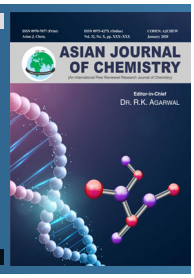
Asian Journal of Chemistry; Vol. 32, No. 6 (2020), 1309-1313

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2020.22533



HPLC Estimation of New Impurity Methyl Ezetimibe in Ezetimibe Drug

DUGGIRALA PARVATHA VENKATA VARDHANI DEVI[1,*], KAPAVARAPU MARUTHI VENKATA NARAYANARAO[1], PULIPAKA SHYAMALA[2], RALLABHANDI MURALI KRISHNA[3] and KOMALI SIVA PRASAD[1]

[1]G.V.K. Biosciences Pvt. Ltd., Plot No. 28 A, IDA Nacharam, Hyderabad-500076, India
[2]Department of Physical and Nuclear Chemistry, Andhra University, Visakhapatnam-530003, India
[3]Department of Chemical Oceanography, Andhra University, Visakhapatnam-530003, India

*Corresponding author: E-mail: dhanimaruthi@gmail.com

Received: 15 November 2019; *Accepted*: 28 December 2019; *Published online*: 30 May 2020; AJC-19875

A new gradient elution mode HPLC method was developed and validated to detect and monitor the novel impurity namely methyl ezitimibe in ezetimibe drug substances. Chromatographic detection and analysis of methyl ezetimibe was performed on XBridge C18 column with mobile phase consisting of 0.02 M phosphate buffer (pH 5) and acetonitrile with 1 mL/min flow rate in gradient elution mode. Methyl ezetimibe was detected and monitored at 248 nm. The calibration curve was linear over range of 0.015 to 0.219% concentration. The limit of detection and quantification were computed as 0.005% (signal to noise ratio 3.60) and 0.015% (signal to noise ratio 15.96), respectively. The precision was 0.97% (%RSD) and accuracy was 93.2 to 98.2% (recovery). The developed method was proved suitable to detect and monitor methyl ezetimibe impurity in ezetimibe drug substances.

Keywords: Methyl ezetimibe, Ezetimibe, Impurity, HPLC.

INTRODUCTION

Ezetimibe, referred chemically as (3*R*,4*S*)-1-(4-fluorophenyl)-3-[(3*S*)-3-(4-fluorophenyl) -3-hydroxypropyl]-4-(4-hydroxyphenyl) azetidin-2-one (Fig. 1), is a cholesterol absorption inhibitor and can be used in humans with primary hypercholesterolemia to decrease cholesterol levels [1-5]. It functions by deterring the bloodstream from consuming cholesterol as well as other plant sterols. The overall impact is a decrease in blood cholesterol levels. Ezetimibe is used to reduce cholesterol in people with heritable hypercholesterolemia in combination with statins such as simvastatin, atorvastatin, *etc*. and a diet that decreases cholesterol.

A drug product′s safety depends not only on the pharmacokinetic characteristics of active drug substance, but also on the effect of impurities produced by different chemical transformations during process. The innovation of the drug substance is therefore incomplete without identifying, characterizing and quantifying the impurities developed during the process [6-8]. The ICH′s stringent regulatory standards have contributed to a growing need to identify, quantify and monitor trace impurities in pharmaceutical substances and drug products for marketing clearance [9,10]. Moreover, detecting the impurities that are produced in very small quantities is more difficult. Impurities that are produced at acceptable limits during process are very difficult to pinpoint and control.

Profiling of impurity is one among the most serious task in analysis during drug substance development. The degree of impurities is strictly regulated for toxicological evaluation and clinical studies by regulatory bodies. As per the guideline of ICH, there is a need for the identification of impurities at or more than 0.1% (or 1 mg overall every day intake) for drug substances with a optimum dose lesser than 2 g per day every day [9,10]. Handful analyses have been recorded concerning the detection, production and characterization of ezetimibe related impurities and their degradants. Filip *et al*. [11] elucidated the structure of (*R*,*R*,*S*) stereoisomer of ezetimibe and ezetimibe degradation product using NMR, IR and MS data . They also developed stability-indicating HPLC method with UV detection for the determination of ezetimibe's stereochemical and chemical purity. Ren *et al*. [12] proposed a practical synthesis approach for two stereoisomers of ezetimibe, *R*,*R*,*S*-ezetimibe and *S*,*R*,*R*-

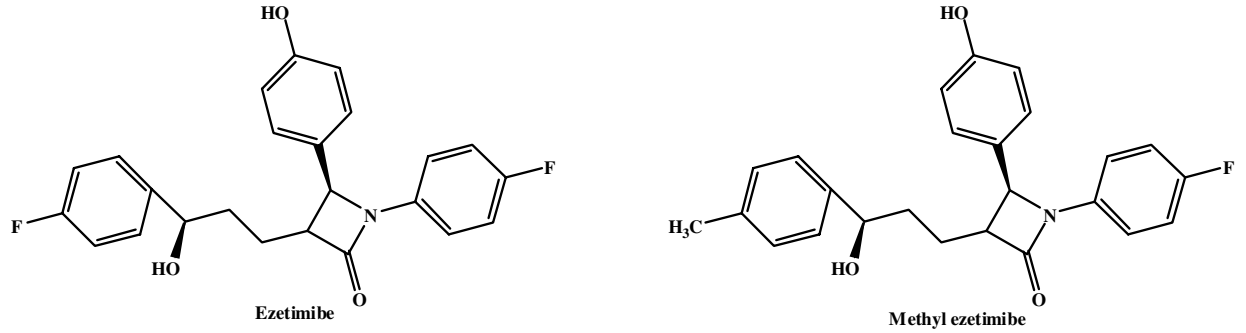


Fig. 1. Structure of ezetimibe and methyl ezetimibe

ezetimibe. Ren *et al*. [12] also provided proof for the existence of *S*,*R*,*R*-ezetimibe through analysis by single-crystal X-ray. An foremost process related impurity, 2-(4-hydroxybenzyl)-*N*,5-*bis*-(4-fluorophenyl)-5-hydroxypentanamide, which related with ezetimibe synthesis was detected by Raman *et al*. [13] using LC-MS. The structure of 2-(4-hydroxybenzyl)-*N*,5-*bis*-(4-fluorophenyl)-5-hydroxypentanamide was explicitly confirmed using data from IR and NMR analyses.

Sánta *et al*. [14] reported the structure of (2*R*,3*R*,6*S*)-*N*,6-*bis*(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-2*H*-pyran-3-carboxamide, which is a main degradant produced during the treatment of ezetimibe with alkali. The details of the structure were obtained from investigations using NMR. Guntupalli *et al*. [15] detected two impurities related to ezetimibe and identified as 2-(4-hydroxybenzyl)-*N*,5-*bis*(4-fluorophenyl)pentanamide and 1-(4-fluorophenyl)-3(3-(4-fluorophenyl)propyl)-4-(4-hydroxyphenyl)azetidin-2-one.

Ren *et al*. [16] synthesized and characterized few key stereoisomers of ezetimibe, namely ezetimibe 4, 5, 6, 7 and 8 during the synthesis process of ezetimibe [16]. Bellur and Karliga [17] identified an ezetimibe related impurity known as (3*R*,4*S*)-3-((*S*)-3-(4-fluorophenyl)-3-hydroxy-propyl)-4-(4-hydroxyphenyl)-1-phenylazetidin-2-one, also called as desfluoroezetimibe [17]. During analyses using HPLC for ezetimibe, a process related impurity in the range of 0.13-0.15% was persistently observed by the authors. Through LC-MS studies, impurity detected during ezetimibe HPLC analyzes was identified as methyl ezetimibe. No analytical method was reported to quantify methyl ezetimibe impurity in the ezetimibe drug substance. In this work, a simple, sensitive, precise and accurate HPLC method is developed and validated for the detection and quantification of methyl ezetimibe impurity in ezetimibe drug substance.

EXPERIMENTAL

The liquid chromatographic system consists of HPLC system was a Waters 2695 alliance with binary HPLC pump, degasser, autosampler and Waters 2998 photodiode array detector. Chromatographic detection and analysis of methyl ezetimibe was performed on XBridge C18 column (150 mm × 4.6 mm, 3.5µ). Integration of data was performed with Waters Empower3 software.

Methyl ezetimibe impurity and ezetimibe were obtained from G.V.K. Biosciences Private Limited, Hyderabad, India. Sodium dihydrogen phosphate monohydrate (analytical grade), sodium hydroxide (analytical grade) purchased from Sd. Fine Chemicals Ltd., Mumbai, India. Acetonitrile (HPLC grade) were purchased from Merck, India. Milli Q water was obtained from Milli Q system, USA.

Mobile phase and gradient elution

Phosphate buffer (0.02 M): To prepare 0.02 M phosphate buffer, dissolved 2.76 g of sodium dihydrogen phosphate monohydrate in 1000 mL of Milli Q water and adjusted pH 5 using dilute sodium hydroxide. The solution was filtered through 0.45µ filter paper and degassed with ultrasonic bath.

Mobile phase A: Mixed 800 mL of 0.02 M phosphate buffer and 200 mL of acetonitrile to prepare mobile phase A (MPA). The solution was filtered through 0.45µ filter paper and degassed with ultrasonic bath.

Mobile phase B: Mixed 850 mL of acetonitrile and 150 mL of Milli Q-water to prepare mobile phase B (MPB). The solution was filtered through 0.45µ filter paper and degassed with ultrasonic bath.

The gradient programme was set as: 0.01 min: 70% MBA, 30% MPB; 20 min: 60% MBA, 40% MPB; 22 min: 10% MPA, 90% MPB; 24 min: 10% MBA, 90% MPB; 24.1 min: 70% MPA, 30% MPB; and 28 min: 70% MPA, 30% MPB.

Conditions for detection and analysis: With the column temperature retained at 45 ºC, the chromatography was performed in gradient mode. The flow rate of 1.0 mL/min has been maintained with a runtime of 28 min. The volume of injection was 10 µL. The methyl ezetimibe impurity was detected at 248 nm and quantified.

Methyl ezetimibe impurity stock solution: Weighed 15 mg of methyl impurity standard accurately into a 100 mL volumetric flask, dissolved and diluted by diluent (acetonitrile) to volume. A 5 mL was transferred to a volumetric flask (100 mL) and diluted by diluent (acetonitrile) to volume (final concentration - 7.5 µg/mL of methyl ezetimibe).

Methyl ezetimibe impurity standard solution: Transfer 5 mL of the above mentioned methyl ezetimibe impurity stock solution (7.5 µg/mL methyl ezetimibe) to 50 mL volumetric flask and dilute to diluent volume (acetonitrile). The final concentration of methyl ezetimibe was 0.75 µg/mL.

Test solution: Weighed approximately 50 mg of test sample (ezetimibe drug substance) precisely in a volumetric flask of 100 mL, dissolve and dilute with diluent to volume.

Procedure to analyze methyl ezetimibe in ezetimibe drug substance: Pre-equilibrate the column with mobile phase for approximately 30 min or till a steady base line is achieved.

10 µL of standard methyl ezetimibe solution was injected in six replicas. Determine the mean peak area of methyl ezetimibe using the suggested chromatographic conditions. A 10 µL of test solution (ezetimibe drug substance) was injected and determine the peak area of methyl ezetimibe using the suggested chromatographic conditions. The percent content of methyl ezetimibe in ezetimibe drug substances was determined using the below formula:

$$\text{Content of methyl ezetimibe (\%)} = \frac{\text{Peak area of impurity in sample}}{\text{Mean peak area of standard}} \times \frac{\text{Weight of standard}}{100} \times \frac{5}{100} \times \frac{5}{50} \times \frac{100}{\text{Weight of sample}}$$

RESULTS AND DISCUSSION

Method development: The separation of methyl ezetimibe impurity from ezetimibe was easily accomplished using the proposed chromatographic procedure. XBridge C18 column (150 mm × 4.6 mm, 3.5µ) showed adequate resolution for the estimation of methyl ezetimibe impurity. A 248 nm wavelength was selected because at this wavelength methyl ezetimibe showed better response. Initially 0.02 M phosphate buffer and acetonitrile in isocratic mode was tried. But methyl ezetimibe was not properly eluted. Then after several chromatographic trails, the gradient elution programme was finalized (Table-1), that gave better peak shape, sensitivity and resolution (Fig. 2).

TABLE-1
GRADIENT PROGRAMME OPTIMIZED

Time (min)	% Mobile phase A*	% Mobile phase B**
0.01	70	30
20.0	60	40
22.0	10	90
24.0	10	90
24.1	70	30
28.0	70	30

*80% 0.02 M phosphate buffer and 20% acetonitrile.
**85% acetonitrile and 15% Milli Q water.

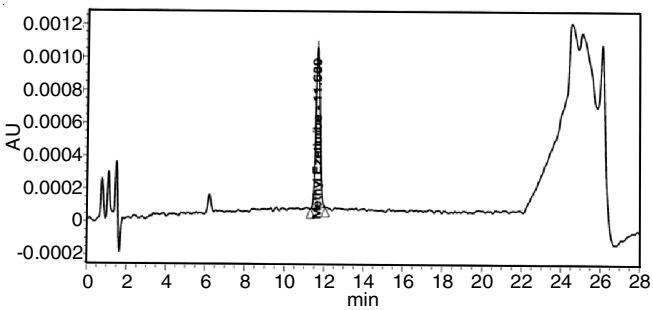


Fig. 2. Chromatogram of methyl ezetimibe (retention time 11.680 min) obtained with conditions maximized

The other conditions like flow rate, column temperature and sample size for injection were optimized as 1 mL/min, 45 ºC and 10 µL, respectively.

Method validation: The method was validated in compliance with ICH directives [18].

Specificity: The selectivity was screened by comparing chromatograms of standard methyl ezetimibe impurity, diluent blank and test sample (ezetimibe). Specificity is indicated by non-obstruction from the test sample and diluent blank at the retention time of methyl ezetimibe impurity peak (Fig. 3a-c). The specificity was also checked by peak purity analysis. The purity angle was lower than purity threshold value of methyl ezetimibe impurity peak (Table-2). This revealed that the peak of methyl ezetimibe was pure without interference from any other peaks (Fig. 3d-e).

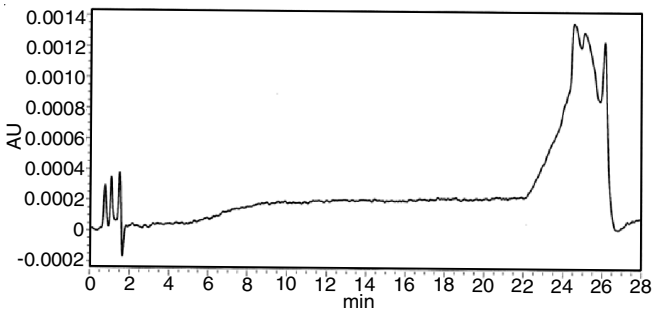


Fig. 3a. Chromatogram of diluent blank

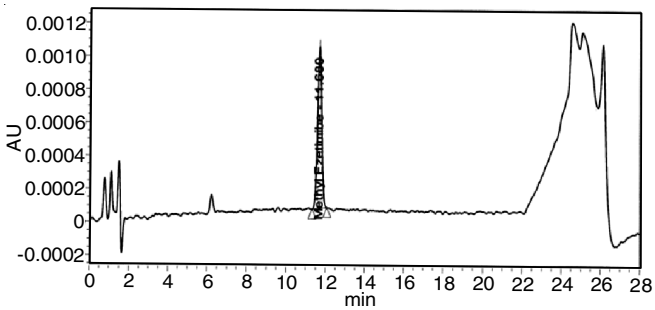


Fig. 3b. Chromatogram of standard methyl ezetimibe impurity

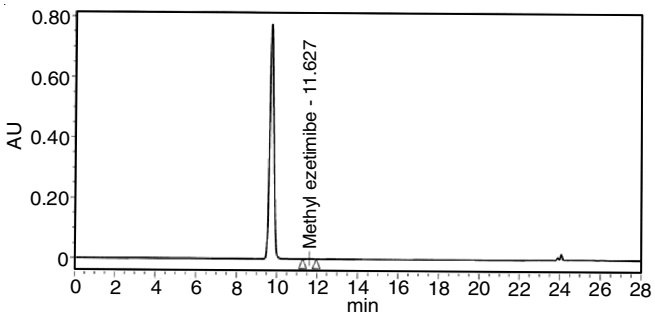


Fig. 3c. Chromatogram of test sample (ezetimibe)

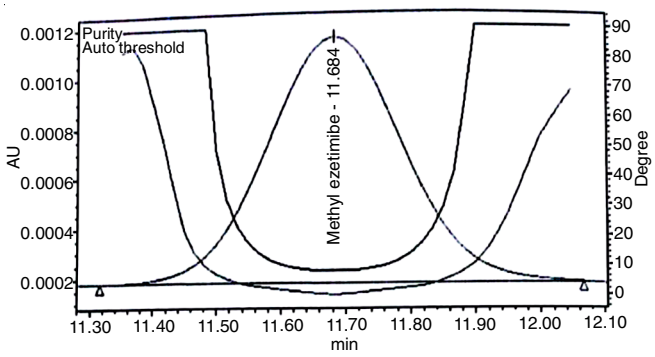


Fig. 3d. Peak purity graph of standard methyl ezetimibe impurity

Linearity: Linearity of response for the methyl ezetimibe impurity was verified. Chromatographed the methyl ezetimibe impurity solutions with concentrations of 0.015, 0.073, 0.117,

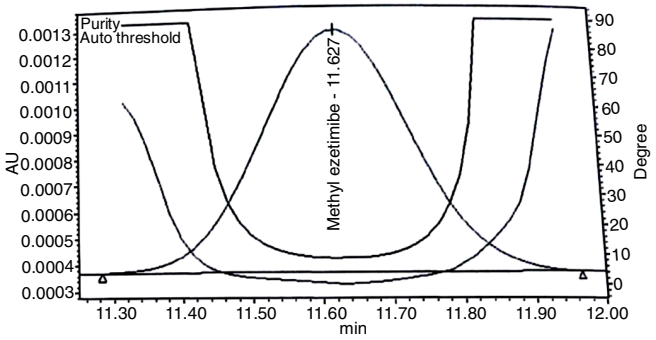


Fig. 3e. Peak purity graph of methyl ezetimibe in test sample

TABLE-2
PEAK PURITY OF METHYL EZETIMIBE IMPURITY

Sample	Purity angle	Purity threshold	Peak purity
Standard methyl ezetimibe impurity	2.144	12.918	Pass
Methyl ezetimibe spiked in test sample	2.183	13.497	Pass

0.146, 0.175 and 0.219 (*i.e.* from LOQ level to 150% of specification level with respect to test concentration 0.15%). The peak area responses obtained were linear (Fig. 4). The regression equation was: y = 88016 x + 54.89 (in this 'y' means peak area and 'x' means concentration of methyl ezetimibe impurity) with correlation coefficient (R^2) = 0.9996.

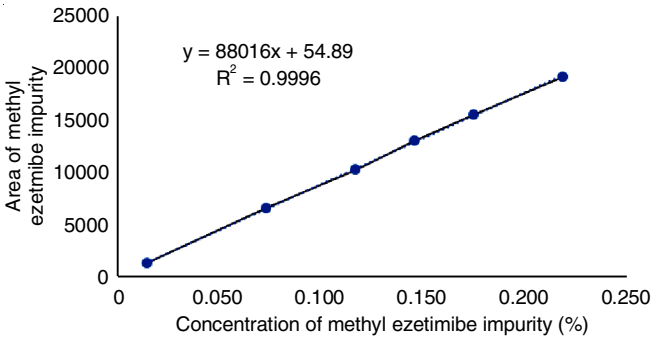


Fig. 4. Linearity of methyl ezetimibe impurity

Limit of quantification and limit of detection: To compute limits of quantification and limit of detection, signal to noise criteria of 3:1 and 10:1 was used. The limits for detection and quantification were computed respectively as 0.005% (signal to noise ratio 3.60) and 0.015% (signal to noise ratio 15.96). The values revealed sufficient method sensitivity (Fig. 5).

Precision: Method precision was checked by analysis of methyl ezetimibe impurity solution (0.146% concentration) six times by the proposed method. The precision was reported as percent relative standard deviation of peak area of methyl ezetimibe impurity. Percent relative standard deviation was less than 10% (Table-3) indicating the good precision of the method.

TABLE-3
PRECISION OUTCOMES FOR THE DEVELOPED METHOD

Sample number	Methyl ezetimibe peak area
1	12613
2	12899
3	12673
4	12791
5	12689
6	12557
Average	12703.7
Standard deviation	123.830
% RSD	0.97

Accuracy: The method accuracy was checked by spiking ezetimibe drug substances containing no detectable quantities of methyl ezetimibe impurity with known quantities of methyl ezetimibe impurity (0.73% which 50% accuracy level, 0.146% which is 100% accuracy level and 0.218% which is 150% accuracy level). Subsequent analysis revealed recovery rates of 93.2 to 98.2% for all concentration levels (Table-4). The results proved good accuracy of the method.

TABLE-4
ACCURACY LEVEL OF THE DEVELOPED METHOD

Sample number	Concentration (%)		Recovery (%)
	Theoretical	Found	
50% accuracy level			
1	0.073	0.068	93.2
2		0.070	95.9
3		0.070	95.9
100% accuracy level			
1	0.146	0.140	95.9
2		0.137	93.8
3		0.139	95.2
100% accuracy level			
1	0.218	0.211	96.8
2		0.209	95.9
3		0.214	98.2

Robustness: Method robustness was studied by making a few deliberate changes in flow rate (± 0.2 mL/min), pH in mobile phase (± 0.2) and column temperature (± 5 ºC). Relative

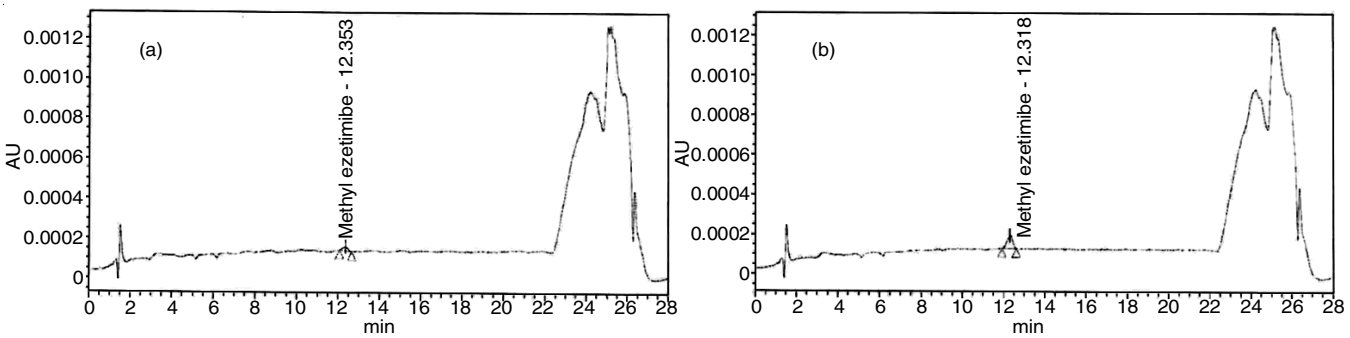


Fig. 5. Methyl ezetimibe chromatogram at (a) limit of detection level concentration and (b) limit of quantification level concentration

retention time of methyl ezetimibe impurity (at 0.146 percent concentration level) was determined in all altered conditions and compared with those acquired with optimized conditions (Table-5). There was no significant difference observed by changing the flow rate, pH in mobile phase and column temperature. Thus, revealed method robustness.

TABLE-5
ROBUSTNESS OUTCOMES FOR THE DEVELOPED METHOD

Parameter	Value studied	RRT* of methyl ezetimibe	Difference (%)
Flow rate			
Optimized value	1.0	1.20	-0.01
Changed value	1.2	1.21	
Optimized value	1.0	1.20	0.01
Changed value	0.8	1.19	
Column temperature			
Optimized value	45	1.20	0.0
Changed value	40	1.20	
Optimized value	45	1.20	0.0
Changed value	50	1.20	
pH of mobile phase			
Optimized value	5.0	1.20	0.0
Changed value	4.8	1.20	
Optimized value	5.0	1.20	0.0
Changed value	5.2	1.20	

*Relative retention time

Ruggedness: Method ruggedness was checked by analyzing 12 methyl ezetimibe impurity standard solutions (0.140% concentration level) by different analyst, column and system. The ruggedness was reported as percent relative standard deviation of methyl ezetimibe impurity concentration found. Percent relative standard deviation was less than 10% (Table-6), indicating the good ruggedness of the method.

TABLE-6
RUGGEDNESS OUTCOMES FOR THE DEVELOPED METHOD

S. No.	Methyl ezetimibe concentration found (%)	S. No.	Methyl ezetimibe concentration found (%)
1	0.140	7	0.141
2	0.140	8	0.139
3	0.140	9	0.133
4	0.139	10	0.137
5	0.138	11	0.142
6	0.139	12	0.137
Average			0.139
Standard deviation			0.002
%RSD			1.439

Solution stability: The stability of methyl ezetimibe impurity solution (0.117% concentration level) was checked by determining the relative difference of percent of known methyl ezetimibe impurity between initial (0 h) and after each time point (24 and 48 h) at room temperature. The relative difference was found below 15% (Table-7), indicating that methyl ezetimibe impurity solution was stable up to 48 h at room temperature.

TABLE-7
STABILITY OF METHYL EZETIMIBE IN SOLUTION

Sample solution analyzed at	Methyl ezetimibe concentration found (%)	Relative difference
Initial h	0.109	–
After 24 h	0.111	1.83
After 48 h	0.115	5.05

Conclusion

A new HPLC method to detect and monitor methyl ezitimibe impurity in ezetimibe drug substances was developed and validated. The quantification of methyl ezetimibe by HPLC for ezetimibe drug substance method was found to be specific, precise, linear, rugged and robust. The limits of quantification and detection values revealed good sensitivity of the method. Stability of methyl ezetimibe in solution was also established and found to be stable up to 48 h at room temperature. Hence, this method can be used for the determination of methyl ezitimibe impurity by HPLC in ezetimibe samples for regular analysis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. R.H., Knopp, H. Gitter, T. Truitt, et al. *Eur. Heart J.*, **24**, 729 (2003); https://doi.org/10.1016/S0195-668X(02)00807-2
2. C. Gagné, H.E. Bays, S.R. Weiss, P. Mata, K. Quinto, M. Melino, M. Cho, T.A. Musliner and B. Gumbine, *Am. J. Cardiol*., **90**, 1084 (2002); https://doi.org/10.1016/s0002-9149(02)02774-1
3. A.C. Goldberg, A. Sapre, J. Liu, R. Capece and Y.B. Mitchel, *Mayo Clin. Proc.*, **79**, 620 (2004); https://doi.org/10.4065/79.5.620
4. J.R. Guyton, D.J. Betteridge, M. Farnier, L.A. Leiter, J. Lin, A. Shah, A.O. Johnson-Levonas and P. Brudi, *Diab. Vasc. Dis. Res.*, **8**, 160 (2011); https://doi.org/10.1177/1479164111406457
5. H.E. Bays, L. Ose, N. Fraser, D.L. Tribble, K. Quinto, R. Reyes, A.O. Johnson-Levonas, A. Sapre and S.R. Donahue. *Clin. Ther.*, **26**, 1758 (2004); https://doi.org/10.1016/j.clinthera.2004.11.016
6. I.V. Sunil Kumar, G.S.R. Anjaneyulu and V. Hima Bindu, *Beilstein J. Org. Chem.*, **7**, 29 (2011); https://doi.org/10.3762/bjoc.7.5
7. C.S. Wu, Z.X. Jia, B.M. Ning, J.L. Zhang and S. Wu, *Chin. Chem. Lett.*, **23**, 1185 (2012); https://doi.org/10.1016/j.cclet.2012.09.001
8. W.L. Wan, Y. He, M. Guan, X.-L. Li, X. Cheng and Y. Wu, *Chin. Chem. Lett.*, **24**, 1118 (2013); https://doi.org/10.1016/j.cclet.2013.07.017
9. Guidance for industry Q3A (R2), Impurities in New Drug Substances, In: International Conference on Harmonization (2006).
10. Guidance for Industry Q3B (R2), Impurities in New Drug Products, In: International Conference on Harmonization (2006).
11. K. Filip, K. Bañkowski, K. Sidoryk, J. Zagrodzka, M. Łaszcz, K. Trzciñska, A. Szyprowska, P. Cmoch and W. Maruszak, *J. Mol. Struct.*, **991**, 162 (2011); https://doi.org/10.1016/j.molstruc.2011.02.020
12. Y. Ren, R.J. Li, Y. Deng, M. Guan, Y. Wu and L. Hai, *Tetrahedron Lett.*, **54**, 6443 (2013); https://doi.org/10.1016/j.tetlet.2013.09.049
13. B. Raman, B.A. Sharma, R. Butala, P.D. Ghugare and A. Kumar, *J. Pharm. Biomed. Anal.*, **52**, 73 (2010); https://doi.org/10.1016/j.jpba.2009.12.021
14. Z. Sánta, J. Kóti, K. Szõke, K. Vukics and C. Szántay Jr., *J. Pharm. Biomed. Anal.*, **58**, 125 (2012); https://doi.org/10.1016/j.jpba.2011.08.041
15. S. Guntupalli, U.K. Ray, N. Murali, P.B. Gupta, V.J. Kumar, D. Satheesh and A. Islam, *J. Pharm. Biomed. Anal.*, **88**, 385 (2014); https://doi.org/10.1016/j.jpba.2013.09.020
16. Y. Ren, Y.J. Duan, R.J. Li, Y. Deng, L. Hai and Y. Wu, *Chin. Chem. Lett.*, **25**, 1157 (2014); https://doi.org/10.1016/j.cclet.2014.03.035
17. A.E. Bellur and B. Karliga, *J. Pharm. Anal.*, **5**, 356 (2015); https://doi.org/10.1016/j.jpha.2015.04.002
18. ICH Expert Working Group. Validation of Analytical Procedures: Text and Methodology Q2 (R1) (2005).