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Research Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF EZETIMIBE IN RABBIT PLASMA USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Objectives: The objective of the present work was to develop an analytical method and validation for the estimation of Ezetimibe in rabbit plasma using high-performance liquid chromatography (HPLC).

Methods: A simple, rapid, sensitive, and accurate HPLC method was developed and validated for the quantification of Ezetimibe concentration in rabbit plasma using metoclopramide as an internal standard. Separation was performed on the Xerra C18 column (250 × 4.6 mm 5 µm) using a mobile phase consisting of 0.1% perchloric acid: acetonitrile(55:45 v/v) at a flow rate of 1 ml/min. Validation of the method was performed to demonstrate its selectivity, linearity, precision, accuracy, ruggedness, recovery, and matrix effect.

Results: The calibration curves of Ezetimibe were linear over a concentration range of 5–1022 µg/mL. The with-in and between-day coefficients of variation were <10%. The extraction recoveries of Ezetimibe at the three levels of quality control samples were 99.961%, 99.767%, and 99.938%.

Conclusion: The method was rapid with a retention time of Ezetimibe and the internal standard observed at 10 min, respectively. The developed method was successfully applied to studying the pharmacokinetics of Ezetimibe in rabbits.

Keywords: Ezetimibe, Phosphate buffer, Internal standard.

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INTRODUCTION

The oral route of administration is most convenient for drug administration compared to all the routes of administration. Orally disintegrating tablets (ODTs) are dosage forms, when placed in the mouth will rapidly disperse and dissolve in the mouth without the need for water [1]. After complete disintegration, the drug solution can be partially or completely absorbed by the sublingual blood vessels and bypass first-pass metabolism by the liver or be absorbed from the gastrointestinal tract after swallowing.

The prescription of ODT products was initially developed to overcome the difficulty in swallowing among pediatric and geriatric populations who have difficulty swallowing conventional tablets and capsules [2]. Oral disintegrating tablets will be more widely available as counter products for the management of many conditions such as lowering cholesterol, heart problems, allergies, and colds. The presence of a highly porous surface in the tablet matrix is the key factor for the rapid disintegration of oral disintegration tablets.

Many methods were reported for solubility and dissolution enhancement of poorly soluble drugs such as mechanization, complexation, solid dispersions, and kneading methods. Solid dispersions are a technique that depends on the melting or dissolution process to disperse one or more active ingredients in a carrier or matrix in the solid state. This ensures increased drug wettability and reduction of particle aggregation and hence increased drug dissolution [3].

Pediatric and geriatric patients may have difficulties in swallowing or chewing pharmaceutical dosage forms for oral administration. Tablets that rapidly dissolve on contact with the buccal cavity could present a solution to those problems, and so there is an increased interest fast dissolving dosage forms for buccal, sublingual, and oral administration. Fast-dissolving/disintegrating tablets are a perfect fit for those patients as they immediately release the active drug when placed on the tongue by rapid disintegration [4]. Hence, in the present investigation, rapidmelts of ezetimibe were prepared. Ezetimibe is widely used in the treatment of hyperlipidemia. It acts as a cholesterol absorption inhibitor. Hyperlipidemia drugs are mainly used to reduce cholesterol levels in patients at risk of cardiovascular disease. Statins generally work through nuclear receptors, statins may have benefits other than just lowering cholesterol, and they have anti-inflammatory properties, which help stabilize the lining of blood vessels [6]. Ezetimibe is practically insoluble in water and crystalline compound. Dissolution is the rate-limiting step that controls oral absorption. Therefore, improvement in solubility and dissolution rate is essential to enhance drug bioavailability. As ezetimibe comes under Biopharmaceutical Classification Class II drug, solid dispersions of ezetimibe were prepared using different polymers in different ratios using different techniques to enhance the solubility of the drug. Then, those solid dispersions were formulated as rapidmelts using different super disintegrants using the direct compression method. To improve the porosity, volatile substances such as subliming agents can be used in the tableting process, which sublimated from the formed tablet. Ezetimibe rapidmelts were prepared using direct compression and sublimation techniques [5]. The optimized formulation was evaluated using rabbit plasma. However, there was no standard method for Eztimibe estimation in rabbit plasma by high-performance liquid chromatography (HPLC). Hence, an attempt was made to develop a new method for the estimation and validation of Ezetimibe in rabbit plasma by the USFDA guidelines.

MATERIALS AND METHODS

Materials

Ezetimibe API samples were obtained from MSN Laboratories Ltd., Hyderabad, India. All the chemicals and solvents such as distilled water, acetonitrile, phosphate buffer, methanol, potassium dihydrogen orthophosphate buffer, and orthophosphoric acid were obtained from RANKEM-Mumbai, India.

Methods

Analytical method development

A Water Alliance 2695 separation module equipped with a ultraviolet detector was employed throughout the study. The column employed in this method was Xterra C18 (4.6×5 mm). The samples were injected with an automatic detector. The 10 µL volume of the sample was injected. The input and output operations of the chromatographic system were monitored by waters empowered software. The flow rate selected was 1.0 mL/min. The detection was done at 233 nm. The temperature and run-time were monitored at 25°C and 10 min, respectively. The Optimized Chromatographic conditions were included in Table 1 and the Model Chromatogram was represented in Figure 1. The Tablet Composition was represented in Table 2.

Preparation of standard solutions

For the standard curve, plasma standards were prepared by spiking 0.5 mL of blood plasma with appropriate volumes of Ezetimibe standard solution to produce concentrations ranging from 3, 6, 9, 12, 15, 18, 21, and 24 ng/mL. The calibration curve was obtained by plotting the chromatographic peak against the concentration of ezetimibe added.

Preparation of phosphate buffer (pH)

1.36 g of potassium dihydrogen phosphate was taken in 1000 mL volumetric flask and 800 mL of HPLC grade water and kept in a sonicator for 5 min and made up the volume with water to produce a final 0.01N KH_2PO_4 buffer.

Preparation of mobile phase

The mobile phase was prepared by mixing 750 mL of 0.1% perchloric acid and 250 mL of acetonitrile in a 1000 mL clean and dry flask. The mixture was degassed in ultra sonicator and the resultant mobile phase was filtered through a 0.45 µ membrane filter under a vacuum.

Preparation of diluent

The diluent was prepared by mixing 0.1% perchloric acid and acetonitrile (HPLC grade) in a ratio of 50:50. This solution was used for diluting the drug solution in the study.

Preparation of standard solution

Take 10 mg of Ezetimibe in a 100 mL volumetric flask add 70 mL of diluent and go for the sonication and make the volume with diluent. From the above stock solution, 1 mL was transferred to a 10 mL volumetric flask and the volume produced 10 µg/mL.

Method validation

The optimized chromatographic method was completely validated according to the procedures described in USFDA guidelines for the validation of analytical methods and stability testing of the new drug, respectively. The method was validated for different parameters such as system suitability, sensitivity, linearity, precision, accuracy, ruggedness, recovery, and matrix effect.

Linearity

To find out the linearity range of the proposed HPLC method in plasma, the standard calibration curve was taken into consideration. The results were given in Table 1 & Figure 2.

Precision

The precision evaluation was assessed by the repeated analysis of rabbit plasma samples containing different concentrations of ezetimibe on separate occasions. A single run consisted of a calibration curve, and six replicates of the lower limit of quantification (LLOQ), lower quality control (LQC), middle quality control (MQC), and higher quality control (HQC) samples. The %RSD for inter-day precision was found to be in the range of 0.12–0.67 for ezetimibe. The %RSD for intraday precision was found to be in the range of 0.10–0.44 for ezetimibe The Results were given in Tables 2 and 3.

Table 1: Optimized chromatographic conditions

Column: Xterra C18 column (250×4.6 mm, 5 µ)
Mobile phase composition: 0.1% Perchloric acid buffer: acetonitrile (55: 45)
Flow rate: 1 mL/min
Injection volume: 50 µL
Run time: 12 min
Detection wavelength: 233 nm
Column temperature: 25°C
Diluent: 0.1%perchloric acid: acetonitrile (50: 50)

Recovery

Recovery of ezetimibe was evaluated by comparing the mean analyte responses of six extracted samples of LQC, MQC, and HQC samples to the mean analyte response of six replicate injections of unextracted quality control samples. For ezetimibe, mean recovery values are 99.90%, 99.86%, and 99.92% at the LQC, MQC, and HQC, respectively. The Recovery Results were represented in Table 4.

Pharmacokinetic study

Healthy rabbits (New Zealand Albino) of either sex weighing 2.5–3.0 were selected and housed with CPCSEA (1722/RO/Ere/13/CPCSEA) guidelines, fasted overnight, and had free access to drinking water.

Experimental design

Animals were separated into three experimental groups, each group consisting of three animals (n=3). The test formulation of batch (E12) was compared with (the marketed formulation) with the following treatment schedule under the fasted condition:

- Group I – (Normal Control) – Received placebo
- Group II – (Positive control) – Marketed formulation
- Group III – Ezetimibe formulation (E12) used as a test.

The optimized formulations were administrated through oral gauge at a dose of 0.53 mg/kg of ezetimibe. Blood samples (each of about 1–2 mL from each animal) were withdrawn from the marginal ear vein at regular time intervals after administration. During each period, approximately 1 mL of blood was collected from a marginal ear vein of a rabbit into microcentrifuge tubes containing EDTA. Blood samples were collected at 0, 0.25, 0.5, 0.75, 1, 3, 6, and 9 h in a heparinized centrifuge tube. The pharmacokinetic parameters such as C_{max}, t_{max}, K_a, area under curve (AUC), and biological half-life were estimated after analysis of the plasma samples.

Extraction of plasma

The samples were centrifuged immediately and the plasma separated was stored at 20°C till the time of analysis. The drug was extracted from the plasma. To 10 µL/mL of plasma 50 µL/mL of 10 µg/mL of drug solution was added in a stoppered test tube. This was kept in a cyclone mixer for 15 min. To that, 2 mL of acetonitrile was added and vortexed for 2 min and centrifuged at 3200 rpm for 15 min. The aqueous layer was collected and drug concentration was determined using reversed-phase HPLC (RP-HPLC).

Determination of various pharmacokinetic parameters

Different pharmacokinetic parameters such as C_{max}, $t_{max,}$ AUC, elimination rate constant (ke), absorption rate constant (ka), and elimination half-life are useful for the assessment of the bioavailability of a drug from its concentration.

Table 2: Composition of ezetimibe rapidmelts by sublimation method

Compound Name	E10	E11	E12	E13	E14	E15	E16	E17	E18
Ezetimibe (mg)	10	10	10	10	10	10	10	10	10
Camphor (mg)	5	10	15						
Urea (mg)				5	10	15			
Ammonium bicarbonate (mg)							5	10	15
Crospovidone (mg)	4	4	4	4	4	4	4	4	4
Aspartame (mg)	2	2	2	2	2	2	2	2	2
Mg stearate (mg)	2	2	2	2	2	2	2	2	2
Talc (mg)	1	1	1	1	1	1	1	1	1
Mannitol (mg)	176	171	166	176	171	166	176	171	166
Total weight (mg)	200	200	200	200	200	200	200	200	200

Table 3: Estimation of ezetimibe by HPLC for plasma samples

Concentration in ng/mL	Peak Area, n=3
0	0
3	109467±100.02
6	221345±250.02
9	356578±105.50
12	473216±500.01
15	594321±199.23
18	720987±200.03
21	836543±896.34
24	957856±758.25

HPLC: High performance liquid chromatography

Table 4: Inter-day precision of the HPLC method for the estimation of ezetimibe in rabbit plasma

Inter-day precision				
Spiked concentration	**LLOQ 3 µg/mL**	**LQC 9 µg/mL**	**MQC 15 µg/mL**	**HQC 21 µg/mL**
Concentration found	2.98	8.99	14.99	20.96
	2.97	8.98	14.96	21.02
	3.01	8.94	14.98	20.96
	2.982	8.986	14.99	20.99
	2.95	9.01	14.98	20.98
	2.99	8.92	15.02	21.02
Mean	2.98	8.971	14.98	20.98
SD	0.020	0.033	0.019	0.027
%RSD	0.671	0.378014	0.131	0.129

LLOQ: Lower limit of quantification, LQC: Lower quality control, MQC: Middle-quality control, HQC: Higher quality control, HPLC: High-performance liquid chromatography

Table 5: Intraday precision of the HPLC method for the estimation of ezetimibe in rabbit plasma

Intraday precision				
Spiked concentration	**LLOQ 3 µg/mL**	**LQC 9 µg/mL**	**MQC 15 µg/mL**	**HQC 21 µg/mL**
Concentration found	2.98	8.99	14.99	20.96
	2.98	8.98	14.96	21.01
	3	8.94	14.98	20.96
	2.982	8.986	14.99	20.98
	2.96	9.01	14.98	20.98
	2.99	8.92	15.01	21.02
Mean	2.982	8.971	14.985	20.985
SD	0.013	0.033	0.016	0.025
%RSD	0.444	0.378	0.109	0.119

LLOQ: Lower limit of quantification, LQC: Lower quality control, MQC: Middle-quality control, HQC: Higher quality control, HPLC: High-performance liquid chromatography

Table 6: Recovery of ezetimibe after adding pre-analyzed plasma sample

Spiked concentration	LQC-9 µg/mL	MQC-15 µg/mL	HQC-21 µg/mL
	Conc. Found	Conc. Found	Conc. Found
Concentration found	8.98	14.96	20.98
	8.99	15.01	21.04
	9.01	14.98	20.96
	8.99	14.96	20.99
	9.02	14.98	20.96
	8.96	14.99	20.98
Mean	8.99	14.98	20.985
SD	0.021	0.018	0.026
%Recovery	99.90	99.86	99.92

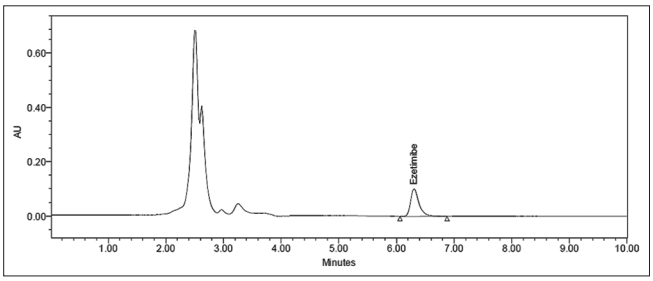

Fig. 1: Model chromatogram for ezetimibe

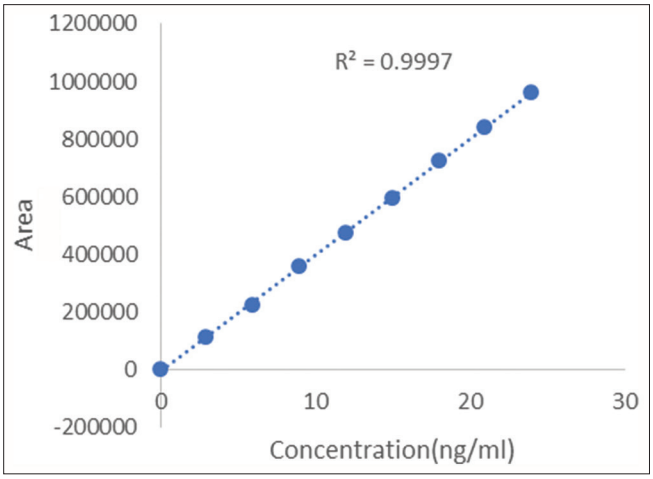

Fig. 2: Standard graph for estimation of ezetimibe by high performance liquid chromatography for plasma samples

Determination of C_{max} and T_{max}

From the time versus plasma concentration curves, the maximum peak plasma concentration (C_{max}) and time at which peak occurred (T_{max}) are recorded.

Table 7: Plasma concentration of ezetimibe following the oral administration of rapidmelts of optimized and marketed formulation in rabbits

Time (h)	Optimized formulation (E12)	Marketed formulation
0	0	0
1	48.96±0.02	21.12±0.20
2	62.54±0.21	65.32±0.53
4	94.73±0.82	35.12±0.12
6	39.61±1.02	20.02±1.52
8	25.85±0.81	12.12±0.20
10	14.13±0.12	1.05±0.03
16	1.09±0.01	0.02±0.82
24	0.09±0.25	0

Table 8: Pharmacokinetic parameters for ezetimibe rapidmelts in rabbit

Pharmacokinetic parameter	Marketed formulation	Optimized formulation (E12)
C_{max} (ng/mL)	65.12	94.73
T_{max} (h)	2.0	4.0
$AUC_{(0-24)}$ (ng.h/mL)	246.12	500.02
Ke (/h)	0.77	0.351
Biological half-life ($t_{1/2}$) (h)	0.9	1.97
Ka (/h)	40.55	93.87
t_a (h)	0.113	0.049
Vd (L)	0.052	0.056
MRT (h)	3.83	4.88

p=0.019, Considered significant

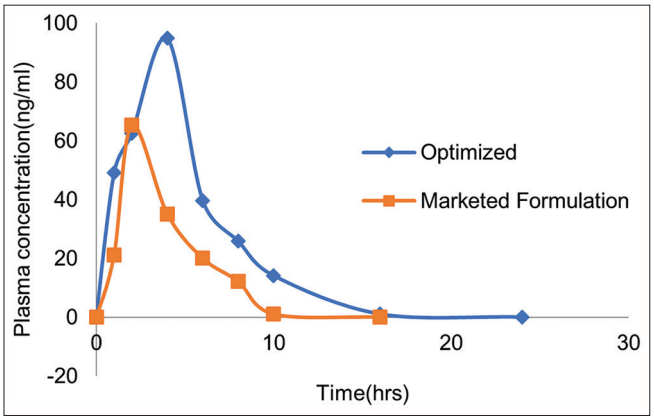

Fig. 3: Mean plasma concentration-time profile of ezetimibe following the oral administration of optimized and marketed formulation

Determination of rate of elimination (ke) and biological half-life ($t_{1/2}$)
Time versus plasma concentration data were plotted on a semilogarithmic graph paper. The corresponding biological half-life is calculated using the equation.

Elimination half-life = 0.693/ke

Determination of rate of absorption (ka) and absorption time (ta)
The absorption rate constant was measured using the method of residuals.

Absorption time = 4.61/ka

Estimation of AUC
AUC was calculated from the trapezoidal rule.

Other pharmacokinetic parameters were calculated from KINETICA software.

Statistical analysis of the pharmacokinetic parameters
Statistical evaluation of different parameters was calculated by GraphPad Instat software.

DISCUSSION

The pharmacokinetic parameters were calculated for ezetimibe (reference) and optimized formulation (E12) from the plasma concentration-time data. From Table 7, it was observed that the amount of drug released from the optimized formulation was more compared to reference formulation at 1 h. Fig. 3 shows that ezetimibe was immediately released from the E12 tablets and subsequently absorbed *In vivo*. The absorption of ezetimibe from reference was rapid but the amount of drug absorbed was less compared with optimized formulation.

The mean peak plasma concentration (C_{max}) of the ezetimibe optimized formulation was 94.73 ng/mL, while reference at the same dose produced C_{max} of 65.12 ng/mL. This showed that optimized formulation effectively increases the amount of drug release than the reference. The Tmax of ezetimibe (reference) was 2 h, while in the optimized formulation T_{max} was 4 h. The $AUC_{(0-24)}$ of the optimized formulation and reference at the same dose has shown 500.02 ng.h/mL and 246.12 ng.h/mL, respectively. The results indicate there is a 2fold increase in AUC compared with the reference sample. The absorption rate constant was calculated from the method of residuals. The rate constant of the optimized and marketed formulations was found to be 93.87/h and 40.55/h. It indicates there is a 2.2-fold increase in the absorption rate constant. From the rate constant (ka), the time of absorption was calculated. From the result, it was observed that the time of absorption (ta) was 2 times faster than the reference sample. The rate of elimination was less for the optimized formulation whereas $t_{1/2}$ was two-folds more than the reference sample the results and MRT were also enhanced for the optimized formulation. From the result, it was concluded that the optimized formulation has shown more MRT, t_a, C_{max}, AUC, and $t_{1/2}$ compared with the marketed formulation. The results also proved that rapid melt preparation by the sublimation method enhanced both the solubility and bioavailability of ezetimibe. The results are given in Table 8.

CONCLUSION

A Simple, sensitive, accurate, and precise HPLC method was developed and validated for the estimation of Ezetimibe in rabbit plasma. The present method was successfully applied in the pharmacokinetic study of Ezetimibe in rabbit plasma, in which all the pharmacokinetic parameters were determined. C_{max}, T_{max}, AUC, Kel, and $t_{1/2}$ were measured. C_{max} and $t_{1/2}$ were high compared to the reference formulation. From the results of pharmacokinetic studies, it was thus concluded that the test formulation was better than the reference formulation as it showed a greater extent of absorption.

AUTHOR'S CONTRIBUTIONS

We here with to submit a manuscript entitled: "Analytical Method Development and Validation For Estimation of Ezetimibe In Rabbit Plasma Using RP-HPLC" author by Neelimarani. T and Indira Muzib. Y for the consideration for the journal as a research paper in the journal Asian Journal of Pharmaceutical and Clinical Research. Neelima Rani Tumma analyzed the laboratory work, analyzed the data, and wrote the manuscript. Both the authors read and approved the manuscript. All authors are the guarantors.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest for the publication of the paper.

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