LC–MS–MS Simultaneous Determination of Atorvastatin and Ezetimibe in Human Plasma

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Atorvastatin and ezetimibe are lipid-lowering drugs prescribed for the treatment of hypercholesterolemia. An LC–MS–MS method has been developed and validated for the simultaneous estimation of atorvastatin and ezetimibe in human plasma using pitavastatin as an internal standard. Liquid–liquid extraction was used for the purification and preconcentration of analytes from human plasma matrix. The chromatographic separation was achieved within 3.0 min by an isocratic mobile phase consisting of 0.2% formic acid in water–acetonitrile (30:70, v/v), flowing through Agilent Eclipse-plus C18, 100 × 4.6 mm, 3.5 μm analytical column, at a flow rate of 0.6 mL min⁻¹. Multiple reaction monitoring transitions were measured in the positive ion mode for atorvastatin and internal standard, while ezetimibe was measured in negative ion mode. A detailed validation of the method was performed as per US-FDA guidelines and the standard curves were found to be linear in the range of 0.2–30.0 ng mL⁻¹ with a mean correlation coefficient >0.999 for both drugs. In human plasma, atorvastatin and ezetimibe were stable for at least 36 days at −70 ± 5°C and 6 h at ambient temperature. After extraction from plasma, the reconstituted samples of atorvastatin and ezetimibe were stable in an autosampler at ambient temperature for 6 h. Also, the cited drugs were stable in plasma samples upon subjecting to three freeze thaw cycles. The method is simple, specific, sensitive, precise, accurate and suitable for bioequivalence and pharmacokinetic studies of this combination.

Introduction

Atorvastatin (ATO), \( [R(R, R)]-2-(4-fluorophenyl)-\beta, \beta, \delta\)-dihydroxy-5-(1-methylethyl)-3-phenyl-4-(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid (Figure 1.1), and ezetimibe (EZE), \( [1-(4-fluorophenyl)-3-[3-(4-fluorophenyl)-3-hydroxy-propyl]-4-(4-hydroxyphenyl)]-azetidin-2-one (Figure 1.2), are oral antihyperlipidemic drugs. ATO is a member of the class of statins that lowers cholesterol levels by inhibiting the enzyme HMG-CoA reductase; this enzyme catalyzes the conversion of (HMG-CoA) to mevalonate, an early and rate-limiting step in cholesterol biosynthesis (1, 2). EZE is a drug that lowers cholesterol by preventing the absorption of cholesterol from dietary and biliary sources through blocking the transport of cholesterol through the intestinal wall (3). Since decrease in low-density lipoprotein (LDL) receptors and high-density lipoprotein cholesterol is observed in hyperlipidemia, the use of both ATO and EZE in combination produces additive effects in hyperlipidemia. ATO when used in combination with EZE causes manifold reduction in LDL cholesterol levels when compared with double the dose of the individual drug when used alone (4). Moreover, the use of EZE with ATO allows for an enhanced effect of the statin at a lower dose and reduction in associated side effects (4). Thence, the combination is used as an effective treatment for primary hypercholesterolemia and for homozygous familiar hyperlipidemia.

Several methods have been reported for the estimation of ATO in biological matrices. Those methods include high-performance liquid chromatography (HPLC) operated with UV detection (5–7), gas chromatography/mass spectroscopy (8) and HPLC equipped with tandem mass spectroscopy (9–11). Similarly, a survey of analytical literature for the determination of EZE in biological samples revealed methods based on UV detection using HPLC technique (12), gas chromatography/mass spectroscopy (13) and HPLC equipped with tandem mass spectroscopy (14–17).

HPLC methods with UV detection are not sensitive enough to measure ATO or EZE in human plasma, while the use of GC–MS–MS requires derivatization steps prior to analysis. Among the reported LC–MS–MS methods, only one developed method for simultaneous determination of ATO and EZE in human plasma was described. However, the published article has not mentioned any details of the analytical method used (limit of detection, linearity range, retention time of analytes, run time, stability of analytes, validation of the analytical method) nor any details of plasma sample preparation procedure (18). Thus, the aim of the present work was to develop a highly selective and sensitive LC–MS–MS method with good precision and accuracy, and simple sample treatment, for the simultaneous estimation of ATO and EZE in human plasma, using pitavastatin (Figure 1.3) as the internal standard (IS). A detailed validation of analytical method was performed in accordance with US-FDA guidelines (19) to yield reliable results. Moreover, the developed method can be successfully applied for further research as to access their pharmacokinetic evaluation of ATO and EZE in a combination form.

Experimental

Materials and reagents

Working standards of ATO calcium (certified to contain 99.81%) was supplied by Marceyl-Egypt, and EZE (certified to contain 99.93%) was supplied by Merck Sharp & Dohme-Egypt. Pharmaceutical grade pitavastatin calcium (certified to contain 99.80%) was supplied by Lilly-USA. Acetonitrile HPLC grade was obtained from Sigma-Aldrich, Germany. Formic acid was obtained from El-Nasr Pharmaceutical Chemicals Co., Egypt. Ethyl acetate extra pure grade was purchased from SD Fine-Chem Limited (India). Deionized and purified water produced in-house from Millipore’s Milli-Q System (USA) was used throughout the analysis.
**Instrument and conditions**

An Agilent 1260 infinity liquid chromatograph–mass spectrometer (Germany), equipped with vacuum degasser, gradient quaternary pump VL, autosampler ALS, column oven TCC and Agilent (6460 Triple Quadrupole) LC–MS–MS system. Separation was carried out on a C18 column (100 mm × 4.6 mm, 3.5 μm)—Zorbax Eclipse Plus, USA.

Chromatographic elution was performed with a mobile phase consisting of 0.2% formic acid in water–acetonitrile (30:70, v/v) pumped through the column at a flow rate of 0.6 mL min⁻¹. The column temperature was maintained at 40 °C. Under these conditions, the retention times for the IS, EZE and ATO were around 2.058, 2.361 and 2.680 min, respectively (Figure 2). The injection volume was 5 μL, and the injector needle wash solvent was acetonitrile–water (50:50, v/v). The Agilent 6460 LC–MS/MS system was operated in the positive ion mode for the detection of ATO and IS, while operated in the negative ion mode for the detection of EZE. The drying gas flow was set at 9.0 L min⁻¹ with nebulizer pressure of 40 psi and temperature at 350 °C. Multiple reaction monitoring (MRM) transitions measured at positive mode at m/z 559.0 → 440.0 for ATO and m/z 422.0 → 290.0 for IS, while EZE was measured at negative mode at m/z 408.0 → 271.0 with a 300-ms dwell time of all drugs (Figure 3). The capillary voltage was set at +4000 V for both ATO and IS, while it was set at −4000 V for EZE. The sheath gas flow was set at 12.0 L min⁻¹ and temperature at 350 °C with nozzle voltage of 2000 V. Collision energy was set at 10.0 V while fragmentor voltage was set at 135 V. Quantitation of the analytes in human plasma was based on the peak area ratio of cited drugs versus IS.

Data acquisition was performed using Agilent MassHunter Workstation software (B.03.01), and data processing was subsequently performed using MassHunter Quantitative analysis software (B.04.00).

**Standard stock solution preparation**

Standard stock solutions of 100 μg mL⁻¹ of each cited drug were prepared separately in acetonitrile–water (50:50, v/v), by dissolving an accurately weighed amount of the drug in the selected solvent, sonicated and completed to volume in a 100-mL volumetric flask. Working solutions of ATO and EZE were prepared at 20, 40, 60, 100, 500, 1000, 1500, 2000, 2400, 2800 and 3000 ng mL⁻¹ by serial diluting the standard stock solution of each drug with the selected solvent. IS was also prepared in same solvent mixture at concentration of 1000 ng mL⁻¹. All solutions were stored under refrigeration (2–8 °C) when not in use.

**Calibration samples and quality control samples preparation**

Eight non-zero calibration standards ranging from 0.2 to 30.0 ng mL⁻¹ were prepared by adding 10 μL of known working solution of ATO and EZE, with 10 μL of IS solution to 980 μL of drug-free human plasma. Quality control (QC) samples were prepared in the manner similar to the calibration standard at three concentration levels—low, medium (mid) and high (0.6, 15.0 and 24.0 ng mL⁻¹). All samples were vortexed to ensure complete mixing. During each run, six replicates of QC samples were extracted along with the calibration standards to verify the integrity of the method.
Sample preparation
To 1000 μL of each spiked calibration plasma standards or QC samples, 6 mL of ethyl acetate was added. It was then vortexed for 1 min and centrifuged at 5000 rpm, at 4°C for 5 min. Approximately 4.0 mL of supernatant was collected and evaporated to dryness under nitrogen at 40 ± 5°C. The residue was reconstituted in 500 μL of the mobile phase and transferred to a glass vial for LC–MS–MS analysis (Figure 4).

Validation
Linearity
To establish the linearity, a series of calibration standards (0.2, 0.4, 1.0, 5.0, 10.0, 20.0, 28.0 and 30.0 ng mL⁻¹) were prepared by
adding 10 μL of respective working solution of both of ATO and EZE, and 10 μL of 1000 ng mL$^{-1}$ of IS solution to 980 μL of drug free human plasma and analyzed. Five linearity curves containing eight non-zero concentrations were analyzed. A correlation of more than 0.99 was desirable. The lowest standard on the calibration curve was to be accepted (19) as the lower limit of quantitation (LLOQ) if the analytes response in the standard was five times more than that of drug-free (blank) plasma. In addition, the analyte peak in LLOQ sample should be identifiable, discrete and reproducible with a precision of $\pm 20.0\%$ and accuracy within 80.0–120.0%. The deviation of standards other than LLOQ from the nominal concentration should not be more than $\pm 15.0\%$. It was desirable that a minimum of six non-zero standards, including LLOQ, met the above criteria.

**Specificity**

Six randomly selected drug-free human plasma samples were processed by the similar liquid–liquid extraction procedure and analyzed to determine the extent to which endogenous plasma components may contribute to the interference at retention time of analytes and IS.

**Recovery (extraction efficiency) from plasma matrix**

Recovery of ATO and EZE was evaluated by comparing the mean peak responses of five extracted QC samples of low, medium and high concentrations to mean peak responses of five plain standards of equivalent concentration. Similarly, the recovery of IS was also evaluated at the concentration of 10 ng mL$^{-1}$. As per the acceptance criteria (19), the recovery of the analytes do not need to be 100.0%, but the extent of recovery of an analyte should be consistent, precise and accurate.

**Matrix effect**

Matrix effect caused by ionization competition occurring between analytes of interest and endogenous co-eluting components was evaluated at two concentration levels (QC high and QC low), by comparing the peak response of ATO, EZE and IS in extracted samples of blank plasma, spiked after extraction, with the corresponding response obtained by direct injection of standard solution of same concentration. Also, the matrix effect for IS was evaluated by applying the same procedure at concentration of 10 ng mL$^{-1}$.

**Accuracy and precision (inter- and intra-day)**

Intra-day accuracy and precision were evaluated by five-replicate analysis of ATO and EZE mixture at concentrations of low, medium and high QC samples in human plasma. The inter-day accuracy and precision were assessed by analysis of low, medium and high QC samples for ATO and EZE on three consecutive days. The precision of the method was determined by calculating the percent coefficient of variation (% CV) for the concentrations obtained for different determinations. For the evaluation of precision, the deviation of each concentration level from the nominal concentration was expected to be within $\pm 15.0\%$. Similarly, the mean accuracy should not deviate by $\pm 15.0\%$ of the nominal concentration (19).

**Stability**

Three aliquots of each of the low and high QC samples were stored in a freezer at $-70 \pm 5^\circ C$ for 36 days. The samples were processed along with precision and accuracy batch and concentrations obtained were compared with nominal concentrations.
to determine the long-term stability of ATO and EZE in human plasma.

In order to determine the short-term stability of plasma samples, three aliquots of each of low- and high-unprocessed QC samples were kept at ambient temperature (23–30 °C) for 6.0 h. After 6.0 h, the samples were processed, analyzed, and compared with nominal concentrations.

Autosampler stability was determined by analyzing three aliquots of low- and high QC samples that were processed and reconstituted before storing at 10 °C for 6.0 h. After completion of 6.0 h, samples were reanalyzed and concentrated compared with the freshly prepared control samples for cited drugs and the area of IS obtained from analysis of control samples after 6.0 h was compared to area of IS obtained from freshly prepared control samples analysis.

For the determination of working solutions stability, 10 ng mL\(^{-1}\) working solutions of each of ATO, EZE and IS were kept at 2–8 °C for 6 days. Also, to access the working solution of cited drugs and IS working solution stability at room temperature, the same procedure was done with keeping 10 ng mL\(^{-1}\) of each solution at room temperature (23–30 °C) for 6 h. Thereafter, the mean area of cited drugs and IS from three replicate chromatographic runs was compared with freshly prepared 10 ng mL\(^{-1}\) of same solution.

Effect of freeze and thaw cycles on stability of plasma samples after three freeze and thaw cycles was also determined. Three aliquots of each of low- and high-unprocessed QC samples were stored at −70 ± 5 °C and subjected to three freeze thaw cycles. After the completion of third cycle, the samples were processed and analyzed, and results were compared with nominal values. All the stability samples were considered stable if the deviation from the nominal concentration was within ± 15%.

Table I

<table>
<thead>
<tr>
<th>QC sample</th>
<th>Concentration of drug in plasma (ng mL(^{-1}))</th>
<th>Mean recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATO</td>
<td>EZE</td>
<td>ATO</td>
<td>EZE</td>
</tr>
<tr>
<td>Low</td>
<td>0.6</td>
<td>0.6</td>
<td>76.06</td>
</tr>
<tr>
<td>Medium</td>
<td>15</td>
<td>15</td>
<td>77.83</td>
</tr>
<tr>
<td>High</td>
<td>24</td>
<td>24</td>
<td>75.07</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; N, number of determinations.

Discussion

Method development

The aim of the present work was to simultaneously determine both ATO and EZE using an HPLC equipped with tandem mass spectrometry to attain higher sensitivity and selectivity for the detection and quantitation of cited drugs in human plasma with a fast and cheap extraction process, allowing the method to be of high use in evaluation of the pharmacokinetics of both drugs either alone or in combination.

During the optimization cycle, several chromatographic conditions were attempted using C18 column (250 mm × 4.6 mm, 5 μm)—Nucleosil and C18 column (100 mm × 4.6 mm, 3.5 μm)—Zorbax Eclipse Plus. Various mobile phase compositions of ammonium acetate buffer, 0.2% formic acid in water, methanol and acetonitrile, in different proportions were tried in an isocratic mode. It was found that acetonitrile allowed faster elution of analytes with better response. The use of 0.2% formic acid was to obtain higher detection response through assisting the ionization of cited drug molecules.

The use of C18 column with 3.5 μm particle size of packing material under controlled temperature (40 °C) column oven, achieved the desirable peak shape and elution of ATO, EZE as well as for IS. It is worthy remarking that upon the use of mobile phase containing high organic portion of acetonitrile at a flow rate of 0.6 mL min\(^{-1}\), a shorter run time was achieved when compared with the use of mobile phase containing methanol at a flow rate of 0.2 mL min\(^{-1}\) as described in a published article (18). Hence, the developed analytical method in this work has the advantage of rapid batch analysis of human plasma samples.
Bio-analytical method validation

Linearity and limit of quantitation
Calibration curves for ATO and EZE were constructed by plotting the peak area ratio of the cited drug to the IS versus the concentration of the cited drug. The constructed calibration curves were found to be linear and precise over the linearity range of 0.2–30.0 ng mL⁻¹ for both ATO and EZE. The LLOQ was 0.2 ng mL⁻¹ (lowest standard level) with an accuracy of 99.77% and 95.38% for ATO and EZE, respectively. The regression equation for each cited drug was also computed and the correlation coefficient was found to be 0.9998 and 0.9997 for ATO and EZE, respectively. Back calculations were made from the calibration curves to determine the accuracy of each calibration standard.

Specificity
There was no significant interference at the retention times for ATO, EZE or IS from six different batches of drug-free human plasma used for analysis (Figures 4 and 5).

Recovey (extraction efficiency) from plasma matrix
The mean recovery for cited drugs in human plasma ranged between 75.07–77.83% and 68.89–70.49% for ATO and EZE, respectively (Table I). IS recovery from plasma was found to be 66.25 ± 0.84 (mean ± SD, %).

Matrix effect
Matrix-dependent signal suppression or enhancement may have a major effect on the quantitative analysis using liquid chromatography coupled to electron spray ionization mass spectrometry. In the present work, evaluation of matrix effect on the analysis of cited drugs and IS was performed, and the results obtained (Table II) show that the matrix effect was negligible for the determination of ATO and EZE in human plasma by applying the proposed extraction procedure and chromatographic conditions.

Accuracy and precision (inter- and intra-day)
For ATO, the coefficient of variation for intra-day was between 2.22 and 4.72% and the accuracy values were found to be between 100.92 and 106.06%, whereas for EZE the coefficient of

### Table II

<table>
<thead>
<tr>
<th>Drug Conc.</th>
<th>ATO QC-low</th>
<th>ATO QC-high</th>
<th>EZE QC-low</th>
<th>EZE QC-high</th>
<th>IS QC-low</th>
<th>IS QC-high</th>
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<tbody>
<tr>
<td>Mean %</td>
<td>96.58</td>
<td>97.90</td>
<td>103.61</td>
<td>101.32</td>
<td>112.22</td>
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<tr>
<td>± SD</td>
<td>4.20</td>
<td>2.31</td>
<td>3.81</td>
<td>2.24</td>
<td>2.75</td>
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</table>

SD, standard deviation.

### Table III

<table>
<thead>
<tr>
<th>Accuracy and precision</th>
<th>QC sample</th>
<th>Conc. of ATO in plasma (ng mL⁻¹)</th>
<th>Mean conc. found of ATO (ng mL⁻¹)</th>
<th>± SD</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day Low</td>
<td>0.6</td>
<td>0.61</td>
<td>0.02</td>
<td>3.28</td>
<td>101.67</td>
<td>5</td>
<td></td>
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<tr>
<td>Medium</td>
<td>15</td>
<td>15.91</td>
<td>0.71</td>
<td>4.46</td>
<td>106.07</td>
<td>5</td>
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<tr>
<td>High</td>
<td>24</td>
<td>24.81</td>
<td>0.55</td>
<td>2.22</td>
<td>103.39</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Inter-day Low</td>
<td>0.6</td>
<td>0.61</td>
<td>0.03</td>
<td>4.92</td>
<td>101.67</td>
<td>15</td>
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<tr>
<td>Medium</td>
<td>15</td>
<td>15.75</td>
<td>0.74</td>
<td>4.70</td>
<td>105.00</td>
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<tr>
<td>High</td>
<td>24</td>
<td>24.65</td>
<td>1.05</td>
<td>4.26</td>
<td>102.71</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

SD, standard deviation; CV, coefficient of variation; N, number of determinations.
Summary of Stability Data of Atorvastatin in Human Plasma

<table>
<thead>
<tr>
<th>Stability term</th>
<th>Conc. of ATO (ng mL(^{-1}))</th>
<th>Mean conc. of ATO found (ng mL(^{-1}))</th>
<th>± SD</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long term (36 days)</td>
<td>0.6</td>
<td>0.58</td>
<td>0.02</td>
<td>3.45</td>
<td>96.67</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>23.48</td>
<td>0.26</td>
<td>1.11</td>
<td>−1.41</td>
<td>3</td>
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<tr>
<td>Short term (6 h)</td>
<td>0.6</td>
<td>0.60</td>
<td>0.01</td>
<td>1.67</td>
<td>−0.13</td>
<td>3</td>
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<tr>
<td></td>
<td>Autosampler (6 h)</td>
<td>0.6</td>
<td>0.59</td>
<td>0.01</td>
<td>1.69</td>
<td>−0.69</td>
</tr>
<tr>
<td></td>
<td>Freeze and thaw</td>
<td>0.6</td>
<td>0.59</td>
<td>0.01</td>
<td>1.69</td>
<td>−1.16</td>
</tr>
</tbody>
</table>

SD, standard deviation; CV, coefficient of variation; N, number of determinations.

Summary of Stability Data of Ezetimibe in Human Plasma

<table>
<thead>
<tr>
<th>Stability term</th>
<th>Conc. of EZE (ng mL(^{-1}))</th>
<th>Mean conc. of EZE found (ng mL(^{-1}))</th>
<th>± SD</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long term (36 days)</td>
<td>0.6</td>
<td>0.58</td>
<td>0.02</td>
<td>3.45</td>
<td>−3.35</td>
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<td>24</td>
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<tr>
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<td>3</td>
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<tr>
<td></td>
<td>Autosampler (6 h)</td>
<td>0.6</td>
<td>0.59</td>
<td>0.02</td>
<td>3.39</td>
<td>−1.32</td>
</tr>
<tr>
<td></td>
<td>Freeze and thaw</td>
<td>0.6</td>
<td>0.59</td>
<td>0.01</td>
<td>1.69</td>
<td>−0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>23.82</td>
<td>0.17</td>
<td>0.71</td>
<td>−0.39</td>
</tr>
</tbody>
</table>

SD, standard deviation; CV, coefficient of variation; N, number of determinations.

Stability

ATO and EZE were stable at −70 ± 5°C for 36 days (long-term stability) in human plasma. The percent changes (bias) in ATO concentration over the stability-testing period of 36 days in deep freezer at −70 ± 5°C were −3.35 and −1.16% at the concentrations of each of low and high QC samples, respectively. While the percent changes (bias) in EZE concentration over stability testing for the same period under same freezing conditions were −3.32 and −3.08% at the concentrations of each of low and high QC samples, respectively (Tables V and VI).

Both ATO and EZE were found to be stable over 6.0 h in human plasma at room temperature (23–30°C). The percent bias observed for ATO was −0.13 and 0.33% at the concentration of 0.6 and 24.0 ng mL\(^{-1}\) (low and high QC samples), respectively. While the percent bias observed for EZE was 0.67 and −0.16% under same conditions at the concentration of 0.6 and 24.0 ng mL\(^{-1}\), respectively (Tables V and VI).

In the autosampler at 10°C, reconstituted samples of ATO and EZE were stable for 6.0 h after sample processing. The percent bias for reconstituted samples after 6.0 h for ATO was −0.69 and −0.80% at low and high QC levels, respectively. While the percent bias for reconstituted samples at same conditions for EZE was −1.32 and −1.50% at low and high QC levels, respectively. Also, the stability of IS in reconstituted samples at same conditions was evaluated, and the percent bias was found to be 0.28 and 0.46% at the concentration of 10 ng mL\(^{-1}\) in the low and high QC levels of cited drugs, respectively (Tables V and VI).

Frozen plasma samples containing both ATO and EZE were found to be stable even after subjecting to three freeze–thaw cycles. The percent bias observed for ATO was −1.16 and −0.55% at low and high QC levels studied, respectively. While the percent bias for EZE after subjecting plasma samples to three freeze–thaw cycles was found to be −0.45 and −0.39% at concentrations of at low and high QC samples, respectively (Tables V and VI).

Working solutions of ATO and EZE, and IS were found to be stable for 6 days at 2–8°C and at room temperature (23–30°C) for 6 h at the tested concentration of 10 ng mL\(^{-1}\) of each (Table VII).

Conclusions

The bio-analytical methodology described in this article is specific, sensitive, accurate and precise enough to be successfully applied to human clinical pharmacology, bioavailability and bioequivalence studies requiring pharmacokinetic evaluation. The method employed sample preparation by liquid–liquid extraction with adequate recovery, followed by isocratic HPLC coupled with tandem mass spectrometric detection (LC–MS–MS). The LC–MS–MS method was rapid enough and capable of simultaneous estimating ATO and EZE up to 0.2 ng mL\(^{-1}\) accurately in human plasma with accuracy and precision.

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