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RESEARCH ARTICLE

Development and validation of a simple RP-HPLC method for simultaneous estimation of ezetimibe and atorvastatin calcium in pharmaceutical dosage form and spiked human plasma

Yehia Z. Baghdady[1], Medhat A. Al-Ghobashy[2,3], Abdel-Aziz E. Abdel-Aleem[2] and Soheir A. Weshahy[4]

1. Analytical Chemistry Department, Faculty of Pharmacy, Modern University for Technology and Information, Cairo, Egypt.

2.Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

3. Biotechnology Centre, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

4. Pharmaceutical Chemistry Department, Faculty of Pharmaceutical Sciences and Pharmaceutical Industries, Future University, Cairo, Egypt.

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Abstract

A simple, sensitive, and specific method was developed for simultaneous determination of ezetimibe (EZB) and atorvastatin calcium (ATVC) by high performance liquid chromatography without previous separation. Satisfactory resolution was achieved using a RP-C18 chromatographic column, Kromasil RP-18 column (250 mm x 4.6 mm i.d) and a mobile phase consisting of acetonitrile: water (6: 4, v/v) at a flow rate 0.7 mL/min and the wavelength detection was 260.0 nm. The retention time for EZB and ATVC was 7.47 ± 0.02and 4.67 ± 0.02 min; respectively. The described method was linear over a range of 10-100 µg /ml for EZB and 5 -100µg/ml for ATVC. The mean percent recoveries were 99.87 ± 0.769 and 100.04 ± 0.480 for EZB and ATVC, respectively. Method was validated according to ICH guidelinesand successfully applied for analysis of bulk powder, pharmaceutical formulations and spiked human plasma.

1.Introduction

Ezetimibe (EZB) is a highly potent and selective cholesterol absorption inhibitor, decreasing the delivery of intestinal cholesterol to the liver, and atorvastatin calcium (ATVC) is a lipid-lowering agent that inhibits ß-hydroxy-ß-methylglutaryl-coenzyme A(HMG-CoA) reductase. The co-administration of both drugshas been recently introduced to the market and offers a well-tolerated andhighly efficient treatment option for patients with dyslipidemiawhich helps in prescribing a low dose ATVC,reducing the side effects (Ballantyne, et al., (2003)*)*. 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)-β,δ-dihydroxy–5-(1–methylethyl)-3–phenyl–4-[(phenylamino)carbonyl]-1H-pyrrole–1– heptanoic acid –calcium salt (2:1) trihydrate(O'Neil, (2006)*)*. The chemical structures of ATVC and EZB are shown in Figure 1.

A survey of the literature revealed several analytical techniques concerned with the simultaneous determination of EZB / ATVC mixture,spectrophotometry(Baldha, et al., (2009), Deshmukh, et al., (2008), Maher, et al., (2010), Patel, et al., (2010), Rajamanickam, et al., (2010), Sonawane, et al., (2006), (2007), Belal, et al., (2012), Baghdady, et al., (2013), Godse, et al., (2009)*)*, HPTLC (Rajamanickam, et al., (2010), Baghdady, et al., (2013), Aiyalu and Mani, (2012), Dhaneshwar, et al., (2007), Chaudhari, et al., (2006)*)*, HPLC (Patel, et al., (2010), Rajamanickam, et al., (2010), Sonawane, et al., (2006), Sama, et al., (2010), Bhatt, et al., (2010), Chaudhari, et al., (2007), Seshachalam and Kothapally, (2008), Qutab, et al., (2007), Choudhari and Nikalje, (2010)*)*, UPLC (Goel, et al., (2013), Abdelbary and Nebsen, (2013)*)* and Capillary electrophoresis (AlShehri, (2012)*)*.

Unfortunately, not all publishedmethods are reliable for analysis protocols. In many cases, theyare not properly validated and problems arise upon methodtransfer to quality control labs. Moreover, High performance liquid chromatography (HPLC) is probably the most powerful and versatile tool for the combined separation and quantitative analysis of many individual components present in a mixture in one single procedure(Lindsay and Barnes, (1992)).

In this work, the resolution efficiency of HPLC technique is utilized for the development of orthogonal, simple, sensitive and validatedmethod for the simultaneous determination of EZB and ATVC in their pharmaceutical preparation and spiked human plasma.

2.Experimental

2.1. Instruments

- An isocratic pump (Model SP 930D, Young Lin Acme 9000 series).
- An ultraviolet detector (Model UV 730D, Young Lin Acme 9000 series).
- A Rheodyne injector (Model 7725i, Rohnert park, CA, USA) equipped with 20 µL injector.
- Hamilton syringe (100µl).
- Kromasil RP-18 column (250 mm x 4.6 mm I.D.), particle size is 5 µm (Eka Chemicals AB, Bohus, Sweden).

2.2. Pure Drugs and Samples

Marcyrl Pharmaceutical Industries (El-Obour City, Egypt) was the supplier for EZB and ATVC pure standards. Their purity were found to be 99.85% and 100.35%, respectively, according to the absorptivity values reported in reference methods (Rajamanickam, et al., (2010), Sonawane, et al., (2006)). Samples of Atoreza® tablets (Marcyrl); batch no.1030599, labeled to contain 10 mg Ezetimibe and 10 mg Atorvastatin, per tablet were obtained from the market.

2.3. Materials and Reagents

- Acetonitrile, HPLC grade (Sigma Aldrich, Germany).
- Methanol, HPLC grade (Sigma Aldrich, Germany).
- De-ionized water (Otsuka pharmaceutical Co., Egypt).
- tert-Butyl methyl ether (Sigma Aldrich, Germany).
- Human plasma was obtained from VACSERA.

2.4. Standard Solutions

EZB and ATVC stock solutions (1000 µg/mL) were prepared by weighing accurately 250 mg of each powder into two separate 250-mL volumetric flasks. Methanol (50 mL) was added, shaken for a few minutes and completed to volume with the same solvent. A set of laboratory prepared mixtures were prepared by transferring different volumes of each of EZB and ATVC stock solutions into 10-ml volumetric flasks and diluting to volume with methanol.

2.5. Chromatographic Conditions

Using Young Lin instrument, the chromatographic analysis was carried out under the following instrumental parameters: flow rate: 0.7 mL/min; temperature: ambient temperature; detection wavelength: 260.0 nm; run time: 10 min; injection volume: 20 µL; mobile phase: acetonitrile: water (6: 4, v/v) and column: Kromasil RP-18 column (250 mm x 4.6 mm I.D.), particle size is 5 µm (Eka Chemicals AB, Bohus, Sweden).

3.Procedures

Aliquots from EZB and ATVC stock solutions (1000 µg/mL) equivalent to 100 – 1000 µg of EZB and 50 – 1000 µg of ATVC were transferred into two separate sets of 10-mL volumetric flasks and completed to the mark with mobile phase. 20 µL samples were analyzed by HPLC conditions mentioned under "Chromatographic conditions". Calibration curves for both EZB and ATVC were plotted and the corresponding regression equations were calculated.

4. Application to pharmaceutical formulations

Ten tablets were accurately weighed and finely powdered. An amount equivalent to one tablet (containing10 mg of EZB and 10 mg of ATVC) was taken, sonicated in 30 mL of methanol for 30 minutes and filtered into a 100-mL volumetric flask. The residue was washed three times with methanol and the combined filtrate and washings were made up to the mark with methanol to a final concentration of 100 µg/mL of each drug. A suitably diluted sample was measured as mentioned under the procedure of the method. Interference from dosage form additives was investigated using the standard addition

technique.

5. Application to spiked human plasma

To a 1 mL plasma sample, containing different concentrations of binary mixtures of EZB and ATVC in a series of centrifuge tubes, 3 mL of tert-butyl methyl ether was added, vortex-mixed for 1 minute and centrifuged at 4000 rpm for 10 minutes. The upper layer (tert-butyl methyl ether) containing EZB and ATVC was quantitatively separated and another 3 mL of tert-butyl methyl ether was added to the same plasma sample, vortex-mixed for 1 minute, centrifuged for 10 minutes and separated, then all the organic layer from both extractions was evaporated using vacuum concentrator (Eppendorf Concentrator 5301, Germany) till dryness at 45°C. The residue was reconstituted with the mobile phase and injected into the liquid chromatograph.

6. Results and discussion

A simple HPLC method was adopted for the simultaneous determination of EZB and ATVC in bulk powder, pharmaceutical formulation and in spiked human plasma.

Concerning the mobile phase, different systems were tried using variable organic and inorganic solvents with different ratios such as: acetonitrile: water: methanol, methanol: water and acetonitrile: water. The best one was found to be a mixture of acetonitrile with water in ratio of 6: 4, v/v. This system gave an excellent resolution and sensitivity with retention time of 7.47 ± 0.02 minutes for EZB and 4.67 ± 0.02 for ATVC as shown in Figure 2.

System suitability was checked by calculating the capacity factor, tailing factor, column efficiency (N) and the selectivity factor (resolution), where the system was found to be suitable. Results are summarized in Table 1.

Linear relationship was obtained between the peak areas and concentration in the range of 10 – 100 µg/mL for EZB and in the range of 5 – 100 µg/mL for ATVC. The regression equation was computed and found to be:

Y = 43.1234 C + 17.9005 r = 0.9999 for EZB

Y = 35.5508 C + 1.7299 r = 1.0000 forATVC

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

The accuracy of the proposed method was determined by analyzing different concentrations of the drugs in bulk powder as shown in Tables 2 and 3. The mean percentage recoveries were 99.87 ± 0.769 and 100.04 ± 0.480 for EZB and ATVC, respectively.

The specificity of the proposed method was emphasized when analyzing laboratory-prepared mixtures of EZB and ATVC. The method was valid for the simultaneous determination of both drugs with mean percentage recoveries 99.69 ± 1.128 and 100.08 ± 0.789 for EZB and ATVC, respectively, Table 4.

The proposed method has been applied to assay EZB and ATVC in their pharmaceutical formulation. The method was further assessed by application of standard addition technique, as shown in Table 5.

The proposed HPLC method was applied successfully for the analysis of binary mixture of EZB and ATVC in spiked human plasma, where different types of extraction techniques of ezetimibe and atorvastatin calcium from plasma were tried, such as liquid-liquid extraction and protein-precipitation extraction methods.

Liquid-liquid extraction is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase is often the aqueous and second phase is an organic solvent. More hydrophilic compounds prefer the polar aqueous phase, while more hydrophobic compounds will be found mainly in the organic solvents. Analyte extracted into the organic phase are easily recovered by evaporation of the solvent and the residue is then reconstituted in a smaller volume of appropriate solvent (preferably the mobile phase), while analytes extracted into the aqueous phase can often be injected directly on to a reversed-phase column.On the other hand, protein precipitation extraction method is the simple method of extraction as compared to liquid-liquid extraction. This can be carried out by using the suitable organic solvents which has good solubility of the analyte and protein precipitating properties.Acetonitrile is the first choice of solvent for protein precipitation due to its complete precipitation of proteins. After protein precipitation the supernatant obtained can be injected directly in to the HPLC or it can be evaporated and reconstituted with the mobile phase (Wal, et al., (2010)*)*.

In this work protein precipitation extraction method was tried first using perchloric acid or acetonitrile, Figure 3, then liquid-liquid extraction method was tried using ethyl acetate, Figure 4, or tertiary butyl methyl ether, Figure 5, where it was found that on using liquid-liquid extraction method by tertiary butyl methyl ether (Li, et al., (2006))the

best results were obtained regarding resolution and recovery. The mean percentage recoveries were 99.71 ± 1.219 and 99.87 ± 1.323 for EZB and ATVC, respectively, Table 6, as calculated from the following regression equations:

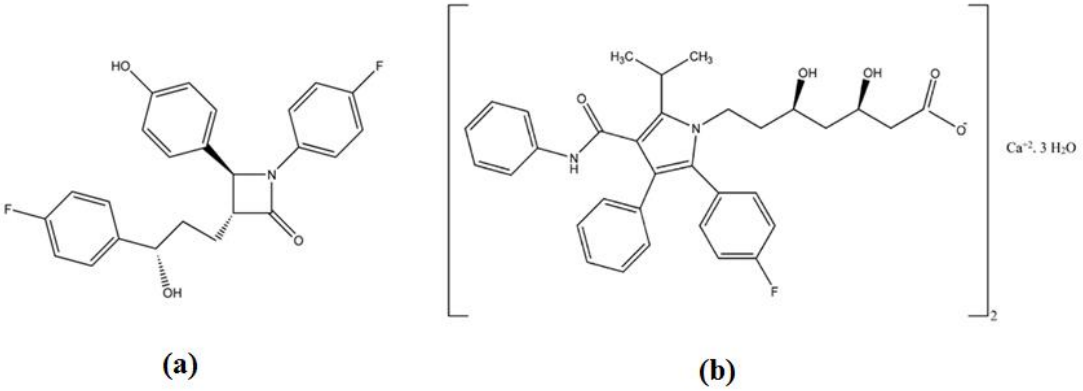
Y = 30.1138C – 1.7828 r = 0.9995 for EZB

Y = 24.1295C + 8.8094 r = 0.9995 for ATVC

The results obtained by applying the proposed HPLC method for the simultaneous determination of EZB and ATVC were statistically compared with the reported method (Godse, et al., (2009)). The t-value and F-value were less than the theoretical ones. This indicates that there is no significant difference between the proposed method and the reported one in accuracy and precision, as shown in Table 7.

The results of assay validation according to ICH 2005 guidelines ((2005))of the HPLC method show that this method is accurate, precise and specific over the specified range, Table 8.

Figure 1. Chemical structures of ezetimibe (a) and atorvastatin calcium (b).



(a) (b)

Figure 2.HPLC chromatogram of ezetimibe (50 µg/mL) (t_R = 7.47) and atorvastatin calcium (50 µg/mL) (t_R = 4.67), using the specified chromatographic conditions.

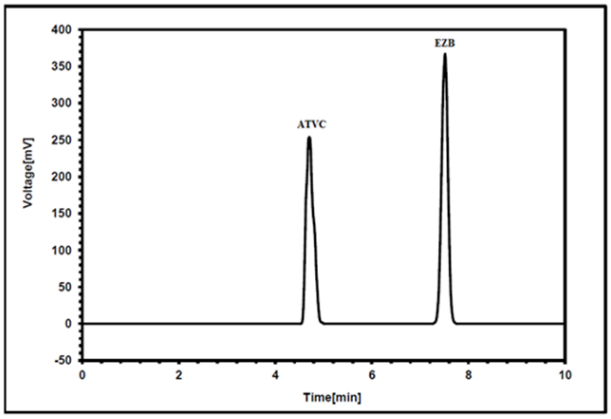


Figure3.HPLC chromatogram of drug-free human plasma spiked with (1:1) mixture containing 30 µg/mL of each of ezetimibe and atorvastatin calcium, using protein precipitation by perchloric acid or acetonitrile for extraction.

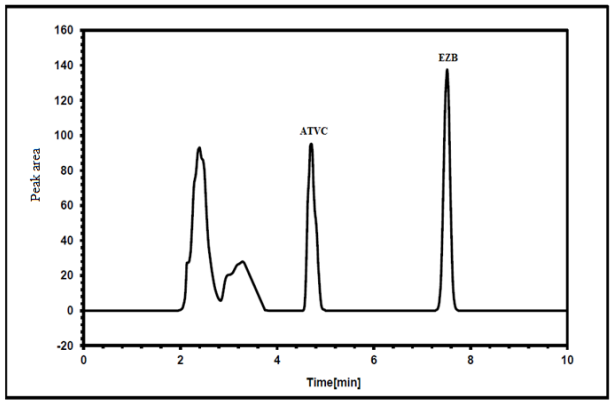


Figure 4.HPLC chromatogram of drug-free human plasma spiked with (1:1) mixture containing 30 µg/mL of each of ezetimibe and atorvastatin calcium, using Liquid-Liquid extraction by ethyl acetate.

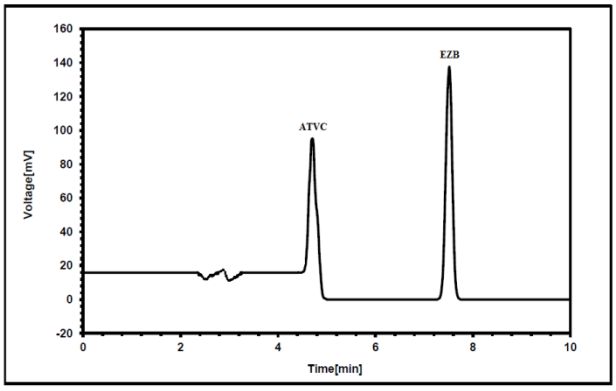


Figure 5.HPLC chromatogram of drug-free human plasma spiked with (1:1) mixture containing 30 µg/mL of each of ezetimibe and atorvastatin calcium, using liquid-liquid extraction by tertiary butyl methyl ether.

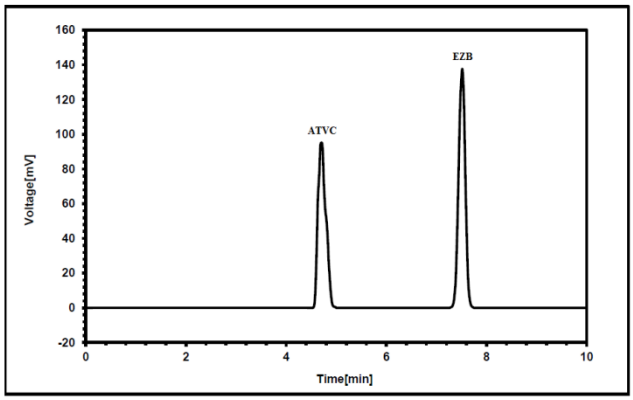


Table 1. Parameters required for system suitability test of the proposed HPLC method.

Parameter	Obtained Value		Reference Value (Andrea and Phyllis, (1997), Adamovics, (1997))
	EZB	ATVC	
Resolution (R)	14.63		R> 0.8
Tailing Factor (T)	0.95	1.14	T=1 for a typical symmetric peak
Relative retention time (α)	1.84		> 1
Column Capacity (K)	4.57	2.49	1-10 acceptable
Column efficiency (N)	5376.60	1384.20	Increases with efficiency of the separation
HETP	0.0046	0.0181	The smaller the value, the higher the column efficiency

Table 2.Determination of ezetimibe in bulk powder by the proposed HPLC method.

Taken µg/mL	**Found* µg/mL**	**Recovery %**
10	9.95	99.47
30	29.61	98.71
50	50.36	100.72
70	70.21	100.30
80	80.41	100.51
100	99.49	99.49
Mean		99.87
SD		0.769
RSD %		0.770

* Average of three determinations.

Table 3.Determination of atorvastatin calcium in bulk powder by the proposed HPLC method.

Taken µg/mL	**Found* µg/mL**	**Recovery %**
5	5.01	100.28
15	14.93	99.55
30	30.22	100.74
50	49.94	99.88
80	79.60	99.50
100	100.30	100.30
Mean		100.04
SD		0.480
RSD %		0.480

* Average of three determinations.

Table 4. Determination of ezetimibe and atorvastatin calcium in laboratory-prepared mixtures by the proposed HPLC method.

Concentration (µg/mL)		EZB Found* µg/mL	ATVC Found* µg/mL	EZB Recovery %	ATVC Recovery %
EZB	**ATVC**				
30	30	29.49	29.88	98.30	99.59
50	50	50.27	49.51	100.53	99.02
70	70	70.78	70.75	101.12	101.07
80	80	79.51	80.21	99.39	100.27
90	90	89.22	90.40	99.13	100.44
Mean				99.69	100.08
SD				1.128	0.789
RSD %				1.132	0.788

* Average of three determinations.

Table 5. Determination of ezetimibe and atorvastatin calcium in Atoreza® tablets by the proposed HPLC method and application of standard addition technique.

Product	HPLC method	Standard addition			
		Taken µg/mL	Added µg/mL	Found µg/mL	Recovery %*
EZB in Atoreza® tablets 10 mg EZB and 10 mg ATVC / tablet B.No. 1030599.	100.79 ± 0.881	30	15	15.02	100.14
			30	30.57	101.91
			45	45.47	101.05
		Mean			101.04
		SD			0.887
		RSD%			0.877
ATVC in Atoreza® tablets 10 mg EZB and 10 mg ATVC / tablet B.No. 1030599.	100.77 ± 0.687	30	15	15.13	100.88
			30	29.92	99.73
			45	45.01	100.01
		Mean			100.21
		SD			0.601
		RSD%			0.599

* Average of three determinations.

Table 6.Determination of ezetimibe and atorvastatin calcium in spiked human plasma by the proposed HPLC method.

Concentration (µg/mL)		EZB Found* µg/mL	ATVC Found* µg/mL	EZB Recovery %	ATVC Recovery %
EZB	**ATVC**				
30	30	29.52	29.42	98.40	98.06
50	50	50.07	50.36	100.15	100.73
70	70	71.03	70.88	101.47	101.25
80	80	79.00	80.28	98.75	100.36
90	90	89.79	89.06	99.77	98.95
Mean				99.71	99.87
SD				1.219	1.323
RSD %				1.222	1.325

*Average of three determinations.

Table 7. Statistical analysis of the results obtained by applying the proposed HPLC and the reported (Godse, et al., (2009))methods for the determination of ezetimibe and atorvastatin calcium.

Value	HPLC method		Reported method*	
	EZB	**ATVC**	**EZB**	**ATVC**
Mean	99.87	100.04	99.69	100.45
SD	0.769	0.480	0.747	0.733
RSD	0.770	0.480	0.749	0.729
n	6	6	6	6
Variance	0.591	0.230	0.558	0.537
Student's t test (2.23)	0.396	1.148	------	------
F value (5.05)	1.060	2.335	------	------

*****Absorbance ratio method (Q-analysis) at 238.6 nm (iso-absorptive point) and 232.6 nm (λ_{max} of EZB).
The values in the parenthesis are the corresponding theoretical values of t and F at (P=0.05).

Table 8. Assay validation sheet of the proposed HPLC method for the determination of ezetimibe and atorvastatin calcium.

Parameter	EZB	ATVC
Accuracy (mean ± SD)	99.87 ± 0.769	100.04 ± 0.480
Specificity	99.69 ± 1.128	100.08 ± 0.789
Precision		
Repeatability*	99.50 ± 0.531	100.08 ± 0.343
Intermediate precision**	99.44 ± 0.716	99.87 ± 0.407
Linearity		
Slope	43.1234	35.5508
Intercept	17.9005	1.7299
Correlation coefficient (r)	0.9999	1.0000
Range	10 – 100 µg/mL	5 – 100 µg/mL
Standard error of the slope	0.25151	0.11689
Confidence limit of the slope	43.1234 ± 0.6983	35.5508 ± 0.3245
Standard error of the intercept	16.16991	6.75713
Confidence limit of the intercept	17.9005 ± 44.8949	1.7299 ± 18.7608
LOD***	3.03 µg/mL	1.54 µg/mL
LOQ***	9.19 µg/mL	4.66 µg/mL

* The intraday (n=3), average of three concentrations (15, 30, 50 µg/mL) for EZB or ATVC repeated three times within the day.
** The interday (n=3), average of three concentrations (15, 30, 50 µg/mL) for EZB or ATVC repeated three times in three successive days.
***LOD and LOQ are determined via calculations(LOD = 3.3xSD/slope and LOQ = 10xSD/slope).

7. Conclusion

The suggested method was successfully applied for the simultaneous analysis of the studied drugs intablets and human plasma. Results demonstrated the lack of interference fromdosage form additives and the usefulness of the method.Hence, the proposed method can be used for the quality control of the cited drugs in ordinary laboratories.

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