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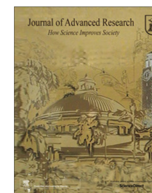

Study of gender-related pharmacokinetics of ezogabine in Egyptian volunteers by a validated LC-MS/MS bioanalytical method

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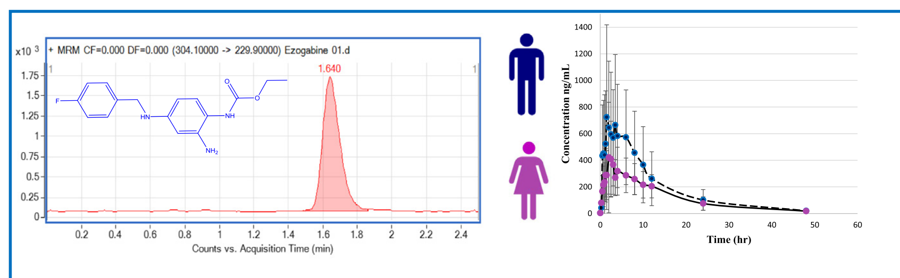
GRAPHICAL ABSTRACT



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ABSTRACT

Gender-based pharmacokinetics and/or pharmacodynamics differences can result in differences in treatment which can accordingly affect the drug safety and/or efficacy. A new validated bio-analytical LC-MS/MS method was developed for the estimation of ezogabine, a third-generation antiepileptic drug, in human plasma using oxcarbazepine as an internal standard (IS) and to study the gender effect on the pharmacokinetic parameters in Egyptian human subjects. Liquid-liquid extraction of plasma samples was performed with diethyl ether: dichloromethane. The separation was accomplished in an isocratic mode with a mobile phase of a mixture of 5 mM ammonium acetate: methanol: acetonitrile pumped on a reversed phase C18 INERTSIL ODS-3 (5 μm, 150 × 4.6 mm). Multiple reaction monitoring was applied and operated by positive mode electrospray ionization. Male and female C_{max} ($p = 0.0308$; CL = 95) and $t_{1/2}$ ($p = 0.0301$; CL = 95) were found to be significantly different using Mann-Whitney *U* test. These findings highlight the difference of ezogabine pharmacokinetics among populations. Further, gender-based ezogabine dose adjustment may be considered.

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Introduction

Epilepsy is a common nervous disorder that affects about 70 million people worldwide [1,2]. Epilepsy disturbs the life quality and can cause health and financial problems for the society [3]. The high incidence of wounds during seizures and the high rate of mortality compared to healthy subjects led to weakening the health quality of people [4,5]. Recently, new approaches assisted in finding new classes of antiepileptic agents, such as ezogabine.

Ezogabine (EZG) (Fig. 1A), N-[2-amino-4-(4-fluorobenzylamino)-phenyl] carbamic acid ethyl ester, also known as retigabine, has been approved in 2011 by the United States Food and Drug Administration (FDA) and the European Medicines Agency [6] and approved and marketed in Egypt in 2017. It is a third-generation antiepileptic drug that acts by activating low-threshold voltage-gated potassium channels in the brain followed

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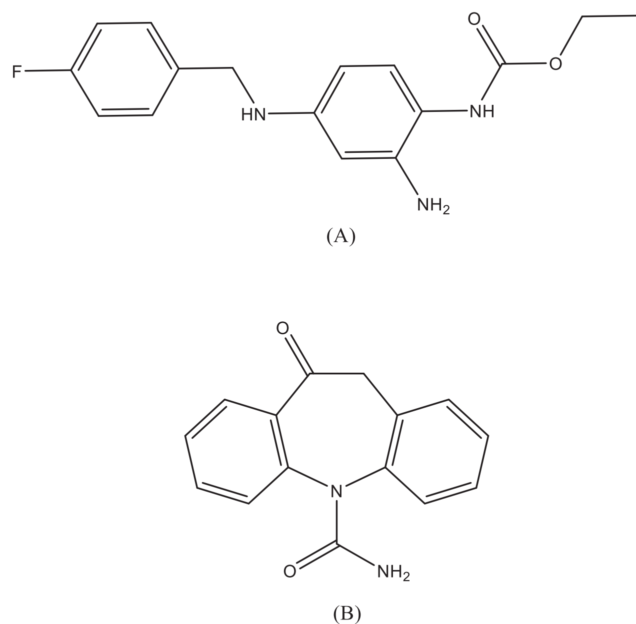

Fig. 1. Chemical structure of (A) ezogabine and (B) oxcarbazepine.

by decreased neuronal excitability with better safety profile [7,8]. It is mainly used for treatment of patients with partial seizures and also useful for treating neuropathic pain and migraine [9–11]. Ezogabine median time to C_{max} is 0.5–2.0 hr. Then, a mono-exponential decline in plasma concentrations occurs with a median half-life of 6–8 hr. Absolute oral bioavailability of ezogabine is ~60%. Its major metabolism happens through N-acetylation and subsequent N-glucuronidation [12].

Pharmacokinetic interindividual variability is mostly the origin of the variation in clinical response to drug administration. In general, this variability may be attributed to the inter-individual variation in the rates the drug is absorbed, distributed and eliminated. Among these factors, gender is quite significant [13,14]. In a previous study, the effect of gender on ezogabine pharmacokinetics in white subjects was evaluated and it was reported the higher AUC and C_{max} values in females than in males [15].

Ezogabine was reported to be estimated by several HPLC methods. Three of these are developed for its determination in pharmaceutical formulations [16–18], four stability-indicating methods by HPLC-UV [19–21], three other methods used LC-MS/MS for its determination in dog plasma [22], in human plasma [23] and for identification of four EZG impurities [24].

Accordingly, the objective of carrying this work was to determine the impact of gender on the pharmacokinetics of ezogabine among Egyptian population and if gender-based dose adjustment is required. A new bioanalytical method was developed and the validation was carried out following US-FDA [25] and EMA [26] guidelines. The developed method was applied to study the difference in the pharmacokinetics of ezogabine between males (n = 10) and female subjects (n = 15).

Experimental

Instrumentation

An Agilent HPLC 1260 series with auto-sampler, gradient quaternary pump vacuum degasser and mixer was used and connected to MS/MS detector (model 6410A)., Agilent MassHunter Workstation software (B.07.00) was used for data acquisition. Other instruments including Vacuum concentrator (Eppendorf, Germany), cooling Centrifuge (Sigma, Germany), Jenway pH-meter (3505, Essex, U.K.), vortex mixer (Stuart, England), and ultrasonic processor (Elma, Germany) were used. Nylon membrane filter (Sigma-Aldrich Co., Germany) was used for mobile phase filtration. The validated WinNonlin 7.0 software (Certara USA) was used for pharmacokinetics calculations.

Material and reagents

Ezogabine was supplied and certified by Optimus, India. Oxcarbazepine (Fig. 1B) was supplied and certified by Amoli organics pvt Ltd, India. Diethyl ether was supplied and certified by Carbon group. Dichloromethane was supplied and certified by Biochem. Methanol and acetonitrile were supplied and certified by Sigma, Germany. Ammonium acetate was supplied and certified by Loba chemie, India. Membrane filters 0.22 μm from ChromTech (UK) were used. All other chemicals and reagents used were of analytical grade unless indicated otherwise. Human plasma specimens acquired from blood bank was used for priori and in-life validation.

LC-MS/MS conditions

Separation was carried out on a reversed phase ZORBAX Eclipse plus C18, 5 μm. The mobile phase was 5 mM ammonium acetate: methanol: acetonitrile, 30:50:20 v/v/v. The column temperature was adjusted at 40 °C at a flow rate of 0.6 ml/min with an injection volume 2 μL. The retention times of Ezogabine and oxcarbazepine were about 1.4 and 1.6 min, respectively within a run time of 3.0 min duration.

Both ezogabine and IS were detected by operating the MS/MS system with the positive ion mode using a spray gas pressure of 45 psi with a nitrogen flow of 11 L/min, capillary voltage (4000 V) and dwell times (200 ms). Fragmentor voltage was set at 130.0 V for ezogabine and at 135.0 V for IS while the collision energies were set at 1.0 V for ezogabine and 5.0 V for IS. MRM transitions were measured at: m/z 304.1 → 229.9 for ezogabine and m/z 253.2 → 236.1 for IS.

Preparation of standard solutions

A solution of ezogabine in 100 ml methanol was prepared (Solution A, 200 μg/mL). Then, it was diluted with diluent I (methanol: water, 50:50, v/v) to prepare Solution B (20 μg/mL) and Solution C (5000 ng/mL). A solution of oxcarbazepine in 100 mL methanol was prepared (Solution D, 100 μg/mL), which was further diluted with diluent I to prepare Solution E (10000 ng/mL). All the standards were stored at −20 °C till the time of analysis.

Calibration and quality control samples preparation

Prepared calibration curves consisted each of 8 calibration standards, a blank sample and a zero sample to be quantified. Control human plasma (450 μL) was spiked with 50 μL of IS stock solution E and 50 μL of Ezogabine standards solutions to prepare the Calibration standards. So, the spiked samples final concentration of calibration standards will be in the range of 10–2000 ng/mL and the QC's are 30, 800 and 1600 ng/mL for QCL, QCM and QCH, respectively, then the samples were mixed by vortex.

Sample preparation

Five mL of diethyl ether: dichloromethane (70:30, v/v) were added to each sample (0.5 mL), then, fifty μL of IS (Solution E 10,000 ng/mL) were added and the mixture was vortexed for 1 min and centrifuged for 10 min at 4500 r.p.m. The organic layer

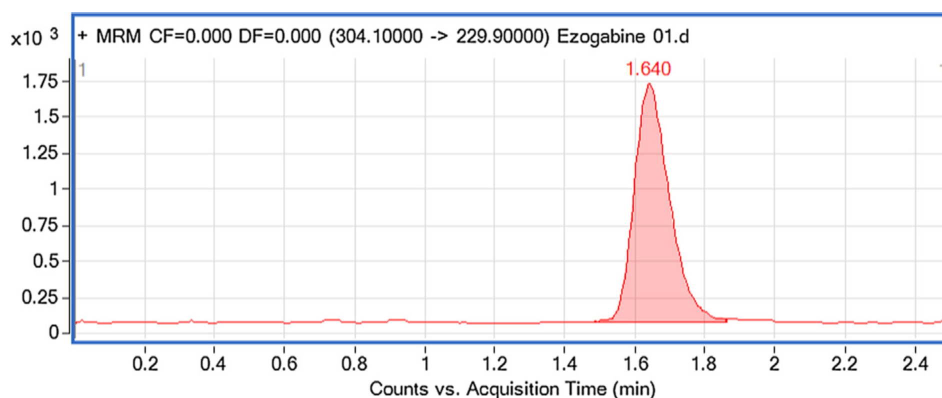

Fig. 2. Chromatogram of Ezogabine (10 ng/mL).

was separated, evaporated under vacuum, then reconstituted in 100 μL of the mobile phase, and injected on the column.

Pharmacokinetic study

The main aim of this study is to explore the impact of gender on the pharmacokinetics of ezogabine in Egyptian volunteers (n = 25; 10 male and 15 female). The review and approval of the experimental procedures and protocols were carried out by the ethics committee of Pharmasolutions CRO, Cairo, Egypt. Male and female volunteers were fasted for 10 h but consuming only water one hour before and two hours after dosing (oral tablet containing 400 mg EZG). Blood samples (5.0 mL) were collected from a forearm vein into polypropylene tubes containing K_2EDTA at 0.00 (pre-dose), 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12, 24 and 48 h after dosing after oral administration of Trobalt 400 mg film coated tablets. The samples were immediately centrifuged, to separate plasma which was stored at −70 °C until time of analysis. For analysis, calibrators and samples were thawed without assistance for about 60 min. Concentrations of EZG in plasma of subjects were calculated using the validated LC–MS/MS bioanalytical method. The pharmacokinetic parameters for EZG were calculated using the validated WinNonlin 7.0 software. Pharmacokinetics Parameters were determined using a non-compartmental approach with a log-linear terminal phase assumption. Linear trapezoidal rule was used to calculate $AUC_{0\text{-}t}$ (AUC from time 0 to the last measurable Cp). The extrapolated AUC (from time of the last measurable Cp to infinity) was estimated from the last measurable Cp divided by λz. $AUC_{0\text{-}inf}$ (AUC from time 0 to infinity) equals to $AUC_{0\text{-}t}$ plus the extrapolated AUC ($AUC_{t\text{-}inf}$). C_{max} was obtained from observed time-Cp profile.

Statistical analysis of the obtained data was performed using GraphPad Prism 7 software.

Results and discussion

Method development

Mass spectrometric detection optimization

Optimization of tandem mass parameters were carried out to obtain the maximum response for both drug and IS. The MRM was selected based on the highest sensitivity. Detection of ezogabine and IS was carried out using the following transitions: m/z 304.1 → 229.9 and m/z 253.2 → 236.2, respectively.

Plasma extraction optimization

Extraction of plasma samples is essential in bioanalytical methods to ensure maximum sample purification from plasma components. Plasma samples were extracted with different solvent mixtures such as dichloromethane, diethyl ether, ethyl acetate or tri-butyl methyl ether. Finally, diethyl ether: dichloromethane (70:30, v/v) resulted in high response and good percentage recovery.

Chromatographic conditions optimization

Chromatographic conditions were optimized by trying various stationary phases and mobile phase composition. Tried columns include C18 Zorbax, Eclipse Plus (1.8 μm, 50 × 2.1 mm) and reversed phase C18 INERTSIL ODS (3.5 μm, 150 × 4.6 mm). For mobile phase optimization, ammonium acetate, ammonium formate buffers or aqueous formic acid were tried in mixture with an organic modifier such as methanol and/or acetonitrile. Good peak shapes and separation were obtained by using methanol and acetonitrile. Ammonium formate buffer (5 mM) was found to be the optimum to obtain the highest detection response (Fig. 2).

Bioanalytical method validation

Pre-study validation for bioanalytical method development assures the suitability of the method for planned application. The following validation parameters are usually evaluated for quantitative procedures: linearity, quantitation limit, matrix effect, selectivity, recovery, precision, accuracy, robustness, and stability and dilution integrity. In-process validation was carried out analyzed using QC samples. With each batch, QC samples were prepared and analyzed along with subjects' samples.

LLOQ of ezogabine is 10 ng/mL with a percentage of nominal concentration of 97.99% and a CV of 9.65%. ULOQ (Upper Limit of Quantification) was 2000 ng/mL.

The concentrations of calibration standards covered the range (10–2000 ng/mL). Weighted linear regression ($1/X^2$) was applied. In the present method, calibration curve was found to be consistently accurate and precise over the concentration range of 10–2000 ng/mL. The mean Coefficient of Determination (R^2) is equal to 0.9989.

Method selectivity was tested by treating and chromatographing six blank samples from different sources. No significant interference was detected in all the plasma blanks at the retention times of ezogabine and IS (Fig. 3).

Recovery of the drug was calculated by comparing mean analyte responses of three processed QC samples obtained by the usual extraction procedure (without addition of internal standard) with working solutions analyzed without processing. The average recovery across the three concentrations was not affected by concentration. Recovery of oxcarbazepine (internal standard) from human plasma by the assay method was assessed by comparing

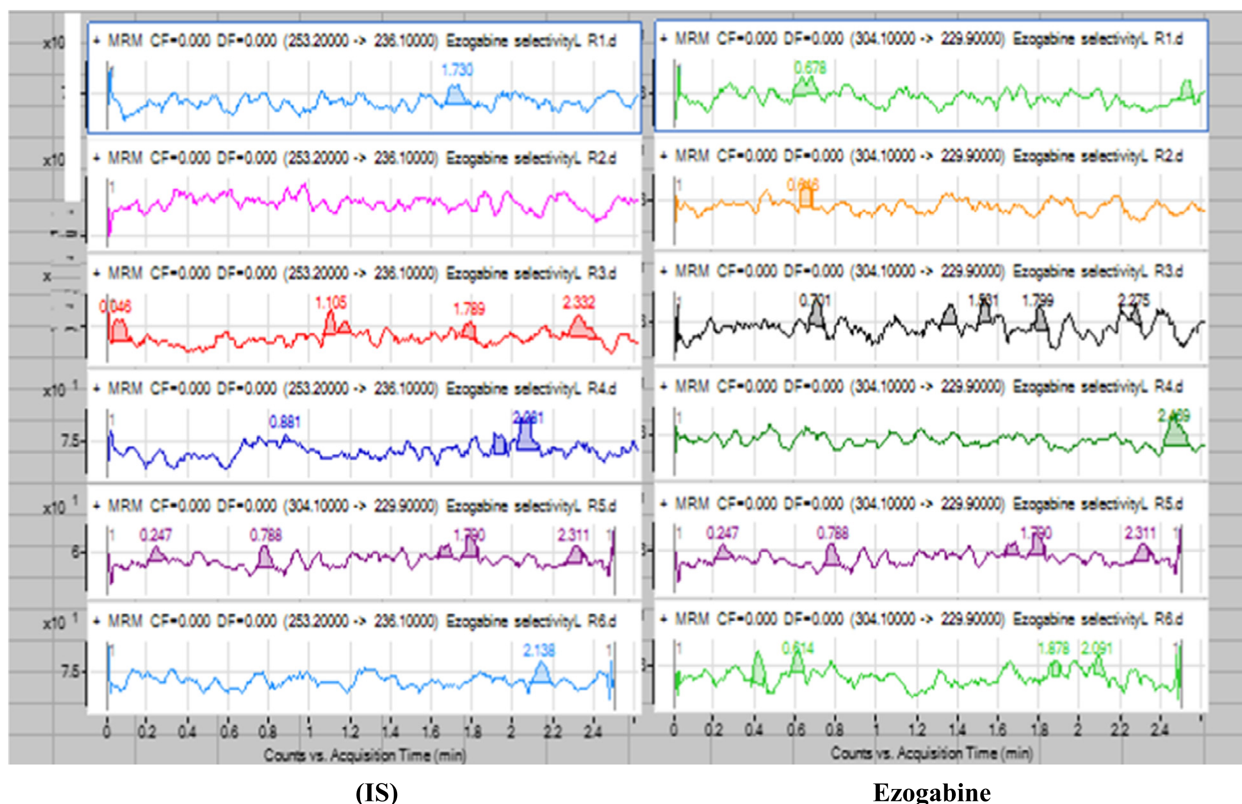

Fig. 3. Processed blank plasma samples chromatograms from six different subjects.

mean IS response from one distinct concentration in plasma (IS working solution) with working solutions of the same concentration. Table 1 shows high recovery results confirming good extraction efficiency.

Matrix effect was calculated using six batches of blank matrix from individual volunteers at low and high QC sample levels. The IS normalized MF and the CV% of the IS-normalized MF are presented in Table S1, See Supplementary File. Moreover, carry over test was carried out by injecting blank sample after each ULOQ calibrator (n = 6) (Table S2, See Supplementary File).

To assess the precision and accuracy, six determinations of LLOQ and quality control samples were analyzed on three different days (Table 1).

In the present method, Dilution integrity quality control samples were prepared by diluting plasma stock with a concentration of 3600 ng/mL. Six samples were diluted twice and six others were diluted four times. Precision and accuracy were confirmed for both dilution factors.

Coefficient of variation% test was 5.051 and 6.328 for two-fold and four-fold dilution, respectively. The accuracy results were

Table 1
A summary of the validation results for Ezogabine.

Parameter	Item	Results		
Linearity: Coefficient of Determination	**R^2**	0.9989		
Calibration Curve Range (ng/mL)		10–2000		
Lower Limit of Quantitation (ng/mL)		10		
		QCL	**QCM**	**QCH**
Inter-day Accuracy (%)	**Accuracy**	108.95	102.46	105.78
Inter-day Precision (%)	**CV %.**	4.42	8.51	4.68
Intra-day Accuracy (%)	**Accuracy**	109.17–111.46	101.93–104.16	102.94–107.63
Intra-day Precision (%)	**CV %**	1.936–6.661	8.733–9.373	4.166–5.081
Recovery of Analyte (%)	**QC mean % recovery**	89.564	90.785	87.675
Dry extract stability (%)	**Accuracy**	95.40		88.33
Short-term stability of analyte in matrix at room temperature (%)	**Accuracy**	102.96		90.47
Long-Term Stability of Analyte in Matrix at −70 °C (%)	**Accuracy**	93.84		98.32
Freeze and thaw Stability of Analyte in Matrix at −70 °C (%)	**Accuracy**	90.87		101.58
Post-Preparative Stability (%)	**Accuracy**	107.60		94.03
Stock Solution Stability of the drug	**Stability %**	**6 hrs**	93.71	
		10 days	99.87	
Stock Solution Stability of the internal standard	**Stability %**	**6 hrs**	102.04	
		10 days	108.53	

QCL, QCM and QCH are quality control samples low, medium and high.
CV% Coefficient of variation.

99.537 and 101.774 for two-fold and four-fold dilution, respectively.

To test short term stability, thawing of triplicates of the low and high QCs (including the addition of internal standard) was carried out at room temperature (22.5 ± 2.5 °C) and then analyzed after keeping it this temperature for 6 h. This was repeated but with freshly processed QC samples with calibration curve and then analyzed for comparison. Results confirm the stability of the analyte and internal standard in processed samples at the room temperature for up to 24 hrs without significant effect on concentration.

Freeze and thaw stability testing was carried out by the storage of samples at two concentrations (QCL, QCH) at −70 °C for at least 12 h followed by thawing unassisted at room temperature for one hour. This was repeated for four cycles. After the third cycle, thawed samples were quantified together with comparison samples.

For long term stability, three replicates were prepared in human plasma at two concentrations (QCL, QCH) and stored at −70 °C (Stability solutions).

Three aliquots of each of the low and high QCs (including the addition of internal standard) were extracted as mentioned before but left at dry state without reconstitution with the mobile phase for 45 h at room temperature.

Results confirm the stability of the analyte and internal standard in the processed samples at room temperature for up to 24 hrs, after three Freeze and thaw cycles, and in the dry extract at the room temperature for up to 45 hrs for the entire period of the study without significant effect on concentration.

Autosampler stability was assessed by preparing QCL and QCH samples and then processed and left for 24 hrs at room temperature (22.5 ± 2.5 °C). Samples were found to be stable for up to 24 hrs.

The stability of the drug and the internal standard stock solutions was evaluated at room temperature (22.5 ± 2.5 °C) for at least 6 h. Results confirm the stability of the analyte and internal standard stock solutions at room temperature for up to 6 hrs and frozen at −20 °C for 10 days (Table 1).

Pharmacokinetic study

Although an equal number of males and females was targeted in the beginning of the study, it ended up to uneven number of the two genders (males, n = 10 and females, n = 15) as a result of inclusion/exclusion criteria. The Mann–Whitney *U* test was used for the statistical comparison of the two groups as it is the true nonparametric counterpart of the *t*-test and it was found that the data do not follow a normal distribution [27].

As shown in Fig. 4, the male and female pharmacokinetic profiles are plotted. The summary of their PK parameters and their statistical description are listed in Table S3, See Supplementary File. Such results show that the difference in ezogabine concentration between males and females have been identified with higher concentrations among males.

The t_{max} and AUC were not significantly different between the two genders. A slight insignificant difference of the average time needed in male and female for attaining maximum plasma concentration (C_{max}) where the t_{max} was 2.92 ± 2.39 h in men and 4.38 ± 3.08 h in women (p = 0.1349; confidence level (CL) = 95). Likewise, the total exposure of ezogabine in both male and female subjects was found insignificantly different (AUC = 9479.62 ± 5928.49 and 6281.37 ± 2514.77 hr.ng/mL, respectively) with moderately increased extent of exposure in men compared with women. No significant difference was found between the weight-normalized CL/F and Vz/F of male and female.

On the other hand, women exhibited significantly longer $t_{1/2}$ (10.13 ± 2.1 h) than in men (8.57 ± 1.18 h), (p = 0.0301; CL = 95).

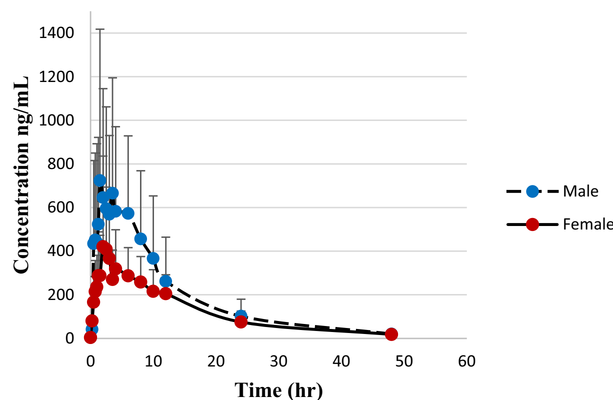

Fig. 4. Mean plasma concentration-time profile of Ezogabine after a single oral dose of Trobalt® 400 mg. Error bars represent standard error of mean.

Similarly, a significant different C_{max} was observed with higher C_{max} in male compared to female (p = 0.0308; CL = 95). These findings are not in agreement with previously reported study on white subjects, where the pharmacokinetic parameters were to some extent higher in women over men [15], which denotes that a gender-based inter-individual variability is observed in the pharmacokinetics of ezogabine among different populations.

Conclusion

Gender-based variation in the pharmacokinetic profile of ezogabine among Egyptian subjects was studied using a validated LC-MS/MS method. The pharmacokinetic profile of the studied Egyptian subjects was highly variable among individuals with significant difference in terms of both C_{max} and $t_{1/2}$ between male and female subjects. Moreover, the results were not in agreement with a previous study on white subjects, highlighting on a gender-based inter-individual variability among populations. This presented the importance of therapeutic drug monitoring to avoid possible side-effects or sub-therapeutic doses and subsequently considering gender-based dose adjustment.

Compliance with ethics requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2019.11.008.

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