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Application of one-step liquid chromatography–electrospray tandem MS/MS and collision-induced dissociation to quantification of ezetimibe and identification of its glucuronated metabolite in human serum: A pharmacokinetic study

Gholamreza Bahrami[a,b,*], Bahareh Mohammadi[a], Pyman Malek Khatabi[b], Mohammad Hosein Farzaei[b], Mohammad Bagher Majnooni[b], Saba Rahimi Bahoosh[b]

[a] *Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran*
[b] *School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran*

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ABSTRACT

A new one-step liquid chromatography–electrospray tandem MS/MS method is described to quantify ezetimibe (EZM) a novel lipid lowering drug in human serum. Also using collision-induced dissociation (CID) of the analyte, identification and chromatographic separation of its major metabolite, ezetimibe glucuronide (EZM-G) is achieved in this study. A thawed serum aliquot of 100 μL was deproteinated by addition of 500 μL methanol containing omeprazole as internal standard (I.S.). Separation of the drug, its metabolite and the I.S. were achieved using acetonitrile–water (70:30, v/v) as mobile phase at flow rate of 0.5 mL/min on a MZ PerfectSil target C18 column. Multiple reaction monitoring (MRM) mode of precursor–product ion transition (408.7 → 272.0 for EZM and 345 → 194.5 for the I.S.) was applied for detection and quantification of the drug while, EZM-G was chromatographically separated and identified using CID. The analytical method was linear over the concentration range of 1–32 ng/mL of EZM in human serum with a limit of quantification of 1 ng/mL. The coefficient variation values of both inter- and intra-day analysis were less than 8% whereas the percentage error was less than 3.7. The validated method was applied in a randomized cross-over bioequivalence study of two different EZM preparations in 24 healthy volunteers.

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1. Introduction

Ezetimibe (EZM) [1-(4-fluorophenyl)-3(*R*)-[3-(4-fluorophenyl)-3(*S*)-hydroxypropyl]-4(*S*)-(4-hydroxyphenyl)-2-azetidinone] (Fig. 1a), the first of a new class of compounds, the 2-azetidinone which selectively inhibit the absorption of biliary and dietary cholesterol as well as phytosterols from the small intestine. EZM inhibits a specific transport process in jejunal enterocytes, which take up cholesterol from the lumen [1]. The drug has not any deleterious effect on absorption of fat-soluble vitamins, triglycerides and bile acids. It lowers LDL-C levels by about 18% and it obtained marketing approval in USA in OCT. 2002 as monotherapy or in combination with statins for the reduction of elevated levels of TC, LDL-C, and Apo B in patients with primary hypercholesterolaemia [2].

Following oral administration EZM is readily absorbed and glucuronidated (EZM-G) in the intestinal epithelium which is pharmacologically active phenolic glucuronide. EZM-G enters an enterohepatic recirculation reaching dual peak blood levels at 4–6 and 10–12 h, suggesting enterohepatic recirculation of ezetimibe and its conjugated form [2–5]. The drug and its metabolite are eliminated slowly; with terminal elimination half-life of 20–30 h [6].

Low blood concentrations are achieved following single oral administration of EZM in normal subjects thus, to describe pharmacokinetics of the drug properly; its quantification method in single dose studies should be accurate and sensitive. EZM has been measured in pharmaceutical preparations using micellar electrokinetic chromatography [7] and high performance liquid chromatography (HPLC) with UV detection either alone [8,9] or in a mixed formulation [10,11] however; more sensitive method is needed to quantify the drug in blood samples. By now three

* Corresponding author at: Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.
Tel.: +98 831 8350197; fax: +98 831 8368410.
E-mail address: gbahrami@kums.ac.ir (G. Bahrami).

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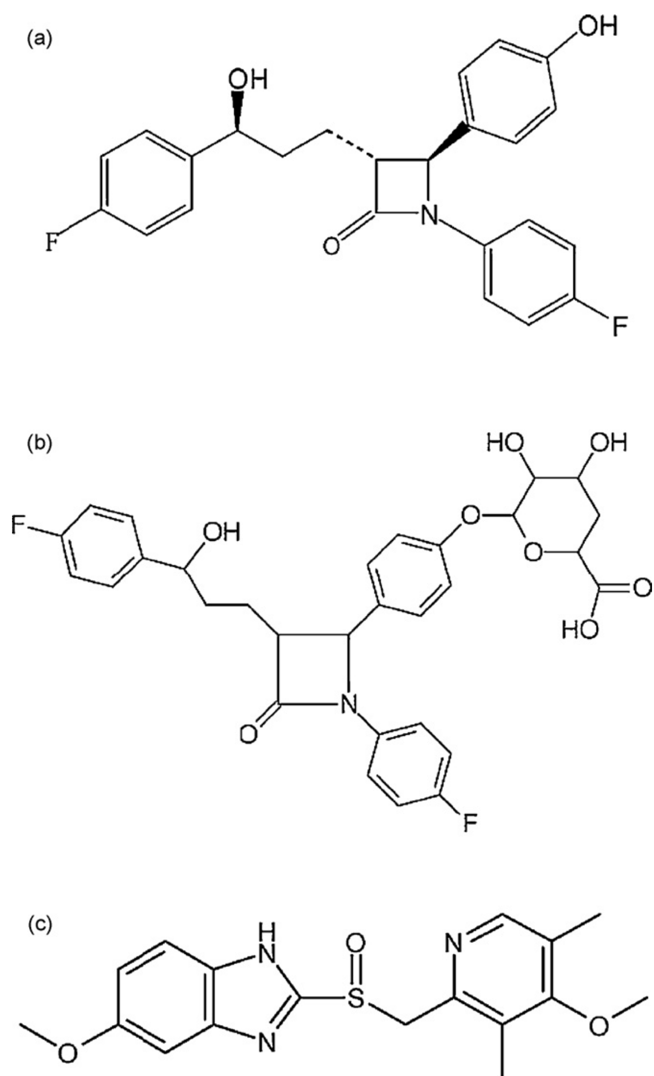

Fig. 1. Chemical structures of (a) ezetimibe (EZM), (b) ezetimibe glucuronide (EZM-G) and (c) the I.S. omeprazole.

analytical methods have been published to determine the drug in human blood samples using LC–MS/MS [11,12] or GC/MS [13] techniques. In these methods however, tedious derivatization procedure [13] or time consuming extraction methods [11,12] are needed. Also in the published LC–MS/MS methods enzymatic cleavage of glucuronide moiety of the analyte has been applied to quantify total EZM. The present paper is the first report of a simple LC–MS/MS procedure to determine EZM in human serum using protein precipitation method in which collision-induced dissociation tandem mass spectrometry (LC-CID-MS/MS) has been used to identify EZM-G. This method was applied and validated in a single dose bioequivalence study, following oral administration of two different EZM preparations in 24 healthy volunteers.

2. Experimental

2.1. Chemicals

EZM (purity 99.3%) was from Ind-Swift Laboratories (Punjab. India) and kindly provided by Exir Pharmaceutical Company (Tehran, Iran). Omeprazole (I.S.; Fig. 1c) was from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany). Water was glass-double distilled and further purified for LC–MS with a Maxima purification system (USF ELGA, England). All other reagents used were of analytical grade.

2.2. Standard solutions and sample preparation

A stock solution of EZM (1000 μg/mL) was prepared in methanol from which the appropriate volumes were diluted with methanol to obtain different working solutions ranging from 10 to 640 ng/mL. Working standard solution of the I.S. (5 ng/mL) was prepared in methanol and was used to precipitate proteins in serum samples. All solutions were stored at 4 °C and were stable for at least 3 weeks except the I.S which was prepared weekly. In a 1.5 mL Eppendorf tube 100 μL serum samples (blank, calibration or unknown) was deproteinated by addition of 500 μL methanol containing the I.S (5 ng/mL). After brief mixing for 10 s on a vortex mixer and centrifugation (1 min at 14,000 × *g*), the liquid phase was removed and transferred to another tube from which 20 μL was injected on to the chromatograph.

2.3. LC–MS/MS equipment and chromatographic conditions

Separation of EZM was carried out isocratically on a MZ Perfect-Sil target C18 column (125.0 mm × 4.0 mm ID., 5 μm) using a C18 guard column (MZ-Anaysentechnik GmbH, Germany). The column temperature was maintained at 38 °C and a mixture of acetonitrile and water (70:30, v/v) was eluted as mobile phase at a flow rate of 0.5 mL/min. Quantitative analysis was performed using an Agilent 1200 series LC system consisting of a quaternary delivery pump, a thermostated column compartment, a degasser (Agilent Technologies, Germany) and a Rheodyne 7725i manual injector valve with a 20 μL sample loop (Cotati, CA, USA). The mass analysis was performed with an Agilent 6410 Triple Quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) which was run by Agilent MassHunter Workstation B.01.03. Ionization was achieved using electrospray ionization (ESI) in the negative mode with the capillary voltage 4000 V. Nitrogen was used as nebulizer gas with nebulizer pressure of 40 psi and source temperature of 100 °C. Drying gas (nitrogen) was heated to 300 °C and delivered at a flow rate of 10 L/min. Fragmentor voltage and collision energy for all analytes were 100 and 3 V, respectively and dwell time was 200 ms. Detection was performed using multiple reaction monitoring (MRM) mode at transitions of 408.7 → 272 for both EZM and EZM-G and 345 → 194.5 for omeprazole (I.S.).

2.4. Validation of the method

2.4.1. Calibration curve and linearity

From the stock solution of EZM (1000 μg/mL) serial dilutions were made to prepare working solutions ranging from 10 to 640 ng/mL. The calibration curves of EZM were prepared at concentrations of 1, 2, 4, 8,12, 16, 24 and 32 ng/mL by spiking the appropriate amounts of the standard solutions in 100 μL pooled human blank serum obtained from the normal subjects. After evaporation of 100 μL from the each working solutions under a gentle stream of nitrogen at 50 °C, the residues were reconstituted in 100 μL of drug-free human serum, mixed for 10 s on a vortex mixer and subjected to protein precipitation and analysis as described above. The linearity of the method was checked in the same day ($n = 6$) and in 6 consecutive days. Calibration curves were obtained using a weighted regression with a weighting factor of $1/(\text{concentration})^2$ by linear least-squares regression analysis plotting of peak–area ratio (EZM to I.S.) versus the drug concentrations.

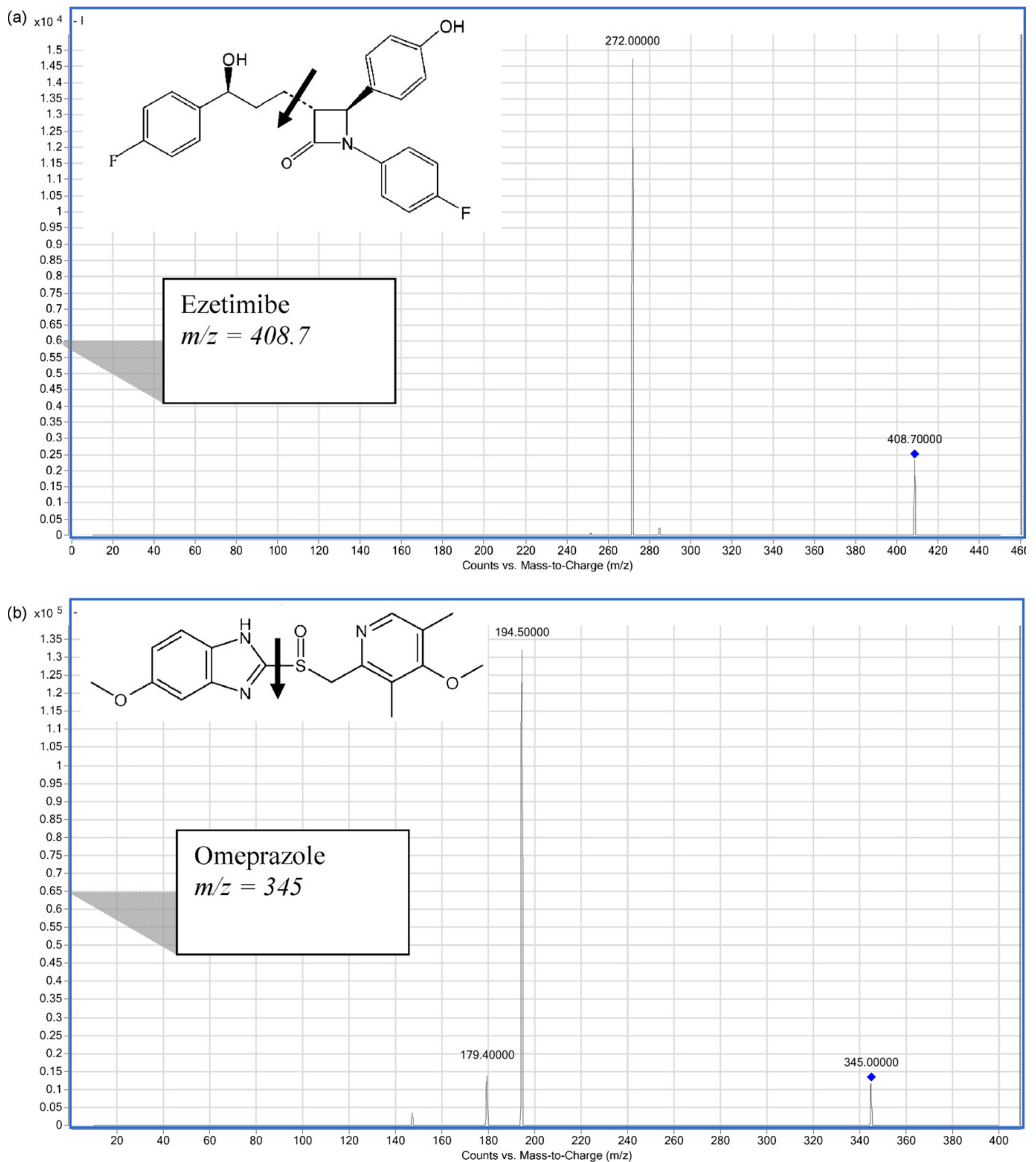

Fig. 2. Structure and product ion spectra of the $[M-H]^-$ ions and the proposed patterns of fragmentation of (a) ezetimibe and (b) the I.S. omeprazole.

2.4.2. Accuracy, precision and sensitivity

Quality control samples used in method validation were prepared with the drug working solutions to make low (1 ng/mL), medium (5 ng/mL) and high (32 ng/mL) concentrations of the analyte. Intra- and inter-day variations were calculated by repeated analysis ($n=6$) of different concentrations of EZM in a single analytical run and in ten analytical runs performed on different days, respectively. The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 3:1. The lower limit of quantification (LLOQ) was defined by calculating precision and accuracy for six samples as the lowest working solution concentration analyzed with accuracy within 80–120% and precision better than 20% R.S.D.

2.4.3. Specificity, matrix effect and stability

The specificity of the method was examined by presence of disturbing endogenous peaks in 24 human serum samples from different volunteers processed by the protein precipita-

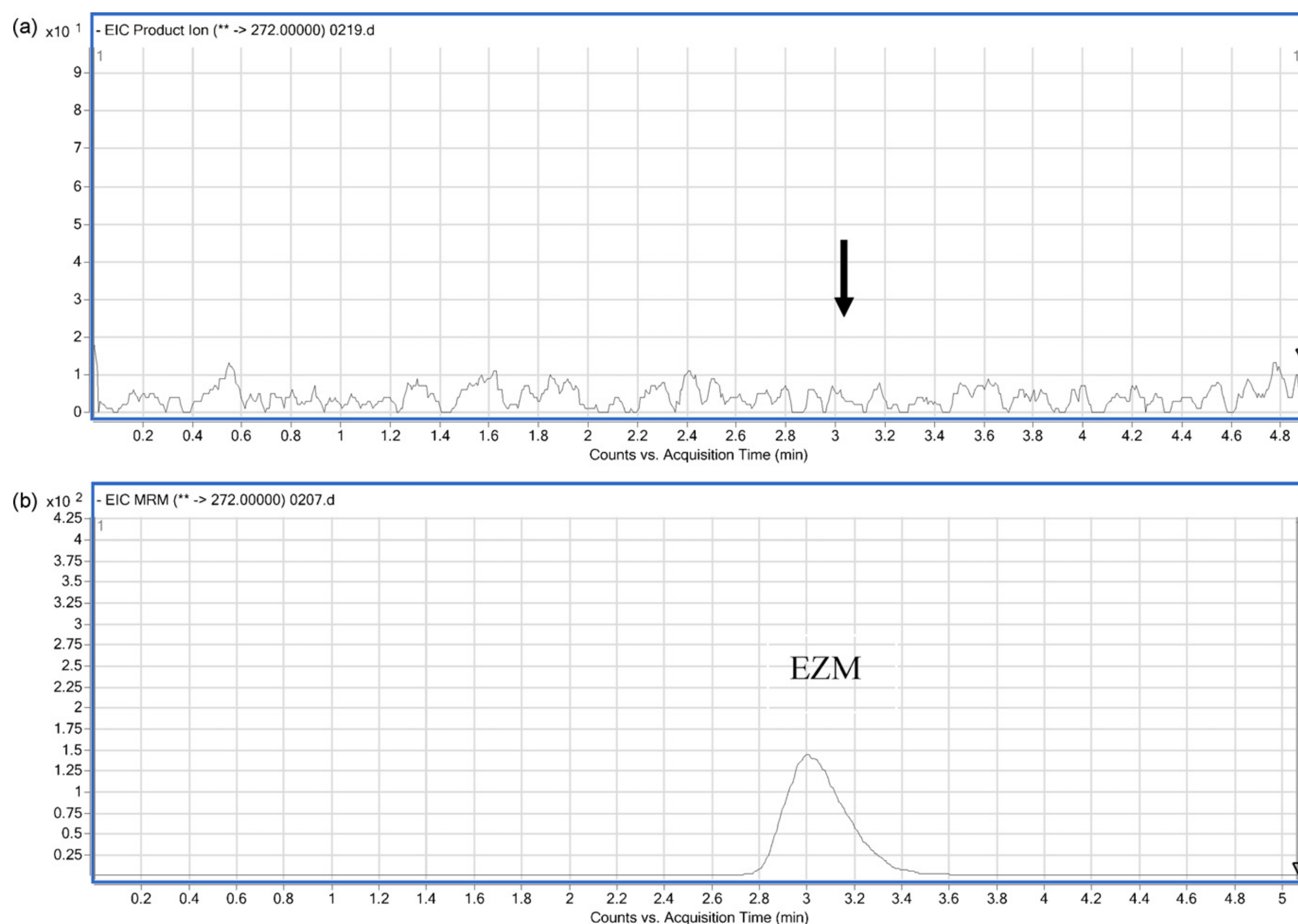

Fig. 3. Multiple reaction monitoring (MRM) chromatograms of (a) human blank serum and (b) human blank serum spiked with EZM at LOQ (1 ng/mL). All chromatograms were obtained by monitoring for m/z transitions 408.7/272 (EZM) and 345/194.5 (omeprazole I.S.) in the negative ion mode.

tion method described above except for the addition of the I.S. The matrix effect of EZM was measured by comparing the peak response obtained from the spike-after-protein precipitation samples at quality control concentration levels with those of the standard solution at the same concentration in the mobile phase. Stability of the quality control plasma samples was subjected to short-term (12 h, 25 °C) at room temperature, three freeze/thaw (−40 to 25 °C) cycles and long-term (30 days, −40 °C) stability tests. Subsequently, the concentrations were measured in comparison to freshly prepared samples. Stability of the solutions of EZM and the I.S. were studied over a period of 3 weeks by comparing of the peak areas at different times.

2.5. Application of the method

The present method was applied to a randomized cross-over bioequivalence study of two different EZM preparations in 24 male healthy volunteers aged 28.5 ± 4.2 years and weighing 72.3 ± 6.8 kg with normal biochemical parameters. Written informed consent was obtained from the subjects and the study protocol was approved by Ethics Committee of Kermanshah University of Medical Sciences. After an overnight fasting, all the volunteers received a single oral dose of 20 mg EZM from either Exir (Tehran, Iran) or MSD SP (Germany) Pharmaceutical Companies on two working days separated by a wash-out period of 3 weeks. Blood sampling were carried out at suitable intervals up to 72 h using disposable glass tubes (100 mm × 16 mm) and the samples were stored at −40 °C until analysis. Pharmacokinetic parameters including maximum concentration (C_{max}), area under the concentration time curve from zero to the time of last sampling (AUC_{0-t}) and area under the concentration time curve from zero to infinity ($AUC_{0-\infty}$) were compared. Student's t-test was used for statistical analysis of the data and statistical significance was defined at the level of $p < 0.05$.

3. Results and discussion

3.1. LC–MS/MS optimization and chromatographic conditions

Both the positive and negative modes of detection were studied and stronger mass response was obtained by EZM in negative electrospray ionization mode hens, acquisition of mass spectrometry data were made in the negative ESI. The mobile phase systems of acetonitrile–water and methanol–water in various proportions were tested. Lower background noise and better symmetric peaks with good ionization properties were obtained by acetonitrile–water (70:30, v/v). The addition of formic acid in different concentrations to aqueous phase not provided any improvement in ionization properties of the analytes.

In order to optimize LC–MS/MS conditions for maximum abundance of the molecular ions of the compounds, acquisition parameters (source temperature, ion spray voltage, collision energy and fragmentor voltage.) were investigated and the highest ion

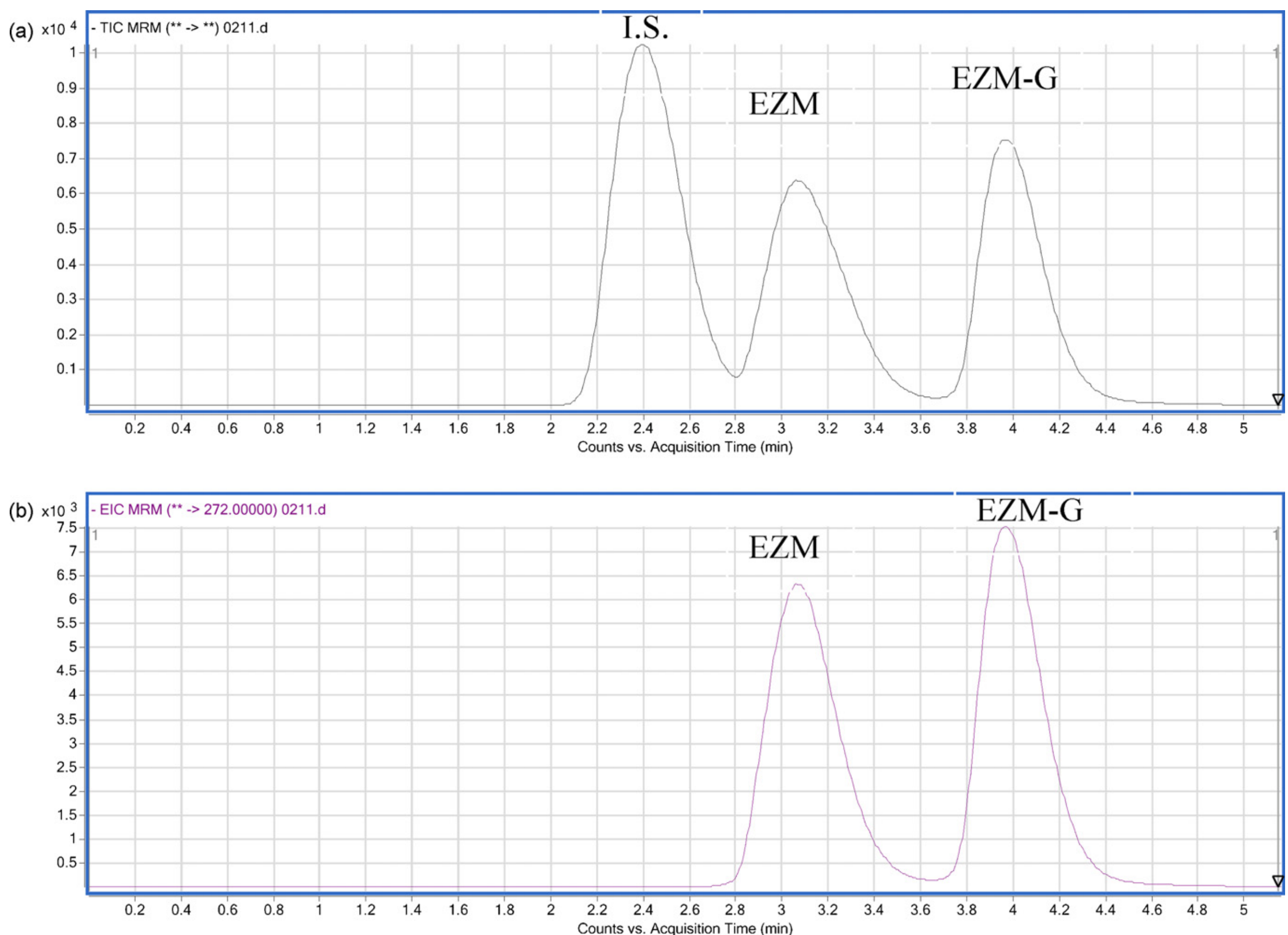

Fig. 4. (a) Total ion chromatogram (TIC) of a sample obtained from a healthy volunteer 12 h post-oral administration of EZM containing the I.S., EZM and EZM-G and (b) their extracted ion chromatogram (EIC) at $m/z = 272$. All chromatograms were obtained by monitoring for m/z transitions 408.7/272 (EZM) and 345/194.5 (omeprazole I.S.) in the negative ion mode.

intensity for molecular ion of the analytes were achieved when the source temperature, ion spray voltage, fragmentor and collision energy were set at 305 °C, 4000 V, 100 and 3 eV, respectively. Using these conditions the negative ion electrospray mass spectrum of EZM and the I.S. produced a molecular ions ($[M-H]^-$) by hydride abstraction at m/z 408.7 respectively corresponding to their molecular weight. After optimization of fragmentor voltage and collision energy, the predominant product ions of m/z 272 (Fig. 2a) and m/z 194.5 (Fig. 2b) were produced from fragmentation of EZM and the I.S, respectively. Therefore, ion transitions of m/z 408.7 → 272 and 345 → 194.5 were selected for the detection of EZM and the I.S., respectively in MRM mode.

Liquid–liquid extraction using different solvents as well as protein precipitation procedure with either methanol or acetonitrile were tested for sample preparation. While time consuming 15 min extraction of the samples with methyl *tert*-butyl ether has been reported by others [11,12], fast analysis is achieved in our study using one-step protein precipitation of the samples by methanol.

In previously published papers after enzymatic cleavage of EZM-G using 1 h incubation of the samples with an enzyme β-glucuronidase, total EZM has been calculated. In our study however, EZM-G was not commercially available thus, considering available data [8] and molecular weight of the glucuronide moiety ($m/z = 176$) we tried to identify EZM-G ($[M-H]^-$) at m/z 584 in serum samples obtained from different volunteers following administration of the drug. But any attempt failed to identify deprotonated precursor of the ion in the samples, using different mobile phases and fragmentor voltage in either full scan or single ion monitoring modes. It has been reported that soft fragmentation of EZM-G produces EZM (m/z 584 → 408) [8]. Hence using transition of m/z 408.7 → 272 and MRM mode, different chromatographic conditions were tested to separate and identify EZM-G in the serum samples. Finally by use of chromatography and LC–MS/MS conditions described above complete resolution of the analytes were achieved, and the I.S., EZM and EZM-G were eluted at 2.4, 3.1 and 3.9, respectively.

Fig. 3 shows the representative multiple reaction monitoring (MRM) chromatograms of (A) human blank serum and (B) human blank serum spiked with EZM at LOQ (1 ng/mL). Total ion chromatogram (TIC) of a sample obtained at 12 h after a single oral dose of 20 mg EZM from a healthy volunteer containing the I.S., EZM and EZM-G has been shown in Fig. 4a, and its extracted ion chromatogram (EIC) at $m/z = 272$ is presented in Fig. 4b. Considering transition of m/z used for analysis of the drug (408.7 → 272) in our method and existence of two separate peaks corresponded with this transition (Fig. 2b) it can be concluded that the peak eluted at 3.9 min is attribute to EZM-G. No endogenous components from serum were found to interfere with the elution of EZM, its metabolite and the I.S.

3.2. Selection of the I.S.

In LC–MS/MS assay especially when matrix effects lead to poor analytical performance, using of an I.S. preferentially deuterated

analogue of the analyte is important to get high accurate and precise results [14]. However, deuterated standard is not always commercially available. Thus a compound with similar structure, extraction recovery, retention and ESI ionization conditions with the analyte may be considered [14]. As there is not any chemical analogue for EZM, in our study various agents from different class of compounds were tested and omeprazole was selected as I.S. due to its appropriate retention time and ESI ionization properties.

3.3. Sensitivity, linearity, specificity and matrix effects

The LOD and LOQ were found to be 0.2 and 1 ng/mL, respectively. The calibration curves were linear over the concentration ranges of 1–32 ng/mL. The correlation coefficients for calibration curves were equal to or better than 0.998. Intra- and inter-day reproducibility for calibration curves were determined on the same day in replicate ($n = 6$) and on different days ($n = 6$) respectively, using the same pooled serum sample. The intra-day average slope of the fitted straight lines was 12.346 ± 0.52 ng/mL (C.V. = 4.5%) and the mean intercept of the calibration curves was 1.839 ± 0.092 (C.V. = 5.2%). The corresponding mean (±SD) coefficient of the linear regression analysis was 0.997 ± 0.011 (C.V. = 0.1%). For calibration curves prepared on different days ($n = 10$), the mean ± SD of results were as follows: slope 12.325 ± 0.055 ng/mL (C.V. = 4.2%), coefficient of the linear regression analysis = 0.9975 ± 0.011 (C.V. = 0.1%) and intercept = 1.60 ± 0.044 (C.V. = 2.7%). There was no significant ion suppression from endogenous substances in matrix and as there was no extraction process in the method, recoveries of the drug and I.S. were not tested.

3.4. Accuracy, precision and stability

The inter- and intra-day accuracy and precision values of the assay method are presented in Table 1. The coefficient variation values of both inter- and intra-day analysis were less than 5% whereas the percentage error was less than 2.9. The stock solutions of EZM and the I.S. were stable for 21 and 7 days, respectively when stored at 4 °C. Stability of the quality control plasma samples were found to be 99.7%, 100% and 100.3% of the initial values, in short-term, three freeze/thaw cycles and long-term stability tests, respectively.

3.5. Application of the method

This method has been successfully applied for the determination of serum concentrations of EZM in a randomized cross-over bioequivalence study following single oral administration of two different preparations in 24 healthy volunteers. Typical serum concentration–time profiles of EZM have been shown in Fig. 5 and resulted pharmacokinetic parameters have been summarized in Table 2. The results show that the preparations were bioequivalent and difference of pharmacokinetic parameters obtained from test and reference were not statistically significant ($p < 0.05$).

Table 1
Precision and accuracy results of the validation. Accuracy has been calculated as a percentage of the nominal concentration.

Known concentration (ng/mL)	Concentration found (mean ± SD)	Coefficient of variation (%)	Accuracy (%mean deviation)
Inter-day ($n = 10$)			
1	1.08 ± 05	4.6	2.9
5	5.11 ± 0.19	3.7	1.8
32	32.64 ± 0.46	1.41	1.3
Intra-day ($n = 6$)			
1	1.04 ± 04	3.8	2.6
5	5.14 ± 0.21	4.1	2.0
32	32.79 ± 0.58	1.80	1.5

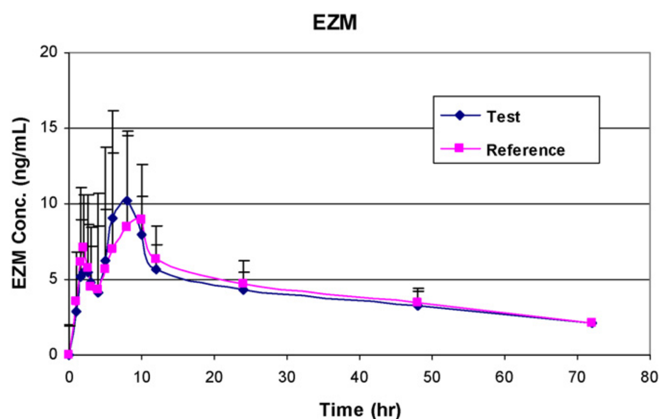

Fig. 5. Mean serum concentrations versus time profiles of EZM for two different preparations in 24 human volunteers after administration of a single 20 mg oral dose.

Table 2
Mean (SD) pharmacokinetic parameters of EZM for two preparations in 24 healthy volunteers after administration of a single 20 mg oral dose. T_{max}, time to maximum concentration; C_{max}, maximum concentration; AUC, area under the concentration time curve; $T_{1/2}$, elimination half-life.

Parameter\Prep.	Test	Reference
T_{max} (h)	4.98(2.09)	4.20(2.63)
C_{max} (ng/mL)	13.6(8.6)	13.4(6.7)
AUC_{0-24} ((ng h)/mL)	186.51(76.9)	193.03(81.5)
$AUC_{0-\infty}$ ((ng h)/mL)	308.88(125.4)	294.75(122.7)
$T_{1/2}$ (h)	36.99(9.7)	32.93(6.9)

4. Conclusion

In conclusion, a new, fast and sensitive method has been validated for the determination of EZM in human serum and identification of its main metabolite using CID/MS. This method which has demonstrated to be suitable for its use in pharmacokinetic studies of EZM, is sensitive with LOQ of 1ng/mL, rapid with simple one-step sample preparation procedure and run time of 4 min.

Acknowledgments

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