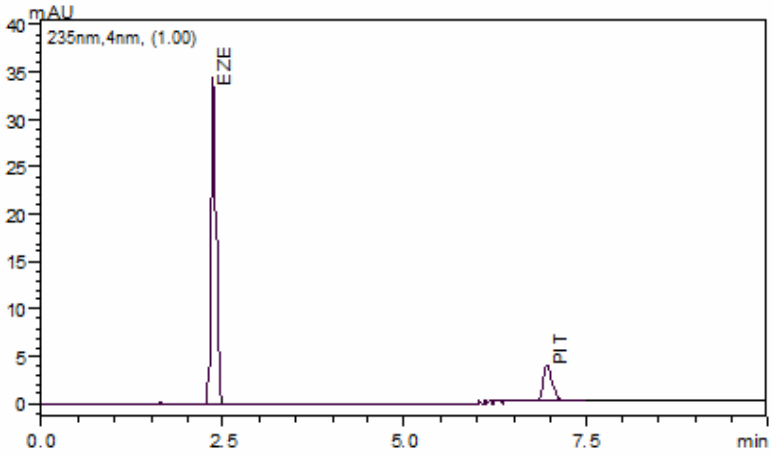

Figure 1: Liquid chromatogram of PIT (0.5 µg/mL) and EZE (2.5 µg/mL) from binary mixture at 235 nm with retention time of 6.98 and 2.36 min, respectively

Table 1: Regression analysis of the calibration curves for PIT and EZE (*n*=3)

Parameter	LC	
	PIT	EZE
Linearity range	0.5-5 µg/mL	0.5-5 µg/mL
Slope	62625.2658	48024.2630
Standard deviation of slope	46.1836	30.7309
Intercept	3132.2301	3334.3205
Standard deviation of intercept	210.8213	127.1639
Correlation coefficient, *r*	0.9999	0.9998

n = Number of determinations

Table 2: Results of recovery study for PIT and EZE (*n*=3)

Drug	Amount taken	Amount added	Recovery, % ± SD	RSD[a], %
PIT, µg/mL	0.5	0	99.38 ± 0.07	0.07
	0.5	0.5	99.57 ± 0.14	0.14
	0.5	1.5	99.46 ± 0.22	0.22
	0.5	2.5	98.99 ± 0.18	0.18
EZE, µg/mL	2.5	0	100.11 ± 0.19	0.19
	2.5	0.5	99.20 ± 0.20	0.20
	2.5	1.5	98.83 ± 0.10	0.10
	2.5	2.5	99.90 ± 0.12	0.12

[a] RSD = Relative Standard deviation, *n* = Number of determinations

Table 3: Result of robustness study for PIT and EZE (*n*=3)

Variable	Optimized value	Range	PIT	EZE
Extraction time, min	15	13	99.65	99.49
		15	99.96	100.10
		17	100.09	99.90
Acetonitrile, %	80	78	100.01	99.99
		80	99.96	100.10
		82	99.99	100.09
Mobile phase pH	3	2.95	99.90	99.93
		3.00	99.96	100.10
		3.05	100.05	100.02
wavelength	235	234	100.10	99.99
		235	99.96	100.10
		236	99.79	100.07
Flow rate, mL/min	1.4	1.2	99.68	100.05
		1.4	99.96	100.10
		1.6	99.53	99.72

Table 4: System suitability test parameters for PIT and EZE

Parameter	PIT ± % RSD[a]	EZE ± % RSD[a]
Retention time, min	6.98 ± 0.06	2.36 ± 0.22
Tailing factor	1.18 ± 0.35	1.32 ± 0.39
Asymmetry	1.21 ± 0.45	1.35 ± 0.56
Theoretical plates	10784.83 ± 0.12	4369.82 ± 1.11

[a] RSD = Relative Standard deviation.

Table 5: Analysis results for PIT and EZE binary mixture (*n*=5)

PIT			EZE		
Labelled amount (mg)	Amount found (mg)	PIT ± SD[a], %	Labelled amount (mg)	Amount found (mg)	EZE ± SD[a], %
2	1.99	99.96 ± 0.14	10	10.01	100.10 ± 0.12

[a] SD = Standard deviation, *n* = Number of determinations

RESULT AND DISCUSSION

The mobile phase consisting of 0.1 % orthophosphoric acid: acetonitrile: triethylamine (19.8: 80: 0.2, v/v/v), pH 3 ± 0.05, at a flow rate of 1.4 mL/min, was found to be satisfactory to obtain good peak symmetry, better reproducibility and repeatability for PIT and EZE. Quantification was achieved with PDA detector at 235 nm based on peak area. The retention times were about 6.98 and 2.36 min for PIT and EZE, respectively (Figure 1).

Method Validation

Both methods were found to be specific as no significant change in the responses of PIT and EZE was observed after 24 h. The excipients present in binary mixture didn't interfere with the chromatographic responses of PIT and EZE, as the peak purities of PIT and EZE from sample solution were >0.99. Peak purity > 0.99 indicates method specificity.

Linear correlation was obtained between peak area and concentration for PIT and EZE each, in the range of 0.5-5 µg/mL. The linearity of the calibration curves were validated by the value of correlation coefficient of the regression (*r*). The regression analysis of the calibration curves is shown in Table 1.

The recovery study was carried out by the standard addition method. The percent mean recoveries obtained for PIT and EZE were 98.99-99.57 % and 98.83-100.11 %, which were satisfactory (Table 2).

The values of % RSD for intraday and interday variations were found to be in range of 0.04-0.37 and 0.14-1.24 for PIT, and 0.09-0.72 and 0.13-1.07 for EZE, respectively. The % RSD values indicate the proposed method is precise.

The LOD and LOQ were found to be 0.0066 and 0.0200 µg/mL for PIT, 0.0130 and 0.0394 µg/mL for EZE.

The methods are found to be robust as the results were not significantly affected by slight variation in the chromatographic conditions such as extraction time by ±2 min, composition of mobile phase by ±2%, pH of mobile phase by ± 0.05, wavelength by ±1nm and flow rate of the mobile phase by ±0.2mL (Table 3).

% RSD for repeatability was found to be 0.11 and 0.14 for PIT and EZE, respectively. System suitability test parameters are listed in Table 4.

Determination of PIT and EZE in binary mixture

The proposed Liquid chromatography was successfully applied for determination of PIT and EZE in binary mixture. The results obtained for PIT and EZE were comparable with the corresponding claim percentage (Table 5).

CONCLUSION

Liquid Chromatographic method was developed for determination of PIT and EZE in combination. The method was validated and found to be simple, sensitive, specific, accurate, precise and robust. Statistical findings of the assay for PIT and EZE in binary mixture indicated satisfactory results. Hence, the method can be used successfully for the routine analysis of combined forms of PIT and EZE.

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