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DEVELOPMENT OF RP-HPLC METHOD FOR DETERMINATION OF EZETIMIBE AND FENOFIBRATE FROM SYNTHETIC MIXTURE

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ABSTRACT

A simple precise, accurate and validated reverse phase HPLC method has been developed for the simultaneous estimation of ezetimibe and fenofibrate in bulk and in synthetic mixture using C18 column (Kromosil, 4.6 mm x 25 cm, 5 µm) with acetonitrile: 0.05 M ammonium acetate buffer (85 : 15 v/v) as a mobile phase, at a flow rate of 1.3 mL/min and detection was done at 253.0 nm. The retention time for ezetimibe and fenofibrate was found to be 2.41 ± 0.011 and 6.03 ± 0.023 min, respectively. Linearity of ezetimibe and fenofibrate was found in the range of 2-20 µg/mL and 16-80 µg/mL, respectively. The percentage assay of ezetimibe and fenofibrate was found between 99% to 101%. The statistical parameters were found within range.

Key words: Fenofibrate, Ezetimibe, RP-HPLC method.

INTRODUCTION

Ezetimibe (EZM) belongs to one of the new class of lipid-lowering agent known as cholesterol absorption inhibitor in hyperlipidemia[1]. Ezetimibe is (3R, 4S)-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxyl-propyl)]-4-(4-hydroxyphenyl)-2-azetidinone. Fenofibrate (FNB) is 1-methyethyl-2-[4-(chlorobenzoyl) phenoxy]-2-methylpropanoate used as antihyper-lipidemic agent[2].

Literature studies on antihyperlipidemic agents showed that the clinical trials done

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with combination therapy of 10 mg of EZM and 160 mg FNB significantly reduced LDL cholesterol levels when compared to either treatment alone. Co-administration of EZM and FNB provides a complementary efficacy and this therapy improves the atherogenic profile of patients with mixed hyperlipidemia[3]. Therefore a synthetic mixture containing both the drugs was prepared in the ratio of 10 : 160 (EZM : FNB) using the most commonly used excipients like magnesium stearate, microcrystalline cellulose and aerosil.

Various methods such as, HPLC[4], HPTLC[5], UV[6-7] spectrophotometry methods have been reported for individual drugs in formulation. An attempt has been made to develop simple, rapid and accurate RP-HPLC method for simultaneous estimation of EZM and FNB from its synthetic mixture.

Material and methods

A HPLC Quaternary gradient system (Lachrom HPLC) consisting of L-7100 Merck Hitachi Pump, UV-visible detector (L-7400), Rheodyne injection syringe with 20 µL was used for analysis. Acetonitrile (HPLC Grade) and ammonium acetate (AR grade) were purchased from Qualigens. Standard gift sample of ezetimibe was kindly supplied by Lupin Ltd., Mumbai (India) and Fenofibrate supplied by Cipla Ltd., Mumbai (India).

Preparation of mobile phase

The mobile phase consisting of acetonitrile: 0.05 M ammonium acetate in the ratio of 85 : 15 was prepared and sonicated for 15 min. and then it was filtered through a 0.45 µ membrane filter paper.

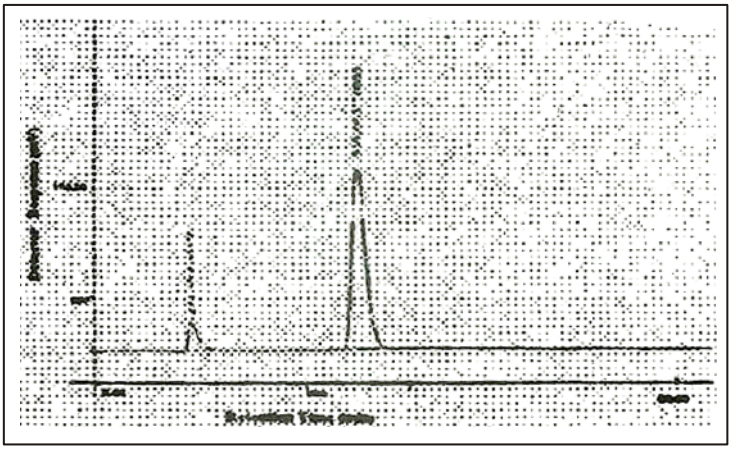

Fig. 1: Typical chromatogram of mixture of standard EZM and FNB

Preparation of standard stock solution of ezetimibe and fenofibrate

About 10 mg of ezetimibe and fenofibrate each was accurately weighed and transferred to 100 mL volumetric flasks, respectively. It was dissolved in methanol and the solution was made upto the volume with methanol to obtain 100 µg/mL of ezetimibe and fenofibrate, respectively.

Preparation of sample solution

The standard stock solution of each drug was suitably diluted with the mobile phase. The solution was kept in an ultrasonic bath for 20 min. and filtered through 0.22 µ membrane filter paper. The sample solution was further diluted with mobile phase in the ratio of 10 : 160 of ezetimibe and fenofibrate, respectively to obtain standard solutions of different concentrations.

Assay of synthetic mixture

The synthetic mixture of both drugs in combination was prepared in the ratio 10 : 160 (EZM : FNB) using most commonly used excipients like magnesium stearate (10%), M.C.C. (Q.S.) and aerosol (1%) required for tablet dosage form. From this mixture amount equivalent to 4 mg of ezetimibe and 64 mg fenofibrate was taken in volumetric flask and diluted up to 100 mL with methanol. The solution was kept for sonication for about 25 min. The solution was then filtered through a 0.22 µ membrane filter. Suitable aliquots of the solution were further diluted with mobile phase to obtain sample solutions within the concentration range for the two drugs. A 20 µL volume of each sample solution was injected into sample injector of HPLC six times under the chromatographic conditions as described above[8-11]. The area under the curve of each peak was measured at 253.0 nm. The amount of each drug present in the sample solutions was determined using the prepared calibration curves of standard ezetimibe and fenofibrate, respectively. Results are given in Table 1.

Table 1: Analysis results for synthetic mixture

Component	Amount present (mg)	Amount found* (%)	Standard deviation*	% RSD*	Standard error*
EZM	4	99.79	0.9486	0.9512	0.3876
FNB	64	99.70	0.2394	0.2403	0.0975

*Denotes average of six determinations. EZM and FNB denotes ezetimibe and fenofibrate

Chromatographic conditions

The mobile phase consisting of acetonitrile: 0.05 M ammonium acetate in the ratio of 85 : 15 was pumped by the dual plunger reciprocating pump (L-7100 Lachrom, Hitachi) at a flow rate of 1.3 mL/min. The separation was carried out on a C_{18} column (Kromasil, 4.6 mm x 25 cm, 5 µm). The column temperature was maintained at 29^{o}C. The sample was injected through a Rheodyne injector and was analyzed by variable wavelength detector set at 253.0 nm. The data was acquired, stored and analyzed with Winchrom software.

Recovery studies

To study the accuracy, reproducibility and precision of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample at three different levels at 80%, 100% and 120%. Percentage recovery and statistical data are given in Table 2.

Precision

From the standard stock solutions, mixed standards containing ezetimibe and fenofibrate in the ratio of 1 : 16 was prepared. Also sample solution was diluted to mixtures containing ezetimibe and fenofibrate in the ratio 1 : 16. Standard and sample solutions (n = 6) were injected using a universal Rheodyne injector with injection volume of 20 µL. From the peak area of ezetimibe and fenofibrate present in the pure mixture, the amount of each drug present in sample (n = 6) was determined. The intra-day and inter-day precision were determined and result of which are given in Table 3 and 4.

Linearity

Aliquots of standard stock solution of EZM and FNB stock solution were taken in 10 mL volumetric flasks and diluted upto the mark with mobile phase in such a way that final concentration of EZM and FNB were in the range of 2-20 µg/mL for ezetimibe and 16-80 µg/mL for fenofibrate, respectively. Triplicate injection of 20 µL were made two times for each concentration of each drug separately and chromatographed under the conditions as described above. Evaluation of two drugs was preformed with the UV detector set at 253 nm and peak areas were recorded. The plot of peak area vs respective concentration of EZM and FNB were found to be linear. Each standard solution was injected six times into the column at a flow rate of 1.3 mL/min in the range of 2-20 µg/mL and 16-80 µg/mL with coefficient of correlation (r^2) 0.9987 and 0.9992 for EZM and FNB, respectively.

Robustness studies

Robustness studies were performed by carrying out deliberate variations of the analytical parameters and effect of the same on the responses such as retention time of the drugs, tailing factor, assay results was examined. Following three factors were selected for changes: flow rate changed (1.3 ± 0.1 mL/min), concentration of acetonitrile (85 ± 2%), and temperature (29 ± 1^{o}C). The solution containing 4 µg/mL of EZM and 64 µg/mL of FNB were injected into the column. a number of analyses (n = 3) were conducted at three level of the factor (-, 0, +). Results are given in Table 5.

Table 2: Recovery studies and its statistical validation data

Level of % recovery	Component	% Recovery*	Standard deviation*	% RSD*	Standard error*
80	EZM	99.45	0.7125	0.7165	0.4118
	FNB	99.31	0.4939	0.4974	0.2864
100	EZM	99.25	0.5646	0.5688	0.2911
	FNB	99.74	0.1721	0.1725	0.0999
120	EZM	99.83	0.3960	0.3974	0.2286
	FNB	99.78	0.1029	0.1205	0.0594

*Denotes average of three determinations at each level of recovery. EZM and FNB denotes ezetimibe and fenofibrate respectively

Table 3: Intra-day precision

Drug	% Mean*	S.D.	% R.S.D.	S.E.
EZM	99.79	0.0884	0.0992	0.0569
FNB	99.65	0.1183	0.1081	0.0621

*Mean of six determinations (n=6). EZM and FNB denotes ezetimibe and fenofibrate respectively

Table 4: Inter-day precision

Drug	% Mean*	± S.D.	% R.S.D.	S.E.
EZM	100.28	0.1554	0.1652	0.0954
FNB	99.97	0.1377	0.1486	0.0854

*Mean of six determinations (n=6). EZM and FNB denotes ezetimibe and fenofibrate respectively

Table 5: Robustness data of EZM and FNB

Level	Flow rate 1.3 ± 0.1 mL/min		% of ACN in mobile phase 85 ± 2%		Temperature 29 ± 1°C	
Response A	**EZM**	**FNB**	**EZM**	**FNB**	**EZM**	**FNB**
-	2.64	6.65	2.44	6.56	2.42	6.04
0	2.43	6.02	2.43	6.02	2.44	6.02
+	2.28	5.84	2.39	5.61	2.49	6.05
Mean ± SD	2.43 ± 0.1752	6.16 ± 0.4500	2.41 ± 0.041	6.09 ± 0.473	2.44 ± 0.200	6.03 ± 0.1526
Response B	1.32	1.22	1.52	0.82	1.22	1.12
	1.21	1.13	1.23	1.13	1.13	1.14
	1.43	0.81	1.64	1.21	1.34	1.13
Mean ± SD	1.31 ± 0.1000	1.03 ± 0.2081	1.43 ± 0.2083	1.03 ± 0.2084	1.21 ± 0.1000	1.12 ± 0.0000
Response C	99.54	98.42	101.04	99.79	99.77	100.54
	100.03	99.82	98.82	100.04	100.02	100.13
	101.52	100.03	100.03	98.45	101.24	98.39
Mean ± SD	100.35 ± 1.038	99.42 ± 0.8854	99.95 ± 1.084	99.42 ± 0.8186	100.32 ± 0.2725	99.67 ± 1.171

Response A – Retention factor Response B – Tailing factor Response C – Assay result
*Average of three determinations

RESULTS AND DISCUSSION

The goal of this study was to develop a rapid and sensitive HPLC method for the analysis of EZM and FNB in synthetic mixture using the most commonly employed C18 column with UV detection.

The mobile phase consisted of acetonitrile, 0.05 M ammonium acetate (85 : 15 v/v). The retention times for EZM and FNB were 2.43 and 6.02 min. respectively (Fig. 1). The peak areas of both the drugs were reproducible as indicated by RSD value which is less than 2%. When the calibration curve of concentration of EZM and FNB and its respective peak areas were plotted, a good linear relationship was observed between the concentration and

their respective peak areas in the range of 2-20 µg/mL for EZM and 16-80 µg/mL for FNB. The results of the assay, recovery studies and its statistical validation data indicate high degree of precision and accuracy of the proposed method. The results of the validation and system suitability parameter are given in Table 6.

Hence it can be concluded that the developed RP-HPLC method can be employed successfully for the estimation of Ezetimibe and Fenofibrate in both bulk and multicomponent formulation.

Table 6: Validation and system suitability studies

Parameter	Ezetimibe	Fenofibrate
Linearity range (µg/mL)	2-20 µg/mL	16-80 µg/mL
Slope ± S.D.*	450112 ± 2778.13	342721 ± 4175.75
Correlation coefficient ± S.D.*	0.9986 ± 0.1254	0.9993 ± 0.2704
Limit of Detection (µg/mL)	0.0031	0.0080
Limit of Quantization (µg/mL)	0.0755	0.0950
Retention time (min.) ± S.D.*	2.41 ± 0.014	6.03 ± 0.021
Resolution factor	5.52	
Tailing factor	1.29	1.18

*Mean of six determinations (n=6) EZM and FNB denotes ezetimibe and fenofibrate respectively

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