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Research Article

Bioequivalence of Atorvastatin and Ezetimibe Including Four-Period Crossover Clinical Design

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Abstract

Atorvastatin (AT) and Ezetimibe (EZ) have high intra-subject variation, making bioequivalence investigation challenging. Herein, the current study has applied a novel clinical design, including a randomized, four-period, two-treatment, two sequences crossover open-label study design, to overcome the intra-subject variation for AT and EZ in bioequivalence investigation, post-oral administration of a single dose for 10/40 mg EZ and AT in forty healthy male adults, under fasting conditions, using a newly developed and validated bioanalytical method, which included a novel LLOQ of 0.04 ng/ml for EZ. Where the LC-MS/MS method has been validated for simultaneous determination of AT and EZ, including free EZ (unconjugated EZ) and total EZ (sum of free EZ and EZ-glucuronide). AT with its labeled internal standards (IS; AT-D5) and EZ with its labeled IS EZ-D4 were extracted from plasma by protein direct precipitation using acetonitrile. The dynamic range was 0.4-60 ng/ml for AT, 0.04-6 ng/ml for free EZ, and 1-120 ng/ml for total EZ. The Cmax and AUC0-t of reference product for AT were 24.89, 124.20, free EZ 4.79, 59.53, and for total EZ were, 32.26, 243.97. All validation results were within the acceptance criteria. The outcome for investigated test product was bio-comparable to the reference product.

Keywords: atorvastatin; ezetimibe; bioequivalence; lc-ms/ms; clinical design

Introduction

Atorvastatin (AT; Figure 1A) and Ezetimibe (EZ; Figure 1B) combo drug is used along with diet to control high cholesterol and triglycerides levels in the plasma, which helps prevent medical problems like a heart attack or stroke caused by clogged plasma vessels in patients at high risk of cardiovascular and cerebrovascular with atherosclerotic diseases (Ai *et al*., 2018; Zhan *et al*., 2018). It is also used for the treatment of homozygous familial hypercholesterolemia conditions. Furthermore, the combination use of EZ with AT helps to enhance the effect of lower doses and reduce the consequent side effects for homozygous familiar hyperlipidemia caused by statin (Hamilton-Craig *et al*., 2010). AT and EZ work in different mechanisms, where AT is a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor "statin class," acts as a lipid regulating drug which prevents the production of cholesterol in the body (Sirtori, 2014; Willey and Elkind, 2010). At the same time, EZ reduces cholesterol absorption from ingested foods (Prasad *et al*., 2013; Wang *et al*., 2013).

Pharmacokinetically, AT exhibits a dose-dependent and non-linear kinetics in human plasma post oral administration; it is very rapidly absorbed to raise its maximum concentration (C_{max}) around 28 ng/ml, which reached within maximum time (T_{max}) 1-2 hr with an AUC about 200 ng·hr/ml, it just has approximately 15% bioavailability due to the high first-pass effect (Lins *et al*., 2003) which lead to high intra-subject variation (Hwang *et al*., 2020) as well as, EZ post oral administration is rapidly absorbed and extensively metabolized (>80%) to the pharmacologically active ezetimibe-glucuronide.

Total EZ (sum of free EZ (unconjugated EZ) and EZ-glucuronide) concentrations reach a maximum of 1-2 hr (Kosoglou *et al*., 2005).

The bioequivalence studies for such variable intra-subject available drugs make clinical application challenging. They need highly sensitive analytical methods to detect the low levels of free EZ, where few studies have reported the bioequivalence of AT and EZ following two periods of crossover design (Abdelbary and Nebsen, 2013; FDA, 2011; Gowda et al., 2007).

To our knowledge, no reported method has applied a four-period crossover clinical design to overcome the intra-subject variation. Therefore, we designed for the first time in the current study a randomized, four-period, two-treatment, two sequences crossover clinical study to investigate the bioequivalence of AT and EZ simultaneously under fasting conditions. On the other hand, many bioanalytical methods for simultaneous determination of AT and EZ have been reported by different techniques, including LC-MS/MS (Abdelbary and Nebsen, 2013; El-Bagary *et al*., 2014; ELAWADY *et al*., 2021; Gowda *et al*., 2007), and the most sensitive reported a bioanalytical method for free EZ determination was not below 0.1 ng/ml for the lower limit of quantification (LLOQ) (Abdelbary and Nebsen, 2013). Herein, we developed and validated a new, more sensitive bioanalytical method, established upon 0.04 ng/ml as LLOQ to quantitate free EZ, including direct protein precipitation as a single extraction step for sample treatment which makes an easy, more economical, and rigid method.

Figure 1:

Experimental

Material and Methods

AT calcium at purity = 92.1% with its labeled internal standard (AT-D5 purity = 86.1%) and EZ at purity = 99.9% with its labeled IS (EZ-D4 purity = 97.0%) were obtained from TRC. β-Glucuronidase-Type HP-2 solution was obtained from Sigma. The collected plasma blank samples were obtained from donors at the Jordan center for pharmaceutical research (JCPR) clinical site. The LC-MS/MS-quality deionized water, acetonitrile, methanol, and acetic acid were purchased from Fisher, Germany; in addition to the other chemicals were all analytical grade.

Instrumentation

The Mass spectrometer was API 6500+, Applied Biosystems, MDS SCIEX, coupled to LC from Agilent 1200 series. Computer System of Windows 10 SP1 and Analyst 1.6.3 software for the data management system.

HPLC Conditions

Chromatographic conditions consisted of a mobile phase of 0.03% acetic acid: acetonitrile (35:65 v/v), pumped in an isocratic mode through a column of Inertsil C8-3, (2.1×50) mm, 5 µm, at a constant flow rate of 0.4 ml/min under the fixed temperature of 35 °C for column oven and samples tray temperature set at 10 °C where the injection volume was 2 µL and total run time of 3.2 min for AT, free EZ and 0.9 for total EZ.

Mass spectrometric conditions

The optimized mass spectrometric (MS) conditions for both AT and EZ were at negative MRM scan mode, where AT MS conditions were DP -43, EP -9, CE -39, and CXP -20, while the optimized MS conditions for EZ were DP -70, EP -9, CE -22.5 and CXP -15. The ion source conditions were curtain gas = 30, CAD = gas 8, gas1 = 45, gas2 50, gas2 = 35, evaporation temperature = 550 °C and the ion source voltage = -4500 V under positive scan mode.

Standard solution

AT and EZ with both IS's major standard solutions were prepared in methanol separately, then the working standard solutions were diluted from the primary solution by 50% v/v methanol in water to prepare the serial dilution that was used for plasma spiking of calibrator samples, where serial solutions of AT were mixed with EZ solutions.

Standard calibration curves and quality control samples for AT and free EZ

The calibrators and quality control samples (QC) for AT and total EZ in human plasma (pooled blank) were prepared by a single spiking step of 20 µL of working mixture solution (contained AT and EZ) into 180 µL of plasma to prepare the calibrators of 0.4, 0.8, 2.0, 4.0, 10.0, 20.0, 40.0 and 60.0 ng/ml, QC low 1.2, QC mid1 7.2, QC mid2 24.0 and QC high 48.0 ng/ml for AT, and free EZ are 0.04, 0.08, 0.20, 0.40, 1.00, 2.0, 4.0 and 6.0 ng/ml, QC low, mid1, mid2 and high are 0.12, 0.720, 2.400 and 4.800 ng/ml, respectively. Serial dilutions for total EZ calibrators are 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 80.0 and 120.0, and 3.0, 15.0, 48.0, and, 96.0 ng/ml for QC low, mid1, mid2 and high, respectively.

The proposed dynamic range of the calibration curve follows the European guideline for bioequivalence studies (European Medicines Agency, 2012) and upon a maximum concentration of AT (Lins *et al*., 2003) and EZ (Kosoglou *et al*., 2005) in human plasma. The lowest calibrator concentration in each dynamic range was considered the LLOQ. The spiked QC samples were divided into aliquots and stored in a deep freezer of −35 to −45 °C until analysis along with the subject's samples under the same storage conditions. The calibration curve in the method validation and routine application was established from a blank sample, zero level (spiked IS in the blank), followed by eight calibrator points, including LLOQ.

β-Glucuronidase-type HP-2 solution

Dilute 13.468 ml from β-Glucuronidase-Type HP-2 (111372 unit/ml) into 150 ml of water to obtain 10000-unit β-Glucuronidase /ml and mix well.

Sample preparation

AT and free EZ with both internal standards (AT-D5 and EZ-D4) were extracted from human plasma samples by protein direct precipitation technique in a single extraction step, where 0.2 ml of plasma (spiked blank or volunteer's unknown sample) was pipetted into an adequately labeled tube, and 50 µL of IS mixture solution was added. Vortex mixed for 10 sec, the precipitation agent 0.5 ml acetonitrile added and vortexed for 0.5 min using a Vibrax Type VX-Z, VXR BasicVortexer (IKA-Werke GmbH & Co., Staufen, Germany) then centrifuged for 5 min at 16000 rpm by Eppendorf centrifuge (Germany). The supernatant was transferred into a glass flat-bottom insert vial for analytical injection.

For total EZ extraction, a 50 µl of sodium acetate (1M, pH 5.0) was added to 0.2 ml of plasma, followed by adding 50 µl β-Glucuronidase (10000 unit/ml), then vortex mix for 30 seconds and incubated at 50 °C for 60 min, after cooling at room temperature, 100 µl of sodium carbonate (0.2 M) was added and vortexed for 30 seconds, then 6 mL of extraction solvent (MTBE) was added and vortexed for 3.0 min, then centrifuged for 5 min at 4400 rpm, the samples were then transferred to the deep freezer for 40 min at -40 ˚C, then the supernatant decanted into a clean labeled evaporating glass tube, after evaporation by compressed air in a water bath at 40 °C, the residue was reconstituted by 300 µl of mobile phase and vortexed for 1 min, the supernatant was then transferred into an autosampler vial.

Bioanalytical method validations

The developed method for simultaneous determination of AT and free EZ in human plasma was fully validated separately from another fully validated method for the determination of total EZ in concordance with the European (Products, 2019) and US FDA (Food and Drug Administration, 2018) guidelines for bioanalytical methods validation. Both methods were validated by the mean specificity, LLOQ, carryover, sensitivity, response linearity, accuracy, precision, dilution integrity, matrix effect, recovery, and stability.

Specificity and carryover

The specificity of the method was confirmed by replicate analysis of plasma blank from 6 different sources. Interference was compared to LLOQ for each AT, total EZ, and free EZ. The carryover effect was estimated in blank samples and then injected into higher-concentration samples to ensure that the rinsing solution could clean the injection needle. According to the guideline, interferences or carryover shouldn't exceed 20% of the LLOQ and 5% for the IS.

Linearity, precision, and accuracy

A linear response was obtained from the AT, total EZ, and free EZ to the corresponding IS peak area ratio versus its concentration over the dynamic range using the 1/X weighted factor. The slopes and intercepts for representative analytical runs of AT, total EZ, and free EZ were reported. The precision and accuracy of within-run evaluation were calculated via an analytical sequence containing 6 replicates of LLOQ, and each level of QC sample with calibration curve included zero and blank. Between-run linearity, precision, and accuracy were estimated by analysis of three sets of intra-run sequences on three different days, where each precession run was freshly prepared.

Recovery and matrix effect

The recovery of AT, total EZ, and free EZ by protein direct precipitation extraction procedure was evaluated from the peak area of AT, total EZ, and free EZ in extracted spiked samples for QC samples at low, Mid1, Mid2, and high with comparison to corresponding AT, total EZ and free EZ peak area in unprocessed samples of spiking supernatant at corresponding QC samples with. Matrix factor (MF) was investigated in 6 different lots of plasma for AT, total EZ, free EZ, and IS, where the ratio of the peak areas in the presence of matrix (prepared by spiking of AT, total EZ, and free EZ in the extracted blank) to the peak area in the absence of matrix (corresponding concentration of true AT, total EZ, and free EZ solution). IS normalized MF was also calculated by dividing the MF of AT and EZ over the MF of IS. CV% for IS-normalized MF was applied on 6 lots of plasma from QC low and QC high not exceeding 15%.

Stability

The stability of AT, total EZ, and free EZ in plasma was evaluated upon triplicate analysis of low and high QC samples, which are analyzed immediately after each applied stability factor of storage conditions to avoid overlaps of stability factors. All QC samples representing stability factors were analyzed against a freshly spiked calibration curve, then compared (stability samples) to freshly spiked corresponding QC samples, where the mean concentration at each stability level was not exceeding ±15% of true concentration. Short-term and long-term validations were applied to the stock solution and spiked plasma. The stock solution and spiked samples were kept at room temperature for 22 h and 15 h, respectively, to evaluate short-term stability. Then further estimation was applied after 8 days (under 2-8 °C) for a stock solution and 16 days under −20 °C for spiked samples parallel with subject samples as a long-term storage test. Short-term stability for spiked QC samples was also estimated under preparation till injection conditions, where dry extract samples were studied for 55 h, the reconstituted samples were examined for 15 h, and a further test was applied under autosampler cooling conditions (10 ˚C) for 26 h. Freeze and thaw stability cycles for AT, total EZ, and free EZ in plasma samples was studied over four freeze and thaw cycles, including at least 12 h refreezing duration that separates thawing cycles at room temperature of 1 h.

Clinical application

This study was applied in a clinical site of JCPR (Amman, Jordan), that was conducted in compliance with Good Clinical Practices (GCP), and the final report was generated by ICH Topic E3 structure and content of clinical study reports (ICH, 1995) adopted by the European agency for the evaluation of medicine (EMEA) (Products, 2019).

A total number of forty healthy subjects were randomized, enrolled, and completed the trial.

Clinical objective

The study was designed for a comparative the bioavailability of an open-label, randomized, single oral dose of one tablet of test product that contains 10/40 mg EZ and AT with concurrent administration of individual tablets of the two reference drugs under fasting conditions, four periods of study of the tested drug EZ + AT film-coated tablet (10+40) mg/tab manufactured by One Pharma, Greece. Verisfield S.M.S.A, Greece as the marketing authorization holder and the alternative batch releaser of the product versus the reference drugs Ezetrol®, EZ tablet 10 mg/tablet manufactured by MSD, Belgium, and Lipitor®, AT film-coated tablet 40 mg/tablet manufactured by Pfizer, Germany.

Subject selection criteria

Healthy subjects aged 18-50 years, normal weight according to Body Mass Index (BMI); (18.5 to 30.0) Kg/m^2 (minimum of 50 kg weight for male and 45 kg weight for female).

Clinical procedure

a single oral dose was administered in each study period. Study subjects were randomized into two treatment sequence groups, sequence 1 (RTRT) and sequence 2 (TRTR) (where R represents the reference product and T represents the test product); study periods were separated by 14 days washout periods. In each study period, each subject received in a fully replicated cross-over design and under fasting conditions, a single dose of either the test drug EZ + AT (10+40) mg/tab or coadministration of one tablet of Ezetrol® (10 mg EZ) and one film-coated tablet of Lipitor® (40 mg AT) of the reference drugs, with about 240 ± 5 ml of water after minimum 10 hours fasting period. In each study period, a (2 x 5 ml) blood samples were collected pre-drug administration and a series of 26 x 4 ml blood samples were collected at the following times: 0.25, 0.5, 0.75, 1, 1.33, 1.66, 2, 2.5, 3, 3.5, 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 12, 24, 48, 72 and 96-hours post drug administration.

Blood Hematology and Blood Chemistry were done during the screening and follow-up examinations. Liver function tests and CPK were done during a screening examination stage, before each period in the study, and during the follow-up investigation. Physical assessment and clinical evaluation were done at screening and follow-up examinations. Serology and Urinalysis were done during the screening examination. ECG was done at the screening examination and follow-up examination. COVID-19 PCR test was done at day 0 in each period.

Pk parameters and statistical analysis

The C_{max}, AUC_{0-t}, AUC_{0-inf}, T_{max}, $K_{el,}$ and $t_{½}$ for free EZ, total EZ, and AT were evaluated separately. The statistical analysis of C_{max} and AUC_{0-t} comprised the analysis of variance with sequence, subject (sequence), product, and period effects for all untransformed pk parameters and after a logarithmic transformation of the data. Point estimates and 90% confidence intervals for the mean ratios of pk parameters were calculated after a logarithmic transformation of the data. The following products were compared: test product vs. reference product.

Result And Discussion

LC-MS/MS analysis

The optimized tandem MS parameters exhibited a high quantitative detection efficiency. The molecular ion for AT and EZ were detected with their daughter fragment upon +MRM scan Mode at the mass transition of m/z 559.1 → 466.15 and 408.5→271.1, respectively. The optimized chromatographic conditions were also efficient enough to separate AT (RT 2.75 min) and EZ (RT 0.68 min for total and free EZ) from the plasma matrix with an excellent quantitative peak as seen in Figure 2B for AT LLOQ and Figure 2D for free EZ LLOQ, where AT and free EZ with their ISs eluted within the total run time of 3.2 min.

Figure 2:

Specificity and carryover

The extraction method from human plasma was specific to quantitate AT, total EZ, and free EZ over IS, and no endogenous peaks were observed through validation and routine analysis as seen from chromatograms in Figure 2A for AT blank and 2C for total EZ blank, where a chromatogram for the extracted plasma's blank sample shows no any prominent peak compared to the LLOQ in Figure 2B and 2D, respectively.

Furthermore, the carryover test exhibited an efficient washing system for the injection port. All injected subsequent blank samples to the high concentration didn't contain any AT, EZ, or IS residual peaks.

Standard calibration curve and linearity

The peak area ratio of each AT, free EZ, and total EZ to IS in human plasma was linear over the selected dynamic range of 0.400 to 60.000, 0.040 to 6.000, and 1.000 to 120.000 ng/ml, respectively. Where the R^2 for all curves were ≥ 0.999, and the mean (n=10) corresponding calibration function is y = 0.00152 (±4.7) x + 0.00097, y = 0.01045 (±3.2) + 0.00165, y = 0.00014 (±2.9) + 0.00091, respectively.

Within- and between-run sensitivity (LLOQ), accuracy, and precision

The within- and between-run accuracy and precisions for analysis of AT and free EZ and total EZ in plasma (spiked QC including LLOQ) were all within the acceptance criteria, as shown in Table 1.

Table 1:

Recovery and matrix effect

The recovery values obtained from the protein direct extraction procedure were all above 92% in TA and fee EZ plasma extraction as presented in table 2, while the recovery values obtained from the liquid-liquid extraction procedure that followed in total EZ extraction from plasma were above 19%, which validated properly and passed all validation sections successfully. summarizes the complete recovery for AT, EZ, and IS obtained by directly comparing the peak areas of extracted QC samples with unprocessed spiked post-extraction from human plasma.

Table 2:

The effect (matrix factor; (MF)) of extracted plasma matrix on AT, free EZ, and total EZ were all below 12%, as well as the IS- normalized MF was less than 4%, as examined through 6 different plasma sources at both QC low and high and given by the mean of IS-normalized MF.

Stability

AT, free EZ, and total EZ stability were examined out- and within the plasma matrix in the short and long-term using both QC low and high, and all results for each corresponding stability condition were above 91%.

Clinical application

During clinical application, there were no clinically relevant abnormalities at physical examination, all findings were normal for all participant volunteers, and there were no safety concerns during the study. For AT, free EZ, and total EZ, no significant difference was found between the C_{max} and AUC_{0-t} of the plasma for either treatment. The **c**oncentration-time profile for analysis of AT, free EZ, and total EZ in human plasma post oral administration of the test drug product AT+EZ (40+10) mg/ tab compared to the reference product under fasting conditions is presented in Figure 3 for fourth participant volunteers derived from the two sequences in the comparative form of test (T) vs. reference (R) drug products.

Figure 3:

Table 3 summarizes the statistical analysis for major pharmacokinetic (PK) parameters for the test product of AT, free EZ, and total EZ compared to the corresponding reference product.

Table 3:

Based on the pk parameters of C_{max} and AUC_{0-t} derived from measurements of AT, free EZ, and total EZ in human plasma for each drug product, the test product was bioequivalent with the reference product of Ezetrol® (EZ tablet 10mg/tablet) and one film-coated tablet of Lipitor® (AT film-coated tablet 40mg/tablet).

Conclusion

The described method for determination of AT, free EZ, and total EZ in human plasma by tandem MS was successfully validated and used to estimate clinical bioequivalence of EZ + AT (10+40) mg/tab that coadministration as one tablet of Ezetrol® (EZ tablet 10mg/tablet) and one film-coated tablet of Lipitor® (AT film-coated tablet 40mg/tablet), which were bioequivalent related to C_{max} and AUC_{0-t} of AT, free EZ, and total EZ.

Authors' Contributions

Ahmed Abu-awwad: Writing original draft – review & editing; Data curation.

Khaled W. Omari; Investigation, Review & editing.

Basel Arafat: Methodology; Data curation; Formal analysis; Resources.

Eyad Mallah: Software; Supervision.

Mona Bustami: Formal analysis, Software.

Eleni Loukeri: Funding acquisition.

Tawfiq Arafate: Project administration, Methodology, Validation, Investigation, Resources.

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Conflicts Of Interest

The authors report no financial or other conflicts of interest in this work.

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Ethical Approval

Ethical approval was obtained and kept for this study.

Data Availability

All data generated and analyzed are included in this article.

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