Research Article

Application of Multivariate Calibration Methods, in Dissolution Testing and Simultaneous Determination of Atorvastatin and Ezetimibe in Their Combined Solid Dosage Form

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A B S T R A C T

Background: Two simple, precise and accurate multivariate calibration methods, partial least square (PLS) and principal component regression (PCR) have been applied for the simultaneous determination and dissolution profile evaluation of atorvastatin (ATV) and ezetimibe (EZT) in their binary mixtures and commercial tablets. Due to the closely overlapping spectral bands of the mentioned drugs, simultaneous determination without previous separation is not possible by conventional spectrophotometric methods. In the proposed methods (PLS and PCR) determination of chemicals was performed by the use of a full-spectrum multivariate calibration method.
Methods: The experimental calibration matrix was designed orthogonally with 16 samples composed of different mixtures of both compounds in related mediums. The simultaneous determination of ATV and EZT was accomplished in mixtures through recording the absorption spectra within a range of 210 to 300 nm.
Results: The concentration of ATV and EZT were considered in the linear range, between 8 to 14 $\mu g.ml^{-1}$. The specificity of the methods was evaluated by analyzing laboratory prepared mixtures of the mentioned drugs in specific proportions.
Conclusion: The applied methods were successfully employed in simultaneous spectrophotometric determination and dissolution profile evaluation of ATV and EZT in their prepared mixtures and pharmaceutical formulation.

Introduction

Atorvastatin (ATV, Figure 1), a member of statins family acts by competitive inhibition of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase in the liver, the rate-limiting enzyme in cholesterol biosynthesis. It reduces the plasma cholesterol levels by decrease in hepatic cholesterol synthesis as well as increase in hepatic uptake of cholesterol.[1] Ezetimibe (EZT, Figure 2), a cholesterol absorption inhibitor, seems to bind to a critical mediator of cholesterol absorption, the Niemann-Pick C1-Like 1 (NPC1L1) protein on the epithelial cells of gastrointestinal tract along with hepatocytes.[2,3] ATV and EZT are antihyperlipidemic agents, which have been co-formulated and administrated in the treatment of hyperlipidemia. Recent studies showed that ATV plus EZT provided more effective treatment than uptitration of ATV to higher doses in older at-risk patients.[4] Therefore, their combination seems to be beneficial in the management of hyperlipidemia in elderly patients. Accordingly, there is an interest in developing of an efficient simultaneous analysis methods for both of these compounds. According to strong overlap between the absorbance spectra of these compounds, their direct determination by conventional spectrophotometric methods is not possible. Regarding literature review the individual analysis of ATV and EZT has been reported by several methods; however, limited methods have been reported for their simultaneous determination. Most of the developed methods are time consuming,[5] need complicated and expensive equipment[6-8] or in some cases (univariate methods)[5,9,10] have not enough sensitivity and accuracy. In addition to simultaneous drug determination, dissolution testing is considered as one of the most important tests in quality control laboratories. Determination of drug dissolution profile is of particular importance in evaluation of *in vivo* performance as well as bioavailability of the drugs. Dissolution testing contribute useful information about formulation efficiency, and thus it could be helpful in the selection of appropriate formulation excipients in

order to improve the efficiency of dosage form.[11] In the present study, spectrophotometric method was suggested for direct and simultaneous determination and dissolution test of ATV and EZT using full-spectrum multivariate calibration methods, partial least-squares (PLS) and principal component regression (PCR) without any pre-separation step. The principal component regression (PCR) is a principal component analysis followed by a regression step. These methods are helpful chemometric techniques for the calculation of one component concentration in multicomponent mixtures. The offered methods are sensitive, simple, accurate, time saving, well-organized and are suitable for routine quality-control experiments.[12] In this study, simultaneous determination and drug-dissolution monitoring was performed using PLS and PCR as most widely used multivariate calibration methods in binary mixtures of ATV and EZT as well as their combined tablet dosage form. It is noteworthy that simultaneous determination of ATV and EZT by the chemometrics-assisted methods has not been reported by other researchers.
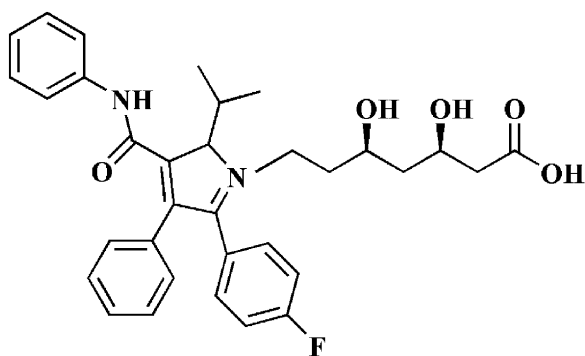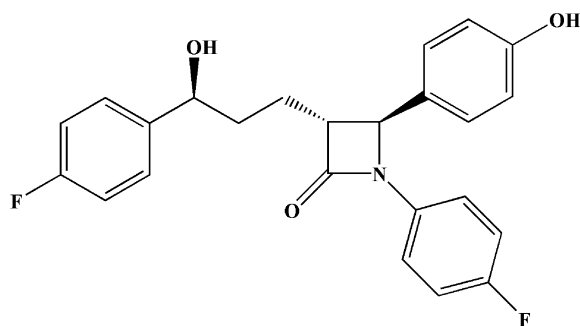

Figure 1. Chemical structures of ATV.

Figure 2. Chemical structures of EZT.

Experimental
Material and Reagents
ATV (PubChem CID: 60823) and EZT (PubChem CID: 150311) powders were supplied from Abidi chemical company (Iran) and Abureyhan pharmaceutical company (Iran), respectively. Tablets of Avas-EZ® (Micro Labs, India); batch no. AVZD0021, labeled to contain 10 mg atorvastatin and

10 mg ezetimibe per tablet were obtained from the market. Methanol (PubChem CID: 887) was HPLC grade and was supplied from (Caledon Labs, Ontario, Canada). Dissolution medium was prepared freshly using double distilled water. All other reagents were of analytical grade.

Apparatus and software
All spectrophotometric measurements were carried out using Shimadzu UV-1800 spectrophotometer, by 1.0 cm quartz cells. All saved spectra were transformed to Excel and MATLAB formats for subsequent manipulation by either PLS or PCR programs. The obtained data were managed using MATLAB software (6.1 version) and PLS and PCR were employed by PLS-Toolbox.[13]
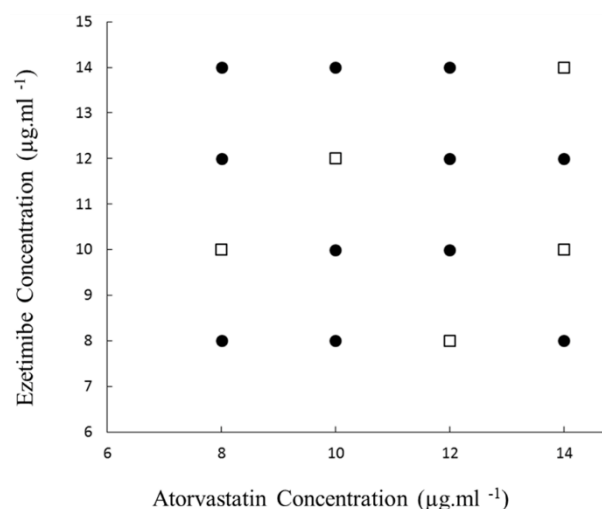

Figure 3. Composition of calibration (●) and prediction (□) sets for the spectrophotometric method.

Calibration procedure for the simultaneous spectrophotometric determination of ATV and EZT
Binary mixtures of ATV and EZT were prepared as follows: specified volumes of the stocks (drug solutions) were transferred into a 25 ml volumetric flask, shaken for a short time and made up to the mark with an appropriate medium separately. The used medium for drug content estimation and dissolution profile experiment was methanol and tween containing phosphate buffer, respectively. The final concentrations were in the ranges of 8-14 $\mu g.ml^{-1}$ for both ATV and EZT. The calibration matrix was obtained using the absorption spectra (between 210 and 300 nm) from different mixed concentrations of ATV and EZT. The composition of calibration and prediction set for spectrophotometric technique has been shown in Figure 3. The optimized calibration matrix, calculated by application of the PLS and PCR multivariate methods and was applied to determine the drug content and dissolution profile of ATV and EZT from analysis of obtained spectra.

Estimation of drug content

In order to simultaneous determination of ATV and EZT in their combination tablet, ten tablets were weighed, their average weight determined, and crushed to fine powder. An accurately amount, equivalent to one tablet (containing 10 mg ATV and 10 mg EZT, according to label claim) was transferred to a 50 ml volumetric flasks (six in each case) and extracted with methanol using magnetic stirrer for 45 minutes (n=6). The excipients were separated by centrifugation for 10 min at 10,000 rpm (Eppendorf 5810R centrifuge). In the case of laboratory prepared mixtures, an accurately weigh amount of ATV and EZT (for each 10 mg) were completely dissolved in 25 ml methanol. Then, appropriate aliquots (within the linearity range) were subjected to the PLS and PCR multivariate methods and the amount of ATV and EZT were determined spectrophotometrically.

Dissolution testing and conditions

The dissolution test was performed in compliance with FDA recommended condition,[14] using apparatus II of United State Pharmacopoeia (USP, paddle method) in tween containing phosphate buffer medium (0.2% w/v tween 80, pH 6.8). Media volume of 900 mL was filled in six dissolution cells and one cell was used as blank for replenishing. The medium temperature was set at 37±0.5°C and the paddle speed was 75 rpm. At specified time intervals (5-45 min), 2mL aliquot from each sample (n=6) was taken at predetermined time intervals, then filtered through a 0.45 μm Millipore filter.[15,16] Due to an immediate release property of these tablets, the earlier time intervals provided more distinguishable profiles. The filtrate was assayed spectrophotometrically and the amount of released drug was determined. For better comparison between release profile of pure drugs powder and commercial tablets, commercial tablets were crushed to fine powder prior to dissolution test. The percent of dissolved drug was plotted versus time and the dissolution profiles of pure drugs were compared with those of commercial tablet.

Results and Discussion

Due to the extensive overlap of ATV and EZT UV spectra (Figure 4), analysis of ATV and EZT by conventional spectrophotometric methods in their binary mixtures is impossible. Their simultaneous determination by derivative and derivative ratio spectrophotometric methods is also faced with considerable difficulty by strong spectral overlap through the wavelength range. Full-spectrum multivariate calibration methods such as PLS and PCR are gaining extensive approval for analysis of pharmaceutical mixtures.[17] We expected that application of these chemometric algorithms, will possibly overwhelmed the spectral overlapping problem and might be applied to simultaneous determination of both drugs with reasonable accuracy and precision. To study the possibility of simultaneous determination of ATV and EZT in their mixtures, the working conditions were adjusted as mentioned before. Method development was made by two calibration models, one for the drug content analysis and the other for the dissolution testing. Optimal conditions are achievable within the range of maximum absorbance spectra of ATV and EZT. The characteristics of calibration graph and the statistical parameters for determination of ATV and EZT with spectrophotometric method under optimum conditions are summarized in Table 1.

Multivariate analysis

In multivariate calibration methods such as PCR and PLS, experimental data (for instance spectrophotometric data in this study) decomposes into the systematic variations (principal components or factors) that explain the observed variance in data. The object of both methods is construction of a calibration model between the concentration of the analyte and the factors of the data matrix.[18] Spectral decomposition is performed in both PLS and PCR; however, the decomposition step is carried out differently. In PCR, the spectra are decomposed on the basis of the maximum variance between spectral data without using the information about the concentrations, whereas PLS uses both spectral and concentration data in modeling.[19,20] Building the calibration matrix for the binary mixtures is the primary step in simultaneous determination via PLS and PCR methods. In this study calibration sets were optimized with the aid of orthogonal design method.[21] Figure 3 shows the composition of the calibration and prediction samples. The considered intervals were at the linear ranges of the ATV and EZT. The calibration process of PLS and PCR is associated

Table 1. Analytical data from the calibration graphs of ATV and EZT by spectrophotometry.

Parameters	ATV		EZT	
Medium	In methanol	In phosphate buffer	In methanol	In phosphate buffer
λmax (nm)	246	255	233	253
Linearity range (μg.ml^{-1})	6-20	6-20	6-20	6-20
Regression equation	y=0.045X-0.016	y=0.035X-0.009	y=0.056X-0.040	y=0.036X-0.020
R^2	0.9993	0.9967	0.9981	0.9973
C $_{LOD}$(μg.ml^{-1})	0.511	0.716	0.859	0.497
C $_{LOQ}$(μg.ml^{-1})	1.703	3.388	2.864	1.657

LOD: Limit of detection and LOQ: Limit of quantification.

Building the calibration matrix for the binary mixtures is the primary step in simultaneous determination via PLS and PCR methods. In this study calibration sets were optimized with the aid of orthogonal design method.[21] Figure 3 shows the composition of the calibration and prediction samples. The considered intervals were at the linear ranges of the ATV and EZT. The calibration process of PLS and PCR is associated with the selection of optimal number of factors, which related to the number of independently varying chemical components as well as the presence of other sources of systematic spectral variation, such as any interactions between the chemical components which changes the shape of the component peak and detector noise.[20] Figure 4 shows the individual spectra, mixtures and sum of the spectra for ATV and EZT in methanol and dissolution medium, respectively. According to the figures, there was no interaction between analytes, and the signals appeared with the great additive properties.

In order to select the number of factors in PLS and PCR, a cross-validation method,[22] put out one sample at a time, was applied. Using this calibration, the concentration of the compounds was predicted in the sample left out during calibration. This process was repeated 20 times until each sample had been left out once. The predicted concentrations of the analytes in each sample were compared with the known concentrations of the compounds in this reference sample, and the prediction error sum of squares (PRESS) was calculated. This procedure was repeated for each component. For finding the smallest model (the fewest number of factors), the F-test was used to carry out the significance determination.[23] Figure 5 shows a plot of the PRESS against the number of factors for each individual analyte.

In all cases, two factors were selected as optimum number of factors. Furthermore, when PC2 versus PC1 (corresponding to a PCA model) was plotted (Figure 6), the score matrix was certainly rotated without noticeable distortion with respect to the calibration matrix (Figsure 3). This confirms that two factors were enough to construct a PCR calibration model. The same results were obtained from PLS method.[24]
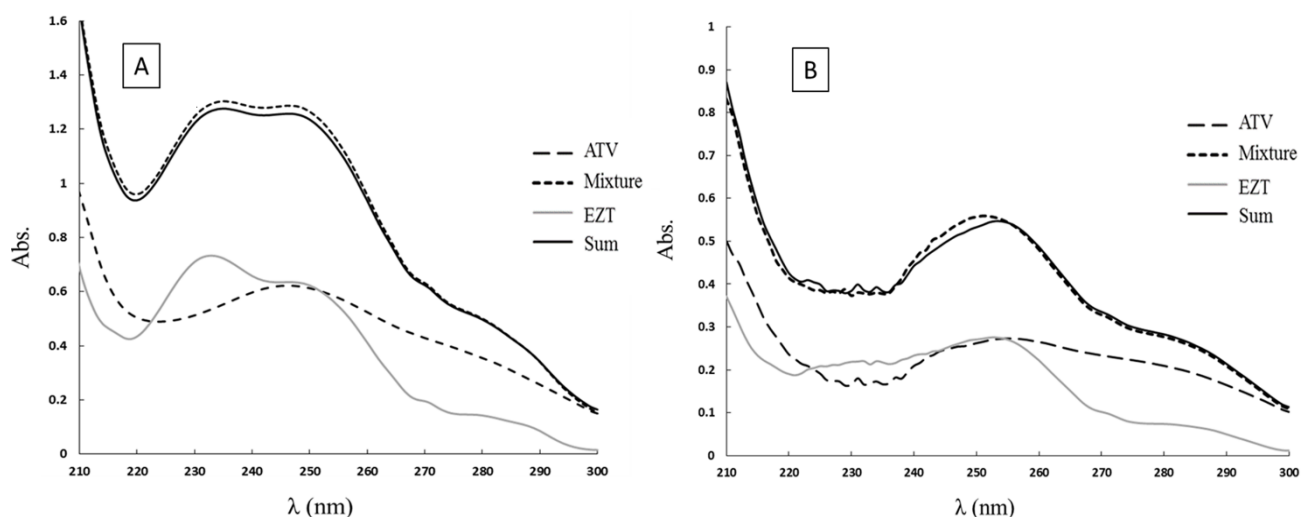

Figure 4. Absorbance (Abs.) spectra of ATV and EZT in theoretical mixture (Sum) and optimum experimental (Mixture) conditions in methanol (both 14 µg.ml^{-1}) (A) and respective dissolution medium (both 8 µg.ml^{-1}) (B).
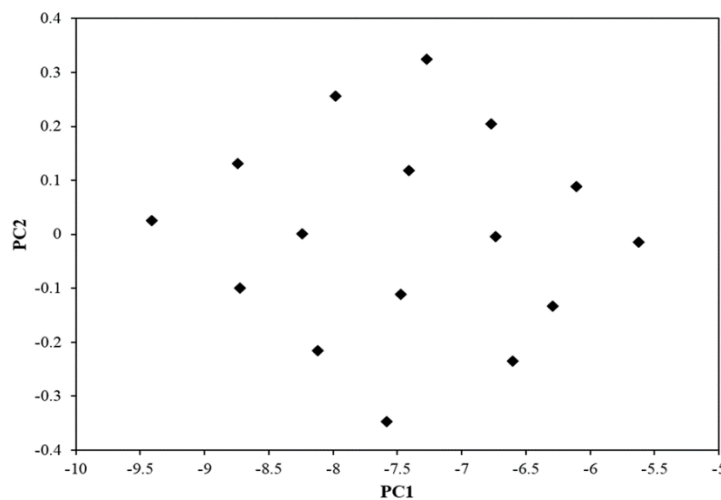

Figure 5. Plot of PRESS against the number of components for ATV and EZT.
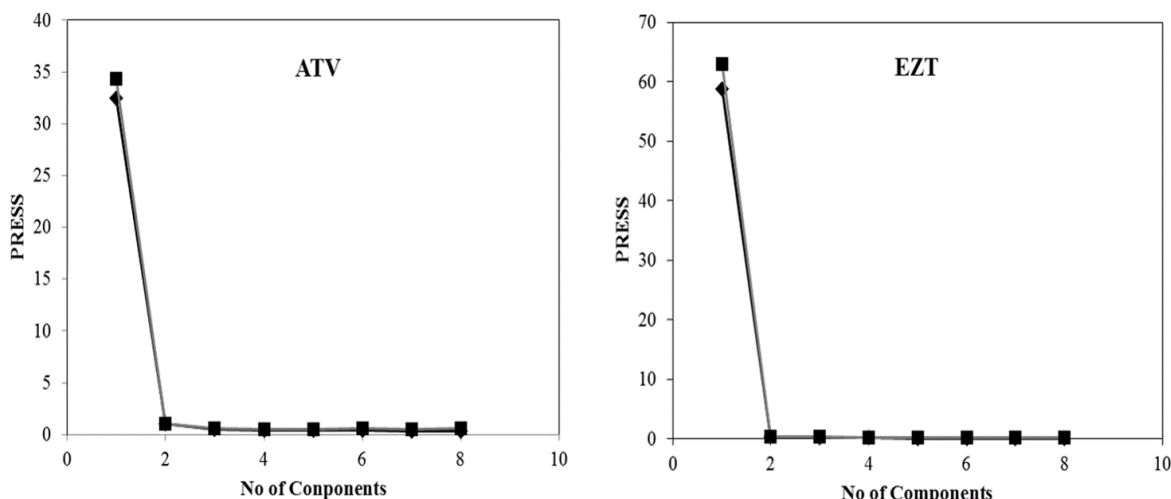
Figure 6. Scores plot of the two first PCs (PCA model) for calibration samples of ATV and EZT mixtures.

Estimation of drug content in mixtures and real samples

The proposed methods were applied to drug assay as well as evaluation of dissolution profiles in the laboratory prepared mixtures and commercial tablets (Avas-EZ®) of ATV and EZT. Table 2 shows the results obtained by the application of the PCR and PLS models on the laboratory prepared mixtures, and pharmaceutical solid dosage form (Avas-EZ® tablet).

Six replicate determinations were performed for each experiment. The drug content determination results for the commercial tablets were in the range of 90 to 110 % of the label claim with reasonably good relative standard deviation (RSD).The high accuracy and excellent RSD of the laboratory prepared samples results was indicative of the high precision and validity of the proposed methods when applied to tablets.

Table 2. Determination of ATV-EZT content in laboratory prepared mixtures (LPM) and commercial tablets (CT) using PLS and PCR.

Samples	Found ± RSD% Percentage of relative error (PE)			
	ATV (mg)		EZT (mg)	
	PLS	PCR	PLS	PCR
LPM				
(Found (mg) ± RSD %)	9.77±0.34 %	9.77±0.36 %	9.81±0.60 %	9.79±0.29 %
(PE)	2.23 %	2.24 %	1.80 %	2.07 %
CT				
(Found (mg) ± RSD %)	10.75 ±2.41 %	10.89±0.64 %	9.41±2.01 %	9.41±2.01 %
(PE)	7.5 %	8.9 %	5.8 %	5.8 %

The nominal value of both ATV and EZT was 10 mg.

Table 3. Dissolution data of ATV and EZT obtained from laboratory prepared mixtures (LPM) and commercial tablets (CT) using PLS and PCR methods.

Time intervals (min)	Components			
	ATV ($\mu g.ml^{-1}$)		EZT ($\mu g.ml^{-1}$)	
	PLS	PCR	PLS	PCR
5 min (LPM)	8.3709	8.3949	5.3167	5.3663
5 min (CT)	9.9636	10.0062	6.4894	6.5625
10 min (LPM)	9.1769	9.1859	5.3246	5.3452
10 min (CT)	11.0108	11.0266	6.9744	6.9921
20 min (LPM)	10.3605	10.3675	6.1505	6.1636
20 min (CT)	11.1894	11.1993	7.8609	7.8643
30 min (LPM)	10.7113	10.7203	6.4284	6.4452
30 min (CT)	11.1927	11.2041	8.5079	8.5148
45 min (LPM)	11.0055	11.0103	6.7147	6.7195
45 min (CT)	11.2885	11.2965	8.7542	8.748

Each data was obtained from the average of six repeated measurements and all of the standard deviations were less than 1.5.
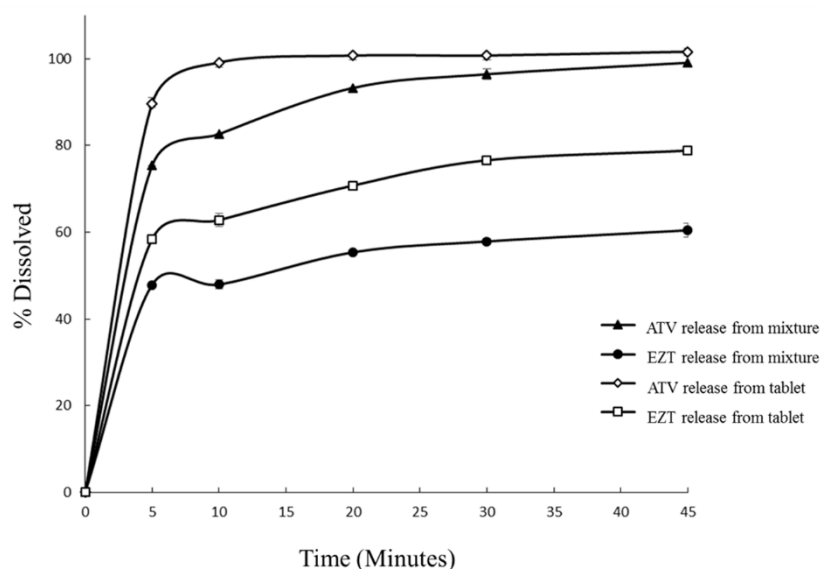
Figure 7. Dissolution profiles of atorvastatin (ATV) and ezetimibe (EZT) obtained from their pure mixture and commercial tablet using PLS method.

Dissolution studies of commercial tablets and binary mixtures

As mentioned before, UV spectra of ATV and EZT overlapped completely in proposed dissolution medium. Therefore, simultaneous determination of these components with conventional spectrophotometric methods is impractical. Table 3 represents the dissolution profiles of the prepared laboratory mixtures and commercial tablets of ATV and EZT obtained from PLS and PCR method. The obtained results from PLS and PCR methods were comparable (Table 3). Figure 7 shows the dissolution profiles of ATV and EZT in commercial tablets and binary mixtures. The dissolution profiles revealed that the commercial tablets had faster dissolution rates than binary mixture in proposed medium.

Conclusion

In conclusion, new suitable analytical method was developed for simultaneous determination of ATV and EZT both in their binary mixtures and their pharmaceutical dosage forms. This study proposed that, under the right conditions, simultaneous determination of the active drug content was possible in combined mixtures of ATV and EZT using PLS and PCR methods. Regarding our findings, both methods produced similar result. The most striking features of the methods are their simplicity, accuracy and speed, which render them suitable for routine quality control analysis of combined pharmaceutical dosage forms in control laboratories.

Conflict of interests

The authors claim that there is no conflict of interest.

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