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Research Article

ABSORBANCE SUBTRACTION AND AMPLITUDE MODULATION AS NOVEL SPECTROPHOTOMETRIC METHODS FOR THE ANALYSIS OF BINARY MIXTURES

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ABSTRACT

Objective: To develop two novel,simple, specific and accurate spectrophotometric methods for simultaneous determination of binary mixtures, without prior separation steps.

Methods: Method A is absorbance subtraction (AS) and method B is amplitude modulation (AM)

Results: Calibration graphs were established in the range of 2-20 µg/mL for both simvastatin (SM) and ezatimibe (EZ) with good correlation coefficients. The developed methods have been successfully applied for the simultaneous analysis of both drugs in their pharmaceutical dosage forms. The methods were validated as per ICH guidelines; accuracy, precision and repeatability are found to be within the acceptable limit.

Conclusion: The two novel spectrophotometric methods are simple, accurate, precise, reproducible, economic and valid for application in laboratories lacking liquid chromatographic instruments.

Keywords: Simvastatin, Ezetimibe, Absorbance subtraction; Amplitude Modulation, absorbance factor, isoabsorptive point, isosbestic point

INTRODUCTION

Simvastatin(SM) (Fig. 1), a hypolipidemic drug belonging to the class of pharmaceuticals called statins is chemically designated as [(1S,3R,7R,8S,8aR)-8-[2-[(2R,4R)-4-hydroxy-6-oxo-oxan-2-yl]ethyl]-3,7-dimethyl 1,2,3,7,8,8ahexahydronaphthalen -1-yl]2,2-dimethylbutanoate. It is used for the treatment of hypercholesterolemia [1]. Following conversion of this lactone prodrug to its hydroxyl acid form, the compound is a potent competitive inhibitor of HMGCoA reductase, the rate limiting enzyme in cholesterol biosynthesis[2]. Different analytical methods have been reported for the determination of simvastatin, which include HPLC[3-6], HPLC-MS/MS[7], derivative spectrophotometry[8] and voltammetric techniques[9].

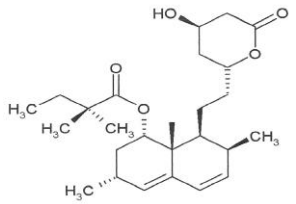

Fig. 1: Chemical structure of Simvastatin

Ezetimibe(EZ) (Fig. 2), a selective inhibitor of intestinal cholesterol and related phytosterol absorption, is designated as 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]- 4(S)-(4-hydroxyphenyl)-2-azetidinone. It prevents transport of cholesterol through the intestinal wall by selectively blocking the absorption of cholesterol from dietary and biliary sources. This reduces the overall delivery of cholesterol to the liver, thereby promoting the synthesis of LDL receptors and a subsequent reduction in serum LDL-C [10,11]. Very few HPLC methods for the determination of ezetimibe are reported in literature[12-13].

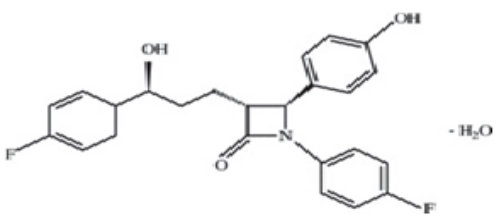

Fig. 2: Chemical Structure of Ezetimibe

Very few methods were reported for the simultaneous determination of these components in their pharmaceutical formulations[14-24].

The main problem of spectrophotometric multicomponent analysis is the simultaneous determination of two or more compounds in the same mixtures without preliminary separation. Several spectrophotometric determination methods have been used for resolving mixtures of compounds with overlapping spectra. Derivative spectrophotometry offers a range which is more reliable with respect to utility and sensitivity than normal spectrophotometry[25], simultaneous equation and absorbance ratio(Q analysis) [26-28],Partial least squares regression (PLS) [29], principal component regression (PCR) [30], multi-wavelength linear regression analysis (MLR) [31], H-point standard addition method (HPSAM) for binary [32] and ternary[33] mixtures have been proposed. Salinas et al.[34] proposed the derivative ratio spectrophotometry, for the simultaneous determination of two compounds in binary mixtures. Berzas Nevada et al. [35] developed a new method for the analysis of ternary mixtures by derivative ratio spectra-zero crossing. In this method, the simultaneous determination of three compounds in ternary mixtures is realized by the measuring of the amplitude at the zero-crossing points in the derivative spectrum of the ratio spectra [36-39]. While, Dinc et al [30,39] proposed a the double divisor-ratio spectra derivative method which is based on the use of the coincident spectra of the derivative of the ratio spectra obtained by using a "double divisor" and measuring at either the maximum or minimum wavelengths. Lotfy[23] developed constant center method utilizing the constants in the ratio spectra to obtain both components in its zero order absorption spectra and measure at their λ_{max}, Lotfy and Hegazy[24,40] introduced two novel methods namely, ratio difference and extended ratio subtraction methods and the last one coupled with a well established ratio subtraction method[41]for simultaneous determination of two components in binary mixtures

The literature review revealed the use of isosbestic point either in the zero order absorption spectrum or in the ratio spectra [41-44] for the determination of total concentrations of both components in the binary mixture and it should be accompany with a complementary method for the determination of one of them.

The aim of this work is to develop a novel method namely; absorbance subtraction (AS) and amplitude modulation (AM) spectrophotometric method acts as a new approach of isosbestic

point by using of smart original mathematical techniques utilizing the absorption factor [24,45] in zero order or constants present in the ratio spectra which could be adapted to isosbestic point analysis for separate quantitative estimation of each drug in their mixture using an unified regression equation.. The novel methods were very simple, accurate, precise, with minimum manipulation steps and without the need of any complementary method to analyze the mixture and did not require any sophisticated apparatus or computer programs.

Theory of absorbance subtraction method (AS)

This method based on the same principles as the absorption factor method [24,45]and it depends on that, If you have a mixture of two drugs X and Y having overlapped spectra intersect at isoabsorptive point and Y is extended more than X, while X doesn't show any absorbance(A_2) at another wavelength (λ_2).

In this method the isoabsorptive point λ_{iso} could be used for separate quantitative estimation of each X & Y in their mixture (X+Y). The determination can be done using mathematically calculated factor of one of these components. By simple manipulation step, we can get the absorbance value corresponding to X and Y, separately. So, the concentration of each component could be obtained via the isoabsorptive point regression equation without any need for a complementary method.

The absorbance values corresponding to X and Y at λ_{iso} were calculated by using absorbance factor {A_{iso} / A_2} which is a constant for pure Y representing the average of the ratio between the absorbance values of different concentrations of pure Y at λ_{iso} (A_{iso}) to those at λ_2(A_2)

$$\text{Absorbance of Y in the mixture at}\lambda_{iso} = \frac{\text{abs1}}{\text{abs2}} \times \text{abs}\,\lambda_2(\text{X+Y})$$

$$\text{Absorbance of X in the mixture at}\lambda_{iso} = \text{abs}\,\lambda_{iso}\,(\text{X+Y}) - \frac{\text{abs1}}{\text{abs2}} \times \text{abs}\,\lambda_2(\text{X+Y})$$

Where; abs_1,abs_2 is the absorbance of pure Y at λ_{iso} and λ_2; $\frac{abs1}{abs2}$ is called the absorbance factor and abs $_{\lambda iso}$(X + Y) and abs $_{\lambda 2}$(X + Y) are the absorbance of the mixture at these wavelengths($\lambda_{iso,}\lambda_2$)

The concentration of each X or Y, separately, is calculated using the isoabsorptive point unified regression equation {obtained by plotting the absorbance values of the zero order curves of either X or Y at isoabsoptive point (λ_{iso}) against their corresponding concentrations X or Y respectively }.

Theory of amplitude modulation method (AM)

The amplitude modulation method is a novel method of ratio spectra manipulation using a normalized spectrum as a divisor, If we have a mixture of X and Y where Y is extended over X and the spectra of X and Y shows isoabsorptive point at the zero spectrum and consequently retained as an isosbestic point at the ratio spectrum.

The absorbance of the zero order absorption spectrum at of mixture of X and Y at isoabsorptive point as follows:

$$[A_m] = [\,a_X C_X] + [\,a_Y C_Y] \quad (1)$$

Dividing eq (1) with normalized spectrum of Y as a divisor (1µg/mL), to get ratio spectrum with isosbestic point (at the same wavelength of the zero order) so the following equation was obtained:

$$[A_m] / [\,a_Y C_{Y'}] = [\,a_X C_X] / [\,a_Y C_{Y'}] + [\,a_Y C_Y] / [\,a_Y C_{Y'}] \quad (2)$$

$$[A_m] / [\,a_Y C_{Y'}] = [\,a_X C_X] / [\,a_Y C_{Y'}] + \text{Constant} \quad (3)$$

$$P_m = P_X + P_Y$$

Where, (P_m) is the amplitude of ratio spectrum of the mixture, (P_X) is the amplitude of component X and (P_Y) is the amplitude of component Y

i.e the recorded amplitude at isosbestic point of the ratio spectrum is equal to the sum of amplitude corresponding to X and that corresponding to Y

The amplitude representing the component Y(P_Y) was the constant [$a_Y C_Y$] / [$a_Y C_{Y'}$] and it can be measured directly from the spectrum at the straight line that is parallel to the wavelength axis in the region where Y spectrum is extended.

Since, we use normalized divisor of Y so, $C_{Y'}$ = 1µg/mL

$$P_Y = [\,a_Y C_Y] / [\,a_Y C_{Y'}]$$

$$P_Y = [\,C_Y] \quad (4)$$

∴ The recorded amplitude of the constant was modulated to concentration so it was representing the concentration of Y [C_Y], ($C_{Recorded}$ of Y)

For determination of amplitude of X in the mixture, If we subtract the measured value of the constant from that of the mixture at isosbestic point of the ratio spectrum Eq.(2);

$$P_X = P_m - P_Y$$

$$P_X = \{[\,a_X C_X] / [\,a_Y C_{Y'}] + \text{Constant}\} - \text{Constant} \quad (5)$$

$$P_X = [\,a_X C_X] / [\,a_Y C_{Y'}] \quad (6)$$

∴ At that isosbestic point $a_X = a_Y$ and normalized divisor of Y $C_{Y'}$ = 1µg/mL

$$P_X = [\,a_X C_X] / [\,a_Y C_{Y'}] \quad (7)$$

$$P_X = [\,C_X] \quad (8)$$

∴ This obtained amplitude of ratio spectrum was modulated to concentration and it was representing concentration of X [C_X], ($C_{Recorded}$ of X)

The corresponding concentration of X or Y could be calculated by using the following regression equation:

$$C_{Recorded} = \text{Slope}\ C + \text{intercept}$$

Slope was found to be approximately one and intercept almost zero

Where; $C_{Recorded}$ represents the recorded amplitudes corresponding to the concentrations of either X or Y that obtained from the ratio spectrum using normalized spectrum of Y (1µg/mL) as a divisor and C represents the corresponding concentration of X or Y.

MATERIAL AND METHOD

Chemicals and Reagents

Standard simvastatin and ezetimibe with claimed purity of 100.41± 0.27 and 100.01 ± 0.35 for SM and EZ, respectively according to reported method[18].Were kindly donated by Global Napi Pharmaceuticals – Egypt. **Inegy tablets**, Batch No. NH49210 (10/10), NH24110 (10/20) and NH49212(10/40) of EZ and SM respectively, manufactured by Global Napi Pharmaceuticals – Egypt and were purchased from local market. methanol E. Merck, Darmstadt, FRG). All other chemicals were of analytical grade.

Apparatus

Spectrophotometric measurements were carried out on Shimadzu 1601 UVPC spectrophotometer, using 1.00 cm quartz cells. Scans were carried out in the range from 200–400 nm at 0.5 nm intervals.

Spectral characteristics

The absorption spectra of the two compounds were recorded over the range 200-290 nm.

Solution and Calibration

Stock solutions of EZ (4 mg/ml) and SM (4mg/ml) were prepared by dissolving the compounds in methanol then completing in 100 ml calibrated measuring flasks. Aliquots of the prepared stock solutions were further diluted with methanol to a final volume of 100 ml. The

diluted solutions were used as the working solutions with concentrations; EZ (40 µg/mL) and SM (40µg/mL).

Linearity

Absorbance subtraction methods (AS)

Standard solutions containing 2-20 µg/mL of EZ or SM respectively, were prepared separately in methanol using their corresponding standard solutions (40 µg/mL). The absorption spectra of the resulting solution were measured and stored in the computer. The absorbance factor of pure EZ at 248.5 nm and 256 nm[$A_{248.5}$ / A_{256}] was calculated, then the concentration of either SM or EZ, was separately calculated using the unified regression equation representing the absorbance of EZ or SM at isoabsorptive point 248.5 nm and the corresponding concentration of EZ or SM.

Amplitude modulation method (AM)

Working standard solutions containing 2-20 µg/mL EZ or SM, were prepared separately in methanol using their corresponding standard solutions (40 µg/mL). The absorption spectra of the resulting solution were measured in the range of 200-290 nm. The scanned spectra of SM or EZ were divided by the normalized absorption spectrum of EZ (1 µg/mL), then the concentration of SM or EZ, was separately calculated using the unified regression equation representing the amplitudes of ratio spectra of either EZ or SM at the isosbestic point 248.5 nm and the corresponding concentration of EZ or SM.

Assay of Laboratory Prepared Mixtures

Absorbance subtraction method (AS)

Into a series of 10mL volumetric flasks, aliquots equivalent to (40 µg -120µg) and (40 µg) of SM and EZ, respectively, were accurately transferred from their standard solutions EZ (40 µg/mL) and SM (40µg/mL) and the volume was completed with methanol. The spectra of the prepared standard solutions from 200-290 nm were scanned and stored in the computer. The absorbance of EZ in the mixtures by using absorbance factor then subtract from the recorded absorbance at 248.5 nm to get the absorbance related to SM. The concentrations of either EZ or SM were calculated by using the unified regression equation at 248.5 nm.

Amplitude modulation Method (AM)

Into a series of 10-ml volumetric flasks, transfer accurately aliquots equivalent to (20 µg) SM and (20 µg -80µg) of EZ and respectively from their stock working solutions EZ (40 µg/mL) and SM (40µg/mL) and complete to volume with methanol. The spectra of the prepared standard solutions were scanned and stored in the computer. The absorption spectra of different laboratory prepared mixtures were divided by the spectrum of the EZ (1µg/mL), Figure (3), then the amplitudes in the plateau region at λ above 256nm (the constant) was subtracted from recorded amplitude at the isosbestic point to obtain amplitudes relating to SM. The concentrations of either EZ or SM were separately calculated from the unified regression equation at 248.5 nm.

Application to pharmaceutical dosage forms

The contents twenty tablets were accurately weighed and powdered. An accurate weight of the mixed sample was transferred into a beaker, 50 ml ethanol were added with continuous magnetic stirring for about 10 minutes. The solution was filtered into a 100-ml volumetric flask, and the volume was completed with methanol. The proposed methods was applied for the analysis of the pharmaceutical preparations solutions using the procedures mentioned under analysis of laboratory prepared mixtures for the proposed methods and the concentrations of the cited drugs were calculated from the corresponding unified regression equation for each method.

RESULTS AND DISCUSSION

Analytical studies related to the quality control and routine analysis of a commercial product in the research or industry laboratories use spectrophotomeric methods such as derivative spectrophotometry, ratio spectra spectrophotometric and other chemometric spectral calibration techniques. These spectrophotometric methods are found to be preferable instead of hyphenated analytical instrumentations or techniques such as liquid chromatography-mass spectroscopy, gas chromatography mass spectroscopy,liquid chromatography-nuclear magnetic resonance, etc., due to the fast quantitative resolution of samples containing two or more substances without needing any chemical pretreatment. In addition of that, the above mentioned hyphenated techniques require a prior step such as derivatization,extraction and other tedious analytical process during analysis. In some cases, these analytical techniques may not give desirable results for some of complex analytical problems. On the other hand, the related techniques having complex components bring high cost and time consumption.

Taking into account all above arguments, the quantitative spectrophotometric resolution of the mixtures of two or more compounds having overlapped spectra is an interesting issue for the analytical chemistry. Besides, the existing spectrophotometric methods were found to be very easy to apply, very rapid, sensitive and yet very cheap for analysis of mixture

For resolving the complex mixtures, the analytical chemist needs new analytical methods or approaches to obtain accurate, precise and safe results. Therefore, the analytical chemists have focused mainly to the use of new mathematical technique or the combined use of the mentioned approaches together with traditional analytical techniques.

Absorbance subtraction method (AS)

This method is based on the absorption factor method [24,45]and its use to analysis of isosbestic point present in zero order absorption spectra known as the isoabsorptive point, where the components exhibiting this point have equal absorptivities. For the determination of SM and EZ, we will utilize their isoabsorptive point at 248.5 nm, Figure 2. By the analysis of the recorded absorbance at the isoabsorptive point, the absorbance corresponding to SM or EZ,separately, at isoabsorptive point 248.5nm can be calculated using absorbance factor [$abs_{248.5}$ / abs_{256}] which is the average of the absorbance of different concentrations of pure EZ using isoabsorptive point at 248.5nm to that at 256 nm which shows no contribution of SM and then the absorbance of EZ can be obtained after subtraction.

Absorbance of EZ in the mixture at$\lambda_{248.5}$ = $abs_{248.5}$ / abs_{256}× abs λ_{256}(SM+EZ) A

bsorbance of SM in the mixture at$\lambda_{248.5}$ = abs $\lambda_{248.5}$(SM+EZ) - $abs_{248.5}$ / abs_{256} × abs λ_{256}(SM+EZ)

Where, abs λ(SM+EZ) is the absorbance of the binary mixture at 248.5 nm or 256 nm and $abs_{248.5}$ / abs_{256} is the absorbance factor of pure EZ at 248.5nm to 256 nm and it was calculated and found to be 1.225.

The calculated absorbance value corresponding to EZ and SM can be separately used to identify each of their concentration using the unified regression equations using isoabsorptive point 248.5 nm.

The only requirements of this method (AS) are the existence of isoabsorptive point of both components and the extension of the spectra of one component.The advantage of the absorbance subtraction method over the conventional isoabsorpative point is that there is no need for another complementary spectrophotometric method to measure the concentration of one of the two components to get the second by substraction. The disadvantage of (AS) method is the increased risk of error in calculating the absorbance factor in case of low concentrations of the extended component EZ or its low value of absorbance at extension region.

Amplitude modulation method (AM)

The new method is based on two facts;the first that the isosbestic point whenever present in an absorption spectrum will be retained at the same point even after division by a one component as a divisor in the ratio spectrum,while the second that the results of manipulating

ratio spectra techniques are greatly affected by the choice of the divisor. So to eliminate the effect of the divisor, we will use the normalized spectrum of EZ (1 µg/mL)(normalized spectrum is prepared mathematically by using sum of different spectra of EZ and divided them by total concentrations of them). Since the two components exhibiting this point have equal absorptivities,by dividing the spectrum of the binary mixture by the normalized EZ divisor spectrum, we obtain the ratio spectra, Figure 3.At the isosbestic point of ratio spectra the amplitude value was modulated to concentration. The amplitude value of the constant can be determined at the plateau region at 256 nm, which is equal to the amplitude constant value of EZ along the whole spectrum.At the isosbestic point (λ_{iso}) at 248.5 nm,the amplitude of the ratio spectra at this point will be equal to the sum of the amplitudes of SM and EZ. After the subtracting recorded amplitude at 248.5 nm from the previously obtained constant at 256 nm - 260 nm, we get the corresponding recorded amplitude of SM, which is equivalent to recorded concentration of SM in the mixture ($C_{Recorded\ of\ SM}$),while the recorded amplitude of constant value will be directly equal to the recorded concentration of EZ in the mixture ($C_{Recorded\ of\ EZ}$)To eliminate any error due to signal to noise ratio, the actual concentration of SM or EZ could be calculated by using their corresponding unified regression equation at isosbestic point 248.5 nm.

$C_{Recorded}$ = 1.005 C+ 0.002.

Where, $C_{Recorded}$ is the recorded amplitude of ratio spectrum at 248.5 nm and C is the corresponding concentration of SM or EZ

The two main requirements of this method are the existence of isoabsorptive point of both components at zero point and consequently in the ratio spectra, and the extension of the spectra of one component.The advantage of amplitude modulation method over other mathematical techniques utilizing the constant is the reduced manipulation steps and only one divisor is needed in order to determine both components in the mixture. By using the normalized divisor, the results are not affected by the choice of divisor. This method has advantage over the isoabsorptive point at zero order that it measures the concentration of both components with no need for other complementary method to measure one of the components in the mixture. In addition,this method has advantages over the newly developed absorbance subtraction method is that by using the normalized divisor, the obtained amplitude at the ratio spectrum will directly represent the concentration of each component and the risk of error upon the determination of absorbance factor of lower absorbance as well as the manipulation steps will be reduced by elimination of the absorbance factor calculation step. That is why the sensitivity of amplitude modulation method (AM) is better than absorbance subtraction method (AS), as shown in Table I.

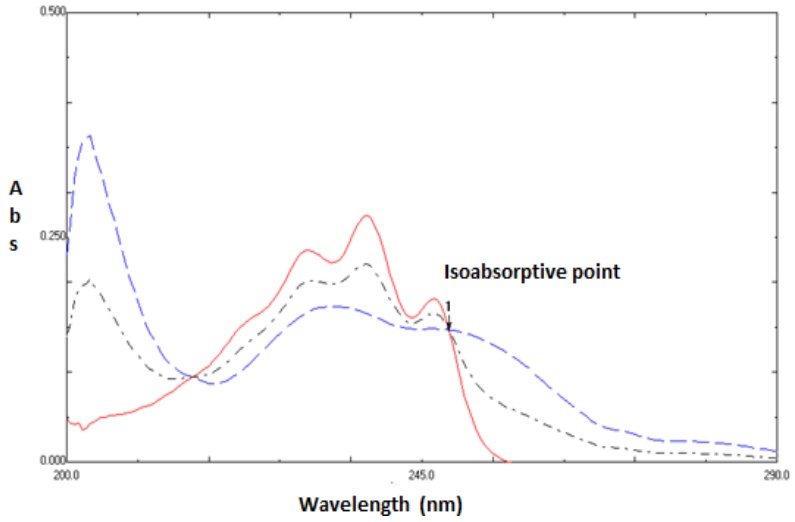

Fig. 3: It shows overlain zero order absorption spectra of 4µg/mLEzetimibe (EZ)(- - -), 4µg/mL Simvastatin (SM)(—) and 2 µg/mL of each (-.-.-) of each in methanol

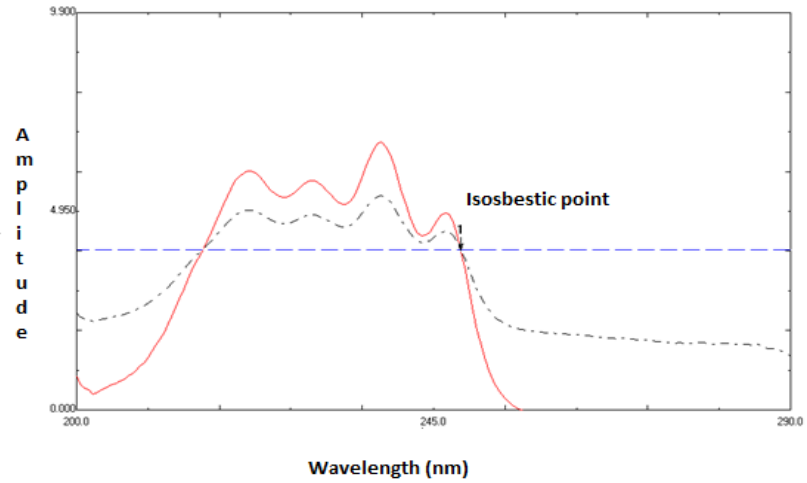

Fig. 4: It shows overlain ratio spectra of 4µg/mL Ezetimibe (EZ)(- - -), 4µg/mLSimvastatin (SM)(—) and 2 µg/mL of each (-.-.-) using normalized spectrum of EZ (1µg/mL)as a divisor.

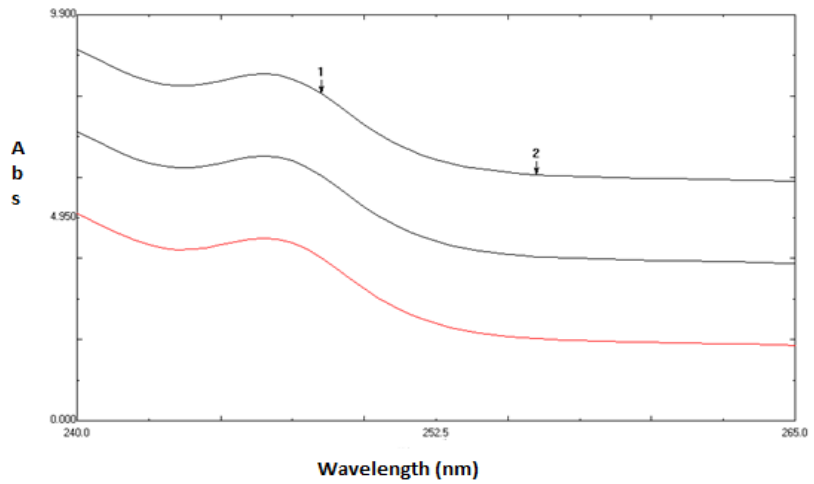

Fig. 5: It shows division spectra of laboratory prepared mixtures 2µg/mL,4µg/ml, 6µg/mL Ezetimibe (EZ) with 2µg/mL Simvastatin (SM) using normalized spectrum of EZ (1µg/ml)as a divisor showing two wavelength 248.5nm and 256nm.

Table 1: It shows assay parameters of Unified Regression parameters and validation sheet

Parameter	Absorbance Subtraction Method Meth at 248.5 nm*	Amplitude Modulation Method at 248.5 nm*
Linearity	2- 20 µg/mL	2-20 µg/mL
Slope	0.0362	1.005
Intercept	- 0.0026	0.002
Correlation coefficient (r)	0.9998	0.9999
Mean + SD	99.98 ± 0.12	100.03 ± 0.08
Accuracy	99.99 ± 0.23	99.98 ± 0.15
RSD% [a]	0.136	0.112
RSD% [b]	0.152	0.126

RSD% [a], RSD% [b]: the intraday and interday respectively (n= 3) relative standard deviation of concentrations EZ or SM(2, 8, 16 µg/mL)

* For both EZ and SM

The proposed method was successfully applied to the analysis of EZ and SM in their laboratory prepared mixtures and in tablet dosage forms. Table 2.

Table 2: It shows results of estimation of pharmaceutical dosage forms by the proposed methods

Sample	Absorbance Subtraction Method		Amplitude Modulation Method	
	Found % ± S.D		Found % ± S.D	
	EZ	SM	EZ	SM
L.P. mixtures (n = 5)*	**100.02 ±0.25**	**99.95 ± 0.22**	**99.99 ±0. 31**	99.97 ± 0.25
Ingey tablets, Batch No. NH49210	**100.26 ±0.87**	**100.28 ± 0.76**	**99.84 ± 0.59**	99.76 ± 0.61
Ingey tablets, Batch No. NH49110	**100.02 ±0.23**	**99.98 ± 0.42**	**100.01 ±0.37**	100.02 ±0.25
Ingey tablets, Batch No. N49112	**99.98 ±0.25**	**99.94 ±0.12**	**100.02 ±0.41**	99.97 ±0.12
Standard addition	99.97 ±0.38	99.98 ±0.52	99.92 ±0.27	100.04 ± 0.46

* Laboratory prepared mixtures (n = 5) 5 sets each of 3 replicates

Method validation

Validation was done according to ICH recommendations [46].

Linearity

The linearity of the methods was evaluated by analyzing six concentrations of SM and concentrations of EZ ranging between 2-20 µg/mL. Each concentration was repeated three times. The assay was performed according to the experimental conditions previously mentioned. The linear equations were summarized in Table 1.

Range

The calibration range was established through considerations of the practical range necessary according to adherence to Beer's law and the concentration of SM and EZ present in the pharmaceutical preparations to give accurate precise and linear results as shown in Table 1.

Accuracy

The accuracy of the results was checked by applying the proposed methods for determination of different blind samples of EZ or SM. The concentrations were obtained from the corresponding regression equations. From which the percentage recoveries suggested good accuracy of the proposed methods were calculated with mean percentage recovery shown in Table 1.

Repeatability

Three concentrations of (2, 8, 16 µg/mL) for either EZ or SM were separately analyzed three times intraday using the proposed method. The relative standard deviations were calculated as shown in Table 1.

Reproducibility (intermediate precision)

The previous procedures were repeated interday on three different days for the analysis of the three chosen concentrations. The relative standard deviations were calculated as shown in Table 1.

Specificity

Specificity of the methods was achieved by the analysis of different laboratory prepared mixtures of SM and EZ within the linearity range. Satisfactory results were shown in Table 1

Stability

EZ and SM working solution showed no spectrophotometric changes up to 3 weeks when stored at 4 °C.

Application of the proposed method for determination of EZ and SM in tablets

The proposed UV methods were applied for the determination of EZ and SM in their combined pharmaceutical formulation Inegy® tablets and the results are shown in table 2 and compared with that of the reported HPLC method[18]. The high percentage recoveries values confirm the suitability of the proposed methods for the routine determination of these components in combined formulation.

Statistical Analysis

Results obtained by the proposed method for the determination of pure samples of EZ and SM are statistically compared to those obtained by the reported HPLC method[18]. The results showed no significant differences between them as shown in Table 3.

As a final conclusion, The absorbance subtraction and amplitude modulation spectrophotometric methods are simple,sensitive and selective could be easily applied in quality control laboratories for the routine analysis of cited drugs in their available dosage forms as it shows equal accuracy and precision compared to the reported HPLC method[18], in contrast it is of lower cost. Furthermore,the proposed methods can be applied for determination of both components in the binary mixtures using an unified regression equation. The proposed methods are also suitable and valid for application in laboratories lacking liquid chromatographic instruments.

Table 3: It shows statistical analysis of pharmaceutical dosage form containing SM and EZ (40:10)

Sample	Absorbance Subtraction Method		Amplitude Modulation Method		Reported HPLC Method[18]	
Parameter	**EZ**	**SM**	**EZ**	**SM**	**EZ**	**SM**
Mean	100.26	100.28	99.84	99.76	99.94	100.10
SD	0.87	0.76	0.59	0.61	0.53	0.65
n	6	6	6	6	5	5
Variance	0.7569	0.5776	0.3481	0.3721	0.2809	0.4225
Student›s test(2.262)*	0.716	0.417	0.293	0.894		
F	2.694 (6.26)*	1.367 (6.26)*	1.239 (5.19)*	1.135 (5.19)*		

* The figures in parenthesis are the corresponding theoretical values at P = 0.05.

REFERENCES

1. Leitersdorf E..Simultaneous estimation of simvastatin and ezetimibe in pharmaceutical formulations by RP-HPLC method. Int. J. Clin Pract 2002; 56 Suppl 2: 116-119.
2. Heek M, Farley C and Compton D. Ezetimibe selectively inhibits intestinal cholesterol absorption in rodents in the presence and absence of exocrine pancreatic function. Br. J. Pharmacol. 2001; 134: 409-417.
3. Singh S, Singh B, Bahuguna R, Wadhwa L and Saxena R. Stress degradation studies on ezetimibe and development of a validated stability-indicating HPLC assay. J. Pharm. Biomed. Anal. 2006; 41 Suppl 3: 1037-1040.
4. Sistla R, Tata V, Kashyap Y, Chandrasekar D and Diwan P. Development and validation of a reversed-phase HPLC method for the determination of ezetimibe in pharmaceutical dosage forms. J. Pharm. Biomed. Anal. 2005; 39 Suppl 3-4:517-522.
5. Mauro V F. Clinical pharmacokinetics and practical applications of simvastatin. Clin. Pharmacokinet. 1993; 24: 195 -202.
6. Alberts A, Chen W J, Kuron G, Hunt V, Huff J, Hoffman, C. et al.A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. 1980;77: 3957 -3961.
7. Carlucci G, Mazzeo P, Biordi L and Bologna M.Simultaneous determination of simvastatin and its hydroxy acid form in human plasma by high performance liquid chromatography with ultraviolet detection. J. Pharm. Biomed. Anal. 1992; 10 Suppl 9: 693 - 697.
8. Ochiai H, Uchiyama N, Imagaki K, Hata S and Kamei T, Determination of simvastatin and its active metabolite in human plasma by column-switching high-performance liquid chromatography with fluorescence detection after derivatization with 1-bromoacetylpyrene. J.Chromatogr. B.; Biomed Appl. 1997; 694 Suppl 1: 211-217.
9. Tan L, Yang L, Zhang X, Yuan Y and Ling S.Determination of simvastatin in human plasma by high performance liquid chromatography. Se Pu. 2000;18 Suppl 3: 232-234.
10. Malenovic A, Ivanovic D, Medenica M, Jancic B and Markovic S. Retention modelling in liquid chromatographic separation of simvastatin and its six impurities applying microemulsion as eluent. J. Sep. Sci. 2004; 27 Suppl 13: 1087–1092.
11. Barrett B, Huclova J, Borek-Dohalsky V, Nemec B and Jelinek I. Validated HPLC-MS/MS method for simultaneous determination of simvastatin and simvastatin hydroxy acid in human plasma. J.Pharm. Biomed.Anal.2006; 41 Suppl 2: 517-526.
12. Wang L and Asgharnejad M. Second-derivative UV spectrometric determination of simvastatin in its tablet dosage form. J.Pharm. Biomed. Anal.2000; 21 Suppl 6: 1243-1248.
13. Coruh O and Ozkan S. Determination of the antihyperlipidemic simvastatin by various voltammetric techniques in tablets and serum samples. Pharmazie. 2006; 61 Suppl 4: 285-290
14. 14.Ozaltin N, Ucakturk E, Ozaltin N and Ucakturk E. Simultaneous determination of ezetimibe and simvastatin in pharmaceutical formulations by dual-mode gradient LC. Chromatographia 2007; 66: 587-591.
15. Dixit P R, Barhate R C,Nagarsenker S M. Stability-indicating HPTLC method for simultaneous determination of ezetimibe and simvastatin. J. Chromatographia 2008; 67 Suppl 1-2:101-107
16. Jain N, Jain R, Swami H, Pandey S, Jain D.K. Spectrophotometric method for estimation of simvastatin and ezetimibe in bulk drug and its combined dosage form. Int J Pharmacy Pharm Sci 2009; 1 Suppl l: 170-175.
17. Kumar D, Sujan D, Viajayasree V and Senshagiriao J.Simultaneous determination of simvastatin and ezetimibe in tablets by HPLC. E- journal of chemistry 2009; 6 Suppl 2: 541-544.
18. Lotfy H M, Aboul alamein A M and Hegazy M A. Quantitative analysis of the cholesterol-lowering drugs ezetimibe and simvastatin in pure powder, binary mixtures, and a combined dosage form by spectrophotometry, chemometry, and high-performance column liquid chromatography.J. of AOAC Inter. 2010; 39 Suppl 5:1844-1855.

19. Ashfaq M, Khana I, Qutab S and Razzaq S. HPLC determination of ezetimibe and simvastatin in pharmaceutical formulations. J. Chil. Chem. Soc. 2007; 52: 1220-1223.
20. Rajput S and Raj H. Indian J. of Pharm. Sci. Simultaneous spectroscopic estimation of ezetimibe and simvastatin in tablet dosage forms.2007; 69:759-762.
21. Palabiyit I, Onur F, Yardimci C and Ozaltin N. Simultaneous spectrophotometric determination of ezetimibe and simvastatin in pharmaceutical preparations using chemometric techniques J. Quimica Nova 2008;31 Suppl 5:1121-1124.
22. Shrestha B, Stephenrathinaraj B, Patel S, Verma N and Mazumder R. Simultaneous HPTLC estimation of simvastatin and ezetimibe in tablet dosage form E-Journal of Chemistry. 2010; 7 Suppl 4:1206-1211.
23. 23.Lotfy H M Determination of Simvastatin and Ezatimibe In Combined Tablet Dosage Forms By Constant Center Spectrophotometric Method. International Journal of Pharmacy and Pharmaceutical Sciences 2012;Vol 4, Suppl 4, 673-679
24. Lotfy H M and Hegazy M A. Simultaneous determination of some cholesterol-lowering drugs in their binary mixture by novel spectrophotometric methods,Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2013; 113: 107–114
25. 25.Dinc E and Onur F. Application of a new spectrophotometric method for the analysis of a ternary mixture containing metamizol, paracetamol and caffeine in tablets. Anal. Chim. Acta 1998; 359: 93 -106.
26. Mali S, Dhabale P, Gonjari I, Deshmukh V and Chanekar P. Simultaneous UV spectrophotometric methods for estimation of atenolol and amlodipine besylate in combined tablet dosage form. Int. J. Pharmacy Pharm.Sci. 2010; 2 Suppl 3:71-74.
27. Likhar A, Gupta K, and Wadodkar S. Spectrophotometric methods for the simultaneous estimation of paracetamol and etoricoxib in tablet dosage forms. Int. J. Pharmacy Pharm. Sci. 2010; 2 Suppl 1:156-161.
28. Vijaya V, Vrushali T, Vrushali K and Dhole S. spectrophotometric simultaneous determination of amlodipine besylate and hydrochlorothiazide in combined tablet dosage form by simultaneous equation, absorption ratio and first order derivative spectroscopy methods. Int. J. Chem. Res.2011; 2 Suppl 1: 7-11.
29. Wold S, Sjostrom M and Eriksson L. A basic tool of chemometrics. Chemom. Intel. Lab.Syst. 2001; 58:109-130.
30. Dinc E, Yucesoy C and Onur F. Simultaneous spectrophotometric determination of mefenamic acid and paracetamol in a pharmaceutical preparation using ratio spectra derivative spectrophotometry and chemometric methods.J. Pharm. Biomed. Anal. 2002; 28:1091-1100.
31. Blanco M, Gene J, Iturriaga H, Maspoch S and Riba, J. Diode-array detectors in flow-injection analysis Mixture resolution by multi-wavelength analysis.Talanta. 1987; 34: 987-993.
32. Bosch-Reig F and Campins-Falco P. Standard addition method Part I. Fundamentals and application to analytical spectroscopy. Analyst. 1988; 113: 1011-1016.
33. Verdu-Andres J., Bosch-Reig F and Compins-Falco P. H point standard addition technique for analyte determination of ternary mixtures. Analyst. 1995; 120: 299-302.
34. Salinas F, Berzas Nevado J, Maspoch S and Riba J. A new spectrophotometric method for quantitative multicomponent analysis resolution of mixtures of salicylic and salicyluric acids. Talanta.1990; 37 Suppl 3: 347-351.
35. Berzas Nevado J, Guiberteau C and Salinas F. Spectrophotometric resolution of ternary mixtures of salicylaldehyde, 3-hydroxy benzaldehyde and 4-hydroxy benzaldehyde by the derivative ratio spectrum zero crossing method. Talanta.1992; 39: 547-553.
36. Berzas Nevado J, Cabanillas C and Contento S. A.Simultaneous spectrophotometric determination of three food dyes by using the first derivative of ratio spectra.Talanta. 1995; 42: 2043-2051
37. Onur F, Yucesoy C, Dermis S, Katral M and Kukdil G. Simultaneous determination of pseudoephedrine sulfate, dexbrompheniramine maleate and loratadine in pharmaceutical preparations. Talanta. 2000; 51: 269-279.
38. El-Gindy A, El-Zeany B, Awad T and Shabana M. Spectrophotometric determination of trifluoperazine HCl and isopropamide iodide in binary mixture using second derivative and second derivative of the ratio spectra methods. J. Pharm. Biomed. Anal. 2001; 26: 203-210.
39. Dinc E, Palabyik I M, Ustundag O, Yustsever F and Onur F. Simultaneous spectrophotometric determination of chlorphenoxamine hydrochloride and caffeine in a pharmaceutical preparation using first derivative of the ratio spectra and chemometric methods.J. Pharm. Biomed. Anal. 2002; 28: 591-600.
40. Lotfy H M. and Hegazy M A. Comparative study of novel methods manipulating ratio spectra: An application on pharmaceutical ternary mixture of omeprazole, tinidazole and clarithromycin. Spectrochimica Acta. 2012; 96: 259–270.
41. 41. El-Bardicy M G, Lotfy H M, El-Sayed M A and El-Tarras M F. Smart stability indicating spectrophotometric methods for determination of binary mixtures without prior separation.J. of AOAC Inter. 2008; 91: 299–310.
42. Abdel-Kawy.M., Amer S M., Lotfy H M. and Zaazaa H E. Simple spectrophotometric analysis of Benazepril hydrochloride or Valsartan in combined pharmaceutical dosages with ydrochlorothazide; Bull. Fac. Pharm. Cairo Univ. 2006; 44(1): 25- 32.
43. El-Ghobashy, M.R. and N.F. Abo-Talib, Spectrophotometric methods for the simultaneous determination of binary mixture of metronidazole and diloxanide furoate without prior separation. Journal of Advanced Research. 2010;1: 323-329.
44. 44.Elzanfaly, E.S., A.S. Saad, and A.B. Abd-Elaleem, Combining the isoabsorptive point in the ratio spectrum and the smart ratio difference methods for a single step determination of compounds with overlapped spectra. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.2012; 95: 188–192.
45. Patel, C.V., et al., Validated Absorption Factor Spectrophotometric and Reversed-Phase High-Performance Liquid Chromatographic Methods for the Determination of Ramipril and Olmesartan Medoxomil in Pharmaceutical Formulations. Eurasian Journal of Analytical Chemistry. 2007; 2(3): 160-171.
46. 46.International Conference on Harmonization (ICH), Q2B: Validation of Analytical Procedures: Methodology, 62, US FDA Federal Register, 1997.