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Simultaneous determination of ezetimibe and atorvastatin calcium in spiked human plasma using SPE coupled to HPLC-MS/MS

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ABSTRACT

Combination drug products containing ezetimibe (EZB) and atorvastatin calcium (ATVC) are widely used for treatment of hyperlipidemia. A rapid, simple and sensitive liquid chromatography–tandem mass spectrometry (LC-MS/MS)method for determination of EZB and ATVC in spiked human plasma has been developed and validated.Plasma samples were subjected to solid phase extraction before analysis with an extraction recovery of 90.59 - 98.02%. Chromatographic separation was performed on a C18 column using isocratic elutionwithin 2 min run time. The mobile phase consisted of acetonitrile: 1% of formic acid in water (8: 2, v/v) and was pumped at a flow rate of 0.8 mL/min. Detection of analytes was achieved by with electrospray ionization (ESI) in negative ion mode for EZB and positive ion mode for ATVC. The calibration curves were linear over the range of 1.67 – 83.33 ng/mL for both drugs. The precision at the lower limit of quantitation for EZB (12.97%) and ATVC (8.99%) was determined. The intra- and inter-day precisions were within 9.16%, while the accuracy ranged from 94.18 to 106.94%. The validated LC-MS/MS method was successfully employed for thedetermination of EZB and ATVC in spiked human plasma for application in therapeutic drug monitoring.

KEYWORDS

LC-MS/MS;
Solid phase extraction;
Ezetimibe;
Atorvastatin calcium;
Bioanalysis.

INTRODUCTION

Ezetimibe (EZB) inhibits the absorption of cholesterol, decreasing the delivery of intestinal cholesterol to the liver. Atorvastatin calcium (ATVC) is a synthetic lipid-lowering agent that inhibits ß-hydroxy-ß-methylglutaryl-coenzyme A (HMG-CoA) reductase. Recently, a combination of EZB and ATVC has been introduced to the market. The co-administration of both drugs offers a well-tolerated and highly efficient treatment option for patients with dyslipidemia and helps in prescribing a low dose of ATVC, which may reduce side effects[1]. The previously reported C_{max} for EZB and ATVC was reported as 5.40 ± 0.64 ng/mL and 14.80 ± 1.72 ng/mL, respectively[2]. Chemically EZB is [(3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3- (4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone], and ATVC is [R-(R\,R\)]-2-(4-fluorophenyl)-b, d-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]- 1H-pyrrole-1 –

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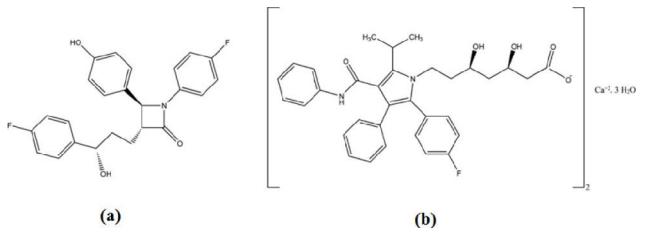
heptanoic acid – calcium salt (2:1) trihydrate[3]. The chemical structures of ATVC and EZB are shown in Figure 1.



(a) (b)

Figure 1 : Chemical structures of Ezetimibe (a) and Atorvastatin calcium (b)

Bioanalytical methods are widely used to quantify drugs and their metabolites in physiological matrices. Such methods are applied to studies in human clinical pharmacology and non-human clinical pharmacology/ toxicology. Bioanalytical method employed for the quantitative determination of drugs in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic and toxicokinetic studies[4]. HPLC coupled to UV or fluorescence has been extensively in use. However, lack of sensitivity and limited selectivity that are required for analysis of co-administered drugto be given at low doses represented the major challenge. Moreover, the relatively long run time, large volume of plasma required for analysis as well as complicated and time-consuming extraction procedures limited the usefulness of these techniques. Recently, HPLC coupled to tandem mass spectrometry (LC-MS/MS)has become the gold standard for the analysis of pharmaceuticals in plasma. The main advantages of LC-MS/MS include extremely low detection limits, minimal sample volume andpre-treatment. The improved selectivity of LC-MS/MS enabled straight forward analysis of multicomponent mixtures of analytesinfew minutes[5].

In tandem MS/MS, the detector may be programmed to select certain ions to fragment. The process is essentially a selection technique. As long as there are no interferences or ion suppression, the LC separation can be quite quick. It is common now to have analysis times of 1 minute or less by MS/MS detection, compared to over 10 minutes with UV detection[6]. However, the task gets complicated if analytesin the sample cannot be determined using the same ionization mode. Modern mass spectrometers allow for positive/ negative polarity switchingand are capable for acquisition of multiple reaction monitoring (MRM) mass spectra in both ionization modes from a single HPLC-MS/MS analysis. However, variability is still a concern and full validation and evaluation of matrix effect is required for both modes[7].

There were few studies available for the determination of either EZB[8-10] or ATVC[11-19] in human plasma using LC-MS/MS method. However, there were no bioanalytical studies for the simultaneous determination of EZB and ATVC in human plasma. In this study, the development of a validated, simple and rapid solid phase extraction (SPE) coupled to LC-MS/MS method for simultaneous determination of EZB and ATVC in spiked human plasma is investigated.

EXPERIMENTAL

Chemicals and samples

EZB and ATVC pure standards were kindly supplied by Marcyrl Pharmaceutical Industries, El-Obour City, Egypt. Human plasma was obtained from VACSERA (Egypt). All other reagents were of HPLC grade and purchased from Sigma Aldrich (USA). Stock solutions (100 µg/mL) of EZB and ATVC were prepared in methanol and stored at 2-8 °C protected from light. Working solutions were prepared fresh as indicated below.

Instruments and chromatographic conditions

The SPE experiments were carried out using a vacuum manifold and manually packed cartridges with HF Bondesil C18 according to manufacturer's specifications (Agilent Technologies, USA). Chromatographic separation was achieved on an Inertsil ODS-3 column, 50 x 4.6 mm, 5 µm (GL Science, Japan). The column oven temperature was maintained at 30 °C and aliquots of 20 µL were injected. The mobile phase consisted of acetonitrile: 1% formic acid in water (8: 2, v/v) at a flow rate of 0.8 mL/min giving a total run time of 2 min. For chromatographic separations, an Agilent 1200HPLC system with G1311A quaternarygradient pump, G1329A auto sampler, G1316A columnthermostat was used (Agilent Technologies, USA). The HPLC system was connected to an Agilent 6410Btriple quadrupole equipped with an Agilent electrosprayion source (ESI, G1948B)

(Agilent Technologies, USA). Data acquisition was performed using theAgilent Mass Hunter workstation software (B.02.01 SP1).The mass selective detector was used in the MRM mode for the highest possibleselectivity and sensitivity. The multimode ion source was operated in the negative ESI mode for determination of EZB and in the positive mode for determination of ATVC. The MSdetector settings were as follows: gas temperature: 350 °C,gas flow: 11 L/min, nebulizer pressure:40 psi, capillary voltage: 4,000 V.Quantitative determinationswere performed in MRM scanmode using the following transitions: m/z 408.1→ 271.1 for EZB and m/z 559.3 → 440.3 for ATVC. The dwell time for eachtransition was 200ms.

Sample pretreatment

SPE was employed for preparation of spiked plasma samples. The plasma samples (0.5 mL) were spiked with suitable concentrations of binary mixtures of EZB and ATVC and 0.5 mL of 2% formic acid in water were added and vortexed for 30 seconds. The content was subsequently transferred to 1 mL SPE cartridges packed with 100 mg of HF Bondesil C18. Cartridges were preconditioned with 2 mL methanol followed by 1.0 mL water. The cartridges were washed with 2.0 mL 2% formic acid in water, and the analytes were eluted with 1.0 mL 0.1 % formic acid in methanol.After the extraction of samples, the extracts were concentrated and evaporated to dryness under a stream of N_2 at 60° C and the residues were reconstituted in 200 µL mobile phase. Resulting solution was vortexed for 30 seconds, prior to transferral to HPLC vials and 20µLwere injected. All samples were kept in ice at all times.

Method validation

Linearity and sensitivity

The optimized method was validated according to the FDA guidelines[20]. Appropriate amounts of EZB and ATVC were added to blank plasma to obtain a concentration range 1.67 - 83.33 ng/mL for both drugs. Samples were then prepared and analyzed using theprocedure described above. The peak areas were calculated from the selected-ion mass chromatogram of analytes. Calibration curves for both EZB and ATVC were plotted and the corresponding regression equations were calculated. Deviation from nominal concentration was then calculated in order to reveal variability in both sample preparation and analysis setps.Sensitivity was measured using Lower limit of Quantification (LLOQ). The accuracy and precision were evaluated at the LLOQ using five replicates.

Accuracy, Precision, Extraction Recovery, Matrix effect and Specificity

Aliquots of each analyte standard solutions of EZB and ATVC were added to six different batches of blank plasma to final concentrations of 5.00, 33.30 and 50.0ng/mL. Sample pretreatment and analysis were carried out as described above.The analyte peak areas were compared to results obtained by spiking in extracted drug-free plasma, immediately prior to chromatography. Therecovery values were calculated and evaluated. Accuracy was calculated as the percentage of determined concentration relative to the nominal concentration. The assay described above was repeated five times within the same day to obtain the intraday precision and over three different days for theinterday precision. Results were expressed as a percentage of relative standard deviation values(RSD%).The matrix effect was assessed at concentrations 5.00, 33.33 and 50.00ng/mL, by comparing mean peak areas of the analytes spiked into plasma extracts to mean peak areas for neat solutions of the analytes. The specificity was assessed using six blank human plasma samples that were subjected to the extraction procedure and chromatographed to determine the extent to which endogenous plasma components could interfere in the analysis of EZB and ATVC. The results were compared to a solution containing 1.67 ng/mL of EZB and ATVC each.

Stability

Short-term, freeze–thaw, and processed sample stabilities of EZB and ATVC in plasma were investigated to assess the analyte integrity throughout the procedure. Both low and highQC sample concentrations (5.00 and 50.00ng/mL) were used. QC samples (n = 5) were analyzed immediately after preparation (100% references) and after storage: (1) short-term stability was assessed after 8 h standing at room temperature, (2) freeze–thaw stability was assesses after three cycles of freezing at – 20 °C and thaw unassisted at room temperature and (3) the stability of the processed samples was determined after storage for 8 h in the autosampler.

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The analytes were considered stable in the matrix when the concentration difference between the fresh samples and the stability testing samples did not exceed 20%. The long-term stability for storage of plasma sample at -80 °C was not assessed because all the real samples were analyzed within 8 h.

RESULTS AND DISCUSSION

LC-MS/MS is a very selective and sensitive technique for quantitation of analytes in highly complex matrices. Characteristic fragments are simultaneously monitored for given analytes[6]. Preliminary studies involving optimization of sample preparation steps and mass detector settings were carried out and the following results were obtained.

Sample pretreatment

In the present study, a SPE extraction procedure for the preparation of human plasma samples prior to LC– tandem MS analysis was optimized. In-house packed SPE cartridges were employed in order to reduce the cost and improve the reproducibility of sample preparation protocol. Plasma samples were mixed with 2% formic acid in order to ensure that the analytes were in the unionized form prior to extraction. Wash steps with solutions containing amounts of organicsolvent such as acetonitrile or methanol in water resulted in a dramaticdecrease in the extraction recoveries of analytes. A wash step with 2% formic acid inwater resulted in cleaner samples and less variable recovery. Elution was carried out using 0.1% formic acid in methanol. Evaporation and reconstitution of the obtained residue was carried out in the mobile phase.

Chromatography and Mass detection

Chromatographic analysis was carried out using a C18 column and a mobile phase of acetonitrile: 1% formic acid in water (8: 2, v/v) at 0.8 mL/min.A total run time of 2 min and retention times ranged from 0.98 to 1.05 min was obtained.These low retention times allowed the rapid determination of the both drugs. Mass detector parameters were optimized and symmetric peaks for EZB and ATVC were obtained.Chromatographic peaks with the highest signal-to-noise ratio were found to be: m/z 408.1→ 271.1 for EZB and m/z 559.3 → 440.3 for ATVC as shown in Figure 2 and 3. The optimized method was validated according to the FDA guidelines[20].

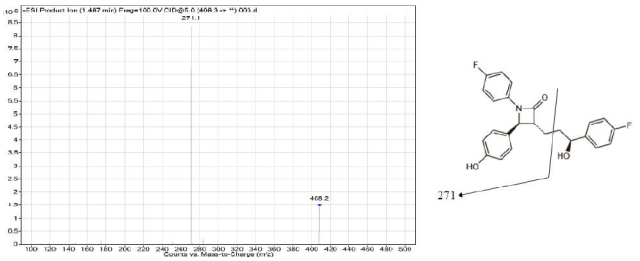

Figure 2 : Product ion mass spectra of the precursor ions and the proposed patterns of fragmentation ofezetimibe [M-H]⁻

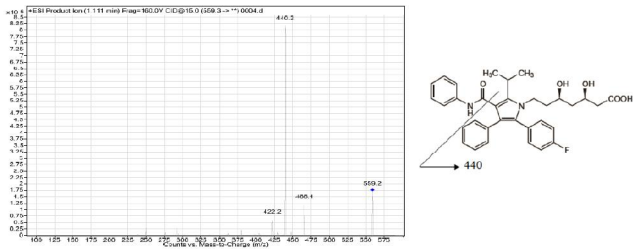

Figure 3 : Product ion mass spectra of the precursor ions and the proposed patterns of fragmentation of atorvastatin [M+H]⁺

Method validation

The coupling of HPLC with MS/MS detection in the MRM mode showed high specificity because only the ions derived from the analytes of interest were monitored. Representative chromatograms of a treated blank plasma sample and a treated plasma sample spiked with either 16.67 ng/mL EZB or 16.67 ng/mL ATVC are shown in Figure 4, 5 and 6, respectively. Results indicated lack of interference by endogenous plasma components at the retention times of the studied drugs. Linear relationship was obtained between the peak areas and concentration in the range of 1.67 – 83.33 ng/mL for both of EZB and ATVC. The deviation from the nominal concentration was less than 8% at all concentrations. The regression equations was computed and found to be:

Y = 17,554.58 C -8,807.56 r = 0.9999 for EZB

Y = 1,351.44 C +1,919.28 r = 0.9999 for ATVC

Where Y is the area under peak, C is the concentration in ng/mL and r is the correlation coefficient.

Since the C_{max} of EZB and ATVC were 5.40 ± 0.64 ng/mL and 14.80 ± 1.72 ng/mL, respectively[2]. The obtained linearity range was considered appropriate for therapeutic drug monitoringapplications upon administration of a fixed dose combination product containing

both drugs, 10 mg each / tablet.Results clearly showed that our method was sensitive enough for determination of EZB and ATVC in human plasma samples. In addition, the sensitivity of the present assay was comparable to the reported studies for the individual determination of either EZB or ATVC[8-19].

At the LLOQ for both drugs (1.67 ng/mL),the precision was found to be 12.97% and 8.99% for EZB and ATVC respectively.On the other hand, the accuracy was 89.44 – 116.11% for EZB and 93.24 – 111.67% for ATVC at the corresponding LLOQ.

The results of assay performance, assessed at three concentrations processed under the same conditions as previously described are summarized in TABLE 1. Analytical accuracy ranged from 94.18% to 106.94%. A higher and more reproducible extraction recovery (90.59% - 98.02%) for both drugs was obtained when compared to conventional liquid-liquid extraction (results not shown). Intra-day precision and inter-day precision ranged from 3.50 - 6.15% and from 4.21 - 9.16%, respectively. All observed data for the intra and inter-assay were below 15.00%. These data indicated that the method provides adequate accuracy and precision for the determination of analytes in plasma samples.Matrix effects caused by endogenous co-eluting components were examined at three different concentration levels. The results for both EZB and ATVC were higher than 93.50% suggesting that there was no signal suppressions during ionization step due to endogenous components.

TABLE 1 : Results of accuracy, extraction recovery and precision of ezetimibe and atorvastatin calcium in human plasma by the proposed LC-MS/MS method.

Analytes	Amount added (ng/mL)	Accuracy(%) (Mean ± RSD%)	Extraction Recovery (%)	Precision (RSD%)	
				Intraday	Interday
Ezetimibe	5.00	94.18 ± 3.11	96.60	3.50	4.21
	33.33	98.08 ± 5.15	97.35	5.60	5.68
	50.00	105.10 ± 4.33	98.02	5.06	7.55
Atorvastatin Calcium	5.00	95.55 ± 5.30	85.59	6.14	6.23
	33.33	98.75 ± 3.15	89.87	5.37	6.36
	50.00	106.94 ± 5.00	88.99	6.15	9.16

Each value is mean of five replicates.

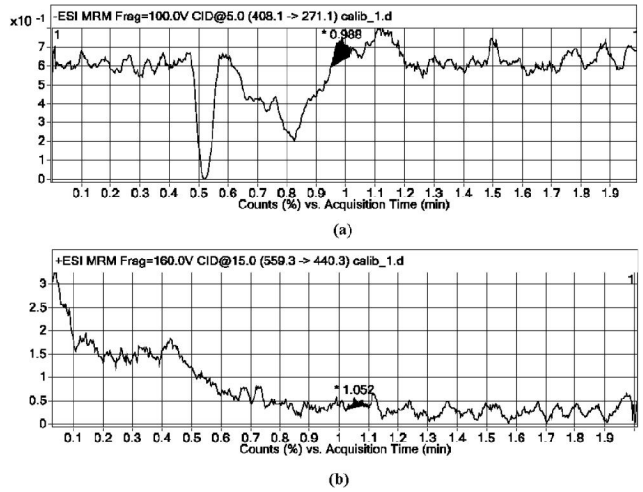

Figure 4 : Representative LC-MS/MS chromatograms of blank plasma for (a) ezetimibe and (b) atorvastatin calcium

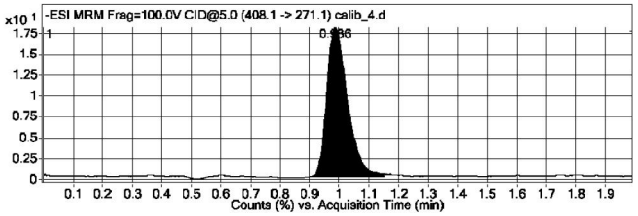

Figure 5 : Representative LC-MS/MS chromatogram obtained from a blank human plasma sample spiked with 16.67 ng/mL ezetimibe, monitored at m/z 271.1

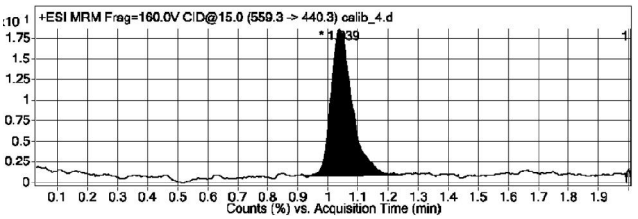

Figure 6 : Representative LC-MS/MS chromatogram obtained from a blank human plasma sample spiked with 16.67 ng/mL atorvastatin, monitored at m/z 440.3

The stability of EZB and ATVC in plasma at room temperature for 8 h did not show significant deviations from the nominal concentrations (less than 10%). Results indicated thatthe analytes in processed samples can remain in the autosampler for at least 8 h without showing significant loss in the quantified values (less than 10%). The stability data of the analytes in plasma over three freeze–thaw cycles indicated a maximal deviation

from the nominal concentration of 13.00%.

CONCLUSIONS

A fast, simple and sensitive method for the determination of EZB and ATVC in human plasma was developed and validated. Simultaneous determination of EZB and ATVC in human plasma using both positive and negative ionization modes was achieved.The results of assay validation of the LC-MS/MS showed that this method is accurate, precise and specific over the specified range. This assay demonstrated a LLOQ smaller than the Cmax for both drugs and is particularily useful in clinical practice for the therapeutic drug monitoring and dose adjustment of EZB and ATVC in hyperlipidemic patients. The short run time and the relatively low flow rate allows the analysis of a large number of samples with minimal mobile phase consumption, proving a cost-effectivebioanalytical tool. Further investigations are required to assess the applicability of the method in pharmacokinetic studies.

REFERENCES

[1] C.M.Ballantyne, J.Houri, A.Notarbartolo, L.Melani, L.J.Lipka, R.Suresh, S.Sun, A.P.LeBeaut, P.T.Sager, E.P.Veltri; Circulation, **107**, 2409-2415 **(2003)**.

[2] K.V.Gowda, D.S.Rajan, U.Mandal, W.D.S.Solomon, P.S.Selvan, A.Bose, A.K.Sarkar, T.K.Chattaraj, T.K.Pal; Asian J. Chem., **19**, 1293 **(2007)**.

[3] M.J.O'Neil; The Merck Index, The Encyclopedia of Chemicals, Drugs and Biologicals, Merck Sharp & Dohme Corp., 668, 864 **(2006)**.

[4] F.Bressolle, M.Bromet-Petit, M.Audran, J.Chromatogr. B, **686**, 3-10 **(1996)**.

[5] P.Wal, B.Kumar, A.Bhandari, A.K.Rai, A.Wal; J Pharm. Sci. Tech., **2**, 333-347 **(2010)**.

[6] D. Zimmer, Chromatographia 57 (2003) 325-332.

[7] H.Trufelli, P.Palma, G.Famiglini, A.Cappiello; Mass Spectrom. Rev., **30**, 491-509 **(2011)**.

[8] S.Li, G.Liu, J.Jia, X.Li, C.Yu; J.Pharm. Biomed. Anal, **40**, 987-992 **(2006)**.

[9] P.R.Oliveira, L.B.Junior, M.Fronza, L.S.Bernardi, S.M.K.Masiero, S.L.Dalmora; Chromatographia, **63**, 315-320 **(2006)**.

[10] J.W.Bae, C.I.Choi, S.H.Park, C.G.Jang, S.Y.Lee; J. Liquid Chromatogr., **35**, 141-152 **(2012)**.

[11] M.Hermann, H.Christensen, J.L.E.Reubsaet; Anal. Bioanal. Chem., **382**, 1242-1249 **(2005)**.

[12] V.Bořek-Dohalský, J.Huclová, B.Barrett, B.Němec, I.Ulč, I.Jelínek; Anal. Bioanal. Chem. 386 (2006) 275-285.

[13] R.V.S.Nirogi, V.N.Kandikere, M.Shukla, K.Mudigonda, S.Maurya, R.Boosi, Y.Anjaneyulu; Biomed. Chromatogr., **20**, 924-936 **(2006)**.

[14] L.Ma, J.Dong, X.J.Chen, G.J.Wang; Chromatographia, **65**, 737-741 **(2007)**.

[15] D.Guillén, F.Cofán, E.Ros, O.Millán, M.Cofán, A.Rimola, M.Brunet; Anal.Bioanal.Chem., **394**, 1687-1696 **(2009)**.

[16] C.Ghosh, I.Jain, S.Gaur, N.Patel, A.Upadhyay, B.S.Chakraborty; Drug Test.Anal., **3**, 352-362 **(2011)**.

[17] N.R.Pilli, J.K.Inamadugu, R.Mullangi, V.K.Karra, J.R.Vaidya, J.Seshagiri Rao; Biomed.Chromatogr., **25**, 439-449 **(2011)**.

[18] Q.Yu, Z.Y.Hu, F.Y.Zhu, J.H.Zhu, L.L.Wan, Y.Li, C.Guo; Chromatographia, **73**, 257-262 **(2011)**.

[19] V.B.Ravi, R.Mullangi, J.K.Inamadugu, N.R.Pilli, R.Gajula, V.Ponneri; Biomed.Chromatogr., **126**, 1436–1443 **(2012)**.

[20] CDER, Guidance for Industry on Bioanalytical Method Validation, U.S. Department of Health and Human Services, **(2013)**.