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Research Article

Simultaneous Determination of Amlodipine Besylate, Valsartan and Its Related Substances in Their Film-Coated Tablets Dosage Form by RP-HPLC Method

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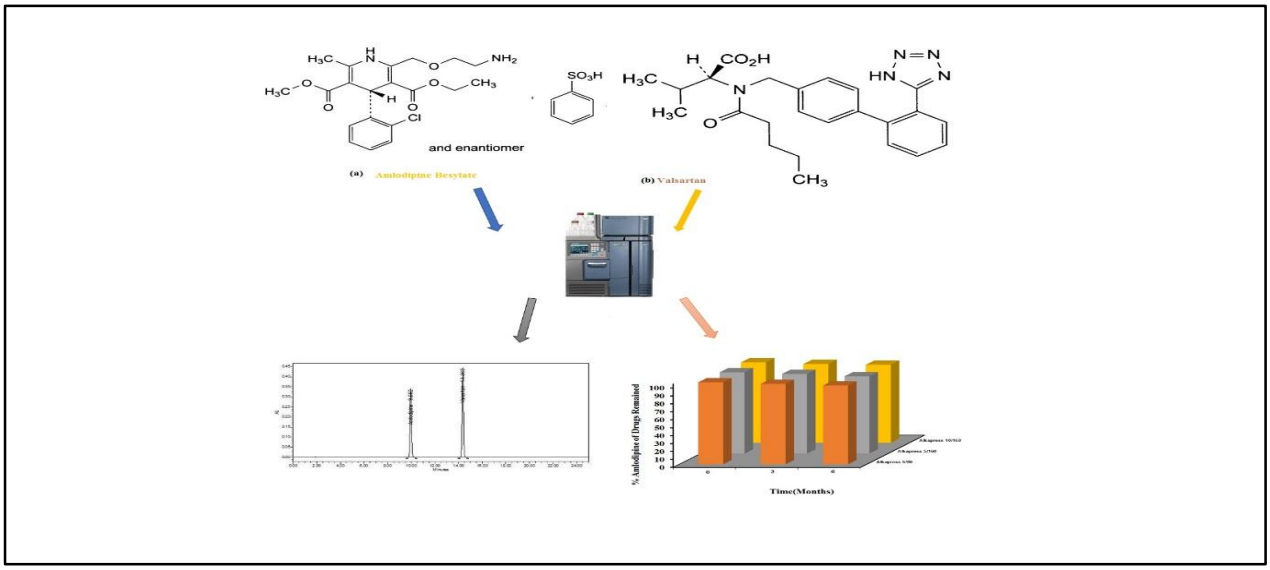
ABSTRACT

The current study aimed to develop and new, simple, accurate, economical and stability-indicating RP-HPLC for simultaneous estimation of Amlodipine Besylate, Valsartan and its related substances in their film-coated tablets dosage form. Chromatographic system was performed on the YMC ODS-A C18 (150 mm × 4.6 mm, 5µm particle size) using a binary gradient elution consist of two solvent systems, solution (A) 0.02 monobasic sodium phosphate Adjust with phosphoric acid to a pH of 2.5 and solution (B) consisting of Solution A: Acetonitrile (45:55). At a flow rate of 1.0 mL/min, injection volume 10 µL, UV detection at 235 nm, column oven temperature 30 °C and autosampler temperature 10 °C. This method was validated according to ICH requirements for new methods, which include accuracy, precision, selectivity, robustness, ruggedness, LOD, LOQ, linearity and range. Linear relationships were obtained in the ranges of 10-300 µg/mL and 5-200 µg/mL with correlation coefficients of 0.9997 and 0.9998 for Amlodipine Besylate and Valsartan respectively. The forced degradation studies as acidity, alkalinity, oxidation, heat, thermal, humidity and photodegradation were performed according to ICH guidelines.

GRAPHICAL ABSTRACT



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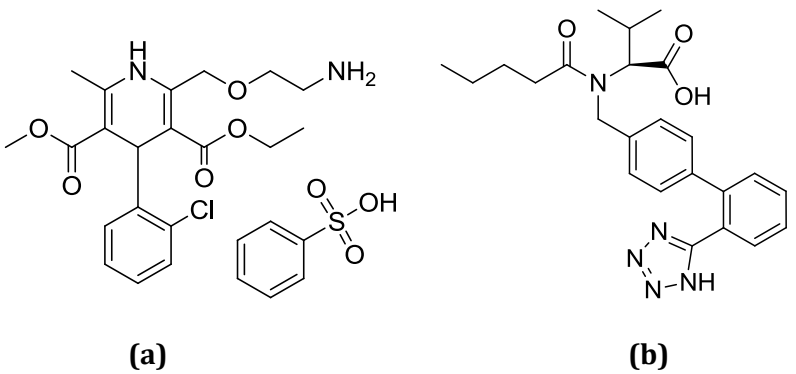
Introduction

Amlodipine (AML) is a dihydropyridine calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. Experimental data suggest that AML binds to both dihydropyridine and no dihydropyridine binding sites. The contractile processes of cardiac muscle and vascular smooth muscle are dependent upon the movement of extracellular calcium ions into these cells through specific ion channels. AML inhibits calcium ion influx across cell membranes selectively, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells AML is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure AML has been demonstrated to block constriction and restore blood flow in coronary arteries and arterioles in response to calcium, potassium epinephrine, serotonin, and thromboxane A2 analog in experimental animal models and in human coronary vessels in vitro. This (Prinzmetal's or variant) angina [1]. AML besylate is the besylate salt of AML, a long-acting calcium channel blocker. AML besylate (Figure 1a) is chemically described as 3-Ethyl-5-methyl (±)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridine dicarboxylate, mono benzene sulphonate. Its empirical formula is $C_{20}H_{25}ClN_2O_5 \bullet C_6H_6O_3S$, AML besylate is a white crystalline powder with a molecular weight of 567.1. It is slightly soluble in water and sparingly soluble in ethanol. [2]. Valsartan (VAL), (Figure 1b) is an Angiotensin II (AT1) receptor antagonist. It is chemically designated as (2*S*)-3-Methyl-2-[pentanoyl[2`-(1H-tetrazole-5-yl) biphenyl-4-yl] methyl] amino] butanoic acid. It has a molecular formula of $C_{24}H_{29}N_5O_3$ and a molecular weight of 435.5.

VAL is white or almost white, hygroscopic powder, practically insoluble in water, freely soluble in anhydrous ethanol, sparingly soluble in methylene chloride [2]. AML and VAL are official in BP and USP [2,3]. Literature review showed that few analytical methods have been described for the estimation of AML and VAL including spectrophotometric [4-7], high-performance liquid chromatography (HPLC) [8–14], spectrofluorimetric method [15–17], electrochemical and voltammetric methods [18-22] had been reported for the estimation of AML and VAL in pure or in dosage forms.



(a) **(b)**

Figure 1. Chemical structures of (a) Amlodipine Besylate and (b) valsartan

According to the best of my knowledge, there is no Stability indicating new-RP-HPLC method for simultaneous determination of Amlodipine Besylate, Valsartan and its related substances in their film-coated tablets dosage form. The current study was designed to develop and validate a simple, sensitive, and accurate stability indicating RP-HPLC method

for the analysis of amlodipine besylate and valsartan and its related substances in Alkapress Plus F.C.T. label claims (5/80 mg) and (5/160 mg) and (10/160 mg) (Amlodipine Besylate/Valsartan) tablet. Validation will be conducted in accordance with the International Conference of Harmonization guidelines (ICH Q2) [23].

Experimental

Materials and methods

Pure sample of AML and VAL were kindly supplied by HIKMA pharma, Egypt with claimed purity of 98.4% and 99.7% respectively. According to manufacturer certificates of analysis (Cadila Healthcare, Hetero Labs limited). Alkapress Plus F.C.T. label claims (5/80 mg) and (5/160 mg) and (10/160 mg) (Amlodipine Besylate/Valsartan) tablet. Acetonitrile, triethylamine, methanol HPLC-grade, water ultrapure and phosphoric acid (analytical grade) were procured from (scharlau, Spain).

Alliance® HPLC system is the flexible and reliable workhorse that meets your fundamental HPLC separation requirements supported with UV/Vis detectors and full integration with Empower® Software for instrument control and data processing. It is 21 CFIR compliance makes it audit friendly too. Stability chamber (VOSTCH VP 1300, Germany). PH meter Mettler Toledo seven compact.

Mobile phase preparation

Solution (A) 0.02 (2.4 gm) monobasic sodium phosphate in 1000 ml water adjust with phosphoric acid to a pH of 2.5. And solution (B) consisting of solution A: acetonitrile (45:55).

Diluent preparation

Solution A and solution B (50:50) (Table 1).

HPLC Chromatographic Conditions

Chromatographic separation was performed on column YMC-ODS-A (15 cm× 4.6 mm) 5 µm. Using a mobile phase mixture as gradient system at column oven temperature 30 °C, flow rate of 1.0 mL/min, Sample cooler temperature 10 °C and UV detection was performed at 235 nm, injection volume was 10 µL and run time was 24 min.

Preparation of Standard Solution

Weigh accurately about 35 mg of amlodipine besylate from amlodipine besylate working standard and 40 mg of Valsartan from Valsartan working standard.

Table 1. Preparation of HPLC standard solution

Time (min)	Solution A (%)	Solution B (%)
0	50	50
3	50	50
15	30	70
20	30	70
20.1	50	50
25	50	50

Transfer completely into a 250 mL volumetric flask, add methanol to 5% of the final volume to dissolve (about 12.5 mL), and add diluent to about 70% of the final volume, sonicate for about 10 minutes, complete to volume using diluent and mix well. Conc. 0.14 mg/mL and 0.16 mg/mL from amlodipine besylate and Valsartan. Filter using 0.45 µm PTFE, Nylon or PVDF syringe membrane filter.

Sample Solution Preparation

For (5/80) Concentration

Sample stock solution: Weigh 10 tablets of Alkapress plus (5/80) and transfer completely into a 500 mL volumetric flask. Initially add water to 10 % of the final volume (about 50 mL) and sonicate to disperse as needed. Add diluent to about 70% of the final volume and shake for up to 45 min to disperse. Following dispersion, Sonicate for 15 minutes. And shake for 30 min. Dilute with diluent to volume to obtain a solution containing known nominal concentrations of 0.1 mg/mL of amlodipine and 1.6 mg/mL of valsartan. Centrifuge the solution for about 10 min at 3000 rpm.

Sample solution A: nominally equivalent to 0.1 mg/mL of amlodipine in diluent from Sample stock solution. (Directly inject after centrifugation and filtration).

Sample solution B: nominally equivalent to 0.16 mg/mL of valsartan in diluent from Sample stock solution. (Accurately transfer 10 ml of sample stock solution after centrifugation to 100 ml volumetric flask and complete volume with diluents).

For (5/160) Concentration

Sample stock solution: Weigh 10 tablets of Alkapress Plus (5/160) and transfer completely into a 500 mL volumetric flask. Initially add water to 10% of the final Volume (about 50 mL) and sonicate to disperse as needed. Add diluent using about 70% of the final volume and shake for up to 45 min to disperse. Following dispersion, sonicate for 15 minutes. And shake for 30 min. Dilute with diluent to volume to obtain a solution containing known nominal concentrations of 0.1 mg/mL of amlodipine and 3.2 mg/mL of valsartan. Centrifuge the solution for about 10 min at 3000 rpm.

Sample solution A: Nominally equivalent to 0.1 mg/mL of amlodipine in diluent from Sample stock solution. (Directly inject after centrifugation and filtration).

Sample solution B: Nominally equivalent to 0.16 mg/mL of valsartan in diluent from Sample stock solution. (Accurately transfer 5 of Sample stock solution after centrifugation to 100 mL volumetric flask and complete volume with diluents).

For (10/160) Concentration

Sample stock solution: weigh 10 tablets of Alkapress plus (10/160) and transfer completely into a 500 mL volumetric flask. Initially add water to 10% of the final volume (about 50 mL) and sonicate to disperse as needed. Add diluent using about 70% of the final volume and shake for up to 45 min to disperse. Following dispersion, sonicate for 15 minutes. And shake for 30 min. Dilute with diluent to volume to obtain a solution containing known nominal concentrations of 0.2 mg/mL of amlodipine and 3.2 mg/mL of valsartan. Centrifuge the solution for about 10 min at 3000 rpm.

Sample solution A: Nominally equivalent to 0.1 mg/mL of amlodipine in diluent from Sample stock solution. (Accurately transfer 10 ml of sample stock solution after centrifugation to 20 mL volumetric flask and complete volume with diluents).

Sample solution B: Nominally equivalent to 0.16 mg/mL of valsartan in diluent from Sample stock solution. (Accurately transfer

5 mL of sample stock solution after centrifugation to 100 mL volumetric flask and complete volume with diluents). The chromatogram obtained was shown in (Figure 2).

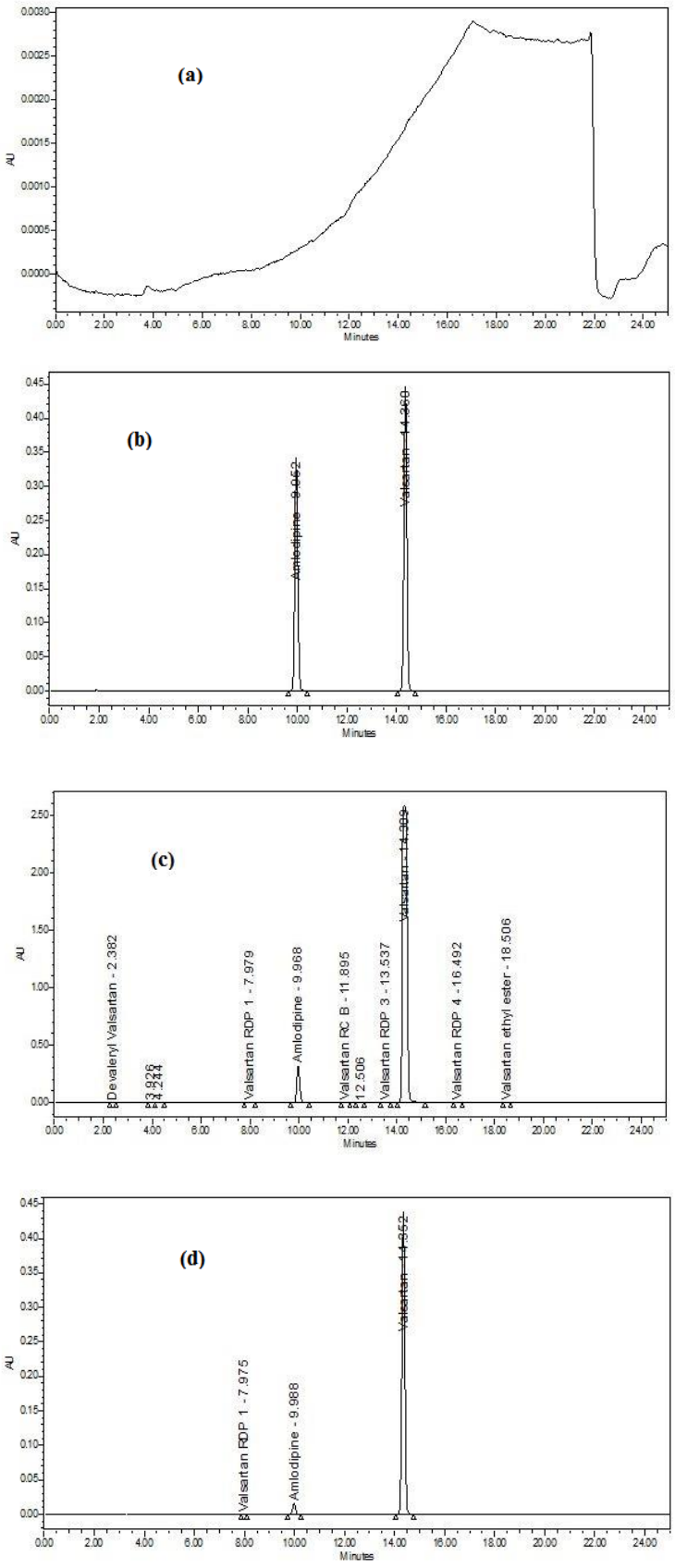


Figure 2. HPLC chromatogram of (a) blank, (b) standard solution of AML (140µg/ml) and VAL (160µg/ml), (c) Sample as Amlodipine and (d) Sample as Valsartan

Construction of calibration curves

Different concentration of AML and VAL equivalent to (10–300 µg/mL) and (5–200 µg/mL) respectively. were separately weighted from their respective stock standard into separate series of 100 mL volumetric flasks, and the volumes were made up to volume with diluent. Duplicate 20 µL injections were made for each concentration maintaining the flow rate at 1.0 mL/min and the effluent was UV-scanned at 235 nm.

Results and discussion

Methods development and optimization

Different developing systems of different compositions and ratios were tried including methanol: water (50:50, v/v), Acetonitrile: water (50:50, v/v). phosphate pH (4.5): ACN (30:70, v/v) gave bad separation, so the choice of binary gradient was useful to separate the impurity of drugs as the best system were solution (A) 0.02 (2.4 gm) monobasic sodium phosphate in 1000 ml water Adjust with phosphoric acid to a pH of 2.5. And solution (B) consisting of Solution A: Acetonitrile (45:55). Different flow rates were tried, scanned wavelengths (210, 235, 254, and 280 nm) were also tried. Preliminary studies involved trying C18 reversed-phase columns. The best developing system was YMC-ODS-A C18 (150 mm × 4.6 mm, 5 µm particle size) using a binary gradient elution consist of two solvent systems, solution (A) 0.02 (2.4 gm) monobasic sodium phosphate in 1000 ml water Adjust with phosphoric acid to a pH of 2.5. And solution (B) consisting of solution A: acetonitrile (45:55). at a flow rate of 1.0 mL/min, injection volume 10 µL, UV detection at 235 nm, column oven temperature 30 ºC and autosampler temperature 10 ºC.

Method validation

The method was validated, in accordance with ICH guidelines (ICH Q2R1), for system suitability, precision, accuracy, linearity, specificity, ruggedness, robustness, LOD, and LOQ [23].

Table 2. Regression and validation parameters of the proposed HPLC method for determination of AML and VAL

Parameter	AML	VAL
Linear range (µg/mL)	10-300	5-200
Slope	685.5703	455.9844
Intercept	147.4020	98.3024
Correlation coefficient	0.9997	0.9998
LOD [a] (µg/mL)	1.33	0.98
LOQ [a] (µg/mL)	4.1	2.94
Repeatability [b]	0.23	0.07

[a] limit of detection (3.3× σ /Slope) and limit of quantitation (10× σ /Slope).

[b] Repeatability for n≥5, RSD ≤2

Repeatability

System precision evaluates the analytical procedure repeatability of the same homogeneous sample when the procedure is applied repeatedly to multiple injections. So the Repeatability of the method was evaluated by calculating the RSD of the peak areas of six replicate injections for the standard concentration (100%) of drugs. Results were examined as % RSD values of concentration of drugs determined. Low values of % RSD (less than 2) indicate high precision of the method as shown in Table 2.

Limit of detection and quantitation

These approaches are based on the standard deviation of the response and the Slope. A specific calibration curve should be studied using samples, containing an analyte in the range of LOD and LOQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation. LOD=3.3×σ /slope and LOQ =10×σ /slope, where σ= the standard deviation of the response Table1.

Accuracy and recovery

Accuracy expresses the closeness of agreement between the value found and the value that is accepted as either a conventional true value or an accepted reference value. Accuracy of the proposed methods was calculated as the percentage recoveries of pure samples of the studied drugs. Accuracy is assessed using three different concentrations (50.0, 100 and 150 µg/mL) (*i.e.* three concentrations and three replicates). Concentrations were calculated from the corresponding regression equations. The mean % recoveries for AML and VAL were between 98.0% to 102% and were shown in Table 3.

Formulation assay

Method precision evaluates the variation experienced by a single analyst on a single instrument. Method precision does not distinguish between variation from the instrument or system alone and from the sample preparation process. Method precision is performed by analyzing multiple replicates of an assay composite sample using the analytical method. The assay is calculated and reported for each value. The results were displayed in Table 4.

Table 3. Data of Accuracy and Recovery for AML and VAL of the proposed HPLC method

Standard Solution(µg/mL)	AML			VAL		
	µg/mL (Injected)	µg/mL (found)	Recovery %	µg/mL (Injected)	µg/mL (found)	Recovery %
50	50	49.96	99.92 %	50	50.10	100.20 %
	50	49.99	99.98 %	50	49.99	99.98 %
	50	49.92	99.84 %	50	50.20	100.40 %
100	100	100.12	100.12 %	100	99.89	99.89 %
	100	100.17	100.17 %	100	99.97	99.97 %
	100	100.25	100.25 %	100	100.06	100.06 %
150	150	150.00	100.00 %	150	149.98	99.98 %
	150	150.30	100.20 %	150	150.11	100.07 %
	150	149.95	99.96 %	150	149.93	99.95 %
Accuracy (Mean±RSD)	100.05±0.14			100.06±0.15		

Table 4. Determination of AML and VAL in pharmaceutical formulation by the proposed HPLC method and application of standard addition technique

Pharmaceutical formulation	Added(µg/mL)		Recovery %		Found %	
	AML	VAL	AML	VAL	AML	VAL
Alkapress Plus F.C.T. label claims (5/80 mg) (AML / VAL)	5		100.20	99.96	102.3±0.8	101.9±0.9
	10		99.98	100		
	15		100.10	100.21		
Mean ± RSD			100.1±0.1	100.1±0.1		
Alkapress Plus F.C.T. label claims (5/160 mg), (AML / VAL)	5		98.88	99.16	103.1±0.2	100.7±0.1
	10		99.17	99.70		
	15		99.80	99.97		
Mean ± RSD			99.3±0.5	99.6±0.4		
Alkapress Plus F.C.T. label claims (10/160 mg) (AML / VAL)	5		100.88	100.23	100.1±0.2	100.8±0.2
	10		100.24	100.35		
	15		99.98	100.21		
Mean ± RSD			100.4±0.5	100.3±0.1		

Intermediate precision (ruggedness)

Intermediate precision refers to variations within a laboratory as with different days, with different instruments, by different analysts, and so forth. Intermediate precision was formally known as ruggedness. A second analyst repeats the method precision analysis on a different day using different conditions and different instruments. The recovery values are calculated and reported. A statistical comparison is made to the first analyst's results in Table 5.

Robustness

The robustness of the proposed methods was evaluated in the development phase where the effects of different factors on method were studied to obtain the optimum parameters for complete separation. Robustness of the method was studied by deliberately varying parameters like flow rate (±0.1 mL/min) and studying the effect of changing mobile phase pH by (± 0.2), acetonitrile composition (±5%) and column temperature changed (±5 °C). The low values of the %RSD, as given in Table 5, indicated the robustness of the proposed methods.

System suitability

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability was checked by calculating tailing factor (T),

column efficiency (N), resolution (Rs) factors. All calculated parameters were within the acceptable limits indicating good selectivity of the methods and ensuring system performance, Table 6.

Table 5. Ruggedness, robustness and stability of analytical solution of the proposed method

Parameter	HPLC		Limit %
	AML	VAL	
Day to Day	0.56	0.87	
Analyst to Analyst	0.76	0.64	
Column to Column	0.86	0.98	
Flow rate change (±0.1 mL/min)	0.57	1.10	
pH changes of mobile phase (±0.2)	1.13	1.21	RSD ≤ 2.0%
Wave length change (210±2.0nm)	0.76	0.86	
Column temperature change (30,25◦C)	0.78	0.93	
Fresh Sample	0.14	0.74	
Stored Sample in fridge	0.32	0.23	
Stored Sample in room temperature	0.34	0.14	

Table 6. System suitability testing parameters of the developed methods

Item	Obtained Value		Reference values
	AML	VAL	
Tailing factor	1.101	0.983	T ≤ 2
Capacity factor(k')	4.3	3.6	k' > 2
Injection precision	0.09	0.05	RSD ≤1%
Retention time ($R_{t)}$	0.12	0.23	RSD ≤1%
Number of theoretical plates(N)	4324	3255	N > 2000

Specificity

Placebo interference

Weigh the equivalent to 10 tablets of Alkapress plus placebo and transfer completely into a 500 mL volumetric flask. Initially add water to 10% of the final Volume (about 50 mL) and sonicate to disperse as needed. Add diluent using about 70% of the final volume and shake for up to 45 min to disperse. Following dispersion, Sonicate for 15 minutes. And shake for 30 min. Dilute with diluent to volume and mix well. Centrifuge the solution for about 10 min at 3000 rpm. Filter using 0.2 µm PTFE syringe membrane filter. The obtained results confirmed that the APIs do not interfere with the placebo and diluent.

Forced degradation

Forced degradation studies are undertaken to degrade the sample (drug product and placebo) deliberately. These studies are used to evaluate an analytical method ability to measure an active ingredient and its degradation products without interference. Samples or drug product and drug substance are exposed to heat, light, acid, base, and oxidizing agent to produce 10%–30% degradation of the active whenever possible. The degraded

samples are then analyzed using the method to determine if there are interferences with the related substances. The results were summarized in Table 7.

Acid degradation

Weigh accurately about 350 mg and 400 mg of AML and VAL. Transfer completely into a 250 mL volumetric flask, add methanol to 5% of the final volume to dissolve (about 12.5 mL), and add diluent to about 70% of the final volume, sonicate for about 10 minutes, complete up to volume using diluent and mix well.

Transfer 10 ml of Standard Stock Solution → HCl, 1.0 N (10 mL), 85 °C, 1h; 50 mL diluent → Complete to 100 mL volumetric flask with diluents

Base degradation

Weigh accurately about 350 mg and 400 mg of AML and VAL. Transfer completely into a 250 mL volumetric flask, add methanol to 5% of the final volume to dissolve (about 12.5 mL), and add diluent to about 70% of the final volume, sonicate for about 10 minutes, complete up to volume using diluent and mix well.

Transfer 10 ml of Standard Stock Solution → NaOH, 0.5 N (10 mL); 50 mL diluent → Complete to 100 mL volumetric flask with diluents

Peroxide degradation

Weigh accurately about 350 mg and 400 mg of AML and VAL. Transfer completely into a 250 mL volumetric flask, add methanol to 5% of the final volume to dissolve (about 12.5 mL), and add diluent to about 70% of the final volume, sonicate for about 10 minutes, complete up to volume using iluent and mix well.

Transfer 10 ml of Standard Stock Solution → H_2O_2, 3% (10 mL), 85 °C, 1h; 50 mL diluent → Complete to 100 mL volumetric flask with diluents

Thermal degradation

Weigh accurately about 350 mg and 400 mg of AML and VAL. Transfer completely into a 250 mL volumetric flask, add methanol to 5% of the final volume to dissolve (about 12.5 mL), and add diluent to about 70% of the final volume, sonicate for about 10 minutes, complete up to volume using diluent and mix well.

Transfer 10 mL of Standard Stock Solution, heat for 1 hrs at 85 °C, Complete to 100 mL volumetric flask with diluents. Add 50 mL diluent.

Accelerated stability study

Accelerated stability studies were performed at storage condition 40±2 OC & relative humidity 75±5% for 6 months. Accelerated stability studies intended to increase the rate of physical change or chemical degradation by using extravagant conditions of elevated temperature and humidity. The obtained results in Table 8 showed that no degradation was occurred during this intervals as shown in (Figure 3).

Table 7. Results of analysis of forced degradation study samples using proposed method, indicating percentage degradation of AML and VAL

	AML		VAL	
Condition	% Degradation	Peak Purity	% Degradation	Peak Purity
Normal	0.00	1000	0.00	1000
Thermal	5.32	1000	3.47	1000
Acidic	12.16	1000	14.67	1000
Basic	16.17	1000	17.56	1000
Oxidative	23.34	1000	25.45	1000

Table 8. Accelerated stability results for analysis of AML and VAL using proposed method

Pharmaceutical formulation	Accelerated Stability Studies						Limit
	Initial		3M		6M		
	AML	VAL	AML	VAL	AML	VAL	
Alkapress Plus F.C.T. label claims (5/80 mg), (AML / VAL)	103	101	101	99	99	97	95-105
Alkapress Plus F.C.T. label claims (5/160 mg), (AML / VAL)	102	100	100	98	97	96	
Alkapress Plus F.C.T. label claims (10/160 mg), (AML / VAL)	101	102	99	100	98	97	

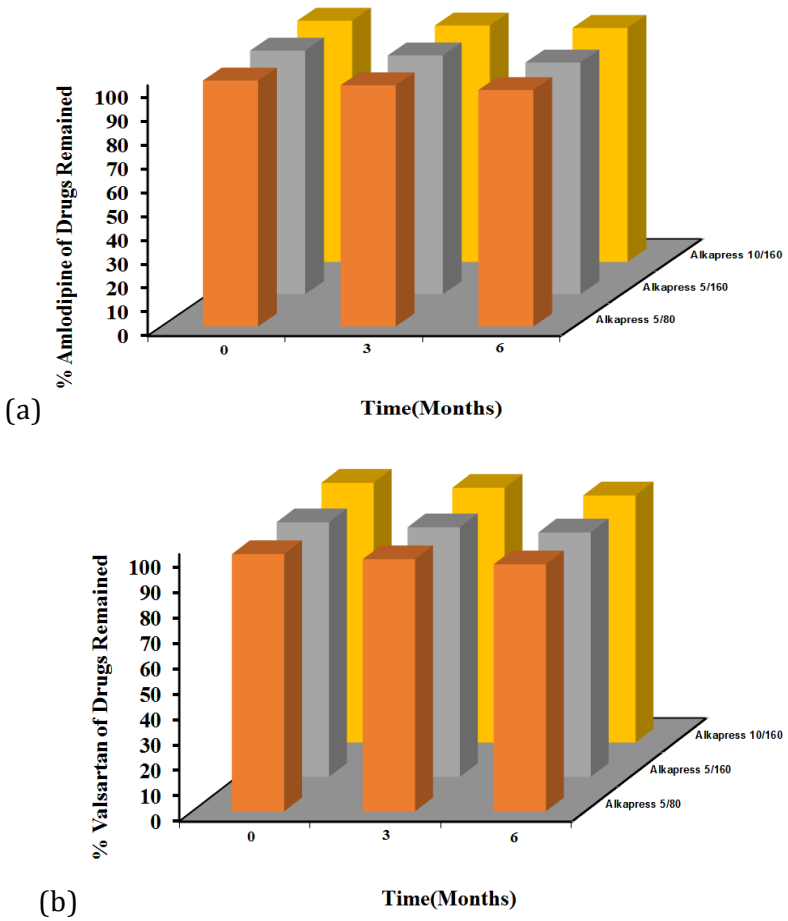


Figure 3. %AML remained of (a) Alkapress (5\80), (5\160), (10\160), (b) %VAL remained of (a) Alkapress (5\80), (5\160), (10\160)

Conclusion

The main objective of this work is to develop accurate, simple, precise, specific, and rapid RP-HPLC method for simultaneous estimation of the analysis of Amlodipine Besylate and Valsartan and its related substances in Alkapress Plus F.C.T. label claims (5/80 mg) and (5/160 mg) and (10/160 mg) (Amlodipine Besylate/ Valsartan) tablet. The results of stress testing that have been undertaken according to the International Conference on Harmonization (ICH) guidelines. Based on the above results, the analytical method is valid, fit for use and can be used for quality control and stability study.

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