ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Spectrophotometric Determination of Ezetimibe with Potassium Permanganate in Bulk and Tablet Dosage Forms.

B Sitaram[1*], P Rama Koteswara Rao[1], Y Pavani[1], P Pavani[1], and C Bala Sekaran[2].

[1]Department of Freshman Engineering, PVP Siddhartha Institute of Technology, Vijayawada, Andhra Pradesh – 520007.
[2]Department of Basic sciences, Nri Institute of Technology, Pothavarappadu, Andhra Pradesh-520010.

ABSTRACT

Two simple and sensitive visible spectrophotometric methods (I & II) are developed for the determination of ezetimibe in pure and tablet dosage forms. The methods I and II are based on the oxidation of the drug by potassium permanganate in alkaline and acidic medium, respectively. In method I, the resulting green colored manganate ions was measured at 610 nm, whereas in method II ezetimibe was treated with a measured excess of permanganate in acid medium and the unreacted permanganate was measured at 550 nm. The optimum conditions for both the methods are established. The calibration curves were found to be linear in the range of 2-16 µg/ml (method I) and 2-40 µg/ml (method II) with molar absorptivity values of 2.415 x 104 L/mole/cm and 1.617 x 104 L/mole/cm, respectively. The Limit of detection was found to be 0.106 & 0.204 µg/ml and Limit of quantification was 0.323 & 0.624 µg/ml for the methods I and II, respectively. The proposed methods have been applied successfully to the assay of ezetemibe in tablet dosage forms and the results are in good agreement with those obtained by the reported spectrophotometric method.
Keywords: Ezetemibe, Potassium permanganate, Spectrophotometric analysis, Validation

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Ezetimibe (EZT)[1-5], chemically known as (3R, 4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl) azetidin-2-one (Fig. 1), is a cholesterol absorption inhibitor and is used to lower high cholesterol level in people with primary hypercholesterolaemia. It works by preventing cholesterol and other plant sterols from being absorbed into the bloodstream. The overall effect is a reduction in cholesterol level in the blood. In conjunction with any of the statins such as simvastatin, atorvastatin etc., and a cholesterol-lowering diet, EZT is used to lower cholesterol in people with inherited familial hypercholesterolaemia.

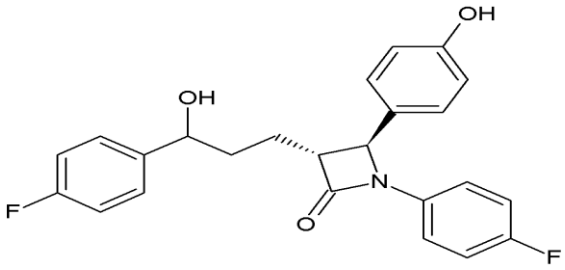

Figure 1: Structure of ezetimibe

The methods reported for quantitative determination of EZT in bulk, pharmaceutical formulations and/or biological fluids include high performance liquid chromatography (HLC) [6-13], thin layer chromatography (TLC) [7], liquid chromatography-mass spectrophotometry (LC-MS) [14-17], voltametry [18] and gas chromatography-mass spectrophotometry (GC-MS) [19]. These methods were time-consuming, tedious, and/or dedicated to sophisticated and expensive analytical instruments.

Spectrophotometry is an analytical technique for the enhancement of sensitivity and specificity in qualitative and quantitative analysis of various compounds including pharmaceuticals. Few visible spectrophotometric methods have been reported for the determination of EZT in pharmaceutical formulations. These methods are based on the redox/complex formation with 1, 10-phenanthroline and hexacyanoferrate (III) in the presence of ferric chloride [20], oxidative coupling with 3-Methyl-2-Benzthiazolinone hydrochloride [21] and formation of condensation product with vanillin in the presence of concentrated H_2SO_4 [6]. Unfortunately, the reported spectrophotometric methods are associated with some major drawbacks such as the lack of selectivity & sensitivity and usage of expensive reagent. UV and derivative spectrophotometric methods [22,23,6,7] were also reported in the literature for the determination of EZT in pharmaceutical formulations. However, these methods are less selective, as the interferences from many excipients present in the formulations will increases in the UV region.

Therefore, the development of new alternative visible spectrophotometric method for the quantification of EZT in tablet dosage forms is very essential. Here, we've reported the development and validation of two simple, sensitive and cost effective visible spectrophotometric methods for the routine analysis of EZT in bulk and tablet dosage forms. The proposed methods are based on the oxidation of EZT with alkaline $KMnO_4$ (method I) and acidic $KMnO_4$ (method II). The reaction is followed spectrophotometrically by measuring the increase in the absorbance at 610 nm (method I) and decrease in the absorbance at 550 nm (method II).

ISSN: 0975-8585

MATERIALS AND METHODS

Apparatus

Systronics UV/VIS double beam spectrophotometer (model SL-2201) with 1cm matched quartz cells was used for all spectral and absorbance measurements.

Reagents and standards

All chemicals and solvents used were of analytical grade and water was double distilled.

Potassium permanganate

An accurately weighed amount (100 mg) of $KMnO_4$ (Merck, Mumbai, India) was transferred into a 100 ml volumetric flask and dissolved in 20 ml of water; the solution was boiled for 10 min to remove any residual manganese ions, cooled, filtered then completed to the mark to provide a stock solution containing 1 mg/ml. The stock solution was diluted with water to get 80 µg/ml for method I and 500 µg/ml for method II. The $KMnO_4$ solution was freshly prepared.

Sodium hydroxide

0.6 M and 0.1 M NaOH solution was prepared by dissolving 2.4 and 0.4 gm of the chemical (Merck, Mumbai, India), respectively in 100 ml of water.

Acetic acid

Prepared by diluting glacial acetic acid (Merck, Mumbai, India) with water in the ratio of 3:2.

Sulphuric acid

1 M H_2SO_4 was prepared by mixing 5.4 ml of concentrated H_2SO_4 in 70 ml of water in a 100 ml volumetric flask and then made up to 100 ml with water.

Ezetimibe standard solutions

Method I

The EZT stock solution (1 mg/ml) was prepared by dissolving 100 mg of the drug in 20 ml of 0.1 N NaOH and then diluted to 100 ml with water. The stock solution was diluted with water to get working concentration of 200 µg/ml EZT.

Method II

Stock solution of EZT (1 mg/ml) was prepared by dissolving 100 mg of EZT in 50 ml of 3:2 diluted acetic acid in a 100 ml volumetric flask and then made up to the mark with solvent. Working standard solution containing 200 µg/ml of EZT was prepared by further dilution of stock solution.

Tablet dosage forms

The commercial tablet dosage forms of EZT, *i.e.,* Ezentia (Sun Pharma, Mumbai, India), Zetica (Torrent Pharma., Ahemdabad, India), Ezzicad (Glenmark Pharmaceuticals Ltd., Mumbai, India) and Ezetib (Unisearch Labs Ltd., Mumbai, India) were purchased from a local pharmacist.

Recommended procedures for the assay of ezetimibe

Method I

Aliquots of 0.1-0.8 ml of EZT (200 µg/ml) were pipetted into a series of 10 ml calibrated flasks and the total volume was adjusted to 0.8 ml with water. To each flask 1 ml of 0.6 M NaOH followed by 1.5 ml of 80 µg/ml $KMnO_4$ were added and then diluted to the mark with doubly distilled water. The contents of each flask were mixed well and the absorbance was measured at 610 nm against the reagent blank prepared similarly omitting the drug.

Method II

Different aliquots of standard solution (0.1-1.0 ml, 200 µg/ml) of EZT were transferred into a series of 10 ml calibrated flasks and the total volume was adjusted to 1 ml with 3:2 acetic acid. Then volumes of 1 ml of 500 µg/ml $KMnO_4$ and 1 ml of 1 M H_2SO_4 were added to each flask accurately, and kept aside for 5 min with occasional shaking before diluting to the mark with doubly distilled water. The absorbance was measured at 550 nm against the water blank.

In both the procedures, the calibration graphs were constructed by plotting the final concentration of the drug in µg/ml *versus* the absorbance values. The amount of the drug was computed either from the calibration graph or from the regression equation.

Assay of ezetimibe in tablet dosage forms

Ten tablets were accurately weighed and ground into fine powder. A portion of tablet powder equivalent to 20 mg of EZT was weighed into a 50 ml volumetric flask, 30 ml of methanol (Merck, Mumbai, India) was added and the mixture was shaken for 20 minutes. The mixture was filtered using Whatman No. 1 filter paper and the filtrate was evaporated to dryness on a water bath. The residue was washed thoroughly several times with water before dissolving it in 20 ml of 0.1 M NaOH. The solution was then transferred into a 100 ml volumetric flask, made up to the mark with doubly distilled water and suitable aliquot was

then subjected to analysis using the procedure described under method I. Another portion of tablet powder equivalent to 20 mg of EZT was accurately weighed into a 100 ml volumetric flask, 40 ml of 3:2 acetic acid was added and shaken for 20 minutes. Then, the volume was made up to the mark with the same solvent, mixed well and filtered using a Whatman No. 1 filter paper. Convenient aliquot was subjected to analysis by the procedure described under method II.

RESULTS AND DISCUSSION

Method development

Being a strong oxidizing agent, $KMnO_4$ has been used in the oxidimetric based analytical methods for the determination of many organic compounds. The oxidation of organic compounds with $KMnO_4$ was found to be pH dependent. During the course of the reaction, the valency of manganese changes with the formation of intermediate ions which in turn act as oxidants. In strong acidic medium, $KMnO_4$ produces the colorless Mn^{2+}, for a net transfer of 5 electrons [24]. In neutral or basic media, colorless manganese dioxide (MnO_2) is formed with corresponding net transfer of 3 electrons [25]. In strongly alkaline solution, green manganate ion (MnO_4^{2-}) is produced [26-28]. The behavior of permanganate was the basis for its uses in the development of visible spectrophotometric method for the determination of drugs in bulk, pharmaceutical formulations and biological samples [29-31].

Method I

The results obtained in method I were due to the formation of green colored manganate ion (MnO_4^{2-}), which resulted as a result of reduction of $KMnO_4$ by EZT in alkaline medium. The green colored manganate ion shows maximum absorption at 610 nm against the reagent blank (Fig. 2). The absorbance of the manganate ion was found to be stable up to 120 minutes. The amount of manganate ions formed corresponds to the amount of EZT (Fig. 3). This has been the basis for the determination of EZT in pure and in tablet dosage forms.

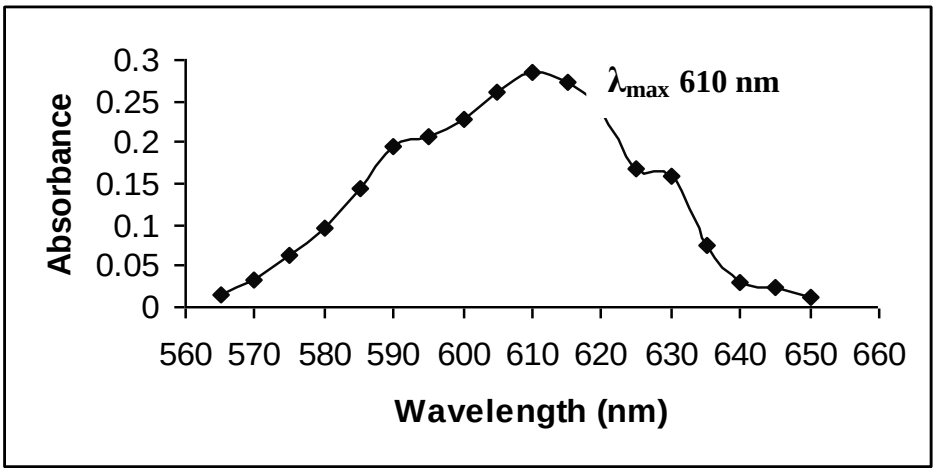

Figure 2: Absorption spectrum of manganate ion

ISSN: 0975-8585

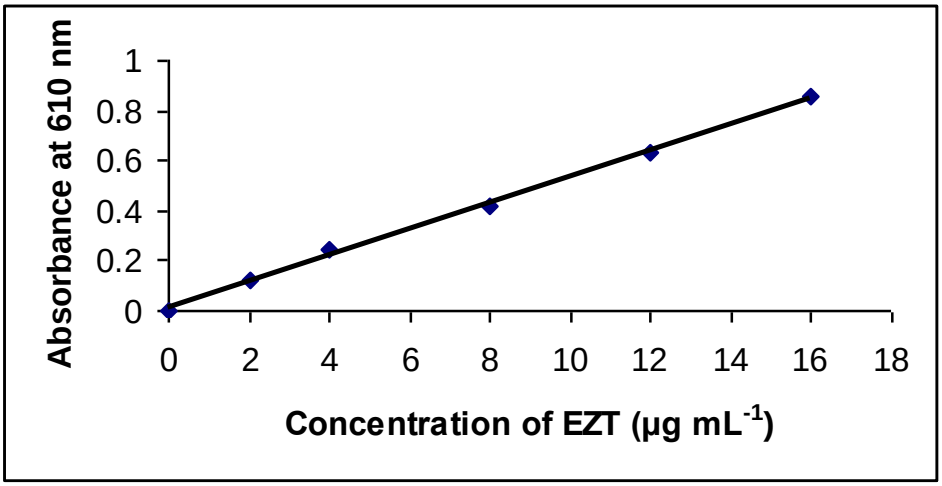

Figure 3: Linearity curve for method I

Preliminary experiments were performed to determine the optimum concentration of the reagents used in the estimation of EZT. The influence of the volume of $KMnO_4$ was observed during the formation of green colored manganate ion (MnO_4^{2-}). To study this, an aliquot of EZT containing 10 µg/ml was pipetted followed by varying volumes (0.5-4.0 ml) of 80 µgml $KMnO_4$ and 1 ml of 0.6 M NaOH. It is evident from Fig. 4 that the maximum absorbance was attained with 1.5 ml of 80 µg/ml $KMnO_4$; above this volume the absorbance decreased. Therefore, 1.5 ml of 80 µg/ml $KMnO_4$ was used in all further measurements.

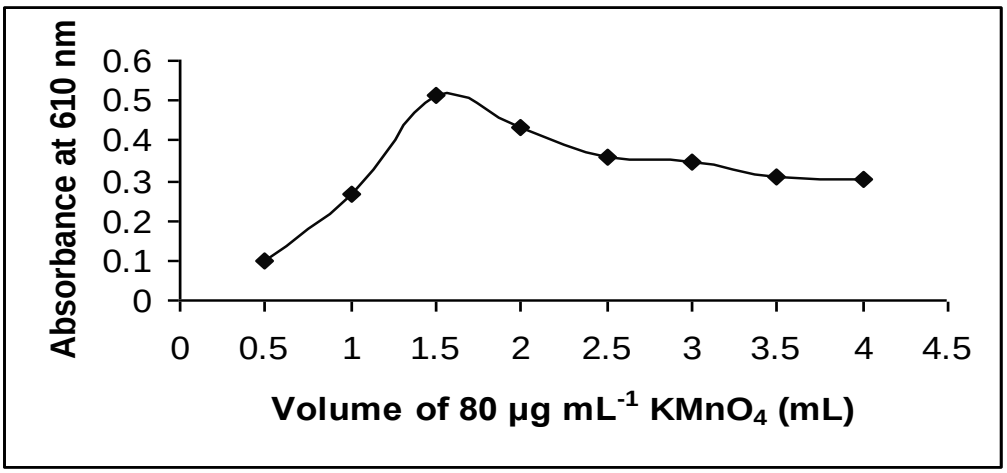

Figure 4: Effect of concentration of $KMnO_4$ on the formation of manganate ion

To investigate the effect of volume of 0.6 M NaOH for green colored manganate ion (MnO_4^{2-}) development, different volumes (0.5-4.0 ml) of 0.6 M NaOH were mixed with 1 ml of EZT (10 µg) and 1.5 ml of $KMnO_4$ (80 µg/ml). The results are presented in Fig. 5, which reveals that the addition of 1 ml of 0.6 M NaOH gave the highest absorbance, which remained constant up to 4.0 ml. Therefore, 1 ml of the 0.6 M NaOH was taken for the determination of the EZT throughout the experiment.

ISSN: 0975-8585

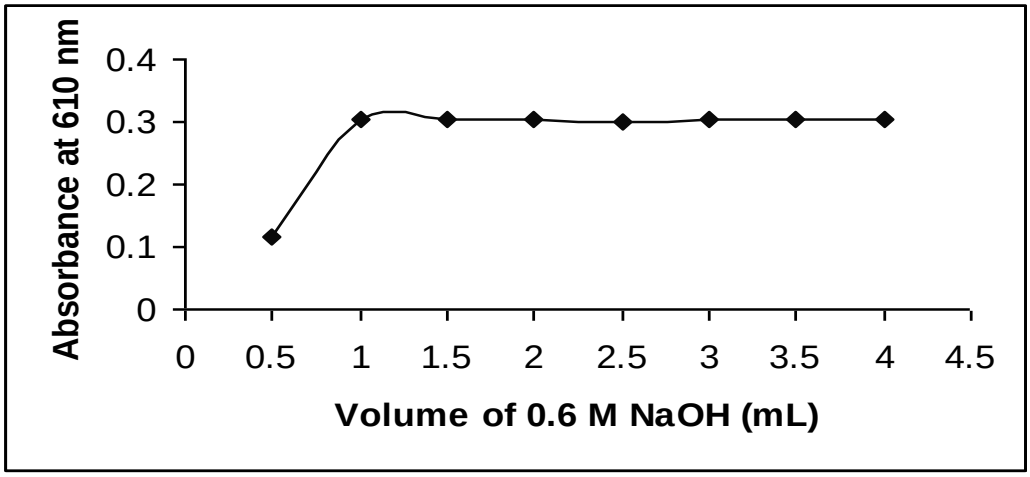

Figure 5: Effect of concentration of NaOH on the formation of manganate ion

Method II

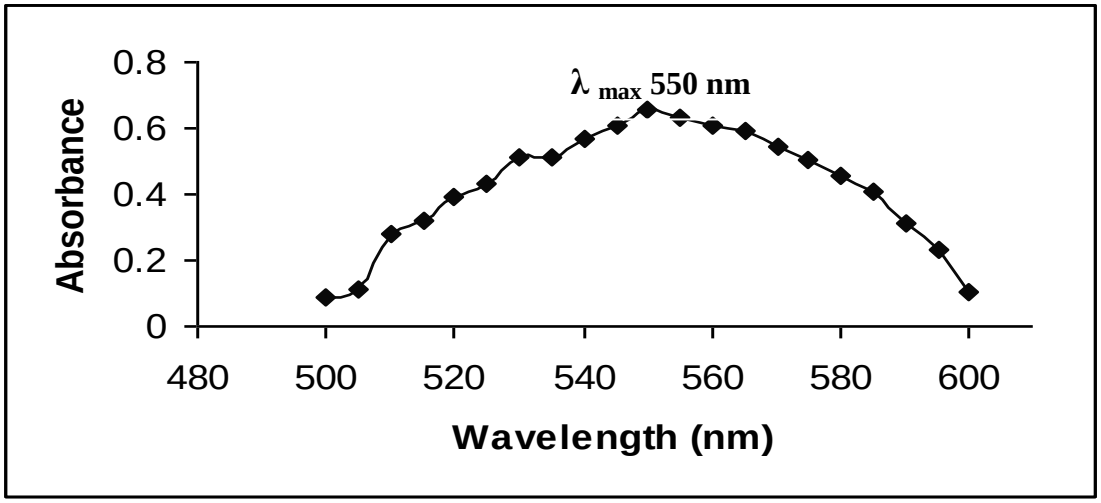

Figure 6: Absorption spectrum of $KMnO_4$ (25 µg/ml) in acid medium

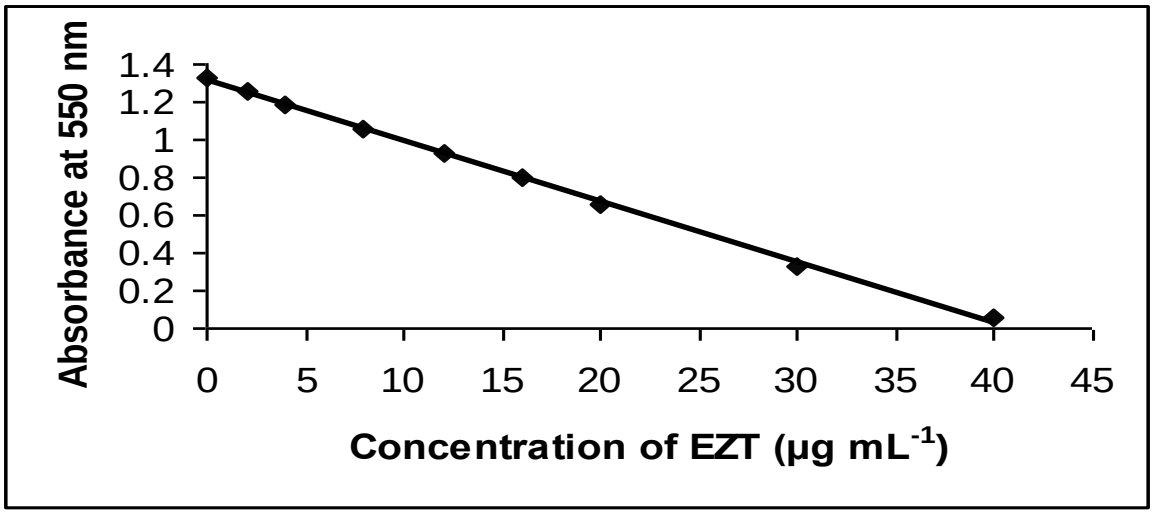

Figure 7: Linearity curve for method II

ISSN: 0975-8585

Absorption maxima of $KMnO_4$ (25 µg/ml) in acid medium was determined by scanning the solution from 400-700 nm and was found to be at 550 nm (Fig. 6). The results obtained in method II were based on the oxidation of ezetemibe by $KMnO_4$ in acid medium followed by measurement of the residual permanganate at 550 nm. The absorbance of the measured unreacted $KMnO_4$ was found to be stable up to 90 minutes. The drug EZT, when added in increasing amounts to a fixed amount of $KMnO_4$ in acid medium, there is a concomitant fall in the concentration of $KMnO_4$ as shown by decrease in the absorbance at 550 nm (Fig. 7). This has been the basis for the quantification of EZT in pure and in tablet dosage forms.

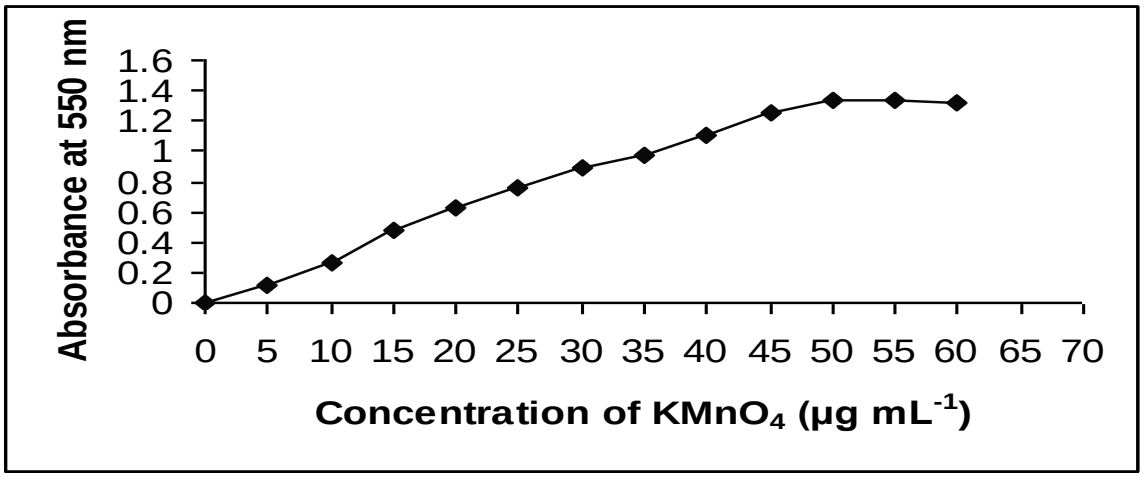

Figure 8: Linearity curve for $KMnO_4$ in 1 M H_2SO_4

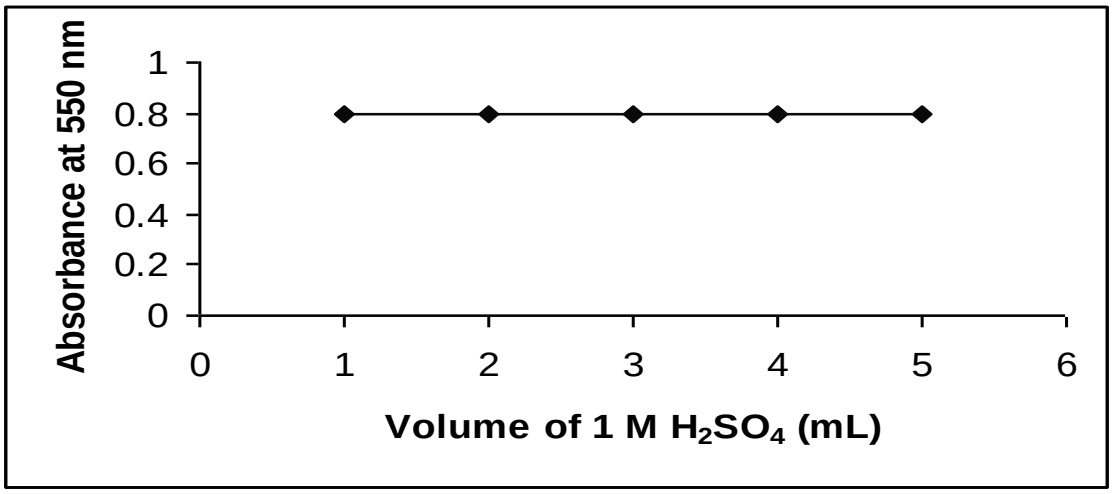

Figure 9: Effect of concentration of H_2SO_4 on the absorbance of $KMnO_4$ at 550 nm

Preliminary experiments were carried out to determine the upper concentration of $KMnO_4$ which gave the maximum absorbance at 550 nm in the acid medium employed and this was found to be 50 µg/ml (Fig. 8). Hence, different concentrations of EZT were reacted with 1 ml of 500 µg /ml $KMnO_4$ to establish the concentration range over which EZT could be determined. One ml of 500 µg/ml $KMnO_4$, in a total volume of 10 ml, must be exactly added in all the reaction flasks; above this volume the absorbance at 550 nm decreased.

The effect of the concentration of acid was studied by treating 1 ml of EZT (15 µg /ml) with 1 ml of 500 µg/ml $KMnO_4$ and varying volumes (1–5 ml) of 1 M H_2SO_4 and the

obtained results are shown in Fig. 9. It is apparent that there was absolutely no change in the absorbance when 1-5 ml of 1 M H_2SO_4 was used. Therefore, 1 ml of 1 M H_2SO_4 was recommended in a volume of 10 ml for the determination procedure.

Method validation

The proposed methods were validated based on linearity, sensitivity, precision and accuracy.

Linearity

After optimizing the reaction conditions, it was found that the relation between the absorbance and final concentration of EZT was linear over the range of 2-16 and 2-20 µg /ml for methods I and II, respectively (Table 1). The linear regression analysis of the results gave the following equation:

Method I: $A = 0.0526\ C + 0.0102$ ($r^2 = 0.9981$)
Method II: $A = 0.0322\ C + 0.0147$ ($r^2 = 0.9989$)

Where A = Absorbance; C = Concentration of drug in µg/ml; r^2 = Regression coefficient.

Sensitivity

Sensitivity parameters such as molar absorptivity and sandell's sensitivity values, the limits of detection and quantification are calculated as per the current ICH guidelines [32]. The results are summarized in Table 1. These results indicate the excellent sensitivity of the proposed methods.

Precision and Accuracy

The precision and accuracy of the proposed methods were ascertained by carrying out six replicate determinations of fixed concentration of EZT, within Beer's law range, by the proposed methods. The standard deviation, relative standard deviation and percentage of error were calculated for the proposed methods and presented in Table 1. The results confirm that the proposed methods are sufficiently precise and accurate. The level of precision and accuracy of the proposed methods were sufficient for the quality control analysis of EZT.

Analytical usefulness of the proposed methods

In order to evaluate the analytical usefulness, the proposed methods were applied to the analysis of four brands of tablet dosage forms, each containing 10 mg of EZT. Excellent recoveries and RSD values were obtained. The results obtained by the proposed method were statistically compared with those of the reported UV-spectrophotometric method [23] by applying Student's *t*-test for accuracy and *F*-test for precision. The results are compiled in Table 2. The results showed no significant difference between the proposed and literature methods with respect to accuracy and precision.

ISSN: 0975-8585

Table 1: Spectral and statistical data for the determination of ezetimibe by proposed methods

Parameters	Method I	Method II
λ_{max} (nm)	610	550
Beer's Limit (µg/ml)	2-16	2-40
Molar Absorbitivity (L mole^{-1} cm^{-1})	2.415 x 10^4	1.617 x 10^4
Sandell's sensitivity (µg cm^{-2}/0.001 Absorbance unit)	0.0169	0.0253
Stability of colored products (minutes)	120	90
Regression equation (A= mC + I)$^{\$\$}$		
Slope (m)	0.0526	0.0322
Intercept (I)	0.0102	0.0147
Regression coefficient (r^2)	0.9981	0.9989
LOD (µg/ml)	0.106	0.204
LOQ (µg/ml)	0.323	0.621
Standard deviation$^{\$}$	0.00178	0.00202
Relative standard deviation (%)	1.460	1.304
% Range of error (Confidence Limits)		
0.05 level	± 1.220	± 1.090
0.01 level	± 1.806	± 1.613

$^{\$\$}$A = mC + I, where A is the absorbance and C is the concentration of drug in µg/ml.
$^{\$}$Average of six determinations

The reliability and reproducibility of the proposed methods were further evaluated by performing recovery studies by standard addition technique. The recovery studies were carried out by adding three different concentrations of bulk samples of EZT to the pre-analyzed formulation and the mixtures were analyzed by the proposed methods. The results of this study are listed in Table 3. It is evident from the Table that the mean recoveries were in the range of 99.66% – 100.20% and 99.84% – 100.50% with RSD in the range of 0.749% – 1.125% and 0.837% – 1.045% for methods I and II, respectively. This is a good evidence of the accuracy and precision of the proposed methods.

Table 2: Results of comparison of the proposed methods with the reference method

Method	Brand name of dosage form	Labeled amount (mg)	Found (mg) ± SD$^{\$}$	Recovery (%)	RSD (%)	t value*	F value$^{@}$
Reference	Ezentia	10	10.03 ± 0.058	100.30	0.578	-	-
	Zetica	10	9.96 ± 0.096	99.60	0.963	-	-
	Ezzicad	10	9.98 ± 0.072	99.80	0.721	-	-
	Ezetib	10	10.07 ± 0.064	100.70	0.635	-	-
Method I	Ezentia	10	10.10 ± 0.101	101.00	1.000	0.128	1.957
	Zetica	10	9.92 ± 0.099	99.20	0.97	0.151	1.059
	Ezzicad	10	9.97 ± 0.042	99.70	0.421	1.536	2.647
	Ezetib	10	10.08 ± 0.086	100.80	0.853	0.549	1.349
Method II	Ezentia	10	9.95 ± 0.075	99.50	0.753	0.937	2.162
	Zetica	10	9.89 ± 0.092	98.90	0.930	0.267	1.672
	Ezzicad	10	10.08 ± 0.026	100.80	0.257	0.395	1.525
	Ezetib	10	10.04 ± 0.031	100.40	0.308	1.065	2.167

$^{\$}$ Mean ± Standard deviation (n = 5)
* Tabulated t-value at 95% confidence level is 2.306, $^{@}$Tabulated F- value at 95 % confidence level is 6.390

Table 3: Analytical results of ezetimibe determination in tablet dosage forms by standard addition method

Method	Labeled amount (mg)	Pure drug spiked (mg)	Found (mg) ± SD$	Recovery (%)	RSD (%)	Error (%)
Method I	10	5	14.95 ± 0.112	99.66	0.749	0.34
	10	10	20.04 ± 0.216	100.20	1.077	0.20
	10	15	25.05 ± 0.282	100.20	1.125	0.20
Method II	10	5	15.05 ± 0.126	100.33	0.837	0.67
	10	10	20.10 ± 0.189	100.50	0.940	0.50
	10	15	24.96 ± 0.261	99.84	1.045	0.16

$ Mean ± Standard deviation (n = 5)

CONCLUSION

Two visible spectrophotometric methods were established and studied for the quantification of EZT. The developed methods with low limits of detection and quantification are more sensitive to previously reported visible spectrophotometric methods for determination of EZT in pharmaceutical dosage form. In addition no sophisticated instrumentation is required. In these methods, high percentage of recovery shows that the EZT was completely extracted from tablet dosage form and the results revealed that the common excipients usually present in the dosage form did not interfere in the assay of the drug. It is concluded that the proposed methods are simple, sensitive, precise, accurate and economical. Therefore, the proposed methods can be successfully applied as an alternative to the existing methods for the determination of EZT in tablet dosage forms.

ACKNOWLEDGEMENTS

The authors express their gratitude to the management Siddhartha Academy, Vijayawada, Andhra Pradesh for providing research facilities.

REFERENCES

[1] Knopp RH, Gitter H, Truitt T, Bays H, Manion CV, Lipka LJ, LeBeaut AP, Suresh R, Yang B, Veltri EP. Eur Heart J 2003; 24: 729-741.
[2] Gagné C, Bays HE, Weiss SR, Mata P, Quinto K, Melino M, Cho M, Musliner, B. Gumbiner TA. Am J Cardiol 2002; 90: 1084-1091.
[3] Goldberg AC, Sapre A, Liu J, Capece R, Mitchel YB. Mayo Clin Proc 2004; 79: 620-629.
[4] Guyton JR, Betteridge DJ, Farnier M, Leiter LA, Jianxin L, Shah A, Johnson-Levonas AO, Brudi P. Diab Vasc Dis Res 2011; 8: 160-172.
[5] Bays HE, Ose L, Fraser N, Tribble DL, Quinto K, Reyes R, Johnson-Levonas AO, Sapre A, Donahue SR. Clin Ther 2004; 26: 1758-1773.
[6] Chetan MB, Jane J, Subrahmanyam EVS. Int J Res Pharm Biomed Sci 2011; 2: 241-244.
[7] El-Moghazy SM, El-Azem Mohamed MA, Mohamed MF, Youssef NF. J Chinese Chem Soc 2009; 56: 360-367.
[8] Sistla R, Tata VS, Kashyap YV, Chandrasekar D, Diwan PV. J Pharm Biomed Anal 2005; 39: 517-522.

[9] Akmar SK, Kothapalli L, Thomas A, Jangam S, Deshpande AD. Indian J Pharm Sci 2007; 69: 695-697.
[10] Ramakrishna K, Kumar ACP, Raju YV, Sunitha G, Shiffali DR, Bhandhavi S. Res J Pharma Bio Chem Sci 2011; 2: 815-821.
[11] Singh S, Singh B, Bahuguna R, Wadhwa L, Saxena R. J Pharm Biomed Anal 2006; 41: 1037-1040.
[12] Doshi AS, Kachhadia PK, Joshi HS. Chromatographia 2008; 67: 137-142.
[13] Basha SJS, Naveed SA, Tiwari NK, Kumar DS, Muzeeb S, Kumar TR, Kumar NV, Rao NP, Srinivas N, Mullangi R, Srinivas NR. J Chromatogr B 2007; 853: 88-96.
[14] Oswald S, Scheuch E, Cascorbi I, Siegmund W. J Chromatogr B Analyt Technol Biomed Life Sci 2006; 830: 143–150.
[15] Li S, Liu G, Jia J, Li X, Yu C. J Pharm Biomed Anal 2006; 40: 987-992.
[16] Oliveira PR, Junior LB, Fronza M, Bernardi LS, Masiero SMK, Dalmora SL. Chromatographia 2006; 63: 315-320.
[17] Bahrami G, Mohammadi B, Khatabi PN, Farzaei MH, Majnooni MB, Bahoosh SR. J Chromatogr B 2010; 878: 2789-2795.
[18] Yola ML, Ozaltin N. Rev Anal Chem 2011; 30: 29-36.
[19] Uçaktürk E, Ozaltin N, Kaya B. J Sep Sci 2009; 32: 1868-1874.
[20] Lakshmi PBS, Ramchandran D, Rambabu C. E-J Chem 2010; 7: 101-104.
[21] Shravya A, Chandan RS, Gurupadayya BM, Sireesha M. Int J Res Ayur Pharm 2011; 2: 521-525.
[22] Kumar P, Kumar A, Juyal V. J Pharma Res 2010; 3: 1334-1337.
[23] Sharma M, Mhaske DV, Mahadik M, Kadam SS, Dhaneshwar SR. Indian J Pharm Sci 2008; 70: 258-260.
[24] Askal HF. Bull Pharm Sci Assiut Univ 1997; 20: 75-85.
[25] Rahman N, Khan NA, Azmi SNH. Pharmazie 2004; 59: 112-116.
[26] Rahman N, Ahmad Y, Azmi SNH. Eur J Pharm Biopharm 2004; 57: 359- 367.
[27] Taha EA. Anal Bioanal Chem 2003; 376: 1131-1136.
[28] Rahman N, Kashif M. Anal Sci 2003; 19: 907-911.
[29] Kalsang T, Basavaiah K, Vinay KB. Jordan J Chem 2009; 4: 387-397.
[30] El-Fetouh AAA, El-Sheikh R, El-Shafey Z, Hossny N, El-Azzazy R. Int J Biomed Sci 2008; 4: 294-302.
[31] Kumar A, Kishore L, Nair A, Navpreet K. Der Pharma Chemica 2011; 3: 279-291.
[32] International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonised Tripartite Guidelines, Validation of Analytical Procedures: Text and Methodology Q2 (R1), Current Step 4 version, Nov. 1996, Geneva, Nov. 2005.