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Optimization of a gas chromatography–mass spectrometry method using chemometric techniques for the determination of ezetimibe in human plasma

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Abstract: A new, rapid, and sensitive gas chromatography–mass spectrometry (GC-MS) method was developed for the determination of ezetimibe (EZE) in human plasma. EZE was derivatized prior to GC-MS analysis. Various derivatization techniques such as acetylation, methylation, and silylation were tried. EZE was extracted from plasma with high recovery (94.39%–97.57%) using methyl tertbutyl ether and carbonate buffer (pH 9). Chromatographic conditions were optimized using chemometric methods. In the first step, optimization with factorial design, chromatographic variables (initial and final column temperature, oven ramp rate, and flow rate of gas) were screened to select important variables for the retention of EZE. In the second step, central composite design was applied to decide on the retention time of EZE. The analysis was achieved in a short period of time (<4 min). The developed method was validated for parameters including specificity, limit of quantitation, linearity, accuracy, precision, recovery, stability, robustness, and ruggedness. The limit of quantitation was found to be 10 ng mL^{-1}. The method was successfully applied to determine total EZE in the plasma of hypercholesterolemic patients.

Key words: Ezetimibe, gas chromatography–mass spectrometry, human plasma, experimental design, validation

1. Introduction

Ezetimibe (EZE) is a synthetic and specific cholesterol absorption inhibitor. It inhibits the absorption of sterols in the intestine by selectively binding to the intestinal cholesterol transporter, Niemann-Pick C1-Like 1.[1,2] 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.[3,4]

Ezetimibe was approved in 2002 by the Food and Drug Administration (FDA). In 2008, the FDA reported early communications about safety concerns regarding EZE, EZE/simvastatin, and simvastatin and urges both healthcare professionals and patients to report side effects EZE. Studies about these safety issues are still being evaluated.[5,6]

Analytical methods for the analysis of EZE in biological samples are required to evaluate its safety and efficiency, to understand its pharmacokinetic profile among various patients, and to determine therapeutic concentration in patients.

In the literature, several analytical methods such as liquid chromatography tandem mass spectrometry (LC/MS/MS), high performance liquid chromatography–ultraviolet detection (HPLC/UV), and GC-MS were

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reported for the analysis of free and total EZE and EZE-glucuronide in biological samples.[7–10] The reported GC-MS method is time- consuming because of the long analysis time (about 15 min) and also recovery of EZE from plasma is low.[10] In the proposed study, different derivatization techniques and derivatization reagents were tried. Solid phase and liquid–liquid extraction were used for extraction of EZE from plasma. Chemometric methods such as full factorial design and central composite design (CCD) were used to optimize the chromatographic variables. After the developed method was fully validated, it was applied to determine total EZE in the plasma of hypercholesterolemic patients.

2. Experimental

2.1. Chemicals and reagents

EZE and oxymetholone (internal standard (IS)) were obtained from Central Institute of Hygiene of Turkey and the Turkish Doping Control Center (Ankara, Turkey), respectively. *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), bis(trimethylsilyl)acetamide (BSA), trimethylchlorosilane (TMCS), N-methyl-N-trimethylsilyl-heptafluorobutyramide (MSHFBA), N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), N-methyl-bis(trifluoroacetamide) (MBTFA), imidazole and β-glucuronidase from *Helix pomatia* (Type HP-2, = 100,000 units/mL of glucuronidase activity) were obtained from Sigma. βMercaptoethanol, ammonium iodide (NH_4I), potassium carbonate, potassium bicarbonate, and methyl tertbutyl ether were purchased from Merck.

2.2. Instrumentation and GC-MS conditions

GC-MS analysis was performed on a 6890 N Agilent GC equipped with a 5973N mass selective detector. A 5% phenyl methylpolysiloxane capillary column (10 m $\times$ 0.25 mm id with 0.25 μm film thickness, Agilent Technologies, USA) was used for chromatographic separation. The initial temperature of the oven was set at 206 °C; then the temperature was increased to 305 °C at a rate of 33.11 °C min^{-1}. The total run time for an injection was 4 min. The mass selective detector was operated in electron impact ionization mode. Selected ion monitoring (SIM) mode was used to determine EZE and IS. The ions of mass-to-charge ratio (m/z) 326 for EZE and m/z 548 for IS were selected for quantitation. The electron multiplier of the MS detector was set to 306 eV. The temperatures of the front inlet, ion source and interface were 280, 230, and 280 °C, respectively.

2.3. Preparation of standard solutions and validation samples

Stock solution of EZE (1000 μg mL^{-1}) was prepared by dissolving 10 mg of EZE in 10 mL of methanol. Working solutions of EZE at concentrations of 10, 1, and 0.5 μg mL^{-1} were used to prepare spiked plasma samples. Working solutions were prepared by serial dilution of stock solution of EZE with methanol. Stock solution was kept at -20 °C until use, while the working solutions were kept at 4 °C.

In order to prepare spiked plasma samples for the validation study, an appropriate amount of working standard solution of EZE and a constant amount of IS were added to 1.5 mL of plasma. The plasma samples were made basic with 500 μL of carbonate buffer (pH 9) and then EZE was extracted with 4 mL of ether. The organic layer was evaporated to dryness under nitrogen. The residue was derivatizated with 40 μL of MSTFA/β-mercaptoethanol/NH_4I.

2.4. Preparation of derivatization reagents

MSTFA/β-mercaptoethanol/NH_4I solution: For the stock solution, 100 mg of NH_4I was added to 5 mL of MSTFA. The mixture was vortexed and kept at 80 °C until the ammonium iodide dissolved; then 300 μL of β-mercaptoethanol was added to it. Working solution was prepared by dilution of 556 μL of stock solution with 5 mL of MSTFA.

MSTFA/Imidazole solution: 0.2 mg of imidazole was added to 5 mL of MSTFA and the mixture was vortexed in order to dissolve imidazole.

BSA/TMCS, MSHFBA/TMCS, MTBSTFA/TMCS solutions: 0.1 mL of TMCS was added to 5 mL of derivatization reagent.

All derivatization solutions were kept at +4 °C in the dark.

2.5. Sample preparation for the analysis of real human plasma

The human blood samples were collected from patients approximately 1 h (t_{max}) after drug administration. The blood samples were placed in a glass tube containing ethylenediaminetetraacetic acid as anticoagulant and then centrifuged at 4000 rpm for 10 min. The supernatants (plasma) were transferred into test tubes. To prepare plasma samples for analysis, firstly acidic hydrolysis was performed to convert EZE-glucuronide to EZE. Hence, 10 μL of IS, 500 μL of sodium acetate buffer (0.5 M, pH 5) and 50 μL of β-glucuronidase were added to 1.5 mL of human plasma samples. They were incubated at 50 °C for 60 min[4] The samples were extracted and then derivatized with 40 μL of MSTFA at 80 °C for 60 min. The solution was injected into the GC-MS system.

2.6. Method validation

The proposed method was validated according to FDA bioanalytical method validation guidelines[11] The following validation parameters were evaluated: specificity, linearity, limit of quantitation, accuracy, precision, stability, recovery, robustness, and ruggedness.

2.6.1. Specificity

Specificity was evaluated by analyzing 6 blank plasma samples obtained from different sources. Chromatograms were investigated for any endogenous interferences at retention time of EZE and IS by monitoring the ions at m/z 326, 416, and 463 for EZE and 548 for IS.

2.6.2. Linearity and limit of quantitation (LOQ)

In order to determine LOQ, spiked plasma samples having decreasing concentration of EZE were analyzed by GC-MS and signal to noise ratio was calculated for each concentration. In addition, precision and accuracy were calculated at LOQ level.[11]

Linearity was performed by analyzing spiked plasma samples prepared at 8 different concentrations of EZE in the range of 10–250 ng mL^{-1}. The calibration curve was constructed by plotting the peak area ratio (peak area of EZE to peak area of IS) versus the concentration of EZE. Standard deviations at each calibration point were evaluated.[11]

2.6.3. Accuracy and precision

Accuracy and precision were evaluated on an intra- and inter.day basis. To determine the precision and accuracy of the GC-MS method, spiked plasma samples were freshly prepared in 6 independent series at 4 concentration levels (10, 20, 150, and 200 ng mL^{-1}) within linear range. Samples were analyzed on the same day (intraday) and on 6 consecutive days (interday). Accuracy and precision were expressed as bias and relative standard deviation (RSD), respectively. The acceptable values of precision and accuracy are 20% for LOQ and 15% for other levels.[11]

2.6.4. Stability

Stability of EZE in plasma was investigated in terms of short-term (for 24 h in room temperature), long-term (for 3 months at –80 °C), freeze-thaw (3 freeze-thaw cycles, for 24 h at –80 °C), and postpreparative (for 24 h in an autosampler) stability.

For short-term and long-term stability, spiked plasma samples prepared at 3 different concentrations were analyzed after storage. The results obtained were compared with those of freshly prepared spiked plasma samples.

Postpreparative stability was evaluated by analyzing the spiked plasma samples before and after the storage in an autosampler for 24 h, and then by comparing the results.

For freeze-thaw stability, spiked plasma samples were stored at –80 °C for 24 h and then thawed at room temperature. This procedure was repeated twice. After 3 cycles, samples were analyzed and the results were compared with those of freshly prepared spiked plasma samples.

2.6.5. Recovery

Recovery was determined at 4 different concentrations (10, 40, 100, and 250 ng mL^{-1}) in terms of absolute and relative recovery. Absolute recovery was calculated as the peak area of EZE spiked in plasma before extraction divided by the peak area of the standard solution of EZE at the same concentration. Relative recovery was calculated as the peak area of EZE spiked in plasma before extraction divided by the peak area of EZE spiked plasma after extraction.

2.6.6. Robustness and ruggedness

Robustness and ruggedness were simultaneously evaluated for the developed method by using a Plackett–Burman design.[12] Effects of 8 variables (initial and final oven temperature, oven ramp rate, flow rate, electron multiplier voltage, different analyst, different brand ether, and MSTFA) were examined. Peak area ratio of EZE to IS was selected as response.

2.7. Statistical analysis

Statistical analysis of the results was carried out using Minitab statistical software. Differences between groups were tested by one-way ANOVA (F-test) at $P = 0.05$.

3. Results and discussion

3.1. Optimization of derivatization conditions

EZE requires derivatization to be stable and volatile at high temperature prior to GC-MS analysis. For this purpose, different derivatization reactions (silylation, methylation, and acetylation) were tried.[13,14] Derivatization reactions were performed at different temperatures (60 and 80 °C) and times (30, 60, and 120 min). The obtained peak area values for each reaction condition were plotted to monitor the yield of the new EZE derivatives.

In the silylation reaction, MSTFA, MSTFA/imidazole, MSTFA/β-mercaptoethanol/NH_4I, BSA/TMCS, MSHFBA/TMCS, and MTBSTFA/TMCS were tried as silylation reagents. With the exception of MTBSFTA/TMCS, these reagents formed a new trimethylsilyl ether (OTMS) derivative by replacing the active hydrogens of EZE with TMS groups. For the silylation reaction, the peak area values at different times and temperatures are provided in Figure 1.

Acetylation was also tried.[13,14] With this technique, active hydrogens of EZE were replaced by a trifluoroacetyl (TFA) group and a new *bis*-OTFA derivative of EZE was obtained. The acetylation reaction was tried at different temperatures (60 and 80 °C) and times (30, 60, and 120 min) and the peak area values were evaluated (Figure 1).

In the methylation reaction, no derivatization product was observed.

Comparing the yield of reactions between the silylation and acetylation reaction, the silylation reaction was seen to be more efficient according to yield of the new EZE derivative. When the responses using MSTFA/imidazole and MSTFA/β-mercaptoethanol/NH_4I at 80 °C for 60 min were statistically compared, no significant differences in the yield of reaction were observed ($P > 0.05$). Repeatability of derivatization ($n = 12$) evaluated by RSD of peak area was 1.10% and 4.68% for MSTFA/β-mercaptoethanol/NH_4I and imidazole/MSTFA, respectively. Therefore, it was decided to derivatize EZE by using MSTFA/β-mercaptoethanol/NH_4I at 80 °C for 60 min.

Solid phase and liquid–liquid extraction were tried for extraction of EZE from human plasma. Different kinds of solid phase sorbents (Oasis HLB, Strata X, C18, and C8) and conditions were examined. It was observed that Oasis HLB and Strata X gave higher recovery (53%–65%) compared to C18 and C8.

In liquid–liquid extraction, 4 mL of hexane, methyl tertbutyl ether, ethyl acetate, and dichloromethane were tried. Ether was chosen as extraction solvent because of the higher recovery. Then extraction was investigated at different pH (5, 8, and 9) using ether. Endogenous interferences from plasma were monitored at pH 5. Recovery values were calculated at pH 8 and 9 and no significant differences were observed (ANOVA test, $P > 0.05$).

It was decided that 500 μL of carbonate buffer (pH 9) and 4 mL of methyl tertbutyl ether were the best choice because of higher (97.66%) and precise (RSD $< 1.94\%$) recovery.

3.2. Experimental design

The optimization of the chromatographic parameters benefited from chemometric methods. Firstly, a 2-level full factorial design was applied to learn the effects of chromatographic variables on retention time of EZE. Mostly, a 2-level full factorial design is used for screening of variables before response surface methodology (RSM).[15,16] Thereby, the large number of variables in RSM is decreased, which makes the evaluation of results simple. Nineteen experimental runs composed of 2^n factorial ($n = 4$) and 3 center points were performed for

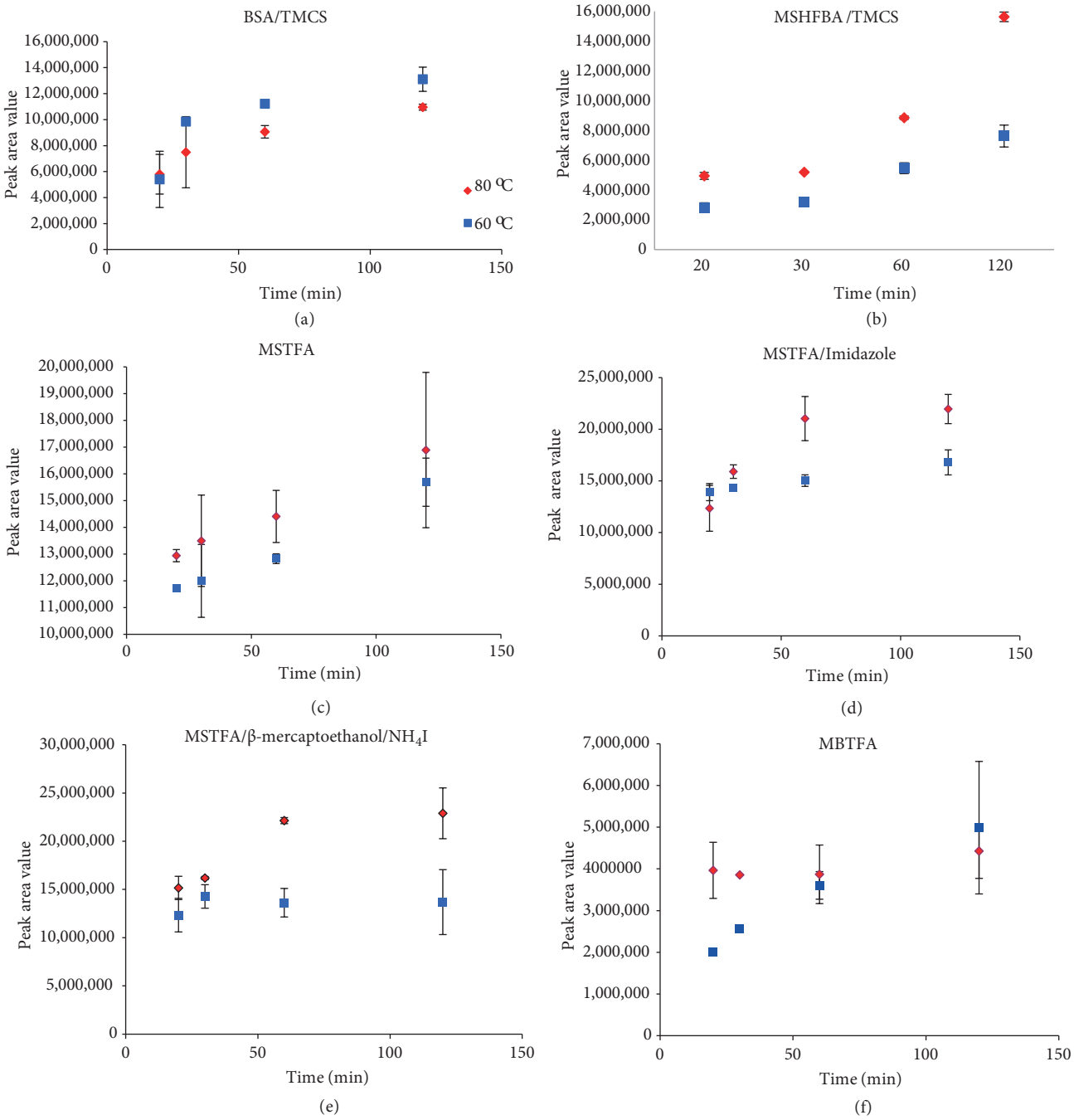

Figure 1. Peak areas of EZE derivative obtained from different derivatization conditions.

full factorial design by selected variables initial (A) and final (B) column temperature, oven ramp rate (C), and flow rate of gas (D). The design matrix and levels of variables are given in Table 1.

Significance of the parameters was determined at the 95% probability level (P = 0.05). Table 2 shows the effects, coefficients, T and P values of each parameter, and interactions between variables. It was seen that the effects of the main variables A, C, and D and one interaction term (AC) were significant but variable B was not.

Table 1. Design matrix and level of variables for full factorial design.

Exp.	Design matrix				Uncoded variables			
	A	B	C	D	A	B	C	D
1	-	-	-	-	180	305	20	0.8
2	+	-	-	-	200	305	20	0.8
3	-	+	-	-	180	315	20	0.8
4	+	+	-	-	200	315	20	0.8
5	-	-	+	-	180	305	30	0.8
6	+	-	+	-	200	305	30	0.8
7	-	+	+	-	180	315	30	0.8
8	+	+	+	-	200	315	30	0.8
9	-	-	-	+	180	305	20	1.2
10	+	-	-	+	200	305	20	1.2
11	-	+	-	+	180	315	20	1.2
12	+	+	-	+	200	315	20	1.2
13	-	-	+	+	180	305	30	1.2
14	+	-	+	+	200	305	30	1.2
15	-	+	+	+	180	315	30	1.2
16	+	+	+	+	200	315	30	1.2
17	0	0	0	0	190	310	25	1
18	0	0	0	0	190	310	25	1
19	0	0	0	0	190	310	25	1

Table 2. Effects, regression coefficients, t values, and significance levels obtained from full factorial design.

	Effect	Coefficient	t value	P
Constant	-	4.9762	4035.12	0.000[1]
A	–0.8301	–0.4151	–336.57	0.000
B	–0.0059	–0.0029	–2.38	–0.140
C	–1.4276	–0.7138	–578.82	0.000
D	–0.2904	–0.1452	–117.73	0.000
AB	–0.0009	–0.0004	–0.35	0.757
AC	0.1639	0.0819	66.44	0.000
AD	0.0006	0.0003	0.25	0.824
BC	–0.0044	–0.0022	–1.77	0.218
BD	–0.0066	–0.0033	–2.69	0.115
CD	–0.0034	–0.0017	–1.37	0.305
ABC	–0.0004	–0.0002	–0.15	0.893
ABD	–0.0001	–0.0001	–0.05	0.964
ACD	–0.0004	0.0002	–0.15	0.893
BCD	–0.0081	0.0041	–3.29	0.081
ABCD	–0.0006	–0.0003	–0.25	0.824

[1]Statistically significant at 95% probability level

Moreover, the normal probability plot of the effects supported these results. As a result, final column temperature (variable B) was kept constant at 305 °C and the variables A, C, and D were selected for further optimization.

In analytical chemistry, RSM is used to find optimal conditions. It fits a second-order regression model containing main effects, interactions, and quadratic terms.[17,18] For the case of 2 variables, a second-order regression model is given below:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon,$$

where y is predicted response from the design; β_0, β_1, β_2, β_{11}, β_{22}, and β_{12} are coefficient of variables; and x_1 and x_2 are experimental variables.

RSM can be applied to different design methods such as central composite design, Box–Behnken design.[19] In this study, CCD was carried out 20 different runs composed of 8 factorial points, 6 axial points and 6 center points (Table 3).

Table 3. Design matrix and level of variables for CCD.

Experiment	Uncoded variables			Design matrix		
	A	C	D	A	C	D
1	180	20	0.8	-	-	-
2	200	20	0.8	+	-	-
3	180	30	0.8	-	+	-
4	200	30	0.8	+	+	-
5	180	20	1.2	-	-	+
6	200	20	1.2	+	-	+
7	180	30	1.2	-	+	+
8	200	30	1.2	+	+	+
9	173.2	25	1	–1.68 ($-\alpha$)	0	0
10	206.8	25	1	1.68 ($+\alpha$)	0	0
11	190	16.6	1	0	–1.68 ($-\alpha$)	0
12	190	33.4	1	0	1.68 ($+\alpha$)	0
13	190	25	0.66	0	0	–1.68 ($-\alpha$)
14	190	25	1.33	0	0	1.68 ($+\alpha$)
15	190	25	1	0	0	0
16	190	25	1	0	0	0
17	190	25	1	0	0	0
18	190	25	1	0	0	0
19	190	25	1	0	0	0
20	190	25	1	0	0	0

ANOVA was used to evaluate the significance of the coefficients of the models. The linear terms A (x_1), C (x_2) and D (x_3), quadratic term C^2 and interaction between A and C were significant with small P value ($P < 0.05$) and the other terms (x_3, x_2x_3, x_1^2, x_3^2) were not significant ($P > 0.05$). The following quadratic equation can be used to calculate predicted response:

$y = 4.8667 - 0.4059x_1 - 0.7118x_2 - 0.1516x_3 + 0.0826x_1x_2 + 0.0008x_1x_3 + 0.0018x_2x_3 - 0.0172x_1^2 + 0.1254x_2^2 + 0.0156\ x_3^2$

Graphical representations of the regression equation are given in Figure 2. It was observed that increasing initial column temperature and rate of temperature have a positive effect on retention of EZE. It was concluded that optimum chromatographic conditions for EZE were initial column temperature 206 °C, rate of temperature 33.11 °C min^{-1} and flow rate of gas 1 mL min^{-1}. Under the optimum conditions chromatographic analysis of EZE was achieved in a short time (<4 min).

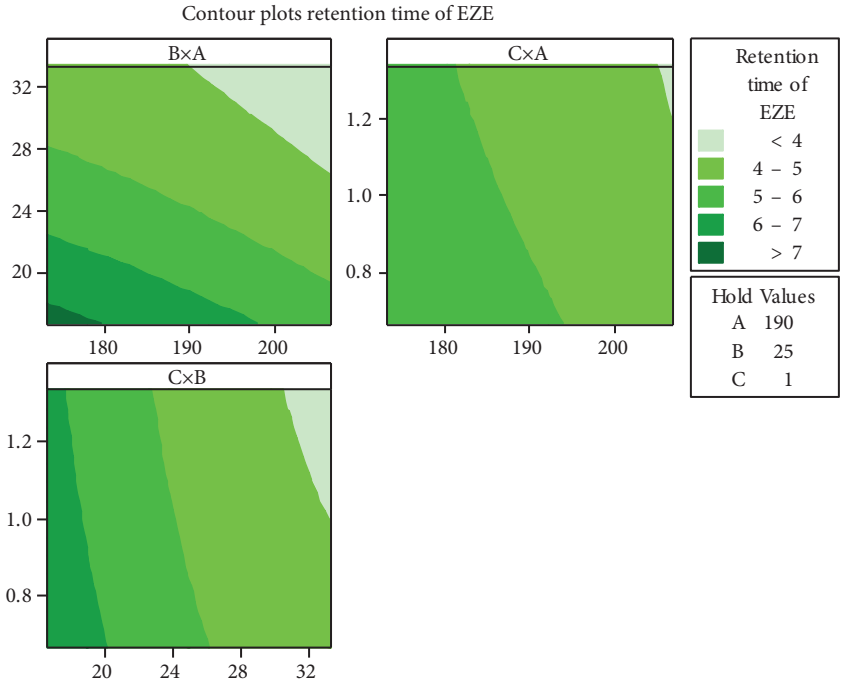

Figure 2. Contour graphs obtained from central composite design.

3.3. Method validation

Evaluating the selected ion chromatograms for selectivity, no interferences were observed with m/z 326, 416, or 463 for EZE or 548 for IS.

LOQ was found to be 10 ng mL^{-1} with acceptable precision (2.77%) and accuracy (102.34%). The developed method was found to be linear over the range of 10–250 ng mL^{-1} with coefficient of determination 0.9986 (R^2). Each concentration on the calibration curve was back-calculated using the calibration equation. The back-calculated concentrations were found within ± 15 of the nominal value.[11]

The values of intra- and inter.day precision and accuracy were <2.77 and <3.23, respectively. These values were within the acceptable ranges. Therefore, it was concluded that the method could produce reproducible and accurate results.

Stability of EZE in human plasma was statistically evaluated by one-sided t-test.[20] The results showed that there were no significant differences in amount of EZE.

Absolute and relative recoveries were found in the range of 94.39%–97.57% for EZE and higher than 98.58% for IS (Table 4).

In the robustness and ruggedness study, evaluating the ANOVA test and the plot of normal probability of standardized effects, it was seen that the effects of selected variables on peak area ratio of EZE to IS were not significant (Figure 3). Therefore, the developed method could be said to be robust and rugged for the variations tested in this study.

3.4. Application of the developed method to real plasma samples

The developed GC-MS method was applied to determine total EZE in human plasma. Human plasma samples were obtained from patients after administration of EZE (10 mg of EZE). Plasma samples from 8 patients

were prepared and then analyzed by GC-MS. The results are given in Table 5. Figure 4 shows the plasma chromatograms of 2 different patients.

Table 4. Recovery of EZE in spiked plasma samples (n = 6).

Nominal amount of EZE (ng mL^{-1})	Absolute recovery (%)	Relative recovery (%)
10	94.44 ± 0.74	94.39 ± 0.69
	RSD: 1.94%	RSD: 1.80%
40	96.87 ± 0.43	96.16 ± 0.55
	RSD: 1.10%	RSD: 1.42%
100	97.04 ± 0.37	96.49 ± 0.44
	RSD: 0.95%	RSD: 1.12%
250	97.24 ± 0.29	97.57 ± 0.68
	RSD: 0.74%	RSD: 1.71%

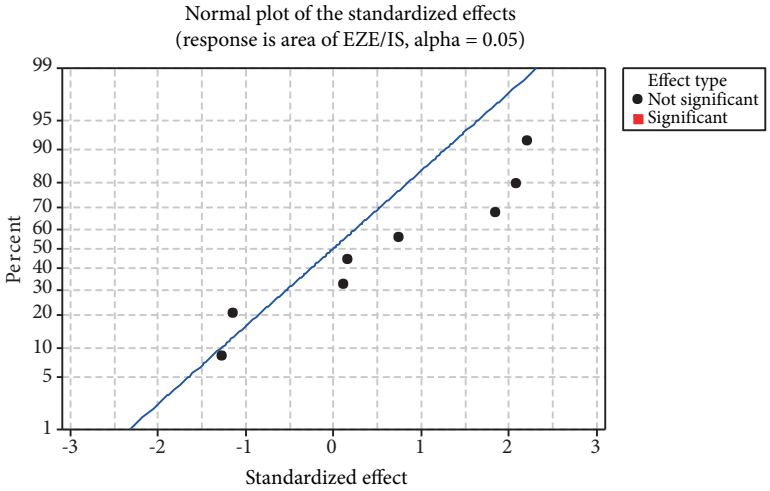

Figure 3. Normal probability graph of standardized effects.

Table 5. Total plasma concentration of EZE in patients receiving a single daily 10-mg dose of EZE.

Patient number	Sex	Amount of total EZE (ng mL^{-1})
1	F	53.07
2	F	40.06
3	F	46.09
4	F	50.20
5	F	59.14
6	F	54.89
7	M	62.09
8	M	55.84

F: Female, M: Male

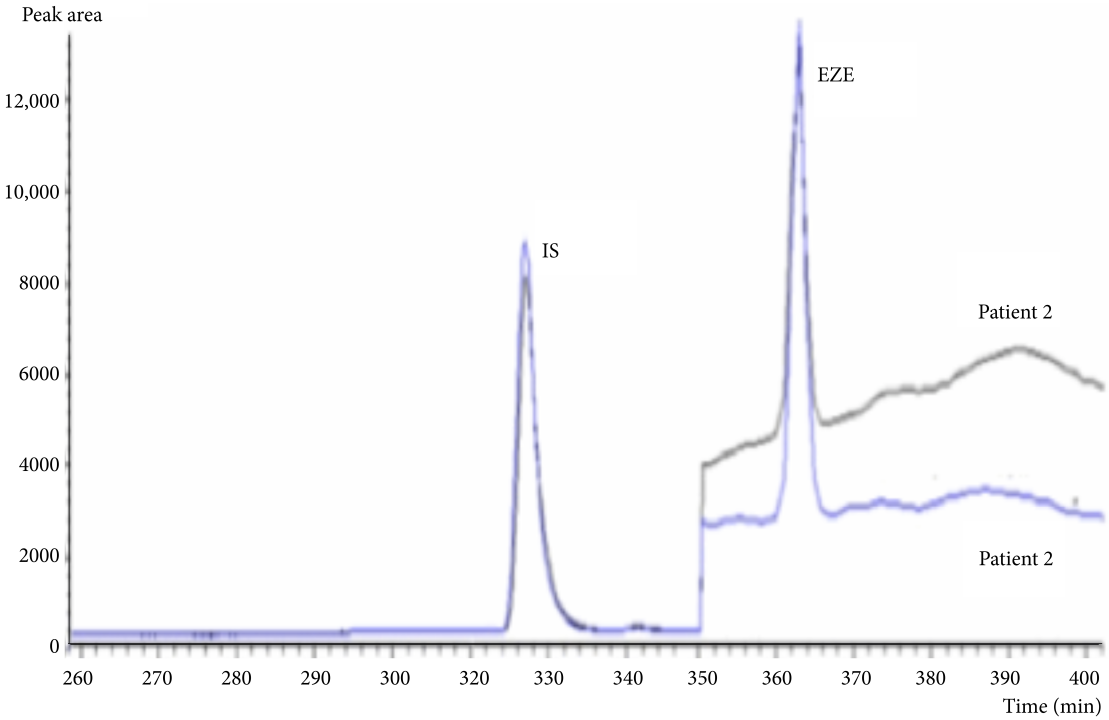

Figure 4. Total ion chromatograms of real human plasma samples from 2 different patients.

4. Conclusion

In this study, a new and fast GC-MS method was developed for the determination of EZE in human plasma. The method has many advantages over the previously reported GC-MS method with respect to total analysis time, limit of quantitation, and recovery.

Extraction of EZE from plasma was achieved with 4 mL of methyl tertbutyl ether after the pH of plasma was adjusted to pH 9 with carbonate buffer. The recovery was greater (97.66%) and the limit of quantitation was lower (10 ng mL^{-1}) compared to the previously reported GC-MS method.[8]

Chemometric methods were used to optimize the chromatographic conditions. Effects of chromatographic variables on retention of EZE were fully investigated by using full factorial and central composite design. The main chromatographic variables, interactions between these variables, and quadratic terms were evaluated with fewer experimental runs.

In optimized chromatographic conditions, total analysis of EZE was achieved in 4 min. This was quicker than the previously reported GC-MS method (about 15 min), which is important for reducing the cost and time in routine analysis of EZE.

The developed method was fully validated to investigate validation parameters such as specificity, sensitivity, linearity, accuracy, precision, recovery, stability, robustness, and ruggedness. It was successfully applied to determine EZE in real human plasma.

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