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Article

Concurrent Estimation of Amlodipine Besylate, Hydrochlorothiazide and Valsartan by RP-HPLC, HPTLC and UV–Spectrophotometry

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Accurate, sensitive and reproducible reversed-phase high-performance liquid chromatography (RP-HPLC), high-performance thin-layer chromatography (HPTLC) and ultraviolet (UV) spectrophopometric methods were developed for the concurrent estimation of amlodipine besylate (AMLO), hydrochlorothiazide (HCTZ) and valsartan (VALS) in bulk and combined tablet dosage forms. For the RP-HPLC method, separation was achieved on a C18 column using potassium dihydrogen orthophosphate buffer (50 mM, pH 3.7) with 0.2% triethylamine as the modifier and acetonitrile in the ratio of 56:44 (v/v) as the mobile phase. Quantification was achieved using a photodiode array detector at 232 nm over a concentration range of 2–25 μg/mL for AMLO, 5–45 μg/mL for HCTZ and 20–150 μg/mL for VALS. For the HPTLC method, the drugs were separated by using ethyl acetate–methanol–toluene–ammonia (7.5:3:2:0.8, v/v/v/v) as the mobile phase. Quantification was achieved using UV detection at 242 nm over a concentration range of 100–600 ng/spot for AMLO, 150–900 ng/spot for HCTZ and 1,200–3,200 ng/spot for VALS. The UV–spectrophotometric simultaneous equation method was based on the measurement of absorbance at three wavelengths; i.e., at 237.6 nm (λ_{max} of AMLO), 270.2 nm (λ_{max} of HCTZ) and 249.2 nm (λ_{max} of VALS) in methanol. Quantification was achieved over the concentration range of 2–20 μg/mL for AMLO, 5–25 μg/mL HCTZ and 10–50 μg/mL for VALS. All methods were validated according to International Conference on Harmonization guidelines and successfully applied to marketed pharmaceutical formulations. Additionally, the three methods were compared statistically by an analysis of variance test, which revealed no significant difference between the proposed methods with respect to accuracy and precision.

Introduction

Amlodipine besylate (AMLO) is a calcium channel blocker that is commonly used in the treatment of hypertension and angina. Chemically, it is (*RS*)-3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate benzene sulfonate (Figure 1A) (1). Various analytical methods have been reported for the estimation of AMLO, alone or in combination with other antihypertensive agents in pharmaceutical formulations. These include ultraviolet (UV) spectroscopy (2–4), high-performance liquid chromatography (HPLC) (5–8), high-performance thin-layer chromatography (HPTLC) (9–10), liquid chromatography–mass spectrometry (LC–MS) (11) and LC–tandem mass spectrometry (MS-MS) (12).

Valsartan (VALS) is a potent specific angiotensin II receptor blocker that is a widely used antihypertensive agent. Chemically, it is *N*-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl) [1,1'-biphenyl]-4-yl] methyl]-L-valine (Figure 1C) (13). Literature has revealed different methods for the quantification of VALS, alone and in combination with other antihypertensive drugs, such as HPLC (14–15), LC–MS (16-18), capillary electrophoresis (20) and simultaneous UV spectrophotometric methods (21–22), and methods in plasma (19).

Hydrochlorothiazide (HCTZ) is a thiazide diuretic used for treatment of high blood pressure. Chemically, it is 2H-1, 2, 4-benzothiadiazine-7-sulfonamide, 6-chloro-3, 4-dihydro-, 1, 1-dioxide (Figure 1B) (23). Many analytical methods have been reported for the quantification of HCTZ, alone or in combination with other drugs, which include spectroscopic and chromatographic methods (24–29). All three drugs can be found in the United States Pharmacopoeia (USP) (30). AMLO and HCTZ are official drugs in both the Indian Pharmacopoeia (IP) (31) and the British Pharmacopoeia (BP) (32).

A literature survey has revealed several methods for the estimation of AMLO, VALs and HCTZ, individually or in combination with other drugs. No method has been reported for the simultaneous estimation of AMLO, VALS and HCTZ in their combined dosage forms by simultaneous equation using UV spectrophotometry. No HPTLC methods have reported for the simultaneous estimation of these three drugs in their combined dosage form.

Two RP-HPLC methods have been reported for this combination. The first method incorporates 55% of organic eluent and the second method utilizes approximately 70% of organic eluent. The present study reports a new RP-HPLC method that uses only 44% of organic eluent. In addition, the retention time of VALS in the proposed method is 10.15 min. With this higher retention time, stability studies for VALS in the presence of AMLO and HCTZ can be conducted. Because degradation products are mostly more polar than the parent compound, they will elute out before the parent drug. In such cases, the proposed method is advantageous, with higher retention time of VALS. Therefore, an attempt was made to develop accurate, precise and sensitive methods using simultaneous equation UV spectrophotometry and HPTLC, plus an alternative RP-HPLC method, for the concurrent estimation of AMLO, VALS and HCTZ in bulk and in their combined dosage form.

Experimental

Chemicals and reagents

Standard drug samples of AMLO, VALS and HCTZ were gifted by Torrent Research Centre (Ahmedabad, India). A marketed

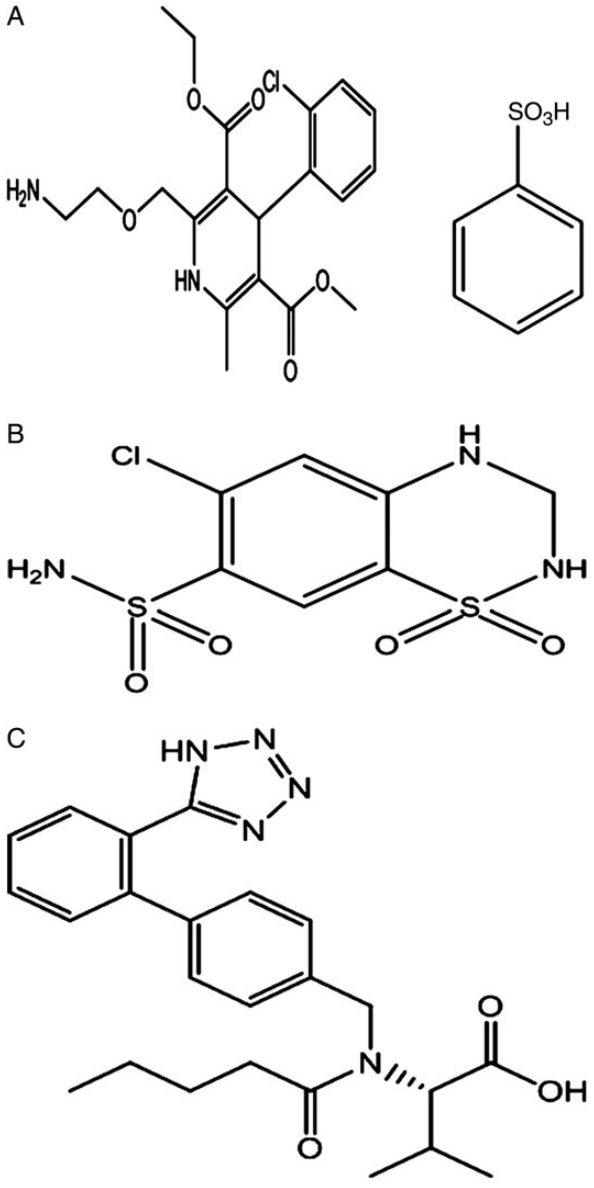

Figure 1. Chemical structures: AMLO (A); HCTZ (B); VALS (C).

tablet formulation, Exforge HCT (Novartis Pharma Stein AG, Stein, Switzerland), containing AMLO (5 mg), HCTZ (12.5 mg) and VALS (160 mg) was purchased from a market in the United States.

For the UV–spectrophotometric method, analytical reagent-grade methanol was purchased from S.D. Fine Chemicals (Mumbai, India). All reagents and solvents that were used in HPTLC (methanol, ethyl acetate, toluene and 25% of ammonia solution) were of analytical grade and were purchased from S.D. Fine Chemicals. For the HPLC method, methanol, acetonitrile, water (HPLC grade), potassium dihydrogen ortho-phosphate, triethylamine and O-phosphoric acid (analytical reagent grade) were purchased from S.D. Fine Chemicals.

Instrumentation

The RP-HPLC method was developed on an HPLC system consisting of a pump (Jasco PU 2080, Japan) equipped with a photodiode array (PDA) detector and a Rheodyne injector with a 20 μL loop. Borwin PDA software was used for computational purposes. A pH meter was used, model 111E/101E (Analabs Scientific Instruments Ltd, India) with a range of pH 0 to 14, resolution of ±0.01 pH and accuracy of ±0.01 pH. The HPTLC instrumentation consisted of a Linomat V sample applicator with a 100 μL Hamilton syringe and a TLC III scanner controlled by WinCATS software (Camag, Muttenz, Switzerland). Merck TLC plates coated with $60F_{254}$ silica gel on aluminum sheets were used as the stationary phase. The plates were developed in a Camag 20 × 10 cm twin trough chamber that was previously saturated for 30 min with the mobile phase. The UVvisible (UV-Vis) spectrophotometric method was developed on a Shimadzu UV-Vis double beam spectrophotometer, model 2400 PC series, with spectral width of 1 nm, wavelength accuracy of 0.5 nm and a pair of 10 mm matched quartz cells (Shimadzu, Japan). All weighing was done on a Citizen electronic balance, model CX 220 (Citizen India, Ltd).

Chromatographic conditions

RP-HPLC method

A Kromasil KR-5 C_{18} column (250 × 4.6mm i.d., 5 μm) was used as the stationary phase. Potassium dihydrogen orthophosphate buffer, 50 mM (pH of buffer adjusted to 3.7 with orthophosphoric acid), with 0.2% triethylamine and acetonitrile in the ratio of 56:44 (v/v) was used as the mobile phase. The mobile phase was filtered through a nylon 0.45 μm, 47 mm membrane filter and degassed before use. The flow rate was 1.0 mL/min. Detection was conducted at 232 nm with a PDA detector. A mixture of acetonitrile and water in the ratio of 50:50 was used as the diluent throughout the HPLC analysis.

HPTLC method

The solutions were spotted in the form of bands of 4 mm width on precoated silica gel $60F_{254}$ aluminium plates by using a Camag 100 μL sample applicator syringe. The plates were activated at 110°C in an oven for 20 min before sample application. A constant application rate of 0.1 μL/s was employed and the space between two bands was 10 mm. The spotted plate was developed in a twin trough chamber, which was previously saturated for 30 min with a mobile phase consisting of ethyl acetate–methanol–toluene–ammonia (7.5:3:2:0.8, v/v/v/v) to a distance of 7 cm. The developed plate was dried in a current of air with an air dryer. The developed spots were scanned at 242 nm, with slit dimensions of 3 × 0.20 mm and a scanning speed of 10 mm/s.

UV spectrophotometric conditions

The solutions of AMLO, HTCZ and VALS were scanned in the spectrum mode from 200 to 400 nm and the overlain UV spectra were measured. From the overlain spectra of these drugs, wavelengths of 237.6 nm (λ_{max} of AMLO), 249.2 nm (λ_{max} of VALS) and 270.2 nm (λ_{max} of HCTZ) were selected for analysis.

Preparation of solutions

Preparation of standard stock solution

AMLO (25 mg), HCTZ (25 mg) and VALS (25 mg) were accurately weighed, transferred to three separate 25 mL volumetric flasks and dissolved in methanol to obtain stock solutions with a concentration of 1,000 μg/mL of each drug. From this stock solution, 1 mL aliquots were transferred to 10 mL volumetric flasks to obtain stock solutions with concentrations of 100 μg/mL of each drug.

Preparation of sample solution

A quantity of tablet powder of 417.89 mg, which was equivalent to 160 mg of VALS, 5 mg of AMLO and 12.5 mg of HCTZ, was weighed and transferred to a 100 mL volumetric flask. Thirty-five milliliters of methanol were added to the flask and the solution was sonicated for 30 min. After sonication, the sample was cooled to room temperature and the volume was completed with methanol. The solution was filtered through Whatman filter paper No. 41. Aliquots (2 mL) were pipetted out, transferred to 50 mL volumetric flasks and diluted with methanol to obtain final concentrations of 2 μg/mL of AMLO, 5 μg/mL of HCTZ and 64 μg/mL of VALS.

Assay method validation

All three developed methods were validated as per the International Conference on Harmonization (ICH) Guidelines for Validation of Analytical Methods (33–34). Various parameters were evaluated, including, specificity, linearity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

Preparation of calibration curve

For the RP-HPLC method, aliquots of the standard stock solution (100 μg/mL) of AMLO, HCTZ and VALS were mixed and diluted to volume with acetonitrile and Millipore water (50:50) to obtain different ternary mixture solutions containing AMLO, HCTZ and VALS in different ratios. Concentrations of solutions were prepared in the range of 2 to 25 μg/mL for AMLO, 5–45 μg/mL for HCTZ and 20–150 μg/mL for VALS to create the calibration curves of these drugs. An aliquot (20 μL) of each solution was injected under the optimized chromatographic conditions and responses were recorded. Calibration curves were constructed by plotting the peak areas versus the concentrations and the regression equations were calculated. Each response was the average of six determinations.

For the HPTLC method, from standard stock solution, suitable aliquots were withdrawn and mixed together to create a ternary mixture of 100 μg/mL of AMLO, 150 μg/mL of HCTZ and 400 μg/mL of VALS in methanol. Concentrations of the solutions were applied on plates in the ranges of 100–600 ng/spot for AMLO, 150–900 ng/spot for HCTZ and 1,200–3,200 ng/spot for VALS. The plates were developed and scanned as described previously. Calibration curves were constructed by plotting peak areas versus concentrations of AMLO, HCTZ and VALS. The regression equations were calculated. Each response was the average of six determinations.

For the UV-Vis spectrophotometric method, the individual solutions were prepared in methanol from standard stock solutions, containing 2, 4, 5, 10 and 20 μg/mL of AMLO; 5, 10, 15, 20 and 25 μg/mL of HCTZ; and 10, 20, 30, 40 and 50 μg/mL of VALS. The solutions were analyzed in methanol at three wavelengths: 237.6 nm (λ_{max} of AMLO), 270.2 nm (λ_{max} of HCTZ) and 249.2 nm (λ_{max} of VALS). The calibration curve was constructed by plotting the absorbance versus the concentration.

Accuracy (recovery)

The accuracy of the method was determined by calculating percentage recoveries for AMLO, HCTZ and VALS by the standard addition method at three different levels (80, 100 and 120%) against a preanalyzed tablet sample. For RP-HPLC, known amounts of the standard solution of AMLO (1.6, 2 and 2.4 μg/mL), HCTZ (4, 5 and 6 μg/mL) and VALS (51.2, 64 and 76.8 μg/mL) were added to a preanalyzed sample solution containing 2, 5 and 64 μg/mL of AMLO, HCTZ and VALS, respectively. For HPTLC, known amounts of the standard solution of AMLO (44, 55 and 66 μg/mL), HCTZ (78.5, 98 and 117.5 μg/mL) and VALS (1,005.6, 1,257 and 1,508.5 μg/mL) were added to a preanalyzed sample containing 55, 98 and 1,257 μg/mL of AMLO, HCTZ and VALS, respectively. For UV spectrophotometry, known amounts of the standard solution of AMLO (4, 5 and 6 μg/mL), HCTZ (8, 10 and 12 μg/mL) and VALS (16, 20 and 24 μg/mL) were added to a preanalyzed sample containing 5, 10 and 20 μg/mL of AMLO, HCTZ and VALS, respectively.

Precision

The intra-day and inter-day precision of the proposed methods were determined by estimating the corresponding responses three times on the same day and on three different days for three different concentrations. For RP-HPLC, AMLO (5, 10 and 15 μg/mL), HCTZ (10, 15 and 20 μg/mL), and VALS (80, 100 and 120 μg/mL) were measured for the precision study. For HPTLC, AMLO (200, 500 and 600 ng/spot), HCTZ (450, 600 and 750 ng/spot) and VALS (1,600, 2,000 and 2,400 ng/spot) were measured for the precision study. For the UV spectrophotometric method, the response of the analytes was recorded at three wavelengths: 237.6, 270.2 and 249.2 nm. The measured concentrations were 2, 4 and 5 μg/mL for AMLO; 15, 20 and 25 μg/mL for HCTZ; and 30, 40 and 50 μg/mL for VALS.

The repeatability was assessed by analyzing one concentration six times. It was performed on concentrations of 5 μg/mL for AMLO, 15 μg/mL for HCTZ and 100 μg/mL for VALS by using the RP-HPLC method. For HPTLC, AMLO (500 ng/spot), HCTZ (500 ng/spot) and VALS (2,000 ng/spot) were measured. For the UV spectrophotometric method, the responses of the analytes were recorded at three wavelengths, 237.6, 270.2 and 249.2 nm, with concentrations of 4 μg/mL for AMLO, 10 μg/mL for HCTZ and 20 μg/mL for VALS, respectively.

LOD and LOQ

The sensitivity of the analytical method was evaluated by determining the LOD and LOQ.

The LOD and LOQ were measured by using the following mathematical equations (34):

$$\text{LOD} = 3.3 \times \sigma/S$$
$$\text{LOD} = 10 \times \sigma/S$$

where σ = standard deviation (SD) of the intercept and S = slope of calibration curve.

Specificity

The separated chromatographic peaks of all drugs were analyzed for peak purity (specificity) by scanning in the range of 200–400 nm with Borwin PDA software, version 1.50, in RP-HPLC and the spectral scanning mode of Wincats software in HPTLC. The specificity of the method was determined by analyzing standard drug and test samples. The peak purities of AMLO, HCTZ and VALS were determined by comparing the spectra at three different regions of the spot, i.e., peak start (*s*), peak apex (*m*) and peak end (*e*) in RP-HPLC and HPTLC.

Robustness

The robustness of the methods was studied by analyzing the same samples of AMLO, VALS and HCTZ with deliberate variations in the method parameters. The changes in the responses of AMLO, VALS and HCTZ were noted. For RP-HPLC, the pH of mobile phase (± 0.2), the flow rate (± 0.2 mL) and the detection wavelength (± 2 nm) were deliberately changed. For HPTLC, various parameters were changed, including detection wavelength (± 2 nm), chamber saturation time (± 3 min) and size of chamber (10 × 10 cm). For the UV spectrophotometric method, the detection wavelength was varied (± 2 nm).

Table I
System Suitability Parameters for the RP-HPLC and HPTLC Methods

RP-HPLC

Parameters	AMLO	HCTZ	VALS
Capacity factor	0.64	0.37	3.41
Tailing factor	1.30	1.41	1.05
Resolution factor	3.175	—	10.37
Theoretical plates	5,378	6,357	4,886
RSD of peak area (%)	0.48	1.85	0.52
HPTLC			
R_f ± SD	0.54 ± 0.01	0.64 ± 0.10	0.23 ± 0.01
Peak area ± SD	2,917 ± 33.30	3,609.78 ± 25.65	7,061.61 ± 99.95

Results and Discussion

The simultaneously conducted analysis of AMLO, HCTZ and VALS in combined their dosage form was a difficult task, because the proportions of these drugs in marketed formulation are in the ratio of 1:2.5:32, respectively.

System suitability test

A system suitability test for RP-HPLC and HPTLC was performed before each validation run. Five replicate injections of the standard preparation were made. Different parameters were monitored for RP-HPLC, including asymmetry of the chromatographic peak, peak resolution, number of theoretical plates and capacity factor. For HPTLC, the parameters monitored were retention factor (R_f) value and peak area (Table I).

Method optimization

RP-HPLC method

Before selection of the final mobile phase, different mobile phases were tested to optimize various RP-HPLC parameters, such as peak shape, peak symmetry, run time and resolution. Symmetrical peaks with good separation (retention time were 3.15 min for AMLO, 10.11 min for VALS and 3.8 min for HCTZ) were achieved on a Kromasil KR-5C 18 column (250 × 4.6 mm i.d., 5 μm) with a mobile phase consisting of acetonitrile–50 mM potassium dihyrogen orthophosphate buffer with 0.2% TEA as a peak modifier in the ratio of 44:56 (v/v). The final pH of the mobile phase was adjusted to 3.7 with ortho-phosphoric acid. Analysis was conducted at a flow rate of 1.0 mL/min. The optimum wavelength for detection and quantification was 232 nm, at which a good detector response was obtained for all three drugs (Figure 2).

HPTLC method

Various experimental conditions, such as the mobile phase and the wavelength of detection, were optimized to achieve

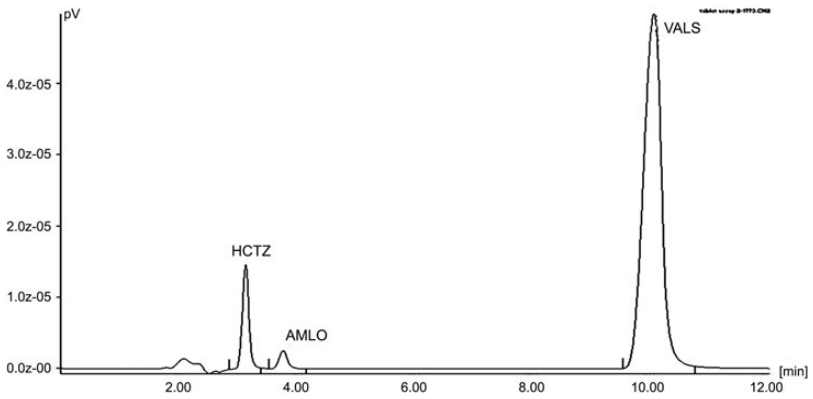

Figure 2. RP-HPLC chromatogram of a standard mixture of AMLO (10 μg/mL), HCTZ (20 μg/mL) and VALS (50 μg/mL).

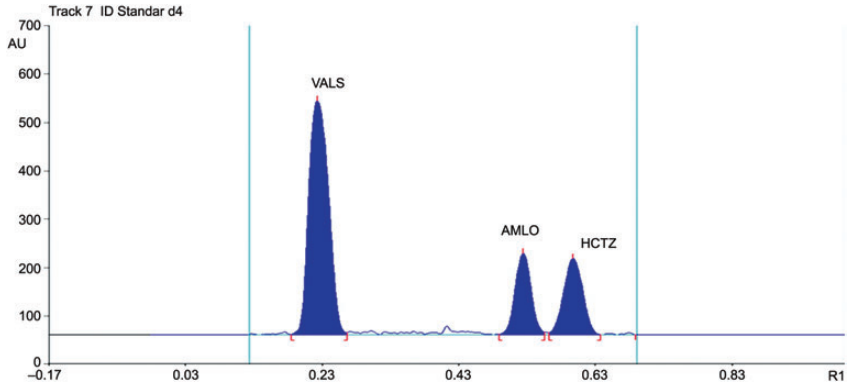

Figure 3. HPTLC chromatogram of a standard mixture of VALS (2,000 ng/spot), AMLO (500 ng/spot) and HCTZ (500 ng/spot).

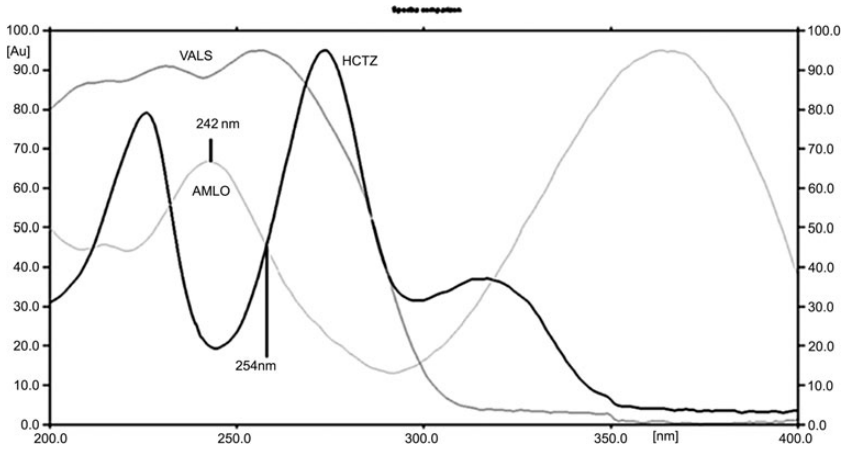

Figure 4. Selection of detection wavelength for AMLO, HCTZ and VALS for HPTLC analysis.

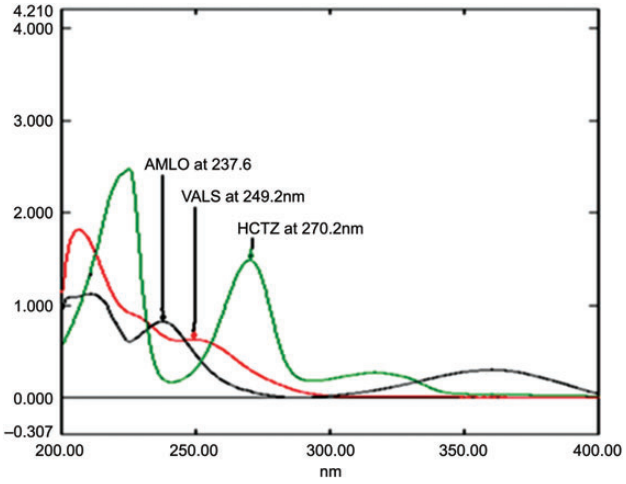

Figure 5. Overlain UV spectra of AMLO, HCTZ and VALS.

accurate, precise and reproducible results for the simultaneous estimation of AMLO, VALS and HCTZ by HPTLC. Good resolution and sharp peaks of these three drugs (R_f values: AMLO = 0.50, VALS = 0.23 and HCTZ = 0.60) with minimum tailing was obtained by using a mobile phase consisting of ethyl acetate–methanol–toluene–ammonia (7.5:3:2:0.8, v/v/v/v) (Figure 3). An isoabsorptive wavelength of 242 nm was selected for the simultaneous quantification of these three drugs. At this wavelength, all three dugs showed promising absorbance (Figure 4).

UV-Vis spectrophotometric method

From the overlain spectra (Figure 5) of the methanolic solution of AMLO, HTCZ and VALS, three wavelengths were finalized for analysis: 237.6 nm (λ_{max} of AMLO), 249.2 nm (λ_{max} of VALS) and 270.2 nm (λ_{max} of HCTZ). The plot of absorbance versus concentration at three wavelengths was plotted and the straight line equation was determined at three wavelengths.

According to the calibration data of all three drugs, the absorptivity coefficients were determined at 237.6, 249.2 and 270.2 nm. The following simultaneous equations were obtained:

$$A_1 = 320C_{AMLO} + 45.88C_{HCTZ} + 320.07C_{VALS} \quad (1)$$

$$A_2 = 177.7C_{AMLO} + 615.55C_{HCTZ} + 141.02C_{VALS} \quad (2)$$

$$A_3 = 178.63C_{AMLO} + 88.086C_{HCTZ} + 295.75C_{VALS} \quad (3)$$

where A_1, A_2 and A_3 represent the absorbance of the sample solution at 237.6, 270.2 and 249.2 nm, respectively. C_{AMLO} is the

Table II
Summary of Validation Parameters for the RP-HPLC Method

Parameter	AMLO	HCTZ	VALS
Linearity range (μg/mL) $n = 6$)	2–25	5–45	20–150
Regression equation	$y = 53047x + 24828$	$y = 135283x + 114584$	$y = 112822x - 83839$
Correlation coefficient (r^2)	0.9945	0.9967	0.9971
Intra-day precision: RSD (%) ($n = 9$)	0.23–1.82	0.5–2.0	0.39–1.54
Inter-day precision: RSD (%) ($n = 9$)	0.03–1.05	0.49–1.26	0.26–0.55
Repeatability: RSD (%) ($n = 6$)	0.5–1.4	0.24–0.36	0.77–1.01
Specificity	Specific	Specific	Specific
LOD (μg/mL)	0.23	0.48	1.1
LOQ (μg/mL)	0.71	0.1.47	3.3
Recovery (%) ± SD ($n = 9$)	99.57 ± 1.33 to 101.42 ± 0.75	98.35 ± 0.19 to 99.16 ± 0.76	99.69 ± 0. 63 to 1002.01 ± 0.07

Table III
Summary of Validation Parameters for the HPTLC Method

Parameter	AMLO	HCTZ	VALS
Linearity range (ng/spot) ($n = 6$)	100–600	150–900	1,200–3,200
Regression equation	$y = 6.534x + 547.48$	$y = 5.931x + 744.6$	$y = 3.48x + 2356$
Correlation coefficient (r^2)	0.9945	0.9926	0.9918
Specificity	Specific	Specific	Specific
Intra-day precision: RSD (%) ($n = 9$)	0.99–2.0	0.67–1.2	0.23–1.83
Inter-day precision: RSD (%) ($n = 9$)	0.48–0.98	0.43–1.92	0.25–1.26
Repeatability: RSD (%) ($n = 6$)	1.98	1.86	0.84
LOD (ng/spot)	2.95	17.84	70.9
LOQ (ng/spot)	8.94	53.9	214.85
Recovery (%) ± SD ($n = 9$)	99.44 ± 0.74 to 100.14 ± 1.32	99.09 ± 0.93 to 100.14 ± 0.67	99.30 ± 0.70 to 101.40 ± 0.77

Table IV
Summary of Validation Parameters of the UV Spectrophotometric Method

Parameter	AMLO	HCTZ	VALS
λ_{max}	237.6 nm	270.2 nm	249.2 nm
Linear range (μg/mL) ($n = 6$)	2–20	5–25	10–50
Correlation coefficient (r^2)	0.9997	0.999	0.999
Repeatability: RSD (%) ($n = 6$)	0.71	0.15	1.41
Intra-day precision: RSD (%) ($n = 9$)	0.52	0.78	0.54
Inter-day precision: RSD (%) ($n = 9$)	0.73	1.02	0.49
LOD (μg/mL)	0.03	0.02	0.03
LOQ (μg/mL)	0.08	0.04	0.09
Recovery (%) ± SD ($n = 9$)	98.4 ± 1.21 to 100.5 ± 1.30	98.9 ± 1.06 to 99.5 ± 1.45	98.53 ± 1.42 to 99.67 ± 1.65

concentration of AMLO, C_{HCTZ} is the concentration of the HCTZ, and C_{VALS} is the concentration of VALS.

Method validation of the proposed methods

The methods were validated in compliance with ICH guidelines. The results of the various parameters are discussed in the following.

Linearity

For the RP-HPLC method, linear correlations were obtained between peak area and concentration for AMLO, VALS and HCTZ in the ranges of 2–25, 20–150 and 5–45 μg/mL, respectively (Table II). For the HPTLC method, linear correlations were obtained between peak area and concentration for AMLO, HCTZ and VALS in the ranges of 100–600, 150–900 and 1,200–3,200 ng/spot, respectively (Table III). For the UV spectrophotometric method, linear correlation was obtained between absorbance and concentration for AMLO, 2–20 μg/mL at 237.6 nm; 5–25 μg/mL at 270.2 nm for HCTZ; and 10–50 μg/mL at 249.2 nm for VALS (Table IV).

Accuracy

The percentage recovery values of AMLO, HCTZ and VALS were obtained in the range of 98 to 102% and the relative standard deviation (RSD) values for all three methods were less than 2%, which confirms the accuracy of method. The values of the accuracy studies for the RP-HPLC, HPTLC and UV methods are shown in Tables II, III and IV, respectively.

Precision

Inter-day and intra-day variation in the quantification of AMLO, HCTZ and VALS showed that the RSD values were always less than 2% during the analysis by all three methods. These low RSD values show that the methods are precise. The values of

Table V
Robustness Study of AMLO, HCTZ and VALS by the HPLC Method

Parameter	AML		HCTZ		VALS	
	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)
Flow rate (± 0.2 mL/min)	$2.92 \pm 0.33 \times 10^4$	1.13	$1.32 \pm 0.14 \times 10^5$	1.03	$1.71 \pm 0.02 \times 10^7$	0.9
PH (± 0.05)	$2.97 \pm 0.48 \times 10^4$	1.64	$1.32 \pm 0.14 \times 10^5$	1.06	$1.27 \pm 0.02 \times 10^7$	1.35
Wavelength (± 2 nm)	$1.27 \pm 0.05 \times 10^5$	0.4	$1.52 \pm 0.21 \times 10^5$	1.37	$1.07 \pm 0.01 \times 10^7$	1.05

Table VI
Robustness Study of AMLO, HCTZ and VALS by the HPTLC Method

Parameter	AMLO		HCTZ		VALS	
	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)	Mean peak area ± SD	RSD (%)
Mobile phase composition: ethyl acetate (± 5%)	1,107.8 ± 14.47	1.3	2,119.9 ± 38.8	1.64	13,749.4 ± 175.3	1.2
Wavelength (242 ± 2 nm)	1,119.63 ± 9.99	0.89	1,743.6 ± 17.2	0.98	1,082 ± 125.29	1.15
Development distance (70 ± 5 mm)	1,073.43 ± 10.6	0.93	1,132.43 ± 10.0	1.02	13,430.1 ± 133	0.99

Table VII
Robustness Study of AMLO, HCTZ and VALS by the UV Spectrophotometric Method

$n = 3$	AMLO			HCTZ			VALS		
Wavelength ± 2 nm	235.6	237.6 (optimum)	239.6	247.2	249.2 (optimum)	251.2	268.2	270.2 (optimum)	272.2
Assay (%)	99.36	98.25	99.12	97.62	98.82	98.14	99.52	98.93	98.23
SD	0.19	0.39	99.54	0.18	0.18	0.41	99.54	0.18	0.18
RSD (%)	0.01	0.07	0.02	0.09	0.04	0.08	0.030	0.04	0.04

the precision studies for the RP-HPLC, HPTLC and UV methods are depicted in Tables II, III and IV, respectively.

LOD and LOQ

For the RP-HPLC method, the LOD values for AMLO, HCTZ and VALS were 0.23, 0.48 and 1.1 µg/mL, respectively; LOQ values for AMLO, HCTZ and VALS were 0.71, 1.47 and 3.3 µg/mL, respectively. For the HPTLC method, the LOD values for AMLO, HCTZ and VALS were 2.95, 17.89 and 70.902 ng/spot, respectively; LOQ values were 8.94, 53.9 and 214.85 ng/spot for AMLO, HCTZ and VALS, respectively. For the UV spectrophotometric method, the LOD values of AMLO, HCTZ and VALS were 0.025, 0.013 and 0.029 µg/mL, respectively; LOQ values for AMLO, HCTZ and VALS were 0.078, 0.041 and 0.089 µg/mL, respectively (Tables II, III and IV).

Specificity

For the RP-HPLC method, the peak purity of AMLO, HCTZ and VALS was assessed by comparing their respective spectra at peak start, apex and end positions of the peak. The peak purity value for all three drugs was more than 995 (ideal value, 1,000), which shows that the peaks were pure with no co-eluting or interfering peaks.

For the HPTLC method, a good correlation (r value more than 0.999) was obtained between the standard and sample spectra of AMLO, HCTZ and VALS, respectively. Acceptable peak purity and correlation values suggest no interference in the quantification of AMLO, HCTZ and VALS in the sample solution. This proves that both methods are specific.

Robustness

The robustness of the methods was studied by performing assays of AMLO, HCTZ and VALS in tablet formulation. The parameters of the optimized method were deliberately varied, and changes in the responses of AMLO, HCTZ and VALS were noted and the assay values were calculated in the changed parameters. The methods proved to be robust, because the assay and system suitability values in the changed parameters were within the accepted range (Tables V, VI and VII).

Assay of marketed formulation

The proposed methods were successfully used for the assay of commercially available combined tablet dosage forms containing AMLO, HCTZ and VALS. Six replicate determinations were performed on accurately weighed tablets. The results for AMLO, HCTZ and VALS were comparable with the corresponding amounts claimed on the label. The assay values obtained by all three methods are presented in Table VIII.

Comparison of proposed methods

A comparison of the developed methods (UV spectrophotometric, HPTLC and RP-HPLC) was performed by applying a Student's analysis of variance (ANOVA) test (single factor) to

Table VIII
Assay of Marketed Formulation by All Three Developed Methods

Name of drug	Assay result (%)	
	UV	HPTLC
AMLO	98.66	100.28
	98.45	101.37
	100.3	98.79
Mean ± SD*	99.14 ± 1.01	100.15 ± 1.30
HCTZ	98.82	101.97
	98.40	101.23
	98.20	99.26
Mean ± SD*	98.47 ± 0.32	100.82 ± 1.40
VALS	98.75	98.59
	98.33	99.15
	99.20	101.61
Mean ± SD*	98.76 ± 0.44	99.78 ± 1.61

*$n = 3$.

Table IX
Results of ANOVA Test for the Three Developed Methods

Drug	Source of variance				
	Between groups	Within group	F_{cal}		F_{crit}
	SS	df	SS	df	
AMLO	3.79	3	6.17	8	1.63
HCTZ	8.45	3	6.13	8	3.68
VALS	5.29	3	11.94	8	1.83

df: degree of freedom; SS: sum of squares.

the assay results. F_{cal} values for AMLO (1.63), HCTZ (3.68) and VALS (1.18) are less than F_{crit} (4.066) at the 95% confidence interval, which reveals no significant difference with respect to accuracy and precision between the proposed methods (Table IX). The UV method is more economic than the HPTLC and RP-HPLC methods. However, all methods are feasible, based upon the requirements and the availability of instruments.

Conclusion

The developed and validated RP-HPLC, HPTLC and UV spectrophotometric methods were found to be simple, rapid, accurate, sensitive, precise and robust for the determination of AMLO, HCTZ and VALS in combined tablet dosage form. The validation data and the recovery studies show that the methods are free from interference from the excipients used in the formulations. A statistical comparison of the assay results for AMLO, HCTZ and VALS in tablet dosage form by the proposed methods indicate no significant differences, and any of the developed methods can be successfully applied for the routine quality control of AMLO, HCTZ and VALS in their combined dosage form.

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REFERENCES

1. O'Neil, M.J.; Merck Index, 13th edition. Merck & Co. Inc., Whitehouse Station, NJ, (2006); pp. 83.
2. Rathee, P., Rathee, S., Thakur, S., Kumar, V.; Simultaneous estimation of amlodipine besylate and lisinopril dihydrate as A.P.I. and in tablet dosage forms by modified form of simultaneous equation method using derivative UV- Spectrophotometry; *International Journal of Chemical Technology and Research*, (2010); 2(1): 556–562.
3. Bhatia, N.M., Deshmane, S.J., More, H.N., Choudhari, P.B.; Simultaneous spectrophotometric estimation of the amlodipine besylate and hydrochlorothiazide in pharmaceutical preparations and biological samples; *Asian Journal of Research in Chemistry*, (2009); 2(4): 393–397.
4. Salve, P., Gharge, D., Kirtawade, R., Dhabale, P., Burade, K.; Simple validated spectroscopic method for estimation of amlodipine besylate from tablet formulation; *Asian Journal of Research in Chemistry*, (2009); 2(4): 553–555.
5. Patil, P.R., Sachin, U.R., Dhabale, P.N., Burade, K.B.; RP-HPLC method for simultaneous estimation of losartan potassium and amlodipine besylate in tablet formulation; *International Journal of Chemical Technology and Research*, (2009); 1(3): 464–469.
6. European Pharmacopoeia, 3rd edition. Council of Europe, Strasbourg, Germany, (2001) pp. 431.
7. Dhorda, V.J., Shetkar, N.B.; Reversed phase high performance liquid chromatographic determination of ramipril and amlodipine in tablets. *Indian Drugs*, (1999); 36: 638–641.
8. Dongre, V.G., Shah, S.B., Karmuse, P.P., Phadke, M., Jadhav, V.K.; Simultaneous determination of metoprolol succinate and amlodipine besylate in pharmaceutical dosage form by HPLC; *Journal of Pharmaceutical and Biomedical Analysis*, (2008); 46(3): 583–586.
9. Ilango, K., Kumar, P.B., Prasad, V.R.V.; Simple and rapid high performance thin layer chromatographic estimation of amlodipine from pharmaceutical dosage forms; *Indian Journal of Pharmaceutical Science*, (1997); 59(6): 171–173.
10. Agrekar, A.P., Powar, S.G.; Reverse phase high performance liquid chromatographic determination of ramipril and amlodipine in tablets; *Journal of Pharmaceutical and Biomedical Analysis*, (2000); 21(6): 1137–1142.
11. Feng, Y., Zhang, L., Shen, Z., Pan, F., Zhang, Z.; Analysis of amlodipine in human plasma by liquid chromatography-mass spectrometry; *Journal of Chromatographic Science*, (2002); 40(1): 49–53.
12. Bhatt, J., Singh, S., Subbaiah, G., Shah, B., Kambli, S., Ameta, S.; A rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the estimation of amlodipine in human plasma; *Journal of Biomedical Chromatography*, (2007); 21(2): 169–175.
13. Goodman and Gillman's the pharmacological basis of therapeutics, 10th edition. McGraw Hill Medical Publishing Division, New York, NY, (2001) pp. 894.
14. Kocyigit, K B., Unsalan, S., Rollas, S.; Determination and validation of ketoprofen, pantoprazole and valsartan together in human plasma by high performance liquid chromatography; *Pharmazie*, (2006); 61: 586–589.
15. Daneshtalab, N., Lewanczuk, R.Z., Jamali, F.; High performance liquid chromatographic analysis of angiotensin II receptor antagonist valsartan using a liquid extraction method; *Journal of Chromatography B*, (2002); 766(2): 345–359.
16. Koseki, N., Kawashita, H., Hara, H., Niina, M., Tanaka, M., Kawai, R., *et al*.; Development and validation of a method for quantitative determination of valsartan in human plasma by liquid chromatography-tandem mass spectrometry; *Journal of Pharmaceutical and Biomedical Analysis*, (2007); 43(5): 1769–1774.
17. Li, H., Wang, Y., Jiang, Y., Tang, Y., Wang, J., Zhao, L.; A liquid chromatography/tandem mass spectrometry method for the simultaneous quantification of valsartan and hydrochlorothiazide in human plasma; *Journal of Chromatography B*, (2007); 852(1-2): 436–442.
18. Senthamil, S.P., Gowda, V.K., Mandal, U., Solomon, W.D., Pal, T.K.; Simultaneous determination of fixed dose combination of nebivolol

and valsartan in human plasma by liquid chromatographic-tandem mass spectrometry and its application to pharmacokinetic study; *Journal of Chromatography B*, (2007); 858(1-2): 143–150.
19. Macek, J., Klíma, J., Ptacek, P.; Rapid determination of valsartan in human plasma by protein precipitation and high performance liquid chromatography; *Journal of Chromatography B*, (2006); 832(1): 169–172.
20. Hillaert, S., Bossche, V.W.; Simultaneous determination of hydrochlorothiazide and several angiotensin-II-receptor antagonists by capillary electrophoresis; *Journal of Pharmaceutical and Biomedical Analysis*, (2003); 31(2): 329–339.
21. Satana, E., Altinay, S., Goger, N.G., Ozkan, S.A., Senturk, Z.J.; Simultaneous determination of valsartan and hydrochlorothiazide in tablets by first-derivative ultraviolet spectrophotometry and LC; *Journal of Pharmaceutical and Biomedical Analysis*, (2001); 25(6): 1009–1013.
22. Tatar, S., Saglik, S.; Comparison of UV- and second derivative-spectrophotometric and LC methods for the determination of VAL in pharmaceutical formulation; *Journal of Pharmaceutical and Biomedical Analysis*, (2002); 30(2): 371–375.
23. Budavari, S.; The Merck Index 14th edition. Merck & Co., Inc., Whitehouse Station, NJ, (2006); pp. 827.
24. Baing, M.M., Vaidya, V.V., Sane, R.T., Menon, S.N., Dalvi, K.; Simultaneous RP-LC determination of losartan potassium, ramipril and hydrochlorothiazide in pharmaceutical preparations; *Chromatographia*, (2006); 64(5): 293–296.
25. Bhusari, K.P., Khedekar, P.B., Dhole, S., Banode, V.S.; Derivative and Q-analysis spectrophotometric methods for estimation of hydrochlorothiazide and olmesartan medoxomil in tablets; *Indian Journal of Pharmaceutical Science*, (2009); 71(5): 505–508.
26. Daniels, S.L., Vanderwielen, A.J.; Stability-indicating assay for hydrochlorothiazide; *Journal of Pharmaceutical Science*, (2006); 70(2): 211–215.
27. Stolarczyk, M., Masalanka, A., Krzek, J., Milczarek, J.; Application of derivative spectrophotometry for determination of enalapril, hydrochlorothiazide and walsartan in complex pharmaceutical preparations; *Acta Poloniae Pharmaceutica, Drug Research*, (2008); 65(3): 275–281.
28. Tian, D., Tian, X., Tian, T., Wang, Z., Mo, F.; Simultaneous determination of valsartan and hydrochlorothiazide in tablets by RP-HPLC; *Indian Journal of Pharmaceutical Sciences*, (2008); 70(3): 372–374.
29. Taomin, H., Zhong, H., Yang, B., Shao, L., Xiaowei, Z., Gengli, D.; Simultaneous determination of captopril and hydrochlorothiazide in human plasma by RP-HPLC from linear gradient elution; *Journal of Pharmaceutical and Biomedical Analysis*, (2006); 41(2): 644–648.
30. United States Pharmacopoeia, 27th edition. United States Pharmacopoeial Convention, Washington, DC, (2009); pp. 1532, 2566, 3842.
31. Indian Pharmacopoeia, Volume II. *Government of India, Ministry of Health and Family Welfare*, Controller of Publication, Delhi, India, (2007); pp. 714, 318.
32. British Pharmacopoeia, Volume I. Her Majesty's Stationary Office, London, England, (2009); pp. 137, 565.
33. ICH Committee S. Validation of Analytical Procedures: Text and Methodology Q2 (R1) Harmonized Tripartite Guideline. International Conference on Harmonization, Geneva, Switzerland, (2005).
34. Taverniers, I., Loose, M.D., Bockstaele, E.V.; Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance; *Trac-Trends in Analytical Chemistry*, (2004); 23(8): 535–552.