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RESEARCH ARTICLE

NATIVE AND SYNCHRONOUS SPECTROFLUORIMETRIC METHODS FOR SIMULTANEOUS DETERMINATION OF AMLODIPINE BESYLATE/VALSARTAN COMBINATION IN TABLETS

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ABSTRACT

Blokatens tablets are novel prescription drug that combines amlodipine besylate (AM) with valsartan (VS), were simultaneously determined by two different spectrofluorimetric methods. The first method depend on measurement of native fluorescence intensity of both drugs at ƛ emission 460nm and 385 nm using ƛ excitation 390nm and 227nm for AM and VS respectively in water. The second method utilizes synchronous fluorimetric quantitative screening of the emission spectra of AM and VS at 375 and 285 nm, respectively using Δλ of 80 nm. The different experimental parameters affecting the synchronous fluorescence intensity of two drugs were carefully studied and optimized. The method was validated according to ICH guidelines. Linearity, accuracy and precision were found to be satisfactory in both methods over the concentration ranges of 0.4-14 and 1.0-22µg/mL for AM and VS, respectively. In the first method, limit of detection and limit of quantification were estimated and found to be 0.165 and 0.5µg/mL for AM as well as 0.495 and 1.5 µg/mL for VS, respectively. Also, limit of detection and limit of quantification were calculated in the synchronous method and found to be 0.148 and 0.45 µg/mL for AM as well as 0.396 and 1.2 µg/mL for VS, respectively.Excellent linearity was observed,careful validation proved advantages of the new methods: high sensitivity, accuracy, selectivity and suitability for quality control laboratories. The results were compared statistically with reference methods and no significant difference was found. The methods were successfully applied for the determination of the two drugs in their co-formulated tablets without pre-separation.

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INTRODUCTION

Amlodipine besylate (AM) Figure 1 is a long-acting calcium channel blocker of dihydropyridine class that is used as an antihypertensive drug and in the treatment of angina pectoris. It inhibits the trans membrane influx of calcium ions into vascular smooth muscle and cardiac muscle thereby reducing blood pressure and increasing blood flow to the heart muscle (Sweet man, 2011). It is chemically described as 3-ethyl-5-methyl (±) - 2- [(2-aminoethoxy) methyl] -4- (o-chlorophenyl) -1, 4- dihydro -6- methyl-3, 5- pyridinedicarboxylate, monobenzenesulfonate. Valsartan (VS) Figure 1 chemically known as L-Valine, N-(1-oxopentyl)-N-[[2`-(1H-trtrazol-5-yl) [1, 1`-bi-phenyl]-4- yl] methyl] is an angiotensin II receptor antagonists used in the treatment of hypertension (Sweet man, 2011). Both drugs are official in the United States Pharmacopeia (USP) (United States, 2014). The USP recommended HPLC methods for determination of AM and VS in drug substance and drug products (United States, 2014). Few analytical methods include high performance liquid chromatography's (HPLC), HPTLC, and capillary electrophoresis weredeveloped for simultaneous quantification of AM and VS in fixed- dose combinations (Ebbed *et al.,* 2014; Tackily, 2013; Sharma *et al.,* 2014; Khalil *et al.,* 2011; Dhaneshwar*et al.,* 2009; Ingot *et al.,* 2003). Only one spectrofluorimetric method has been reported yet for quantification of AM and VS in combined tablet form (Shaolin and Bellas, 2010). To the best of our knowledge, there are no reported direct or synchronous spectrofluorimetric methods for determination AM either alone or in combination with VS without need for derivatization methodologies. Accordingly, the main objective of this investigation was to develop and validate two new methods:native fluorescence and synchronous fluorimetric methodsfor thesimultaneous determination of AM and VS in drug substance and in drug product, offering better sensitivity than the previously reported methods. Literature survey revealed liquid chromatography (LC) (Oz emir and Kayos, 2014; Elshanawane *et al.,* 2014; Aziza *et al.,* 2013; Gizawy *et al.,* 2013), capillary electrophoresis (Salem *et al.,* 2014; Huang *et al.,* 2009; Jankovics *et al.,* 2008), potentiometric (El-Kopsas *et al.,* 2014; Li *et al.,* 2004), andspectrophotometric (Rahman and Nasrul, 2003; Prabhakar and Giridhar, 2002; Dhaka *et al.,* 2002; Rahman and Azmi, 2000; Jain and Agawam, 2000; Sridhar *et al.,* 1997) methods have been reported for determination of

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AM alone or in combination with other antihypertensive agent. In addition, LC (Ebbed *et al.,* 2014; Elshanawane *et al.,* 2014; Li*et al.,* 2013; Amin*et al.,* 2013; Krishnaiah *et al.,* 2010), LC with tandem mass spectrometry (Blix *et al.,* 2015; Gonzalez *et al.,* 2010; Liu *et al.,* 2008), capillary electrophoresis (Hilleary and van den Borsches, 2003; Hilleary and van den Borsches, 200), TLC (Tsvetkova and Obreshkova, 2012; Dhaneshwar *et al.,* 2009), spectrofluorimetric (Cagily *et al.,* 2001; Cagily *et al.,* 2001) and ultraviolet-spectrophotometric (Tatar and Sagle, 2002; Erik, 2002; Astana *et al.,* 2001) methods were reported for estimation of VS alone or in combination with other agents. The emission spectra of AM and VS were overlapped,it was difficult to analyze and determine their contents by conventional fluorimetric method. This observation led us to utilize synchronous fluorescence spectroscopy (SFS) to solve such problem by measuring Synchronous Fluorescence Intensity (SFI) at 375 and 285 nm for AM and VS respectively. Thus the aim of the present study was to develop validated, sensitive, simple, rapid, inexpensive and precise spectrofluorimetric procedures for simultaneous analysis of AM, VS in drug substances and drug products. Synchronous fluorescence spectroscopy (SFS) has several advantages over conventional fluorescence spectroscopy, including simple spectra, high selectivity and low interference (Chen *et al.,* 1990). Because of its sharp, narrow spectrum, SFS serves as a very simple, effective method for achieving data for quantitative determination in a single run (Petra and Mishra, 2002).

Experimental

Instrumental

1-Fluorescence spectra and measurements were recorded using an Agilent Cary Eclipse Fluorescence Spectrophotometer equipped with a 150 Watt Xenon arclamp, gritting excitation and emission monochromatorsfor all measurements and an Agilent Cary Eclipse recorder. Slitwidths for both monochromators were set at 10 nm. A 1 cm quartz cell was used. The SF spectra were estimated at 375nm and 285 nm for AM and VS respectively.

2-Digital pH meter PW 9409 PyeUnicum was used for checking the pH of the buffer solutions used.

Material and Reagents

All the chemicals uses in the present studies were of analytical reagent grade and the solvents were of HPLC grade.

- Amlodipine besylate was kindly supplied by PFIZER Co. Egypt.
- Valsartan was kindly supplied by NOVARTIS Co. Egypt.
- Blokatens 10/160 mg tablets labeled to contain 10 mg AM and 160mg SV per each tablet (B.N. 13140) were purchased from commercial sources in local market.

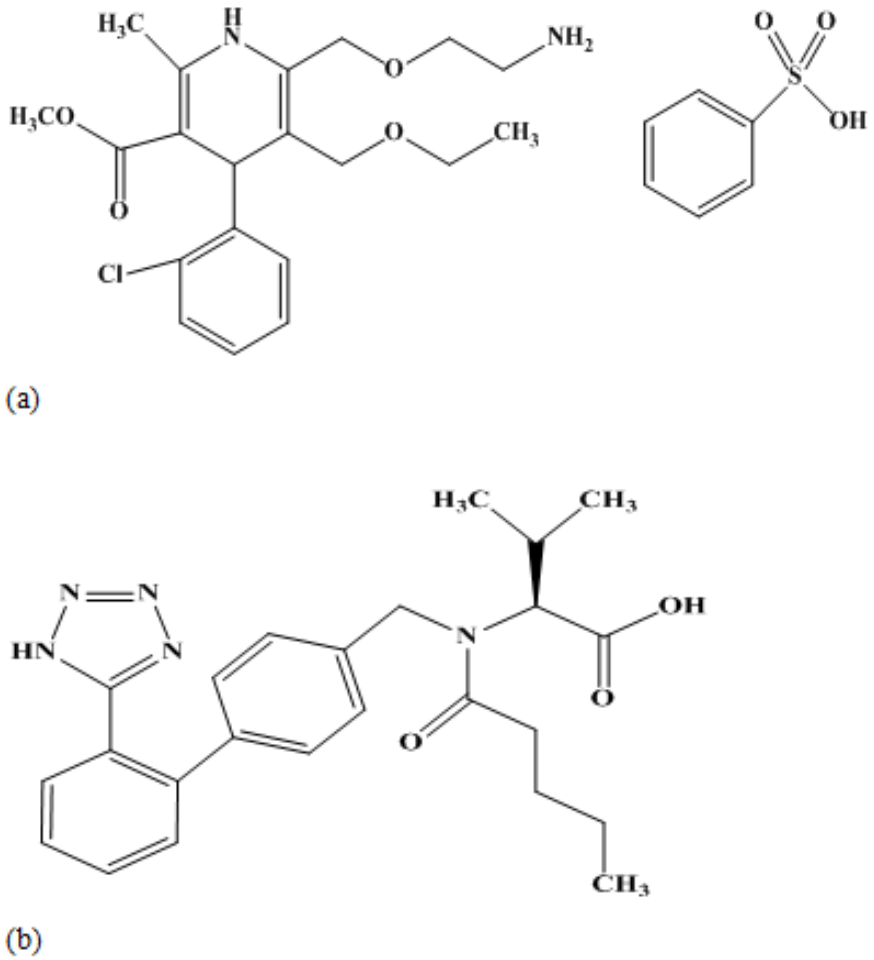

Fig.1. Structural formulae of studied drugs where (a) Amlodipinebesylate (AM), (b) Valsartan (VS)

- McIlavine buffer solution pH3.2 and pH8 were prepared by appropriate volume of 0.2M di-sodium hydrogen orthophosphate anhydrous AR (Brixworth- Northants, U.K, B. No: 700765), and 0.1M citric acid (El-Nasser Pharmaceutical Chemicals Co. Abu Zaabal, Egypt,B. No: 111C00).

StandardStock Solutions Preparation

Ten mg of AM and VS were accurately weighted and transferred separately into 100-ml volumetric flask. Then, they were dissolved and made up to volume with distilled water to give concentration 100µg/ml for each. Further dilutions were made separately using the same solvent to prepare 40µg/ml of AM and VS. The standard solutions were stable for 5 days when kept in the refrigerator.

Recommended Procedures

Construction of the calibration graphs

Aliquots of AM and VS standard solutions covering the working concentration range cited in Table 3 were transferred into a series 10-ml volumetric flasks. Then the solutions were diluted to volume with distilled water and mixed well. Relative fluorescence intensity (RFI) was measured in two methods. In the first method, it was measured directly against blank at 390 and 460nm upon excitation at 227 and 385nm for AM and VS respectively. In the second method, synchronous fluorescence spectra of the solutions were recorded by scanning both monochromators at a constant wavelength difference $\Delta\lambda = 80$ nm and scan rate of 600nm/min using 10nm excitation and emission windows.The intensities of the SFS were estimated at 375nm and 285 nm for AM and VS respectively. A blank experiment was performed simultaneously. The relative fluorescence intensity of the synchronous spectra was plotted v*s* the final concentration of the drugs (µg/ml) to get the calibration curves and regression equations.

Procedure for the synthetic mixture

Aliquots of AM and VS standard stock solutions were accurately transferred into a series of 10-ml volumetric flasks to give final concentration of 0.4, 0.8, 1.2and 6.4, 12.8, 19.2µg/ml for AM and VS. The recommended procedure under Calibration Curve was then performed. The recovery percentage was calculated using the corresponding regression equations.

Procedure for commercial tablets

The films of ten coated (individually weighed) tablets were gently removed with water. The tablets were then dried, weighed, powdered and mixed well. A weighed quantity of the powder equivalent to 1.0 mg of AM and 16 mg of VS (in their ratio 1:16) was transferred into a small conical flask and extracted with 50 ml of water by ultra-sonication for 30 min. the extract was filtered through a What- mann No. 42 filter paper previously moisten with water. The collected filtrate was transferred quantitatively into 100-mL calibrated flask. Aliquots covering the working concentration range were transferred into 10 ml volumetric flasks. The recommended procedure under "Calibration curves" was performed. The content of the Tablets were determined either from a previously plotted calibration curve or using the corresponding regression equation.

RESULTS AND DISCUSSION

Native Fluorescence Spectra of AM and VS

The goal of the present investigation was to develop two validated, sensitive, simple and rapid spectrofluorimetric method for simultaneous determination quantification of AM and VS in drug products. AM and VS are soluble in water, the solutions exhibited an intense native fluorescence in different media, such as water, 0.1M HCl, 0.1M NaOH, and methanol.

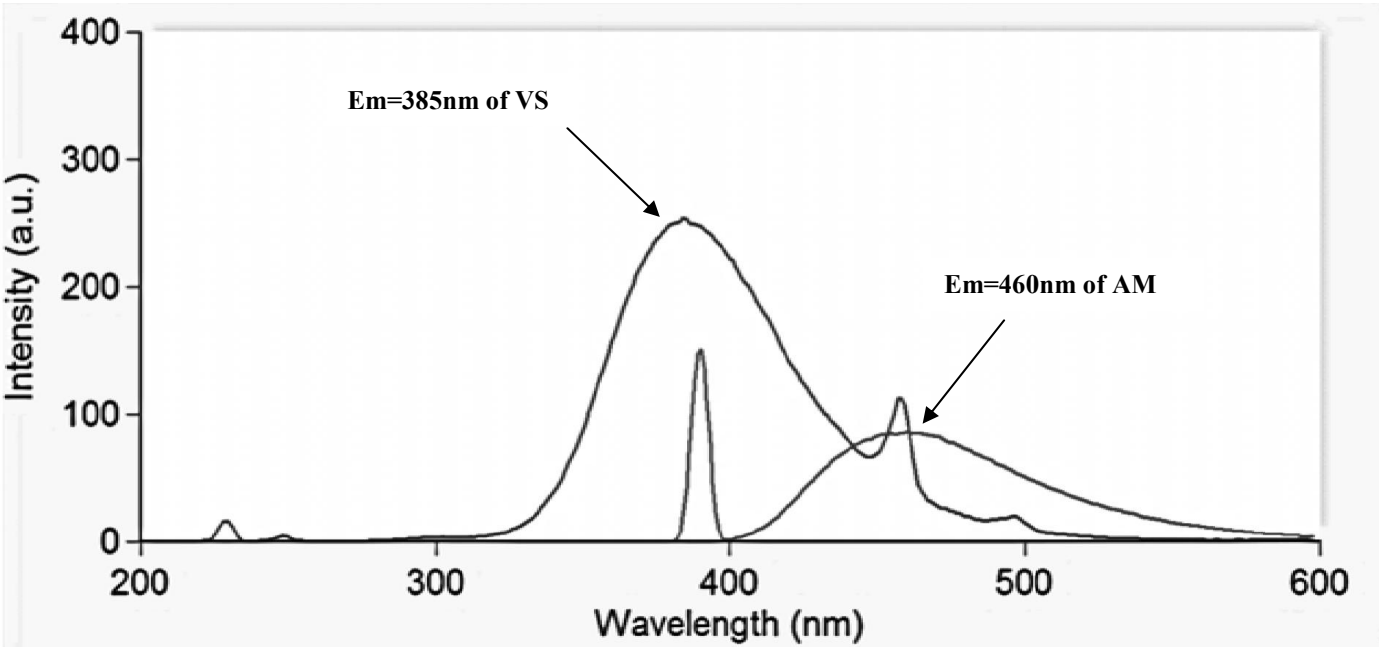

Fig. 2. Fluorescence spectra of: emission spectrum of AM at 460nm (14µg/ml), emission spectrum of VS at 385nm (14µg/ml) in distilled water

The highest fluorescence intensity was obtained for both drugs at λ emission 460nm and 385 nm using λ excitation 390nm and 227nm for AM and VS respectively in water Fig. 2.

Synchronous Fluorescence Spectra of AM and VS

Amlodipine besylate was found to exhibit excitation wave length at 390 nm and emission spectrum at 460 nm. Valsartan was found to exhibit maximum fluorescence intensity at 385nm after excitation at 227nm. The emission spectra of both AM and VS greatly overlapped Fig. 2. This fact hindered the use of direct measurement for the simultaneous determination of AM and VS .this problem is more aggravated if it is desired to determine these compounds in their co-formulated preparations.It was necessary to record first the normal synchronous spectra for both AM and VS. There is no overlap between them after subtracting the value of the blank.Fig.3shows the SFS of different concentrations of AM at 375 nm in presence of constant concentration of VS (20 µg/ml),whereas, Fig. 4 illustrates the SFS of different concentrations of VS at 285 nm in presence of constant concentration of AM (14 µg/ml).

Optimization of Experimental Conditions

Different experimental parameters affecting the performance of the proposed methods were carefully studiedand optimized. Such factors were changed individually while other was kept constant. These factors included Δλ, pH, and type of the diluting solvent.

Selection of optimum Δλ

The optimum Δλ value is an essential factor for performing the synchronous fluorescence scanning method with regards to its resolution, sensitivity and features. It can directly influence spectra shape, band width and signal value. For this reason a wide range of Δλ (50, 60, 70, 80, 90, 100, 110 nm) was examined Fig. 5. It was found that Δλ of 80 nm is suitable simultaneous determination of AM and VS in their medicinally recommended ratio of (1:16). The maximum synchronous fluorescence peaks of AM and VS were at 357nm and 285nm, respectively; no significant interference would occur in their medicinally recommended ratio.

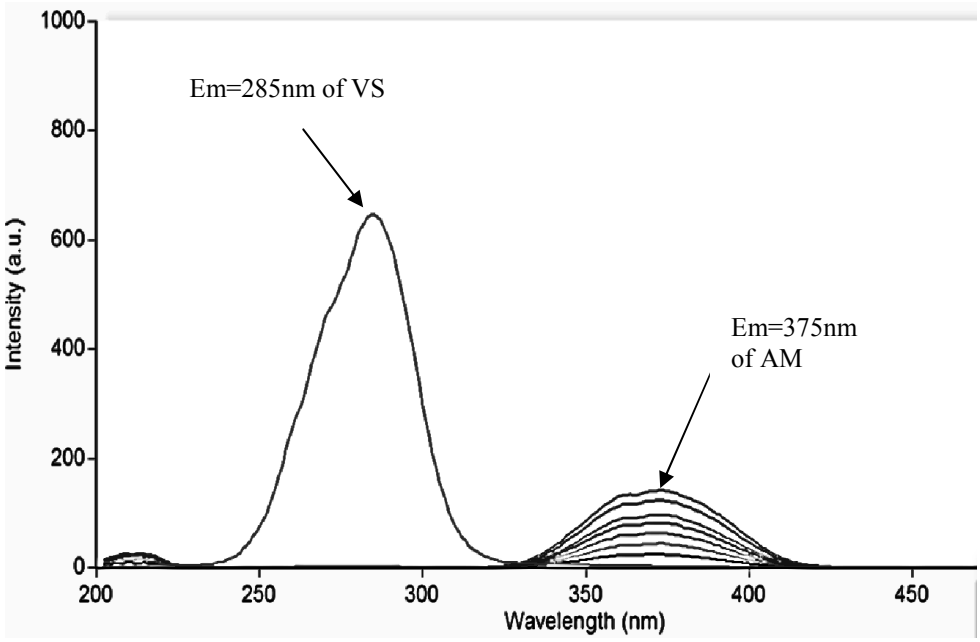

Fig. 3. Synchronous fluorescence spectra of VS (20µg/ml), AM (2, 4, 6, 8, 10, 12, 14µg/ml)

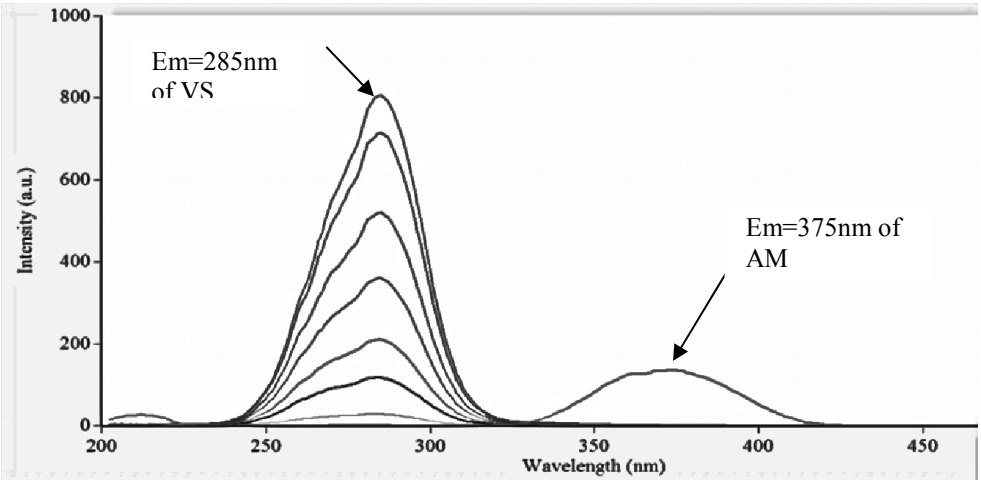

Fig.4. Synchronous fluorescence spectra of AM (14µg/ml), VS (1, 4, 6, 10, 14, 20, 22µg/ml)

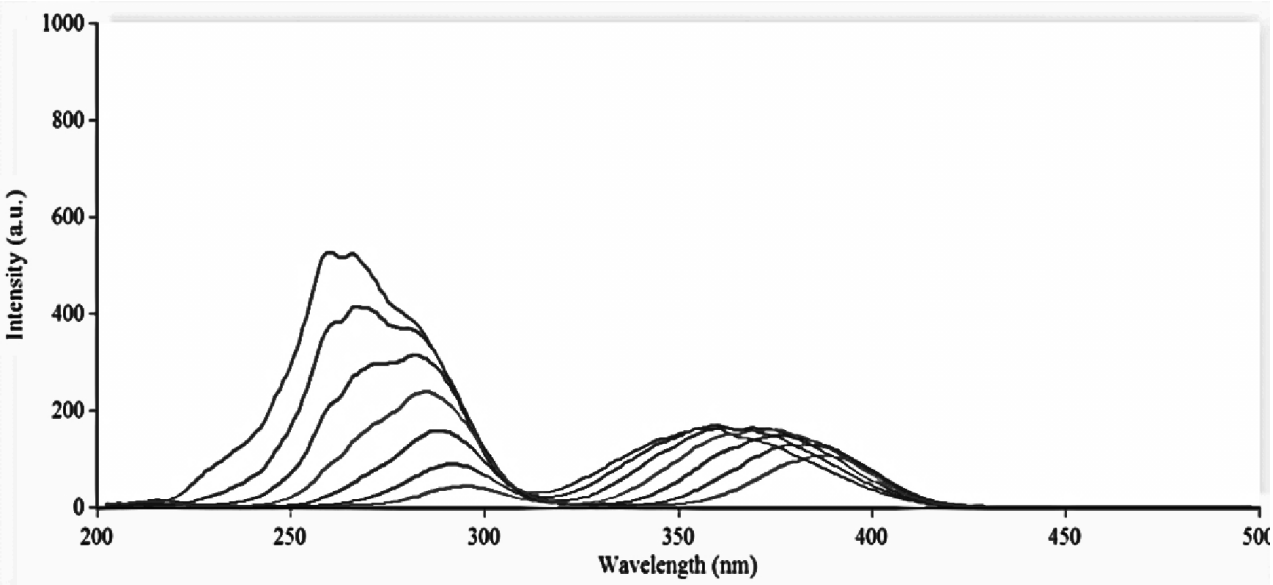

Fig. 5. Synchronous fluorescence spectra of AM and VS at different Δλ (50, 60, 70, 80, 90, 100, and 110 nm)

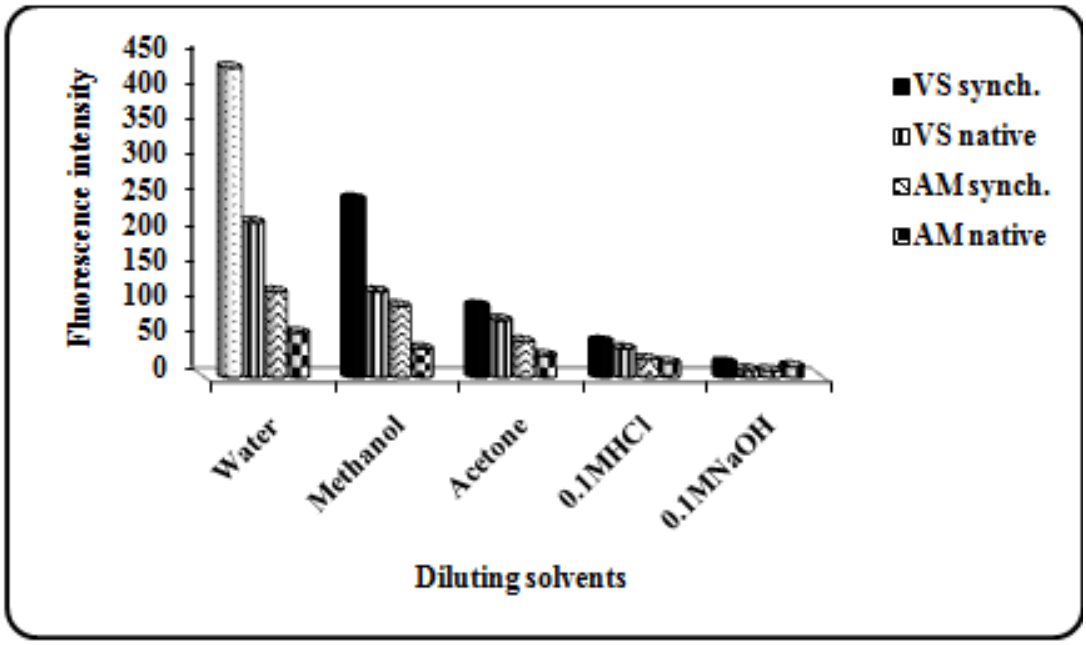

Fig. 6. Effect of diluting solvent on native and synchronous fluorescence intensity for AM and VS

Selection of optimum pH

The influence of pH on the fluorescence intensity of the two drugs was studied using McIlavine buffer covering the whole pH range (pH 3.2 – 8). It was found that, using any of these buffers does not affected the synchronous fluorescence or even decrease it. Therefore, for simplicity of the methods no buffer was used throughout the study.

Effect of diluting solvent

For the native conventional and synchronous spectrofluorimetric procedure, the influence of different diluting solvents on the SFI of AM and VS was investigated using water, methanol, acetone, 0.1M HCl, and 0.1 M NaOH.

Water was chosen as the diluting solvent as it showed a good SFI compared to other solvents, water is friendlier to environment and with no blank readings Fig.6.

Validation of the Methods

Thevalidity of the methods were tested regarding; linearity& range, accuracy, precision, repeatability, and specificity according to ICH Q2B recommendations (Guidance for Industry, 1996).

Linearity and Range

The regression plots showed a linear dependence of RSFI values on drug concentration over the range cited in Table 1. The validity of the methods was proved by statistical evaluation of the regression lines, using the standard deviation of the residuals ($S_{y/x}$), the standard deviation of the intercept (S_a) and the standard deviation of the slope (S_b).

The results are abridged in Table 1. The small values of the figures point out to the low scattering of the points around the calibration curves and high precision.

Limit of quantification (LOQ) and limit of detection (LOD)

LOQs and LODs of each drug were calculated. LOQ and LOD were calculated according to ICH Q2B recommendations (Guidance for Industry, 1996), results are given in Table 1.

$LOQ = 10\ \sigma/S$ $LOD = 3.3\ \sigma/S$

Where: S is the slope, and σ is the standard deviation of the intercept of regression line of the calibration curve.

To prove the accuracy and utility of the proposed method, the result of the assay of AM and VS with the proposed methods were compared with those of the reference methods (United States Pharmacopeia, 5115). Statistical analysis of the results using the Student's t-test and the variance ratio F-test revealed no significant difference between performances of the developed methods and the reference methods regarding the accuracy Table 2.The proposed method was applied to the simultaneous determination of AM and VS in synthetic mixturescontaining different concentrations of both drugs in ratio of 10:160. The relative synchronous fluorescence intensities were measured for both drugs.

Table 1. Performance data and results of the proposed spectrofluorimetric methods for the simultaneous determination of AM and VS

Item	Conventional fluorescence		Synchronous fluorescence	
	AM	VS	AM	VS
Linearity(µg/ml)	0.4 – 14	1 – 22	0.4 – 14	1 – 22
LOD(µg/ml)	0.165	0.495	0.148	0.396
LOQ(µg/ml)	0.500	1.50	0.450	1.20
Regression equation(y)[a]				
Slop(b)	4.961	18.628	9.751	37.671
Standard deviation of slop(S_b)	0.051	0.236	0.145	0.517
Intercept(a)	2.138	- 6.244	3.699	-16.122
Standard deviation of intercept(S_a)	5.235	3.088	1.145	6.759
Regression coefficient(r^2)	0.9999	0.9998	0.9997	0.9997
Standard deviation of residuals($S_{y/x}$)	0.591	8.559	4.792	18.368
% Error	0.118	0.896	1.956	7.500
Results(Recovery percentage ± SD)				
Drug in bulk	99.59±0.236	100.1±0.610	99.51±0.720	99.66±0.521
AM and VS synthetic mixture	99.87±0.324	99.64±0.354	99.98±0.351	99.87±0.351
Blokatens tablets 10/160mg tablets	99.81±0.354	99.54±0.521	99.92±0.810	100.2±0.541

AM amlodipine besilate, VS valsartan
(y)[a]= a + bc where c is the concentration in µg/ml and y is the fluorescence intensity.

Table 2. Statistical comparison analysis results of drug substances using the proposed methods and those of the reference methods

Statistical term	AM			VS		
	Conventional method	Synchronous method	Official HPLC[2]	Conventional method	Synchronous method	Official HPLC[2]
Mean	100.16	99.95	99.41	99.95	99.97	99.47
± SD	0.471	0.188	0.319	0.591	0.470	0.324
± SE	0.192	0.077	0.130	0.240	0.192	0.132
%RSD	0.470	0.188	0.321	0.591	0.470	0.325
n	6	6	6	6	6	6
Variance	0.222	0.035	0.102	0.349	0.221	0.105
t-test	1.321(2.228[a])	1.011(2.228[a])		0.981(2.228[a])	0.894(2.228[a])	
F-test	2.176(5.1[a])	2.914(5.1[a])		3.324(5.1[a])	2.105(5.1[a])	

[a] figures in parentheses are theoretical t and F values at (p = 0.05) and n the number of experimental

Table 3.Application of the proposed method for determination of the studied drugs in their mixtures

sample	Concentration taken (µg/ml)		Concentration found (µg/ml)		Accuracy %	
	AM	VS	AM	VS	AM	VS
AM and VS	0.4	6.4	0.399	6.398	99.98	99.98
mixture	0.8	12.8	0.798	12.57	99.79	99.97
	1.2	19.2	1.197	18.99	99.95	99.96
Mean					99.91	99.97
±SD					±0.102	±0.01
%RSD					0.103	0.02
% Error					0.106	0.05

Each result is the average of three separate determinations.

Accuracy

Accuracy of the results was investigated by calculating recovery percentage of six different concentrations of the two drugs analyzed by the proposed spectrofluorimetric methods.

The RSFI of AM at 375 nm where VS shows nil contribution, similarly, the RSFI for VS was measured at 285 nm where AM shows nil contribution. The concentrations of both drugs in the synthetic mixture were calculated according to the linear regression equation of the calibration graphs.

The results indicate high accuracy of the proposed method as shown in Table 3.

Precision

Repeatability (intraday, n = 3) and intermediate precision (interday, n= 3) were checked using three different concentration at low, medium and high level of the standard curve. Percentageof relative standard deviation was calculated to check the precision of the methods Table 4.

Table 4. Precision data of the spectrofluorimetric methods for determination of AM and VS

Intra-days assay		Inter-days assay	
Conventional method	Synchronous method	Conventional method	Synchronous method
Found± %RSD[a]	Found± %RSD[a]	Found± %RSD[a]	Found± %RSD[a]
Conc. of AM (µg/ml)			
0.5 0.49±0.125	0.485±0.350	0.500±0.624	0.51±0.357
6 5.88±0.235	5.990±0.154	5.887±0.156	6.11±0.611
12 11.80±0.510	11.92±0.341	11.951±0.112	11.99±0.145
Conc. of VS (µg/ml)			
2 1.99±0.610	2.01±0.111	1.97±0.321	1.98±0.181
10 9.87±0.347	9.99±0.210	10.30±0.510	9.98±0.521
20 19.89±0.621	19.97±0.351	19.91±0.136	19.95±0.135

AM, amlodipine besilate; VS, valsartan
[a]All the results are the average of three determination

Specificity

The specificity of the methods was investigated by observing any interference encountered from the excipients of the tablets. It was shown that these compounds do not interfere with the proposed methods. The proposed method was found to be specific for the two studied drugs in their combined tablets without interference from common tablet excipients such as Titanium oxide, anhydrous lactose, colloidal silicon dioxide, and macro gel. These matrix compounds did not interfere with the proposed method.

Stability

Results from the stability studies of the standard stock solutions indicated that they were stable for two weeks at 2-8 ^{0}C with satisfactory recovery percentage of more than 98%. These solutions can therefore be used during this interval of time without the results begin affected.

Conclusion

A new simple, rapid, and sensitive spectrofluorimetric method were developed and validated for the simultaneous determination of AM and VS in drug substances and drug products. The proposed methods have many advantages regarding analysis time, high sensitive, and cost compared with those of the previously reported methods. Lower values of LOQ and LOD could be allowing determination of these drugs separately in biological fluids using the synchronous spectrofluorimetric method. On the other hand, the direct native method showed good tolerance and could be applied to determination of AM and VS in their co-formulated tablets with other drug. Finally, the proposed methods can be used for the quality control of the cited medication in ordinary laboratories.Two new simple, rapid, sensitive and environmentally friendly methods spectrofluorimetric methods were developed and validated for the simultaneous determination of AM and VS in drug substances and drug products.

The proposed methods have many advantages regarding analysis time, high sensitive, and cost compared with those of the previously reported methods. The synchronousspectrofluorimetric method can be applied to the analysis of both drugs in their co-formulated dosage forms without pre-separation. It was possible to measure concentrations as low as (0.5 and 2µg mL^{-1}) for both drugs with good accuracy. Both the methods were validated as per ICHcould be applied to the analysis of both drugs in their co-formulated dosage forms.

Both the methods were validated as per ICH Lower values of LOQ and LOD could be allowing determination of these drugs separately in biological fluids using the synchronous spectrofluorimetric method. On the other hand, the direct native method showed good tolerance and could be applied to determination of AM and VS in their co-formulated tablets with other drug. Finally, the proposed methods can be used for the quality control of the cited medication in ordinary laboratories. Moreover, the proposed methods are reproducible and time saving and could be applied for routine analysis of both drugs in quality control laboratories. These methods are environmentally friendly methods because used water as solvent which is low cost and safe.

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