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Original Paper

Quantitative analysis of ezetimibe in human plasma by gas chromatography-mass spectrometry

A new, specific and sensitive GC-MS method with electron impact ionization technique was developed for quantitative analysis of ezetimibe (EZE) in human plasma. Prior to GC analysis, EZE was derivatized with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), which is a trimethyl silylating reagent. The derivatization reaction was optimized and parameters such as catalyst, derivatization time, temperature, solvent and the volume of silylating reagent were investigated. Trimethylsilyl ether derivative of EZE was determined in selected ion monitoring (SIM, mass-to-charge ratio (*m/z*): 326) mode. The method was validated with respect to LOD and LOQ, precision, accuracy, linearity, specificity, stability, and recovery. The LOQ and LOD were found as 15 and 10 ng/mL, respectively. The linearity of the method ranged from 15 to 250 ng/mL. The correlation coefficient of the calibration curve was 0.9977 ± 0.0004 (± S.E.M.). The intra- and inter-day precisions (RSD) were less than 6% and accuracies (bias) for intra- and inter-day accuracy were found between −4.04 and 9.71% at four different concentration levels (15, 40, 100, 250 ng/mL). The proposed method was successfully applied to real human plasma samples for determination of total EZE.

Keywords: Ezetimibe / GC-MS / Plasma / Silylation / Validation

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1 Introduction

Ezetimibe (EZE) [1-(4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-(4S)-(4-hydroxypropyl)-2-azetidinone] is a new, specific and synthetic cholesterol absorption inhibitor. It inhibits the absorption of sterols such as cholesterol and plant sterol in the intestine by selectively binding to the intestinal cholesterol transporter, Niemann-Pick C1-Like 1 [1, 2]. EZE is used alone or in combination with other anti-cholesterolemic drugs such as statins to reduce blood level of low density lipoprotein cholesterol [3]. After oral administration, EZE is absorbed and extensively converted to EZE ketone, and EZE benzylic glucuronide, minor metabolites, and also the pharmacologically active metabolite EZE-glucuronide by glucuronidation of its 4-hydroxyphenyl group. EZE and its glucuronide are major fragments in plasma. Whereas the amount of unchanged EZE varies from 10 to 20% in plasma, its glucuronide varies from 80 to 90%. After a 10 mg dose of EZE, the mean peak plasma concentration (C_{max}) of EZE and EZE-glucuronide was reported as 3.4 – 5.5 ng/mL within 4 – 12 h and 45 to 71 ng/mL within 1 – 2 h, respectively (Zetia Product information, www.zetia.com/zetia/shared/documents/zetia_pi.pdf). Following the 10 mg oral dosing to healthy volunteers, maximum concentration of total EZE (EZE plus EZE glucuronide) was reached at 1 h [4, 5].

Several analytical procedures based on HPLC using LC/MS/MS and HPLC-UV have been reported for the analysis of free, total EZE and EZE-glucuronide in biological samples such as plasma, urine and bile [6 – 9].

In the literature, determination of EZE was described by GC-MS [10], but this reported analytical method did not include the optimization of derivatization reaction, and parameters that affect the reaction were not investigated. The validity of the method, which is necessary to judge the quality, reliability and consistency of analytical results, was also not demonstrated. In the present study, a new, specific and sensitive method for determination of EZE in plasma is described. The method was used for the analysis of total EZE in human plasma. EZE was derivated using *N*-methyl-*N*-trimethylsilyl-trifluoro-

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Abbreviations: EZE, ezetimibe; **IS,** internal standard; **MSTFA**, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide; ***m/z***, mass-to-charge ratio; **TMS,** trimethylsilyl

acetamide (MSTFA) as a derivatizing reagent prior to GC-MS analysis. Optimization of derivatization reaction, the validation parameters, LOD and LOQ, precision, accuracy, linearity, specificity, stability and extraction recovery are all discussed in detail.

2 Experimental

2.1 Chemicals and standards

EZE and methyltestosterone used as internal standard were supplied from the Central Institute of Hygiene of Turkey and the Turkish Doping Control Center (Ankara, Turkey), respectively. MSTFA, imidazole and β-glucuronidase from Helix pomatia (Type HP-2, =100 000 units/mL of glucuronidase activity) were supplied from Sigma. β-Mercaptoethanol, ammonium iodide and methyl tert-butyl ether were obtained from Merck. All other chemicals were of analytical reagent grade. Drug-free plasma was kindly provided from the Blood Bank of Hacettepe University Hospitals and maintained frozen at −50°C.

2.2 Instrumentation and GC-MS conditions

GC-MS analysis was performed on a 6890 N Agilent GC equipped with 5973N mass selective detector. The separation was carried out on HP-5ms (5% phenyl methylpolysiloxane, 30 m × 0.25 mm id with 0.25 μm film thickness, Agilent Technologies, USA) capillary column. Helium was used as carrier gas at constant flow rate of 0.8 mL/min. Injection of 1 μL was carried out in the splitless mode. The initial temperature of the column was set as 200°C. It was then increased to 250°C at a rate of 50°C/min, then ramped from 250 to 280°C at a rate of 30°C/min, maintained at 280°C for 8 min and then raised to 300°C at a rate of 20°C/min, and held at 300°C for 4 min. The total run time was 15 min. The mass spectrometer was operated in electron impact ionization (70 eV). The detection was operated under SIM mode. The ions of mass-to-charge ratio (*m/z*) 301 for internal standard (IS) and *m/z* 326 for EZE were most abundant and were selected for quantitation. The temperatures of front inlet, ion source and interface were 280, 230, and 280°C, respectively.

2.3 Preparation of standard solutions, calibration and validation samples

Stock standard solutions of EZE and IS with 1000 μg/mL were prepared by dissolving compounds in methanol and were stored at 4°C. The stock solutions were further diluted to desired levels with methanol.

For calibration and validation of samples, a known amount of EZE standard solutions and 10 μL IS (10 μg/mL) were added to 500 μL blank human plasma (drug-free human plasma). Plasma samples were extracted with 4 mL methyl tert-butyl ether for 15 min and then centrifuged at 3500 rpm for 5 min. The supernatant was separated and evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was derivatized as described in Section 2.4. The solution was analyzed by the proposed GC-MS method.

2.4 Preparation of derivatization reagents and trimethylsilyl (TMS) ether derivatives of EZE and IS

The stock solution of MSTFA/NH_4I/β-mercaptoethanol (100:2:6, v/w/v) was prepared by adding 100 mg of NH_4I and 300 μL of β-mercaptoethanol to 5 mL of MSTFA. Derivatization reagent solution (1000:2:6, v/w/v) was prepared by diluting the stock solution with MSTFA. MSTFA/imidazole mixture was prepared by mixing 5 mL of MSTFA and 100 μL imidazole. Solutions were stored at 4°C in the dark.

To prepare the TMS ether derivatives of EZE and IS, MSTFA/NH_4I/β-mercaptoethanol mixture was added to extract residue of standard or spiked plasma samples. This solution was heated to 80°C for 60 min in a heating block.

2.5 Validation of the GC-MS method

2.5.1 LOD and LOQ

The LOD and LOQ were evaluated using the S/N approach (ICH Topic Q2B, Validation of analytical procedures: methodology (CPMP/ICH/281/95)). LOD and LOQ were calculated as a concentration at points of S/G : 3 and S/G : 10, respectively. LOD and LOQ were performed by adding EZE to the blank plasma in decreasing concentrations.

2.5.2 Linearity

Linearity experiments were investigated over a six-day period. Spiked plasma samples were prepared freshly in six sets with eight concentration points in the range of 15 – 250 ng/mL. Each sample was analyzed three times. Calibration curves were constructed by plotting the concentration of EZE *versus* mean peak area ratio (selected ion of EZE (*m/z*: 326)/selected ion of IS (*m/z*: 301)).

2.5.3 Precision and accuracy

Precision and accuracy were assessed as intra- and inter-day variations. To determine precision and accuracy of the method, spiked plasma samples were freshly prepared in six independent series at four concentration levels (15, 40, 100, 250 ng/mL) within linear range for a six-day duration. Samples were analyzed on the same day (intra-day) and on six consecutive days (inter-day) and

were also injected three times. Precision and accuracy were expressed in terms of RSD and %Bias, respectively.

2.5.4 Specificity

Specificity of the present method was tested by the analysis of six blank plasma samples obtained from different male or female volunteers. These blank samples were analyzed by the proposed method. All target ions of SIM were investigated and compared to blank plasma samples. Specificity test was performed again for each blank plasma sample that was obtained from a different source (different patients).

2.5.5 Stability

The stability of EZE and IS stock solutions was assessed under storage conditions (4°C) for 1 month.

To investigate short-term stability, spiked plasma samples were stored at room temperature for 24 h. After storage, samples were extracted and derivatized and then injected into the GC-MS system. Freeze-thaw stability was evaluated after three freeze-thaw cycles. Plasma samples were stored at −20°C for 24 h and thawed unassisted at room temperature. This cycle was repeated three times and the samples were analyzed. The results obtained were compared to freshly prepared samples. The autosampler (post-preparative) stability was examined by reanalyzing extracted and then derivatized spiked plasma samples containing EZE and IS kept under autosampler at 4°C for 24 h.

2.5.6 Recovery

Extraction recovery of EZE from plasma was evaluated in spiked samples of three sets at four different concentrations (15, 40, 100, 250 ng/mL) over the linear range, and samples were analyzed in triplicate. The peak area obtained from samples after extraction were compared to those obtained by direct injection of the same amount of methanolic solutions.

Extraction recovery was calculated using the following Eq. (1):

$$\text{Extraction recovery} = \frac{\text{Peak area after extraction}}{\text{Peak area of methanolic solutions of EZE (IS)}} \times 100 \qquad (1)$$

2.6 Human plasma collection and sample preparation for analysis

The human ethics committee of Hacettepe University Faculty of Medicine approved the study. All patients were fully informed about the study and provided their informed consent to participate.

The blood samples of the patients (eight female and seven male, age: 55 – 70 years) were collected from a vein 1 h (t_{max}) after drug administration. The blood was placed in a glass tube containing EDTA as anticoagulant then centrifuged immediately at 4000 rpm for 10 min. The supernatant (plasma) was then transferred into test tubes and analyzed by the proposed method on the same day.

To prepare plasma sample for analysis, 10 μL IS, 500 μL sodium acetate buffer (0.5 M, pH 5) and 50 μL β-glucuronidase (≥100 000 units/mL) were added to 500 μL human plasma and incubated at 50°C for 60 min according to Patrick *et al.* [5]. The samples were extracted and then derivatized as described in Section 2.4. The resulting solution was injected into GC-MS.

3 Results and discussion

3.1 Selection of optimum derivatization conditions

Since EZE is not stable and has no volatility at high temperature, it was not monitored directly by GC-MS. Its polar hydroxyl groups were derivatized with silylating reagent for analysis. In this derivatization reaction, while the volatility and thermal stability of EZE increased, its polarity decreased.

Silylation is the most widely used derivatization technique for GC-MS analysis, and the TMS group is most frequently used for these purposes. Different properties (volatility, stability, reactivity, by-product formation, *etc*.) of trimethlysilylating reagents were emphasized in the literature [11] (Analytix Notes Derivatization of Drug Substances with MSTFA, 2005). In the present study, MSTFA, which is an effective TMS donor for derivatization, was chosen as silylation reagent. MSTFA reacted with labile hydrogen atoms of EZE and its hydroxyl groups were converted into TMS ether derivates under trimethylsilylation derivatization conditions.

Some of the parameters that affect the derivatization (catalyst, derivatization time, temperature, solvent and the volume of silylating reagent) were investigated. Derivatization reaction was optimized by comparing the peak areas of derivatized EZE. The ion (*m/z* 326) for EZE was monitored to ensure that the derivatization was as efficient as possible.

Different catalysts can be used to increase the silylation power of MSTFA. We used imidazole as the base catalyst in the silylation reaction and mixture of NH_4I/β-mercaptoethanol. MSTFA, MSTFA/imidazole and MSTFA/β-mercaptoethanol/NH_4I were investigated as silylating reagents at different temperatures (60 and 80°C) and time periods (20, 30, 60 min). The results showed that the addition of a small portion of catalysts increased the reactivity of MSTFA and improved the derivatization (silylation) efficiency. The response of the yield was highly increased using both catalyzers with MSTFA (Fig.1).

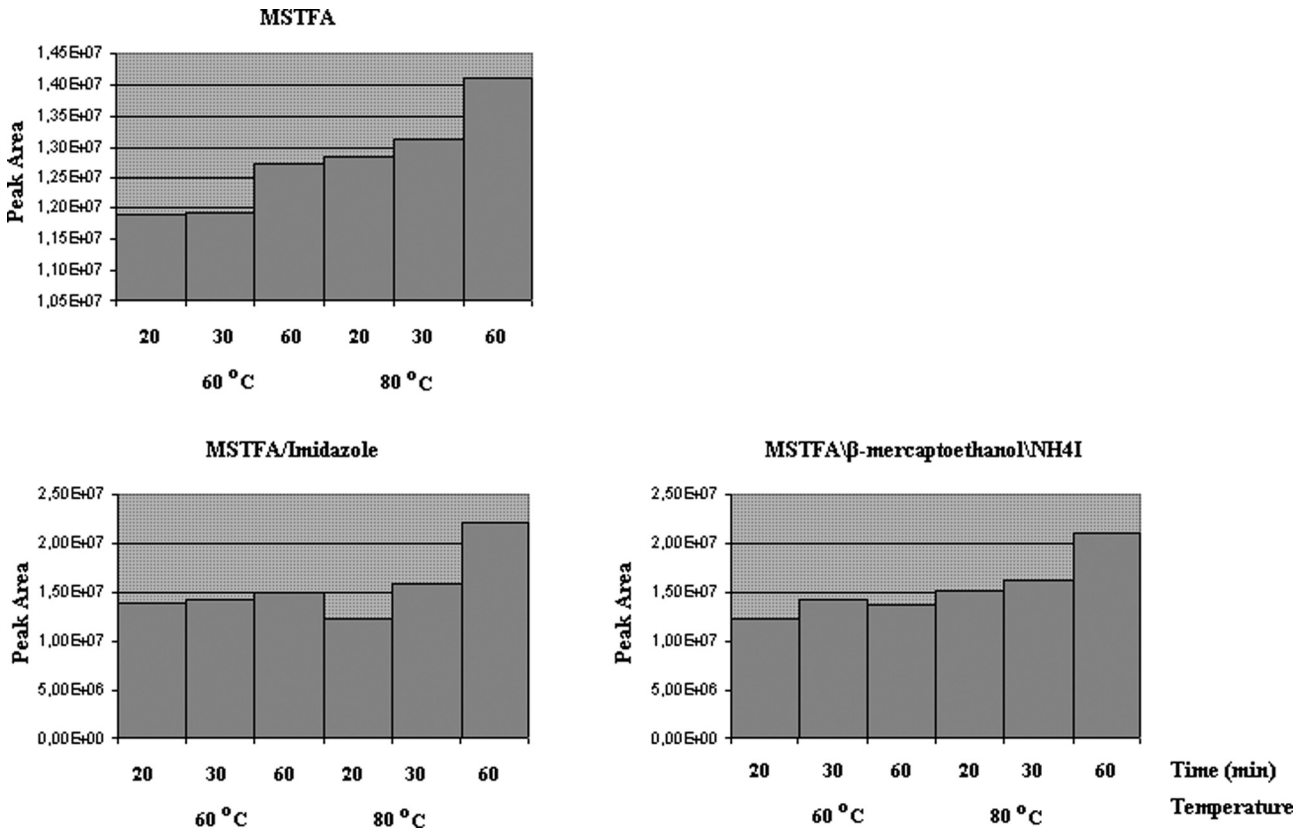

Figure 1. EZE peak areas obtained with MSTFA, MSTFA/imidazole and MSTFA/β-mercaptoethanol/NH_4I at different temperatures and time periods.

The temperature and time of reaction strongly affected the derivatization. The best results of experiments were taken using MSTFA/imidazole and MSTFA/β-mercaptoethanol/NH_4I at 80°C for 60 min. When the responses using those silylating reagents at 80°C for 60 min were compared, no significant difference was observed. Repeatability of derivatization (n = 6) measured by RSD of peak area was 1.10 and 4.68% for MSTFA/β-mercaptoethanol/NH_4I and imidazole/MSTFA, respectively. Therefore, a mixture of MSTFA/β-mercaptoethanol/NH_4I was selected as the derivatization reagent.

The silylating reagent was an adequate solvent in most cases; however, additional solvent was sometimes required due to the kinetics of the silylation reaction, its catalytic properties, and solubilization of the analyte [12]. Therefore, the effects of solvents on the derivatization reaction were investigated. Experiments were performed using a mixture of MSTFA/β-mercaptoethanol/NH_4I at 80°C for 60 min without solvent and using aprotic apolar solvents such as hexane and aprotic polar solvent such as dichloromethane, dimethylformamide and ACN. We observed that when the derivatization reagent was used without solvent, the reaction yield was remarkably increased.

In order to optimize the volume of silylating reagent, 40, 50, 100 and 200 μL reagent were added to the derivatization medium; 40 μL reagent was observed to be sufficient for derivatization.

Finally, it was determined that the optimized derivatization conditions were realized using a 40 μL mixture of MSTFA/β-mercaptoethanol/NH_4I at 80°C for 60 min. The full scan mass spectrum of TMS ether derivative of EZE and IS is shown in Fig. 2.

Methyltestosterone was selected as the IS because of its high extraction efficiency with methyl tert-butyl ether and silylation yield under optimized derivatization condition, and also because of its suitable retention time and no interference at ion m/z 301 [13 – 15].

3.2 Method validation

International Conference on Harmonisation, ICH, and Food and Drug Administration, FDA, method validation guidelines were evaluated and the developed GC-MS method was validated according to following parameters: LOD and LOQ, precision, accuracy, linearity, specificity, stability, and recovery (ICH Topic Q2B, Validation of analytical procedures: methodology (CPMP/ICH/281/95)

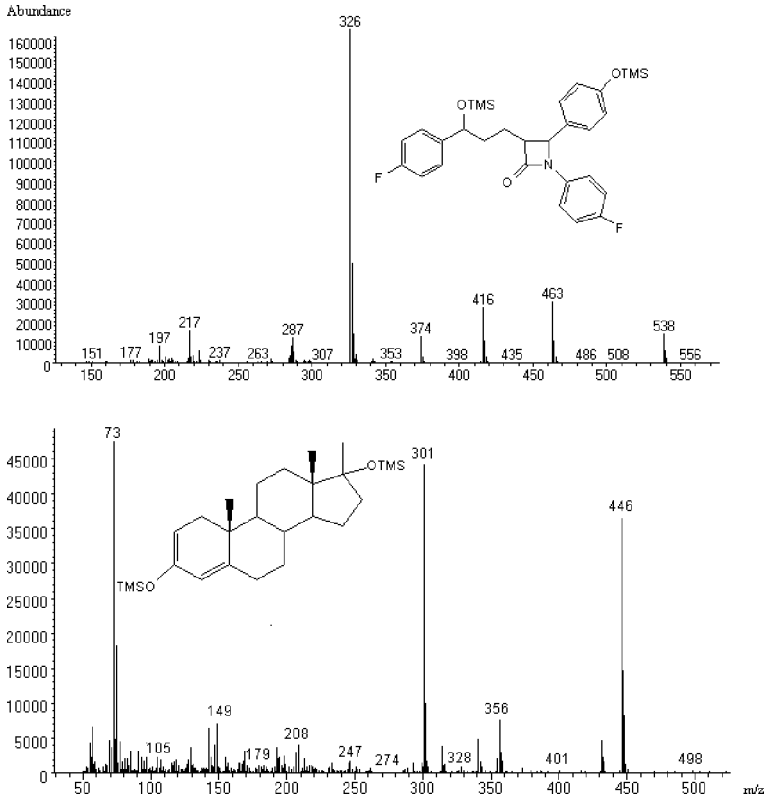

Figure 2. The full scan mass spectrum of TMS ether derivative of EZE and IS.

Table 1. Intra- and inter-day precision and accuracy results for EZE analysis in spiked plasma samples ($n = 6$).

Nominal concentrations (ng/mL)	Intra-day			Inter-day		
	Measured concentration[a)]	Precision (RSD)	Accuracy (Bias[b)] %)	Measured concentration[a)]	Precision (RSD)	Accuracy (Bias[b)] %)
15	16.01 ± 0.24	3.74	6.75	16.26 ± 0.51	5.07	9.7
40	39.57 ± 0.17	1.06	−1.06	39.81 ± 0.28	3.46	−4.04
100	100.70 ± 0.72	1.75	0.70	99.88 ± 1.94	2.64	−0.11
250	250.87 ± 0.18	4.25	0.35	249.21 ± 2.37	5.23	−0.31

a) Mean (± S.E.M.)
b) Bias% : ((found − added)/added) × 100

and FDA Bioanalytical method validation guideline, www.fda.gov/CDER/GUIDANCE/4252fnl.htm).

3.2.1 Linearity and LOQ and LOD

LOD and LOQ were determined for EZE as 10 and 15 ng/mL, respectively. In the literature, EZE was analyzed in plasma by LC/MS/MS and LOQ was found 0.25 and 1 ng/mL [6, 7]. The developed GC-MS method was not highly sensitive compared to LC/MS/MS but it is suitable for quantitation of total EZE in plasma. The linear regression equation of EZE was $y = 0.0012$ (± 4.77E-02)x − 0.0020 (± 0.0001), where y = peak area ratio of EZE to IS and x = concentration of EZE. The correlation coefficient was found as 0.9977 (± 0.0004), demonstrating a linear correlation between concentration and the obtained response.

3.2.2 Precision and accuracy

RSD was <5% for intra-day precision and <6% for inter-day precision. Percentage bias for intra- and inter-day accuracy was found between −4.04 and 9.71%. Results are summarized in Table 1. Also, the retention times of EZE and the IS showed less variability, with a RSD well within acceptable limits of 5%.

3.2.3 Specificity

Representative chromatograms of blank plasma and plasma sample spiked with EZE were shown Fig. 3. No chromatographic interferences were observed with *m/z*

Table 2. Stability of EZE in spiked plasma samples ($n = 6$)

Nominal concentrations (ng/mL)	Short term stability		Post preparative stability		Freeze-thaw stability	
	Remained[a] (%)	RSD	Remained[a] (%)	RSD	Remained[a] (%)	RSD
15	94.61 ± 2.35	6.06	104.91 ± 0.72	1.75	97.63 ± 0.23	0.59
40	97.44 ± 0.88	2.22	105.80 ± 0.45	1.05	98.23 ± 0.62	1.56
250	98.03 ± 0.78	1.94	104.58 ± 0.78	1.82	96.58 ± 0.53	1.34

[a] Remained (%): Peak area of EZE after storage conditions/ Peak area of EZE freshly prepared

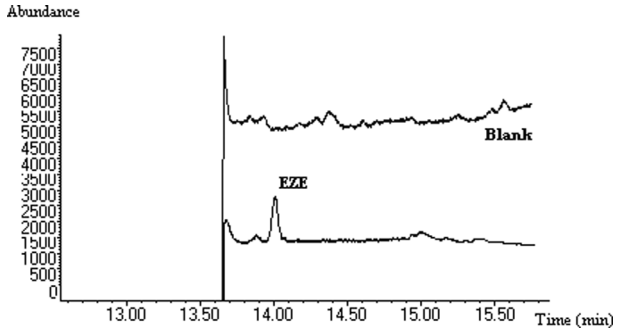

Figure 3. SIM chromatograms of blank plasma and plasma spiked with 15 ng/mL EZE (*m/z*: 326).

326 and 301 as quantifier ions for EZE and IS, respectively.

3.2.4 Stability

On the basis of the results obtained, the working solutions were determined to be stable for 1 month at 4°C. Table 2 summarizes the post-preparative, short-term and freeze-thaw cycle stability results. All the results showed that EZE can be considered stable under these conditions.

3.2.5 Recovery

EZE was extracted from plasma 8 mL of 1-chlorobutan and 4 mL of methyl tert-butyl ether in the literature [5–7]. Addition to these extraction solvents, we tried hexane, isopropanole, chloroform, ethyl acetate. Extraction recovery was found higher using 4 mL methyl tert-butyl ether. The mean extraction percentage recoveries at 15, 40, 100, and 250 ng/mL concentration points were calculated as 70.87 ± 1.05, 81.51 ± 1.08, 84.80 ± 0.31, and 85.06 ± 1.02 (mean ± S.E.M.), respectively. Furthermore, extraction recovery for IS was calculated as 98.57 ± 2.16%.

3.3 Application of the method to real plasma samples

To demonstrate the suitability of the developed and validated method for clinical use, it was applied to quantify total EZE in human plasma. We analyzed plasma obtained from patients in Hacettepe University Department of Cardiology after administration of EZE at a constant daily dose (10 mg EZE). Plasma samples of 15 patients were prepared as described in sections 2.4 and 2.5 and then analyzed by GC-MS. Total EZE plasma con-

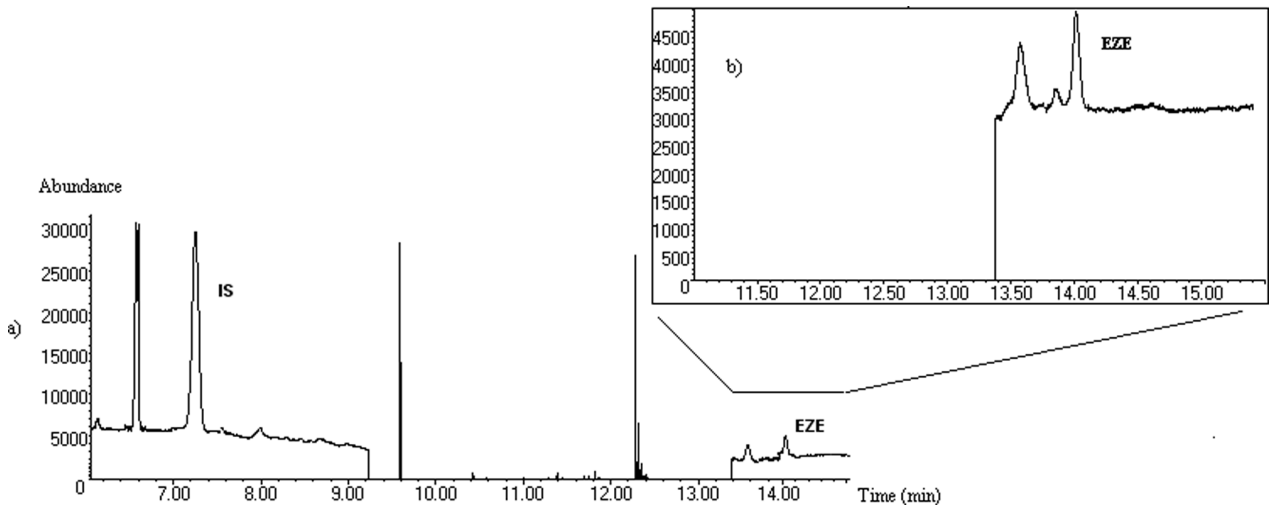

Figure 4. Chromatograms of real human plasma sample: (a) TIC including EZE and IS and (b) SIM chromatogram at *m/z*: 326 for EZE.

centration of patients at C_{max} was found as 43.06 ± 12.79 ng/mL. This value is in good agreement with the literature. Figure 4 shows the GC-MS chromatogram of real human plasma sample with concentration of EZE 43 ng/mL in SIM mode.

4 Concluding remarks

A new GC-MS method for analysis of EZE in plasma was described herein. It involved derivatization of EZE by means of a mixture of MSTFA/β-mercaptoethanol/NH_4I at 80°C for 60 min with high silylation efficiency. Repeatability of the derivatization reaction (RSD: 1.10%) was very acceptable. The method was proven to be specific, sensitive, accurate, and precise according to the validation data.

The developed method was applied successfully to real human plasma samples. Although the method is not sufficiently sensitive for determination of free EZE in plasma, it is suitable for quantitation of total EZE in plasma. When compared to the other methods found in the literature, such as LC/MS/MS, the present method is easy to use and a low-cost method. Therefore, it can be concluded that this proposed GC-MS method is suitable for use in routine monitoring of total EZE in plasma in clinical laboratories and is a good alternative to the high-cost LC/MS/MS method.

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The authors declared no conflict of interest.

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