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DEVELOPMENT AND VALIDATION OF H-POINT STANDARD ADDITION METHOD APPLIED FOR THE ANALYSIS OF BINARY MIXTURE OF AMLODIPINE AND ATORVASTATIN

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ABSTRACT

Simple, specific, accurate and precise spectrophotometric method was developed for the simultaneous determination of Amlodipine besylate (AML) and Atorvastatin calcium (ATV) in tablet dosage forms. The proposed H-Point Standard Addition Method (HPSAM) involves addition of the analyte of interest on the binary mixture, measuring the absorbance at two wavelengths and then the calibration curves are used to estimate the concentration of the main analyte and interfernt one. Two analytical wavelengths selected were 241.0-252.4 nm and 278.0-305.6 nm for the estimation of AML and ATV; respectively. The calibration curves were linear over the concentration range of 4-40 and 8-32 µg/mL for AML and ATV, respectively. This method was tested by analyzing synthetic mixtures of the above drugs and they were applied to commercial pharmaceutical preparation of the subjected drugs. The standard deviation was < 1.5 in the assay of raw materials and tablets. Methods were validated as per ICH guidelines and accuracy, precision, repeatability and robustness were found to be within the acceptable limit.

KEY WORDS: **Spectrophotometry; H-Point; standard addition; Atorvastatin; Amlodipine.**

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1. INTRODUCTION

Amlodipine (AML), 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridine carboxylic acid 3-ethyl 5-methyl ester) [1] (Fig. 1 a), is a dihydropyridine derivative with calcium antagonist activity. It is used in the treatment of hypertension and chronic stable angina pectoris [2]. Atorvastatin (ATV), [*R*-(*R**,*R**)]-2-(4-Fluorophenyl)-β,δ-dihydroxy–5-(1–methylethyl)-3–phenyl–4-[(phenylamino)carbonyl]-1*H*-pyrrole–1–heptanoic acid, [1] (Fig. 1 b), is a selective, competitive inhibitor of HMG-CoA reductase enzyme. It is used to reduce LDL-cholesterol, apolipoprotein B, and triglycerides and to increase ATL-cholesterol in the treatment of hyperlipidaemias [3].

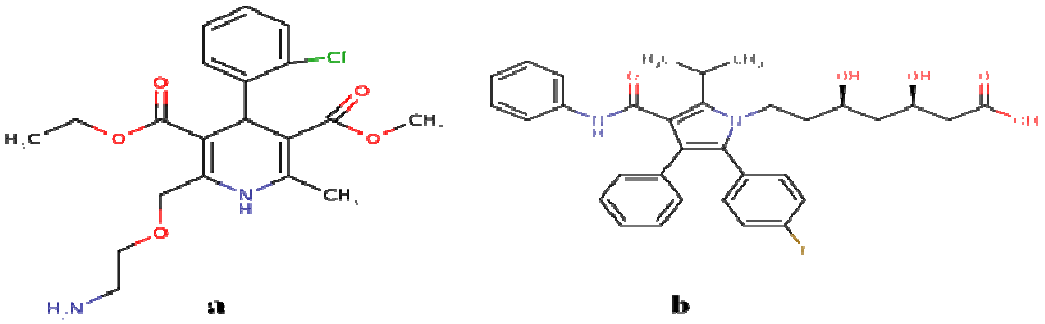

FIGURE 1
STRUCTURAL FORMULAE FOR A) AMLODIPINE AND B) ATORVASTATIN

Caduet® is a dosage form that has been launched by Pfizer Ltd. for the simultaneous treatment of hypertension and dyslipidaemia [4]. Caduet® contains both AML for the treatment of high blood pressure and ATV for the treatment of hypercholesterolaemia. Caduet® tablets are intended for oral administration and are available in several different strength combinations. The literature survey revealed that Amlodipine besylate is official in British Pharmacopoeia [5]. There are reported methods for the determination of AML or ATV in different dosage forms [6-8]. Different methods have been reported for the estimation of AML and ATV in their binary mixture [9-16], these methods include chromatography, spectrophotometry and chemometrics. The H-point standard addition method (HPSAM) permits both proportional and constant errors produced by the matrix of the sample to be directly corrected. It is based on the principle of dual wavelength spectrophotometry and the standard addition method. The method has been applied to binary mixtures of drugs with overlapped absorption spectra [17-20]. In this manuscript the theoretical background of HPSAM was discussed and the method was developed for determination of AML and ATV in their binary mixture and validated according to the ICH guidelines.

1.1 Theory of H-point standard addition method [20]

HPSAM uses the analytical signal at the two accurately selected wavelengths corresponding to constant absorbance of the interfering substance to be plotted versus the added analyte concentration. The following principles have to be followed for selection of appropriate wavelengths for applying HPSAM:

a) At the two selected wavelengths, the signal of interfering substance must remain the same, even if the analyte is changed.
b) The analytical signals of the mixture composed of the analyte and interfering substance should be equal to the sum of the individual signals of the two species.
c) The slope difference of the two straight lines obtained at two selected wavelengths

must be as large as possible in order to get good accuracy.

The great advantage of HPSAM is that, it can remove the errors resulting from the presence of an interfering substance and blank reagent. By plotting the absorbance at the two selected wavelengths versus the added analyte concentration, two straight lines are obtained that have common point with coordinates H ($-C_H$, A_H), where C_H (= C_x) is the unknown analyte concentration and A_H (= A_Y) is the analytical signal due to the interferent [18-20]. Consider an unknown sample containing analyte (X) and interferent (Y). The determination of concentration of X by HPSAM under these conditions requires the selection of two wavelengths; λ_1 and λ_2 at which the interferent (Y) has the same absorbance values. Then known amounts of analyte (X) are successively added to the mixture and the resulting absorbance is measured at the two selected wavelengths and expressed by Equations (1) and (2)

$A_{\lambda 1} = b_0 + b + M_{\lambda 1} C_i$ (1)

$A_{\lambda 2} = A_0 + A^{\backslash} + M_{\lambda 2} C_i$ (2)

Where, b_0 and A_0 ($b_0 \neq A_0$) are the absorbances of X (in the sample) at λ_1 and λ_2, respectively, b and $A^{\backslash}$ are the absorbances of Y (in the sample) at the same wavelengths, $M_{\lambda 1}$ and $M_{\lambda 2}$ are the slopes of the standard addition calibration lines at λ_1 and λ_2, respectively, C_i is the added analyte (X) concentration and $A_{\lambda 1}$ and $A_{\lambda 2}$ are the absorbance measured at the two wavelengths. By plotting the analytical signal versus added analyte concentration, two straight lines are obtained that intersect at the so-called H point ($-C_H$, A_H). At the H-point, since $A_{\lambda 1} = A_{\lambda 2}$, $C_i = -C_H$, so

Equations (3) and (4) can be obtained

$b_0 + b + M_{\lambda 1}(-C_H) = A_0 + A^{\backslash} + M_{\lambda 2}(-C_H)$ (3)

$$-C_H = \frac{[(A_0 - b_0) + (A^{\backslash} - b)]}{M_{\lambda 1} - M_{\lambda 2}}$$

$$C_i = -C_x = -C_H = \frac{A_0 - b_0}{M_{\lambda 1} - M_{\lambda 2}} = \frac{b_0}{M_{\lambda 1}} = \frac{A_0}{M_{\lambda 2}} \qquad (4)$$

If the value of $-C_H$ is included in Equation (4), then

$A_H = b_0 + b + M_{\lambda 1}(-C_H)$

From Equation (4)

$b_0 = -M_{\lambda 1} C_H A_H = b$ (5)

And similarly,

$A_H = A^{\backslash}$ (6)

Therefore, Y can be quantified by considering $A_H = A_Y$ and running a calibration graph at λ_1 (or λ_2) for pure Y. According to the above discussion of H point, C_H is independent from the concentration of the interferent Equations (4) and so, A_H is also independent from the analyte concentration Equations (5) and (6). The basic difference between the HPSAM and the standard addition method lies in the fact that the coordinate of the H point (A_H), i.e. that which provides the concentration of X ($-C_H$), is non-zero as λ_1 and λ_2 are chosen so as to obtain identical absorbance values for Y ($b = A^{\backslash} \neq 0$) [18].

2. EXPERIMENTAL

2.1.Apparatus

SHIMADZU dual beam UV-visible spectrophotometer (Kyoto/ Japan), model UV-1650 PC connected to IBM compatible and a HP1020 laserjet printer. The bundled software, UV- Probe personal spectroscopy software version 2.21 (SHIMADZU) is used. The spectral band is 2 nm and scanning speed is 2800 nm/min with 0.1 nm interval.

2.2. Chemicals and reagents

2.2.1. Pure samples

a) Pure Amlodipine; kindly supplied by Al-Hekma pharmaceutical Company, Cairo, Egypt, its purity was certified to be 99.89 ± 0.691.

b) Pure Atorvastatin; kindly supplied by Al-Delta pharmaceutical Company, Cairo, Egypt, its purity was certified to be 99.79 ± 0.461.

2.2.2. Market samples

Two Caduet® tablet dosage forms, labeled to contain 5(AML)/10(ATV) mg batch number 1030039 and 10(AML)/10(ATV) mg batch number 0795049, manufactured by Pfizer Ltd., Cairo, Egypt.

2.2.3. Methanol

Spectroscopy grade (El-NASR Pharmaceutical Chemicals Co., Abu-Zaabal, Cairo, Egypt).

2.3. Procedures

2.3.1. Standard stock and working solutions

- AML standard stock solution; 1 mg/mL in methanol
- ATV standard stock solution; 1 mg/mL in methanol
- AML standard working solution; 80 µg/mL in methanol
- ATV standard working solution; 80 µg/mL in methanol

2.3.2. Spectral characteristics of AML and ATV

The zero-order (D_0) absorption spectrum of 24 µg/mL AML, 24 µg/mL ATV and a binary mixture of AML and ATV (12 µg/mL for each) were recorded against methanol as a blank over the range of 200-400 nm.

2.3.3. Construction of calibration curves

For AML, synthetic samples were prepared by transferring aliquots equivalent to 80.0 µg of AML and 80.0 µg of ATV into a set of 10-mL volumetric flasks from their respective working standard solutions (80.0 µg/mL), a standard adition of different aliquots of AML in the range of 40.0-180.0 µg to the previously prepared synthetic samples was done and the volumes were completed to the mark with methanol. The °D absorption spectra of the synthetic mixtures were recorded against methanol as a blank. The absorbances at the selected working pair of wavelengths 241.0 and 252.4 nm were measured then plotted against the corresponding added AML concentrations and the regression parameters at the two selected wavelengths were computed. Similarly, for ATV, synthetic samples were prepared by transferring aliquots equivalent to 80.0 µg of ATV and 80.0 µg of AML into a set of 10-mL volumetric flasks from their respective working standard solutions (80.0 µg/mL), a standard addition of different aliquots of ATV in the range of 80.0–220.0 µg to the previously prepared synthetic samples was done and the volumes were completed to the mark with methanol. The °D absorption spectra of the synthetic mixtures were recorded against methanol as a blank. The absorbance at the selected working pair of wavelengths 278.0 and 305.6 nm was measured then plotted against the corresponding added ATV concentrations and the regression parameters at the two selected wavelengths were computed. Four calibration curves were constructed relating the absorbance values at each of the selected wavelengths pairs for each drug to its corresponding added concentrations.

2.3.4. Accuracy

The previously mentioned procedure under construction of calibration curves was repeated for the determination of different concentrations of AML and ATV along their linearity range using different constants of analyte concentrations in the synthetic mixtures, for AML (4.0, 8.0 and 12.0 µg/mL) and for ATV (8.0, 12.0 and 16.0 µg/mL). The concentrations were calculated from the corresponding regression equations.

2.3.5. Specificity

2.3.5.A. Series A: (for AML)

Different sets of solutions were prepared in 10-mL volumetric flasks containing a mixture of the same aliquot (40.0 µg) of AML, as an analyte, and different aliquots of ATV, as an interferent, equivalent to 80.0, 120.0 and 160.0 µg. To those synthetic mixtures, different aliquots from 40.0-180.0 µg of AML were transferred from their corresponding working solution (80.0 µg/mL), and the volume was completed to mark with methanol. The prepared mixtures contain different ratios of the two drugs.

2.3.5.B. Series B: (for ATV)

Different sets of solutions were prepared in 10-mL volumetric flasks containing mixture of the same aliquot (80.0 µg) of ATV, as an analyte, and different aliquots of AML, as an interferent, equivalent to 40.0, 80.0 and 120.0 µg. To those synthetic mixtures, different aliquots from 80.0–

220.0 ug of ATV were transferred from their corresponding working solution (80.0 µg/mL), and the volume was completed to mark with methanol. The prepared mixtures contain different ratios of the two drugs.

2.3.6. Analysis of AML and ATV in laboratory prepared mixtures

Aliquots of AML and ATV were transferred from their standard working solution (80 µg/mL) into a series of 10-mL measuring flasks, completed to volume with methanol to prepare mixtures containing different ratios of AML and ATV. The concentrations of AML and ATV were calculated by the proposed method.

2.3.7. Analysis of AML and ATV in Caduet® tablets

Ten tablets of both Caduet® 5(AM)/10(AT) mg, 10(AM)/10(AT) mg were accurately weighed and finely powdered. An amount of the powder equivalent to 2 mg AT was weighed, dissolved in methanol by shaking in ultrasonic bath for about 30 minutes. The solutions were filtered and transferred quantitatively into two separate 100-mL volumetric flasks. The volume was then completed to the mark with methanol. Then necessary dilutions were made to reach concentrations within the linearity range. The concentrations of AML and ATV were calculated by the proposed method.

3. RESULTS AND DISCUSSION

Many methods have been introduced for the analysis of binary mixtures among which the molecular absorption spectroscopy was the most simple, fast and applicable in almost all laboratories. The use of this technique for pharmaceutical analyses has the inherent constraint that most active drugs absorb in the UV region and exhibit strongly overlapped spectra that impede their simultaneous determination. Several manipulations were performed to enable mixture resolution for example, using different order derivatives [21], derivatives of the ratio spectrum [22], ratio subtraction technique [23] and isoabsorptive method [24]. Many spectrophotometric methods have been developed which depend on measuring the absorbance at two wavelengths, which may improve the accuracy and precision of the methods. Some of these methods, the Dual Wavelength method [25], Bivariate method [26], Absorbance Ratio method [27] the method of Ratio Difference [28-29] and H-Point standard addition method [17]. The zero-order absorption spectra (D_0) of AML and ATV showed overlapping, Fig. 2, which allows the analysis of AML in presence of ATV at 359.4 nm, but prevents the analysis of ATV in presence of AML. In this work, we applied the HPSAM for analysis of both AML and ATV in pure powder form, laboratory-prepared mixtures and Caduet® tablets.

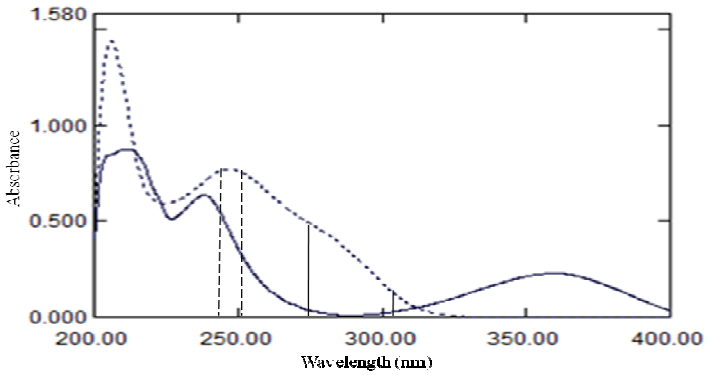

Figure 2
Zero order absorption spectrum of 20µg/mL AML (—) and 20µg/mL ATV (- - -) using methanol as blank showing the selected wavelengths for HPSAM

HPSAM uses the analytical signal data at the two accurately selected wavelengths corresponding to constant absorbance of the interfering substance to be plotted versus the added analyte concentration, two straight lines are obtained that have a common point with coordinates H ($-C_H$, A_H), where, C_H is the unknown analyte concentration and A_H is the analytical signal due to the interferent. Consider an unknown sample containing AML and ATV, the determination of concentration of AML and ATV by HPSAM under these conditions requires the selection of two pair of wavelengths; for AML determination the selected pair of wavelengths was 241.0 and 252.4 nm, at which ATV gives the same absorbance values, Fig. 2**.** On the other hand, the figure shows the other selected pair (278.0 and 305.6 nm) for ATV determination in which AML gives the same absorbance values. Different calibrations were plotted for various synthetic mixtures, of different AML and ATV concentrations, to assess the accuracy and specificity of the selected two pair of wavelengths for the determination of each drug. For AML, different concentrations were added to mixtures of constant ATV concentration (8 µg/mL) and different AML concentrations (4, 8 and 12 µg/mL), and then the absorbance at the two selected wavelengths (241.0 and 252.4 nm) was plotted against the added AML concentrations, to assess the accuracy, Fig. 3**.** While to assist the specificity, different concentrations of AML were added, to different mixtures of constant AML concentration (4 µg/mL) and variable ATV concentrations (8, 12 and 16 µg/mL), then the absorbances were plotted against the added AML concentrations, Fig. 4**.**

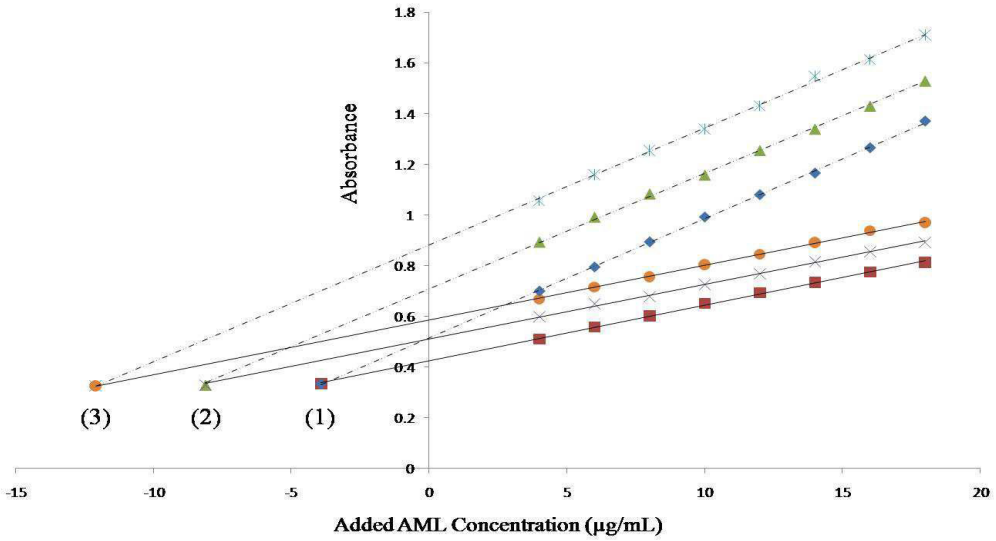

Figure 3

Plots of H-point standard addition method between the absorbance at 241.0 nm (- - -) and 252.4 nm (—) and the added concentration of AML to mixture of fixed ATV (8.0 µg/mL) and AML (1)4.0µg/mL (2)8.0µg/mL (3) 12.0 µg/mL

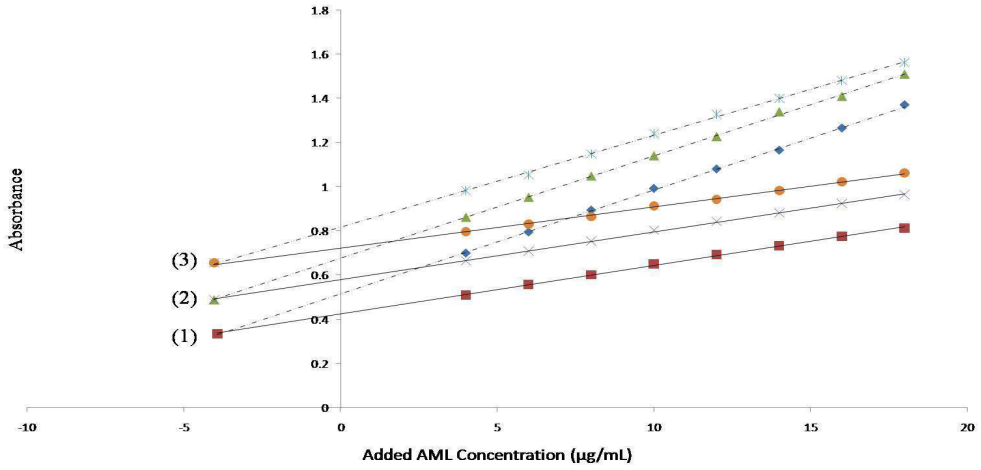

Figure 4

Plots of H-point standard addition method between the absorbance at 241.0 nm (- - -) and 252.4 nm (——) and the added concentration of AML to mixture of fixed AML (4.0 µg/mL) and ATV (1) 8.0 µg/mL (2) 12.0 µg/mL (3) 16.0 µg/mL

Similarly, the accuracy of the selected pair of wavelengths for ATV determination was assessed by adding different concentrations of ATV to mixtures of constant AML concentration (8 µg/mL) and different ATV concentrations (8, 12 and 16 µg/mL), then the absorbances at the two selected wavelengths (278.0 and 305.6 nm) were plotted against the added ATV concentrations, Fig. 5**.** While the same procedure was followed but to different mixtures of constant ATV concentration (8 µg/mL) and variable AML concentrations (4, 8 and 12 µg/mL), then the absorbance was plotted against the added ATV concentrations, to assess the specificity of ATV determination at the selected wavelengths, Fig. 6**.**

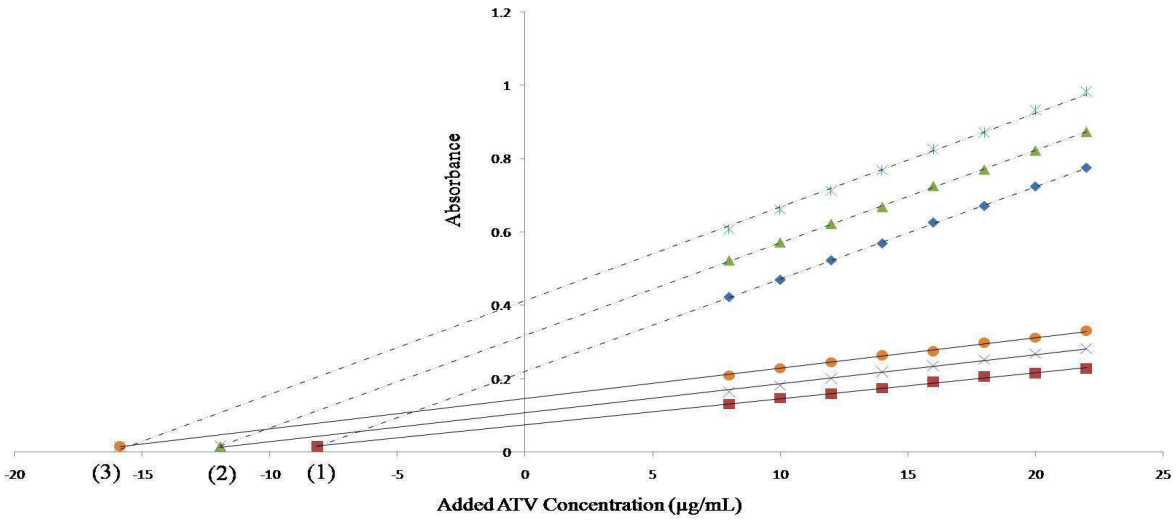

Figure 5

Plots of H-point standard addition method between the absorbance at 278.0 nm (- - -) and 305.6 nm (——) and the added concentration of ATV to mixture of fixed AML (8.0 µg/mL) and ATV (1) 8.0 µg/mL (2) 12.0 µg/mL (3) 16.0 µg/mL.

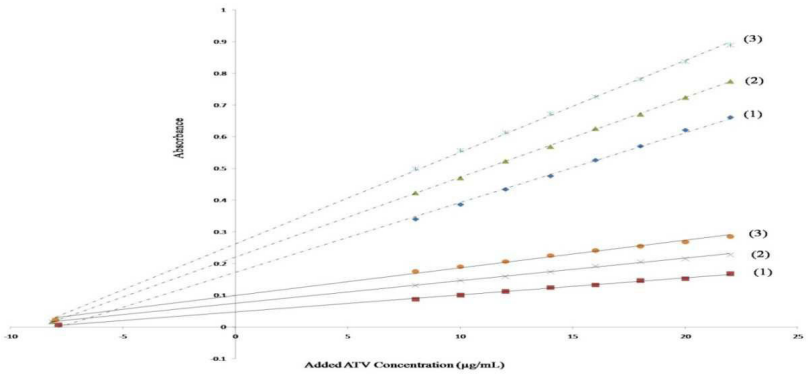

Figure 6

Plots of H-point standard addition method between the absorbance at 278.0 nm (- - -) and 305.6 nm (——) and the added concentration of ATV to mixture of fixed ATV (8.0 µg/mL) and AML (1) 4.0 µg/mL (2) 8.0 µg/mL (3) 12.0 µg/mL

The regression parameters were calculated for each calibration, for AML and ATV Table 1 and 2, respectively. It was evident from the results that the slopes at each of the selected pair of wavelengths were almost equal, and the calculated recoveries of AML or ATV were satisfactory which ensures the accuracy and specificity of the selected pair of wavelengths for each drug.

Table 1

Determination of AML and ATV in synthetic samples by HPSAM.

A-C Equation	r	Concentration (µg/mL)		Found (µg/mL)	
		AML	ATV	AML	ATV*
$A_{241.0}$ = 0.0460C + 0.5149	0.9998	4	8	3.93	8.10
$A_{252.4}$ = 0.0221C + 0.4210	0.9996				
$A_{241.0}$ = 0.0460C + 0.7033	0.9998	8	8	8.12	7.98
$A_{252.4}$ = 0.0219C + 0.5075	0.9995				
$A_{241.0}$ = 0.0460C + 0.8834	0.9998	12	8	12.11	7.91
$A_{252.4}$ = 0.0216C + 0.5880	0.9998				
$A_{241.0}$ = 0.0464C + 0.6765	0.9998	4	12	4.03	11.94
$A_{252.4}$ = 0.0219C + 0.5777	0.9997				
$A_{241.0}$ = 0.0460C + 0.8225	0.9997	4	16	4.03	15.60
$A_{252.4}$ = 0.0219C + 0.7255	0.9997				

****calculated from individual calibration curve of ATV at 252.4 nm, where its regression equation is $A_{252.4}$ = 0.0404C + 0.0071***

Table 2

Determination of ATV and AML in synthetic samples by HPSAM.

A-C Equation	r	Concentration (µg/mL)		Found (µg/mL)	
		ATV	AML	ATV	AML*
$A_{278.0}$ = 0.0250C + 0.2199	0.9999	8	8	8.17	7.87
$A_{305.6}$ = 0.0064C + 0.0680	0.9994				
$A_{278.0}$ = 0.0253C + 0.3178	0.9999	12	8	11.94	7.89
$A_{305.6}$ = 0.0061C + 0.0886	0.9992				
$A_{278.0}$ = 0.0250C + 0.3820	0.9996	16	8	15.93	8.10
$A_{305.6}$ = 0.0060C + 0.0800	0.9996				
$A_{278.0}$ = 0.0257C + 0.2103	0.9997	8	4	7.88	3.89
$A_{305.6}$ = 0.0057C + 0.0527	0.9993				
$A_{278.0}$ = 0.0252C + 0.2249	0.9997	8	12	8.00	11.65
$A_{305.6}$ = 0.0059C + 0.0705	0.9996				

****calculated from individual calibration curve of AML at 278.0 nm, where its regression equation is $A_{278.0}$ = 0.0020C.***

The computed regression equations at the two selected wavelengths could be represented as follows:

$A_{\lambda1} = M_{\lambda1}C_1 + Y_1$

$A_{\lambda2} = M_{\lambda2}C_2 + Y_2$

Where, A is the absorbance, M is the slope, C is the concentration and Y is the intercept. The point at which each pair of calibrations intersect is the H-point with a coordinate ($-C_H$, A_H), where C_H is the concentration of AML or ATV, and A_H is the analytical signal due to the interfering analyte concentration, ATV or AML, respectively. So, the constant analyte concentration in those mixtures (C_H) can be calculated by substituting the measured absorbance at the two selected wavelengths, for each analyte, directly in the previously mentioned equation.

For C_H calculation (for analyte determination)

At H-point $A_{\lambda1} = A_{\lambda2}$

$M_{\lambda1}C_1 + Y_1 = M_{\lambda2}C_2 + Y_2$

$M_{\lambda1}C_1 - M_{\lambda2}C_2 = Y_2 - Y_1$

$C_1 = C_2$, so

$$C_H = \frac{Y_2 - Y_1}{M_{\lambda1} - M_{\lambda2}}$$

While for the determination of the concentration of ATV, the absorbance at H-point (A_H) was calculated and substituted in the individual regression equation of the ATV.

For A_H calculation (For the interferent determination)

$$C_1 = \frac{A_1 - Y_1}{M_{\lambda1}}$$

$$C_2 = \frac{A_2 - Y_2}{M_{\lambda2}}$$

At H-point $C_1 = C_2$

$$\frac{A_1 - Y_1}{M_{\lambda1}} = \frac{A_2 - Y_2}{M_{\lambda2}}$$

$M_{\lambda1}(A_{\lambda2} - Y_2) = M_{\lambda2}(A_{\lambda1} - Y_1)$

$M_{\lambda1}A_{\lambda2} - M_{\lambda1}Y_2 = M_{\lambda2}A_{\lambda1} - M_{\lambda2}Y_1$

$M_{\lambda1}A_{\lambda2} - M_{\lambda2}A_{\lambda1} = M_{\lambda1}Y_2 - M_{\lambda2}Y_1$

$A_H = A_1 = A_2$ so,

$A_H(M_{\lambda1} - M_{\lambda2}) = M_{\lambda1}Y_2 - M_{\lambda2}Y_1$

$$A_H = \frac{M_{\lambda1}Y_2 - M_{\lambda2}Y_1}{M_{\lambda1} - M_{\lambda2}}$$

Then A_H was used for the determination of the concentration of interferent in the prepared sets by substituting in its regression equation of its pure form. The term $M_{\lambda1} - M_{\lambda2}$ determines the sensitivity of the method. A great difference between the two slopes can be achieved by choosing two wavelengths sufficiently distant from the maximum of the interfering analyte. Many wavelength pairs were used for determination of AML and ATV. For determination of AML the wavelength pairs 241-252.4, 238.6-255, 216.6-250, 243.4-250, 223.4-261 and 229.6-261 were tried, and 241-252.4 nm showerd best linearity and sensitivity. While for determination of ATV the wavelength pairs 267.4-321, 257-359 and 278-305.6 nm were tried and the pair 278.0-305.6 nm showed the best results. By plotting the absorbance versus added analyte concentration at the selected pair of wavelengths, two straight lines were obtained that intersect at H-point. Fig. 7 shows this intersection when 241.0 and 252.4 nm were selected for AML analysis. Moreover, the same figure shows the intersection when 278.0 and 305.6 nm were selected for ATV analysis.

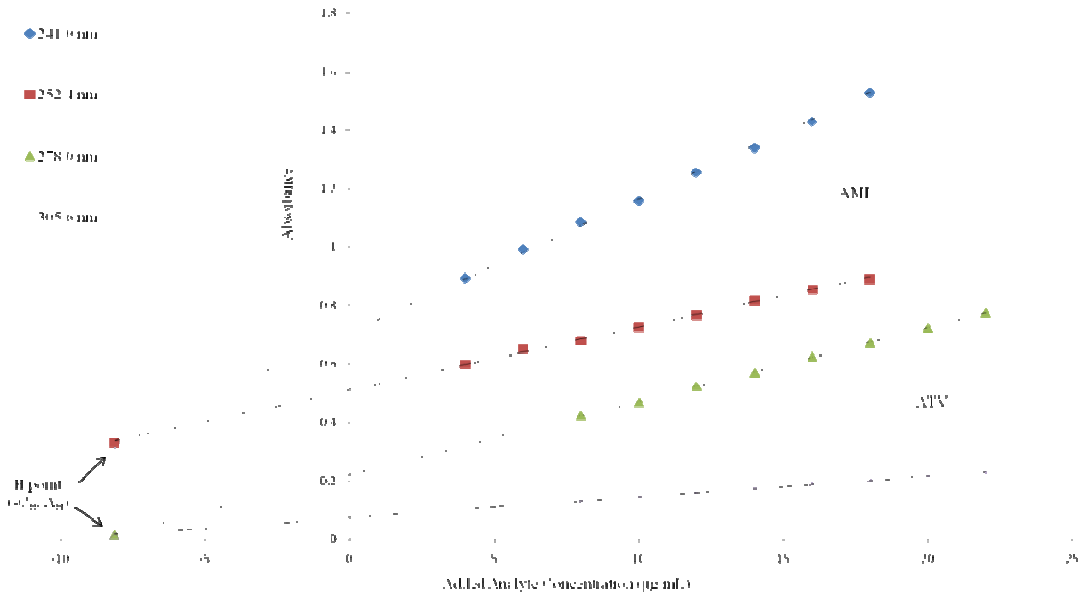

Figure 7

Plots of H-point standard addition method between the absorbance and the added concentration of :-
-AML at 241.0 and 252.4 nm, to mixture of 8.0 µg/mL AML and 8.0 µg/mL ATV
-ATV at 278.0 and 305.6 nm, to mixture of 8.0 µg/mL AML and 8.0 µg/mL ATV

A linear correlation was obtained between the absorbance at the selected wavelengths for each of AML and ATV against its added concentration. The regression equations were computed:

For AML

$A_{241.0}$ = 0.0460C + 0.7033 r = 0.9998 at 241.0 nm

$A_{252.4}$ = 0.0219C + 0.5075 r = 0.9995 at 252.4 nm

For ATV

$A_{278.0}$ = 0.0250C + 0.2199 r = 0.9999 at 278.0 nm

$A_{305.6}$ = 0.0064C + 0.0680 r = 0.9994 at 305.6 nm

Where, A is the absorbance, C is the added drug concentration in µg/mL in the mixtures and r is the correlation coefficient. The proposed method was found to be valid in the range from 4.0-40.0 µg/mL and 8.0-32.0 µg/mL for AML and ATV, respectively. The selectivity of the proposed method was assessed by the analysis of laboratory prepared mixtures containing different ratios of the two drugs, where satisfactory results were obtained over the calibration ranges as shown in Table 3.

Table 3
Determination of AML and ATV in laboratory prepared mixtures by the proposed HPSAM.

Concentration (µg/mL)		HPSAM Found % [a]	
AML	ATV	AML	ATV
8	8	102.38	101.73
16	8	100.11	100.24
24	8	101.02	97.92
8	16	98.64	101.26
8	24	97.66	99.12
16	24	99.33	100.43
24	16	99.55	99.88
Mean		99.81	100.08
SD		1.550	1.282
RSD%		1.553	1.281

[a] *average of three determinations*

The proposed method was also applied for the determination of AML and ATV in Caduet® tablets and the validity of the proposed procedure was further assessed by applying the standard addition technique as presented in Table 4.

Table 4
Determination of AML and ATV in Caduet® tablets by the proposed HPSAM and the reported method [30] and application of standard addition technique.

Product	Drug	HPSAM	Reported method [a]	Standard addition			
				Taken (µg/mL)	Added (µg/mL)	Found (µg/mL)	Recovery% [b]
Caduet® 5/10	AML	99.89±1.114	100.43±0.541	6	4	4.07	101.75
					6	6.10	101.67
					8	7.98	99.75
						Mean	101.06
						SD	1.132
						RSD%	1.120
	ATV	99.50±0.571	99.88±0.347	12	8	8.00	100.00
					12	11.90	99.17
					16	16.28	101.75
						Mean	100.31
						SD	1.317
						RSD%	1.313
Caduet® 10/10	AML	100.78±0.745	101.30±0.761	12	8	8.06	100.75
					12	11.99	99.92
					16	16.19	101.19
						Mean	100.62
						SD	0.645
						RSD%	0.641
	ATV	100.51±0.808	100.79±0.693	12	8	8.03	100.38
					12	12.16	101.33
					16	15.79	98.69
						Mean	100.13
						SD	1.337
						RSD%	1.335

[a] *first derivative spectrophotometry at 340.0 and 295.0 nm for AML and ATV, respectively.*
[b] *average of three determinations*

Results obtained by the proposed method for the determination of the drugs in Caduet® tablets were statistically compared to those obtained by the reported method [30], no significant differences between the results were obtained as presented in Table 5. Validation was done according to ICH recommendations [31] as shown in Table 6.

Table 5

Statistical comparison for the results obtained by the proposed methods depending on isoabsorptive point, HPSAM, bivariate method and the reported method [30] for the analysis of AML and ATV in Caduet® tablets.

Value	HPSAM		Reported Method [30] [a]	
	AML	AML	AML	ATV
Mean	100.34	100.01	100.87	100.33
SD	0.979	0.835	0.746	0.700
RSD%	0.976	0.835	0.739	0.697
N	6	6	6	6
Variance	0.958	0.698	0.556	0.490
Student's t test [b]	1.060 (2.228)	0.731 (2.228)	-----	-----
F value [b]	1.724 (5.05)	1.425 (5.05)	-----	-----

[a] first derivative spectrophotometry at 340.0 and 295.0 nm for AML and ATV respectively.
[b] The values in the parenthesis are the corresponding theoretical values of t and F at P= 0.05.

Table 6

Assay validation sheet of the proposed methods depending on isoabsorptive point, HPSAM and bivariate method for simultaneous determination of AML and ATV.

Parameter	HPSAM	
	AML	ATV
Accuracy (mean ± RSD)	100.22±1.759	100.13±1.416
Precision		
– *Repeatability* [a]	0.985	0.897
– *Intermediate precision* [b]	0.999	1.260
Robustness [c]	1.144	1.030
Linearity		
– *Slope*	0.0460 *(241.0 nm)* 0.0219 *(252.4 nm)*	0.0250 *(278.0 nm)* 0.0064 *(305.6 nm)*
– *Intercept*	0.7033 *(241.0 nm)* 0.5075 *(252.4 nm)*	0.2199 *(278.0 nm)* 0.0680 *(305.6 nm)*
– *Correlation coefficient*	0.9998 *(241.0 nm)* 0.9995 *(252.4 nm)*	0.9999 (278.0 nm) 0.9994 *(305.6 nm)*
Range	4.0-40.0 µg/mL	8.0-32.0 µg/mL

[a] The intraday (n = 3), average of three concentrations (12,16,20 µg/mL) for AML and ATV repeated three times within the day.
[b] The interday (n = 3), average of three concentrations (12,16,20 µg/mL) for AML and ATV repeated three times in three days.
[c] Robustness (n = 3), average of three concentrations (12,16,20 µg/mL) for AML and ATV analyzed using 75 and 70% methanol

CONCLUSION

From the previous discussion, it could be concluded that the proposed method is selective and, unlike the other spectrophotometric methods applied on this mixture, can cancel the matrix effect during the analysis of AML and ATV in their available dosage form. The method is suitable and valid for application in laboratories lacking liquid chromatographic instruments.

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