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ORIGINAL ARTICLE

Validated stability-indicating HPLC-DAD method of analysis for the antihypertensive triple mixture of amlodipine besylate, valsartan and hydrochlorothiazide in their tablets

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Stress degradation;
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Abstract A simple and selective HPLC-DAD stability indicating method was developed for the simultaneous determination of the three antihypertensive drugs amlodipine besylate (AML), valsartan (VAL) and hydrochlorothiazide (HCT) in their combined formulation. Effective chromatographic separation was achieved using Zorbax SB-C8 column (4.6 × 250 mm, 5 μm ps) with gradient elution of the mobile phase composed of 0.025 M phosphoric acid and acetonitrile at a flow rate of 1 mL/min. The multiple wavelength detector was set at 238 nm for measurement of AML and 225 nm for both VAL and HCT. Quantification was based on measuring the peak areas. The three compounds were resolved with retention times of 4.9, 6.4 and 8.3 min for HCT, AML and VAL respectively. Analytical performance of the proposed HPLC procedure was statistically validated with respect to system suitability, linearity, ranges, precision, accuracy, specificity, robustness, detection and quantification limits. The linearity ranges were 5–200, 5–200 and 10–200 μg/mL for AML, VAL and HCT respectively with correlation coefficients > 0.9993. The three drugs were subjected to stress conditions of acidic and alkaline hydrolysis, oxidation, photolysis and thermal degradation. The proposed method proved to be stability-indicating by resolution of the analytes from their forced-degradation products. The validated HPLC method was applied to the analysis of the cited antihypertensive drugs in their combined pharmaceutical tablets (Exforge HCT). The proposed method made use of DAD as a tool for peak identity and purity confirmation.

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1. Introduction

Amlodipine besylate (AML) is a dihydropyridine calcium channel blocker used in the treatment of hypertension and angina pectoris (Sweetman, 2009). Valsartan (VAL) is an

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angiotensin II receptor antagonist used in the management of hypertension, to reduce cardiovascular mortality in myocardial infarction patients and in the management of heart failure (Sweetman, 2009). Hydrochlorothiazide (HCT) is a moderately potent diuretic used in the treatment of hypertension either alone or with other antihypertensives. It is also used to treat edema associated with heart failure and with renal and hepatic disorders (Sweetman, 2009). Fig. 1 shows the chemical structures of the three compounds. In 2009, the US Food and Drug Administration (FDA) and the European Medicines Agency approved a triple fixed-dose combination of AML, VAL and HCT. It was found that the use of this triple combination was generally more effective in reducing blood pressure and providing overall blood pressure control than the dual combination therapies regardless of age, race, gender, ethnicity, or hypertension severity (Calhoun et al., 2009 and Chrysant, 2011).

The simultaneous determination of AML and VAL in their binary combination was addressed in several analytical reports. These reports proposed spectrophotometric (Gupta et al., 2010 and Kul et al., 2010), spectrofluorimetric (Shaalan and Belal, 2010), TLC (Dhaneshwar et al., 2009 and Ramadan et al., 2010) and several HPLC methods with UV detection (Kul et al., 2010, Patel et al., 2009 and Ramadan et al., 2010). In addition, both drugs were determined in human plasma using HPLC with fluorescence detection (Khalil et al., 2011) and capillary electrophoresis (Alnajjar, 2011). On the other hand, the simultaneous determination of VAL and HCT was carried out using various spectrophotometric methods (Deshpande et al., 2012, Dinc et al., 2004, Erk, 2002, Lakshmi and Lakshmi, 2011 and Satana et al., 2001), HPLC (Ahmed et al., 2011, Ivanovic et al., 2007 and Satana et al., 2001), HPTLC (Kadam and Bari, 2007), and capillary electrophoresis (Hillaert and Van den Bossche, 2003). Additionally, HPLC-MS–MS was applied for the quantification of both drugs in human plasma (Li et al., 2007, Liu et al., 2008 and Shah et al., 2009).

Few reports can be found in the scientific literature for the simultaneous determination of AML, VAL and HCT. These reports presented spectrophotometric (Anandakumar and Jayamariappan, 2011, and Nikam et al., 2010) HPTLC (Galande et al., 2011 and Varghese and Ravi, 2011) and some HPLC methods (Anandakumar et al., 2012, Galande et al., 2011, Shaalan and Belal, 2012, Varghese and Ravi, 2011, Vignaduzzo et al., 2011). Specificity was tested in only one previous report (Shaalan and Belal, 2012) by separation of the three analytes from several other pharmaceutical compounds, however, none of these previous reports was selective enough to resolve the three drugs from their potential impurities and forced-degradation products, and consequently, none of these procedures can be considered stability-indicating.

This work describes development, validation, and application of a new simple, selective and reliable HPLC-DAD method for the analysis of AML-VAL-HCT drug combination. The method was validated for its specificity and stability-indicating properties by resolution of the cited drugs from their forced hydrolytic, oxidative, photolytic and dry heat degradation products.

2. Experimental

2.1. Instrumentation

The HPLC-DAD system consisted of Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) (quaternary pump, vacuum degasser and diode array and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with Agilent ChemStation Software. A Rheodyne manual injector with 20 μL loop was used. The column used was Zorbax SB-C8 (4.6 × 250 mm, 5 μm particle size) (Agilent).

2.2. Materials

Amlodipine besylate (AML) was kindly supplied by Pfizer Egypt S.A.E., Cairo, Egypt, valsartan (VAL) was kindly

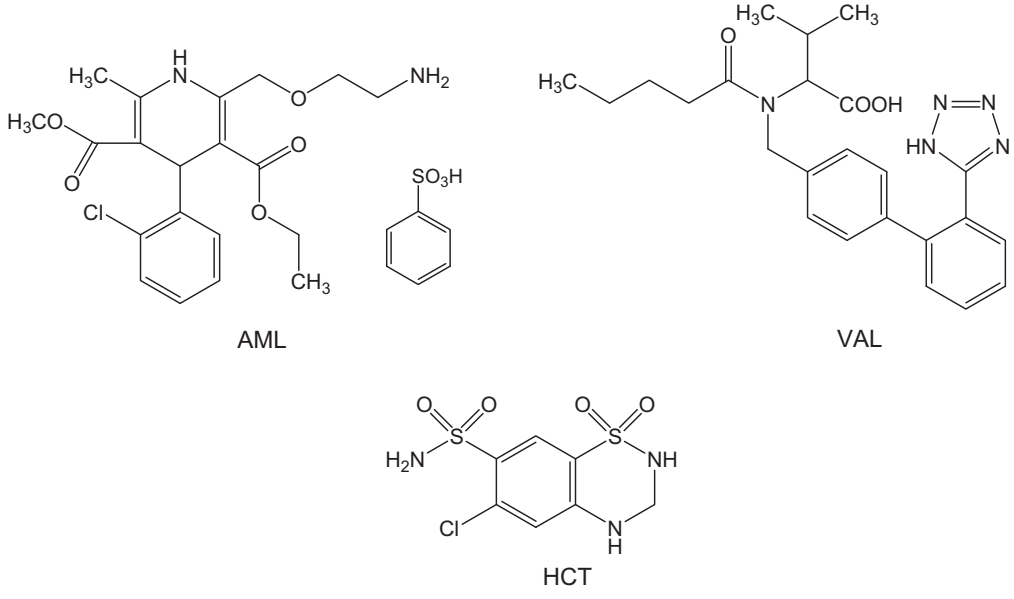

Figure 1 Chemical structures of amlodipine besylate (AML), valsartan (VAL) and hydrochlorothiazide (HCT).

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provided by Novartis Pharma S.A.E., Cairo, Egypt and hydrochlorothiazide (HCT) was kindly donated by Pharco Pharmaceuticals Co., Alexandria, Egypt. HPLC-grade acetonitrile (Scharlau Chemie S.A., Sentmenat, Spain), HPLC-grade methanol (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland), analytical grade of ortho-phosphoric acid, hydrochloric acid, sodium hydroxide, 50% hydrogen peroxide and high purity distilled water were used. Pharmaceutical preparation containing the three drugs is Exforge HCT® tablets labeled to contain 10 mg AML, 320 mg VAL and 25 mg HCT (Novartis Pharma, Switzerland) was purchased from the local market.

2.3. General procedure

2.3.1. Chromatographic conditions

A mobile phase system consisting of 0.025 M phosphoric acid and acetonitrile was used. The separation was achieved with the linear gradient program shown in Table 1. The flow rate was 1.0 mL/min. The injection volume was 20 μL. The eluant was monitored by the diode array detector from 190 to 400 nm, and chromatograms were extracted at the wavelengths of 225 and 238 nm. All determinations were performed at 25 °C.

2.3.2. Standard solutions

AML (1000 μg/mL), VAL (2000 μg/mL) and HCT (1000 μg/mL) stock solutions were prepared in HPLC-grade methanol. The working solutions were prepared by dilution of aliquots of the stock solutions with distilled water to reach the concentration ranges 5–200, 5–200 and 10–200 μg/mL for AML, VAL and HCT, respectively. Triplicate injections were made for each concentration and chromatographed under the previously described LC conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration graphs.

2.4. Assay of commercial tablets

Ten Exforge HCT® tablets were weighed and finely powdered. HPLC-grade methanol (30 mL) was added to a quantity of the powdered tablets equivalent to 5 mg AML, 160 mg VAL and 12.5 mg HCT, the solution was stirred for 10 min then filtered into a 50-mL calibrated flask. The residue was washed with 2×5 mL methanol and washings were added to the filtrate and diluted to final volume with methanol. Aliquots of the tablet solution were diluted with distilled water to obtain final concentrations within the specified ranges then treated as under General Procedure and recovered concentrations were calculated from the corresponding calibration graphs. For standard addition assay, sample solutions were spiked with aliquots of standard solutions of the three compounds to obtain total concentrations within the previously specified ranges then treated as under General Procedure. Recovered concentrations were calculated by comparing the analyte response with the increment response attained after addition of the standard.

Table 1 Gradient program used in the study.

Time (min)	0.025 M H_3PO_4%	Acetonitrile%	Flow (mL/min)
0	75	25	1
4	50	50	1
5	25	75	1
15	25	75	1

2.5. Forced degradation and stability-indicating study

Forced degradation studies were carried out on AML, VAL and HCT standards according to the following conditions:

(a) Acidic and basic conditions: AML, VAL and HCT solutions were treated with 1 mL of 1 M HCl or 1 M NaOH. The solutions were placed in a water bath at 90 °C for 1 h, except for AML solution where the time was reduced for only 10 min at 90 °C in HCl and at 60 °C in NaOH. In case of VAL the time for basic degradation was extended for 2 h at 90 °C. After the specified time, all solutions were neutralized by adjusting the pH to 7.0 and then diluted to volume with distilled water to obtain a final concentration of 20 μg/mL each.
(b) Oxidation with H_2O_2: AML, VAL and HCT solutions were treated with 0.5 mL of hydrogen peroxide 5%. The solutions were placed in a water bath at 80 °C for 1 h, except for AML the time was extended for 2 h. After the specified time intervals, the solutions were diluted to volume with distilled water to obtain a final concentration of 20 μg/mL each.
(c) Photolytic degradation: An amount of each drug powder (100 mg) was subjected to UV irradiation at 254 nm for 60 h. After the specified time, each powder was dissolved in methanol, and aliquots of these methanolic stocks were diluted to volume with distilled water to obtain a final concentration of 20 μg/mL each.
(d) Dry heat degradation: An amount of each drug powder (100 mg) was kept in an oven at 90 °C for 18 h. After the specified time, each powder was dissolved in methanol, and aliquots of these methanolic stocks were diluted to volume with distilled water to obtain a final concentration of 20 μg/mL each.

After the previous treatments, solutions were filtered with a 0.45 μm filtration disk prior to injection to the column.

3. Results and discussion

With the development of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of a stability-indicating assay method (SIAM) has become more clearly mandatory. The guidelines require conducting of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat and others followed by separation of drug from degradation products (ICH, 1993). Because of the requirement of separation of multiple components during the analysis of stability samples, HPLC has gained popularity in stability studies due to its high-resolution capacity, sensitivity and specificity.

The work proposed in this paper was directed toward the study of the chromatographic behavior of the products of stress degradation of the three antihypertensives AML, VAL and HCT; as well as the simultaneous determination of the three drugs in their combined formulation. The fact that up till now the stability indicating analysis of this drug combination has not been reported in the literature has encouraged us to

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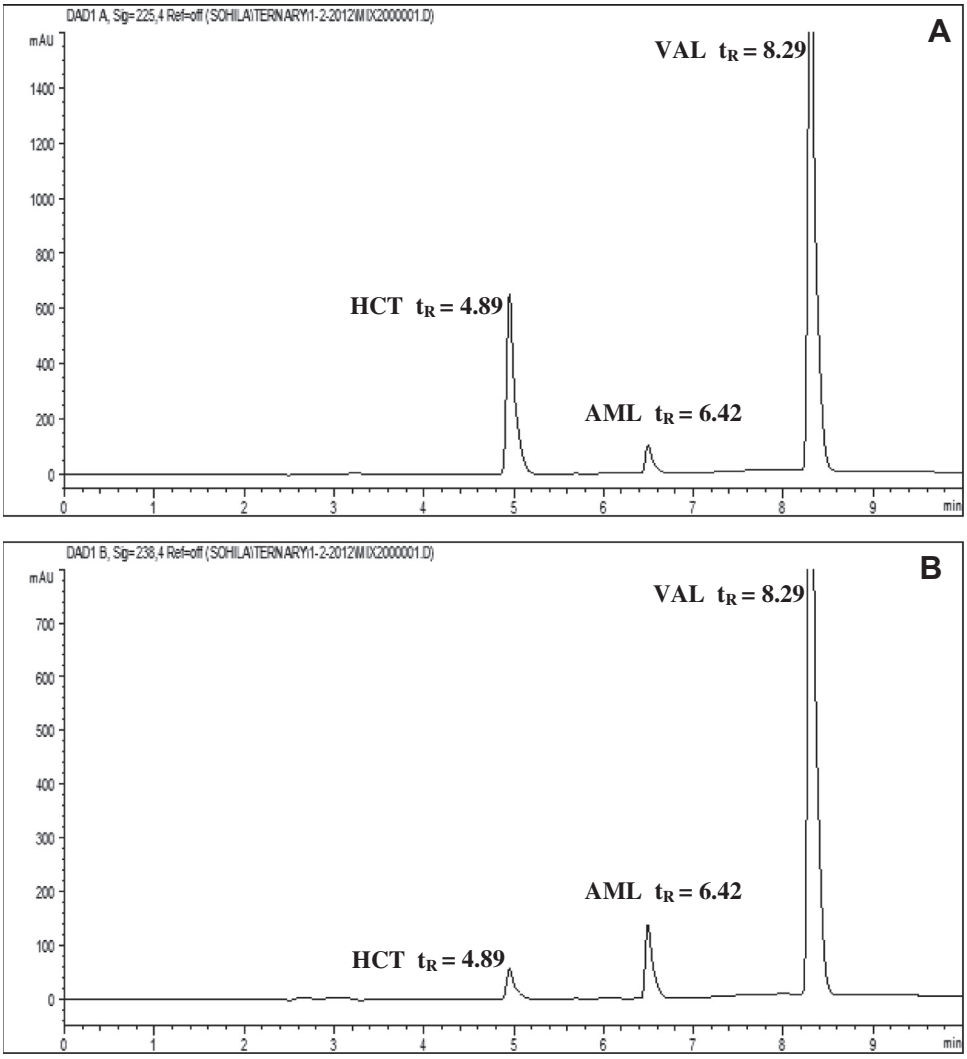

Figure 2 HPLC chromatograms of 20 μL injection of a mixture containing 20 μg/mL AML, 160 μg/mL VAL and 25 μg/mL HCT at 225 nm (A) and 238 nm (B).

Table 2 System suitability parameters for the HPLC-DAD determination of AML-VAL-HCT mixture.

Parameter	HCT	AML	VAL
t_R ± SD (min)	4.89 ± 0.058	6.42 ± 0.018	8.29 ± 0.017
Capacity factor (k')	1.39	2.13	3.04
Theoretical plates (N)	19884	36497	71028
Selectivity (α)	1.53	1.43	
Resolution (R_s)	11.13	13.87	

develop an HPLC- DAD stability indicating assay where the decomposition products were resolved from the intact drugs.

Forced-degradation studies should be considered during development of chromatographic procedures particularly when degraded products are unknown or not available (Klick et al., 2005). Hence, forced-degradation experiments were carried out on AML, VAL and HCT in order to produce the possible relevant degradants and test their chromatographic behavior using the developed HPLC method. Hydrolytic (using both strong acidic and basic media) and oxidative degradation studies were conducted either at room temperature or with the aid of heating. Moreover the dry heat and photolytic stress conditions were applied.

3.1. Optimization of chromatographic conditions

Reviewing the literature revealed that only few papers described the simultaneous determination of AML, VAL, and HCT, one of these reports is a gradient liquid chromatographic method coupled with diode array detection Shaalan

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and Belal, 2012 for the analysis of this ternary mixture in their combined pharmaceutical preparation. The mobile phase system optimized in the aforementioned paper consisted of (A) 0.025 M phosphoric acid and (B) acetonitrile with a linear gradient program and it was taken as an early initiative in our work. Several gradient programs using phosphoric acid and acetonitrile were tried and further optimized, concerning the ratio of acetonitrile to the aqueous phase in the system as well as the time of gradient program, so as to fulfill the requirement of resolving the three drugs from each other and from their stress degradation products. Acetonitrile in high concentration (> 65%, v/v) was an important factor that led to the elution of VAL in a reasonable retention time with acceptable peak asymmetry. On the other hand, using the mobile phase with a high proportion of acetonitrile in the gradient mode resulted in a complication for the quickly eluting HCT peak. To overcome this conflict and to ensure complete resolution of the active ingredients from other extra forced degradation peaks, the best compromise among adequate resolution, reasonable retention times, and tolerable peak asymmetry was achieved using a gradient system starting with 25% (v/v) acetonitrile ramped up linearly to 50% in 4 min, at 5 min acetonitrile was further ramped to 75% and then maintained at this percentage afterward for 15 min (Table 1). The effect of flow rate was studied, and 1.0 mL/min was found optimum regarding run time, peak asymmetry, and column pressure and was kept constant throughout the gradient program. The multiple wavelength detector offers the advantage of measuring each analyte at its maximum absorption wavelength, thus improving sensitivity; AML was measured at 238 nm while HCT and VAL were measured at 225 nm. In addition, diode array detection enhances the power of HPLC and is an elegant option for assessing method specificity by comparison of recorded spectra during peak elution. Quantification was achieved using diode array detection based on peak area measurement.

The previously described gradient program was established with a view to develop a stability indicating assay method where AML, VAL and HCT were resolved with symmetrical peaks, Fig. 2 shows a typical chromatogram for the separation of the three drugs. Moreover, the chromatographic conditions described above gave good separation between each of the three drugs and their stress degradation products. Resolution as well as other system suitability parameters was calculated and they were found acceptable (Table 2).

3.2. Stability indicating aspects

Forced-degradation experiments were carried out on each of the three drugs in this combination in order to produce the possible relevant degradants and test their chromatographic behavior using the developed method. Hydrolytic (using both strong acidic and basic media), oxidative degradation, dry heat and photolytic degradation studies were conducted under different conditions of time and temperature (Table 3). HCT is susceptible to degradation in both acidic and basic media. In strong acidic medium, degradation of HCT was noticed from the decrease of its peak area which reached 10% of the expected area at room temperature, while heating in acidic medium at 90 °C for 1 h led to increased degradation up to 75% of the expected area (Fig. 3A), Similarly, 1 N NaOH caused a diminished degradation at room temperature; while heating at 90 °C for 1 h enhanced degradation (Fig. 3B) and only 70% of the expected area remained. Oxidative H_2O_2 degradation at room temperature revealed 93% from the peak area compared to a standard of the same concentration. The situation changed upon heating at 80 °C for 1 h (Fig. 3C), where the remaining HCT peak eluted with 70% of the expected area. Only one degradation peak can be detected for HCT eluting at a retention time of 4.53 min in both hydrolytic and oxidative degradation. The degradation peak shows an absorption spectrum similar to that of the intact drug. Under UV irradiation

Table 3 Summary of degradation studies of AML-VAL-HCT using the proposed HPLC method.

Analyte	Degradation conditions	Degradation (%)	Retention times of degradation products (min)
1- Acidic degradation			
AML	90 °C for 10 min	30	3.18, 3.40, 4.03, 6.06, 8.87
VAL	90 °C for 1 h	50	4.34, 9.17
HCT	90 °C for 1 h	75	4.53
2- Basic degradation			
AML	60 °C for 10 min	35	5.08, 6.04
VAL	90 °C for 2 h	13	–
HCT	90 °C for 1 h	30	4.53
3- Oxidative degradation			
AML	80 °C for 2 h	25	6.02
VAL	80 °C for 1 h	40	7.49, 7.65, 7.85, 8.04
HCT	80 °C for 1 h	30	4.53
4- Photo-degradation			
AML	UV at 254 nm for 60 h	–	–
VAL	UV at 254 nm for 60 h	6	7.88, 9.22, 9.57
HCT	UV at 254 nm for 60 h	13	4.53, 6.69, 8.85
5- Thermal degradation (dry heat)			
AML	90 °C for 18 h	–	–
VAL	90 °C for 18 h	5	4.38
HCT	90 °C for 18 h	–	–

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at 254 nm the HPLC chromatogram revealed degradation up to 13% after being exposed to the UV light for 60 h, and the degradation products appeared at 4.53, 6.69 and 8.85 min (Fig. 3D). Finally, no signs of degradation of HCT could be observed under dry heat conditions. HCT peak appeared at its specific retention time (4.89 min) with area almost identical

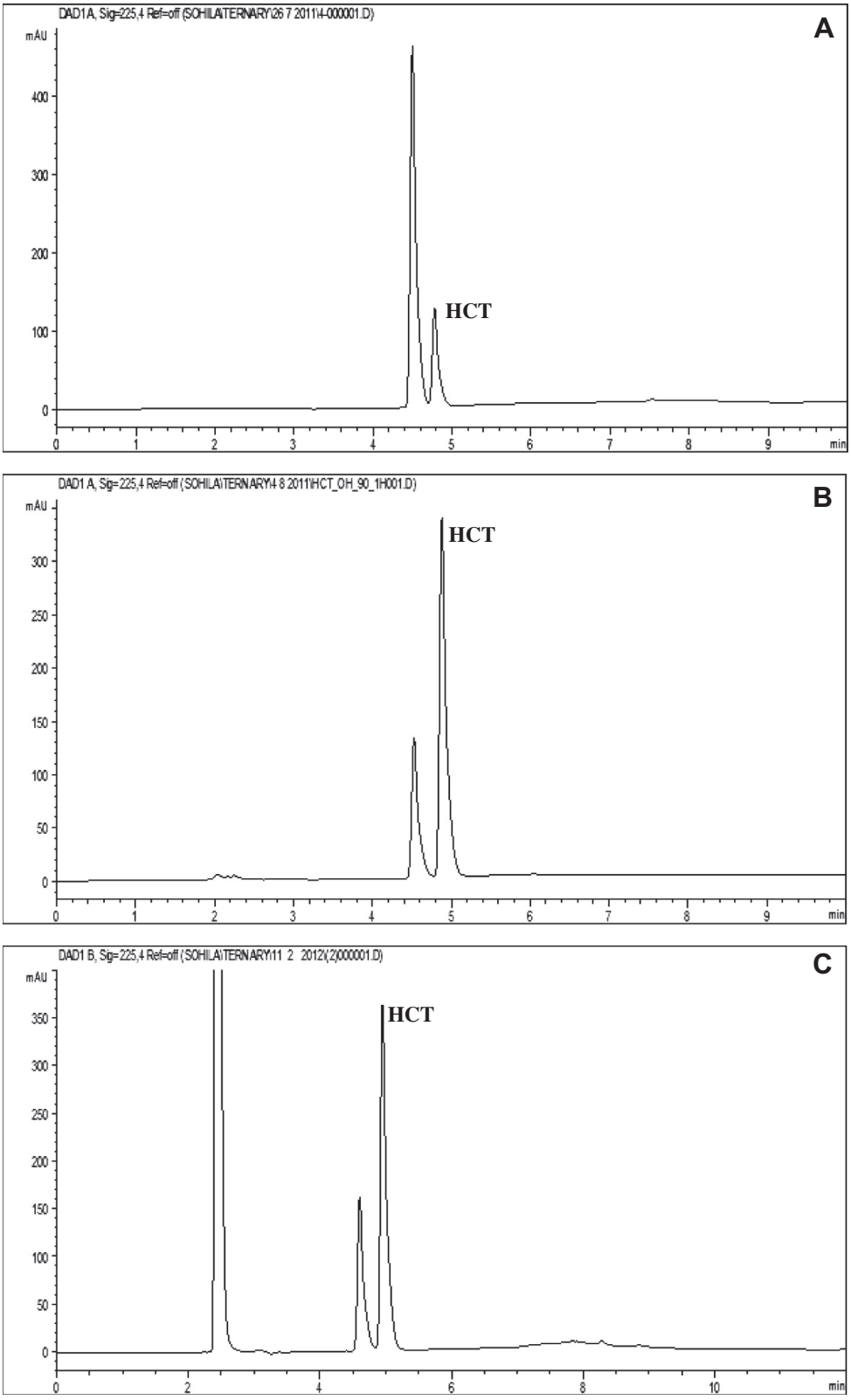

Figure 3 HPLC chromatograms of 20 μg/ml solution of HCT after exposure to (A) acid degradation, (B) base degradation, (C) oxidative degradation, (D) photolytic degradation, and (E) thermal dry heat degradation.

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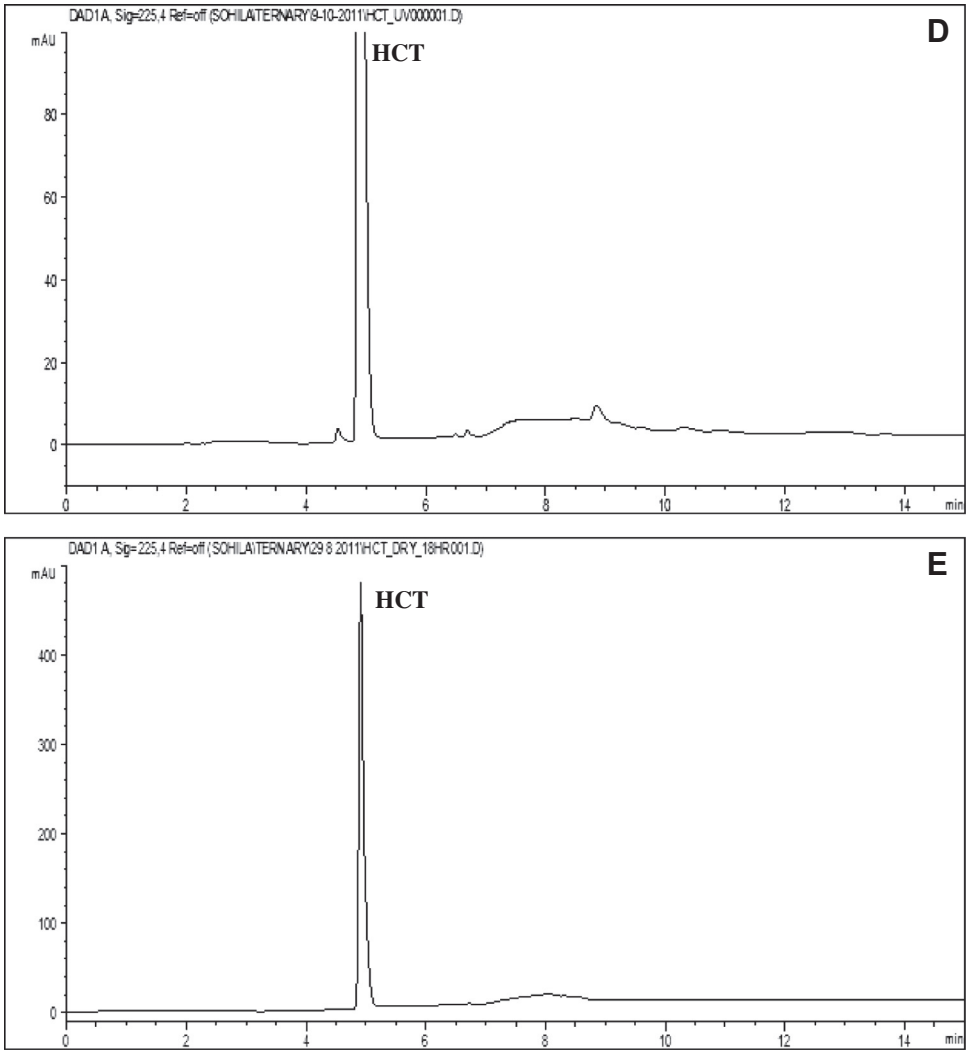

Fig. 3 (*continued*)

to that of standard of the same concentration, additionally, the chromatograms did not show any extra peaks (Fig. 3E).

Similarly, the same stress conditions were applied to test the stability indicating behavior of VAL. In strong acidic medium, 1 M HCl attacked VAL; a residual peak of the drug appeared with less than 50% of the expected area, and degradation products' peaks eluted at about 4.34 and 9.17 min (Fig. 4A).VAL appeared to be stable without any degradation in strong basic medium (1 M NaOH at 90 °C for 1 h) and the chromatogram revealed an essentially intact VAL peak, increasing the heating time to 2 h led to increased degradation up to 13% only, and no degradation peaks appeared in the chromatogram. Fig. 4B shows the chromatogram of VAL after treatment with 1 M NaOH at 90 °C for 2 h. HPLC after oxidative H_2O_2 degradation at room temperature for 24 h revealed an almost intact VAL peak with area about 96% of the intact area. The situation was much different upon heating at 80 °C for 1 h, after which the remaining VAL peak had about 60% of the expected area. Several degradation products' peaks of different intensities eluted at 7.49, 7.65, 7.85 and 8.04 min (Fig. 4C). Photolytic degradation revealed almost intact VAL peak with only 6% reduction in its peak area. Fig. 4D shows the chromatogram of VAL after treatment at 254 nm for 60 h where weak degradation peaks could be detected at 7.88, 9.22 and 9.57 min. Thermal degradation under the conditions stated in Table 3 led to 5% degradation and the emergence of a single degradation peak at 4.38 min. (Fig. 4E).

On the other hand, AML is well susceptible to hydrolysis; acid hydrolysis with1 N HCl at room temperature for 24 h caused about 15% reduction in the peak area of AML, while 1 N HCl with the aid of heating strongly attacked AML, an approximately 30% decrease in the peak area was observed after heating AML with 1 N HCl at 90 °C for 10 min (Fig. 5A). Well resolved minor degradation peaks can be detected at retention times 3.18, 3.40, 4.03, 6.06 and 8.87 min. Excessive heating for longer time or at higher temperatures led to complete attack of the drug, and the chromatograms showed almost no peak for AML. Similarly, sodium hydroxide powerfully attacked AML. The drug readily decomposes in basic medium. Heating AML with 1 M NaOH at 60 °C for only 10 min revealed the remaining AML peak which was about 65% of the expected area, in addition to the appearance of degradation peaks at 5.08 and 6.04 min (Fig. 5B). Oxidative degradation using H_2O_2 was performed at 80 °C for 2 h where the remaining AML peak was 75% and degradation peaks eluted at 6.02 min (Fig. 5C).

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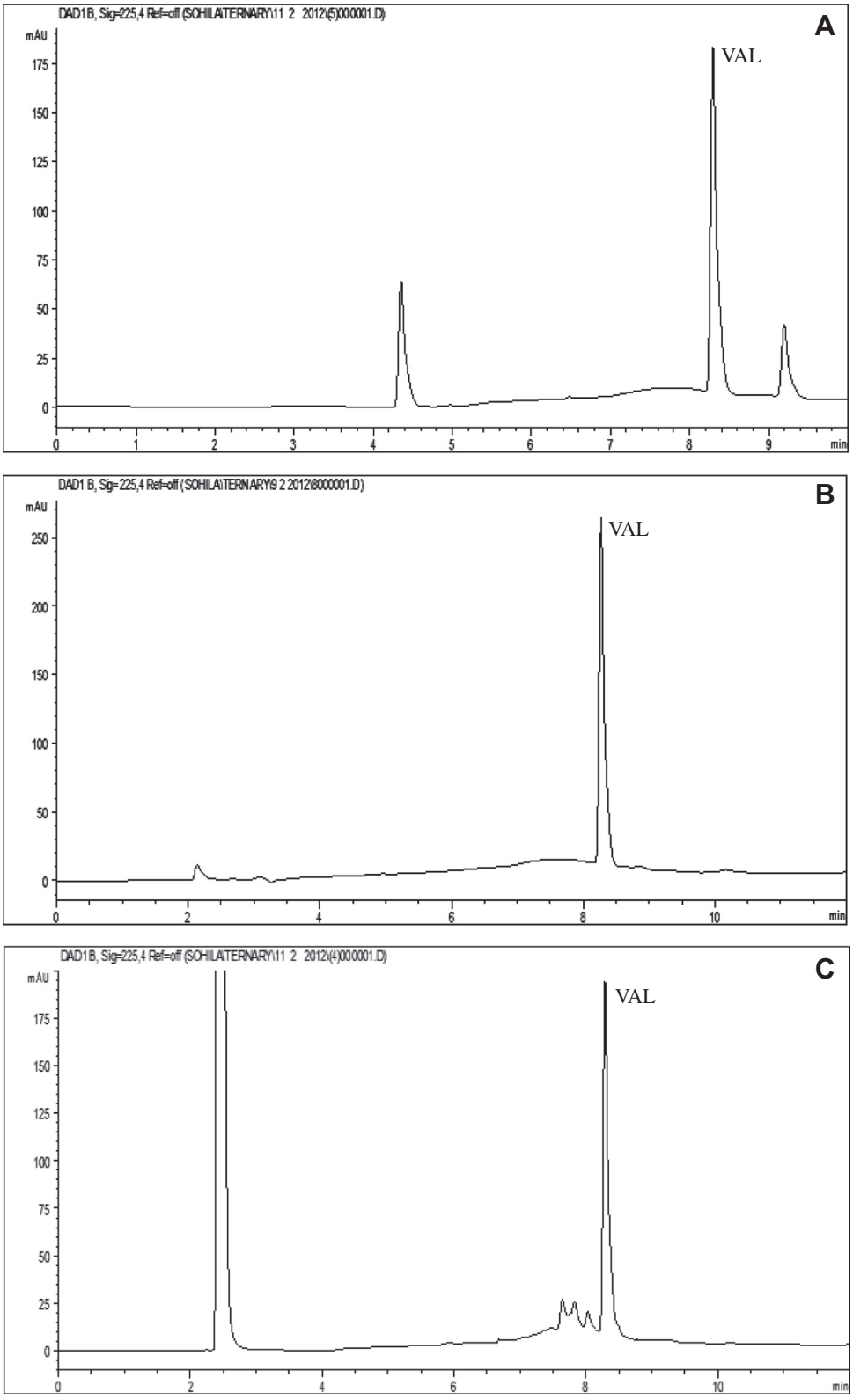

Figure 4 HPLC chromatograms of 20 μg/ml solution of VAL after exposure to (A) acid degradation, (B) base degradation, (C) oxidative degradation, (D) photolytic degradation, and (E) thermal dry heat degradation.

AML appeared to be stable without any degradation when subjected to UV irradiation for 60 h, fig. 5D shows the intact drug with no degradation peaks. Almost the same condition applied for the dry heat degradation where AML peak appeared at its specific retention time (6.42 min) with area almost identical to that of the standard of the same concentration, furthermore, the chromatograms did not show any extra peaks (Fig. 5E).

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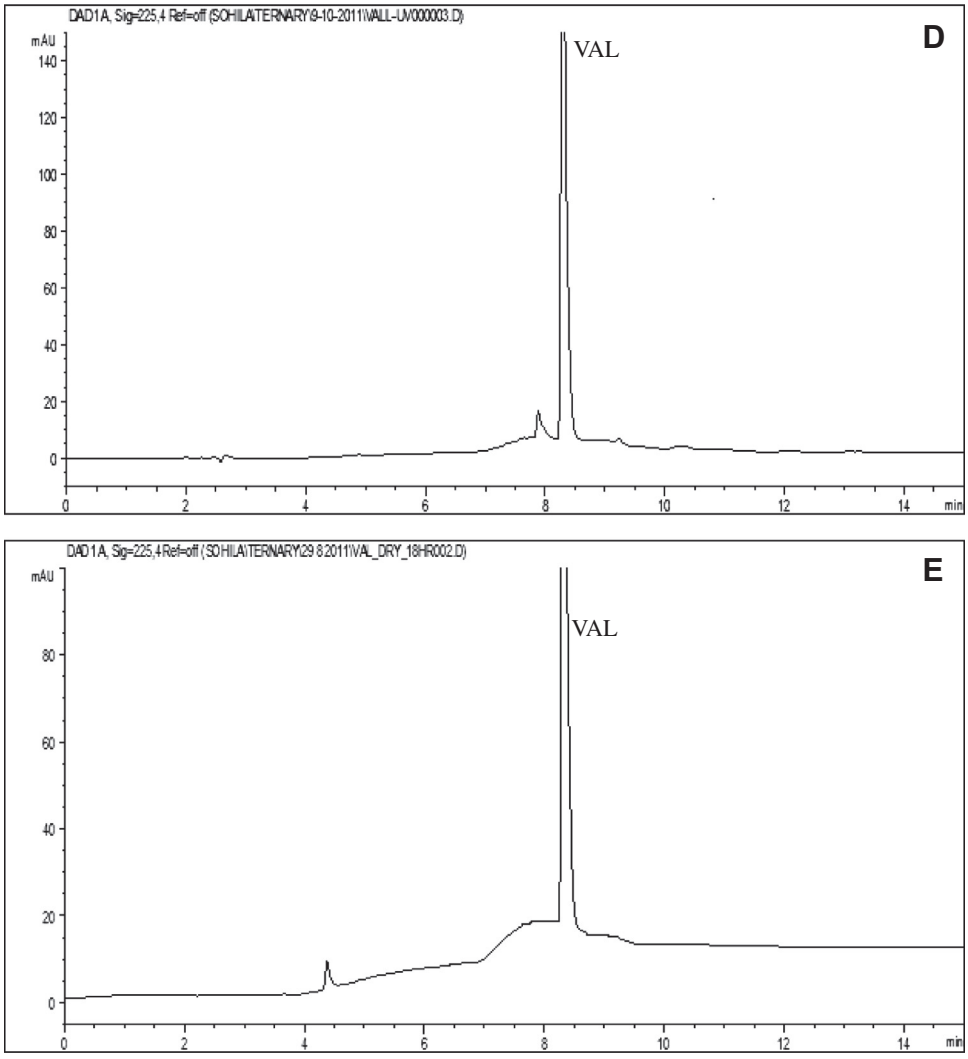

Fig. 4 (*continued*)

In all these experiments, resolution was calculated between any of the three analytes and the nearest degradation products peaks. Resolution was found not <2.23; this implies an adequate baseline separation between the main drugs and any of the degradation products. It is noteworthy to mention that peak purity test results obtained from the diode-array detector (DAD) confirm that AML, VAL and HCT peaks are homogenous and pure in all the analyzed samples subjected to forced degradation conditions.

3.3. Validation of the proposed method

3.3.1. Linearity and concentration ranges

The linearity of the proposed HPLC procedure was evaluated by analyzing a series of different concentrations for each of the three analytes. The linear regression equations were generated by least squares treatment of the calibration data. Under the optimized conditions described above, the measured peak areas were found to be proportional to concentrations of the analytes. Table 4 presents the performance data and statistical parameters including linear regression equations, concentration ranges, correlation coefficients, standard deviations of the intercept (S_a), slope (S_b) and standard deviations of residuals ($S_{y/x}$). Regression analysis shows good linearity as indicated from the correlation coefficient values (>0.9994). In addition, deviation around the slope can be further evaluated by calculation of the RSD% of the slope (S_b%) which were found to be less than 1.1%. The analysis of variance test for the regression lines reveals that, for equal degrees of freedom, an increase in the variance ratio (F values) means an increase in the mean of squares due to regression and a decrease in the mean of squares due to residuals. The greater the mean of squares due to regression, the steeper is the regression line. The smaller the mean of squares due to residuals, the less is the scatter of experimental points around the regression line. Consequently, regression lines with high F values (low significance F) are much better than those with lower ones. Good regression lines show high values for both r and F statistical parameters (Armitage and Berry, 1994).

3.3.2. Detection and quantification limits

According to the pharmacopoeial recommendations (USP, 2011), the limit of detection is defined as the concentration that

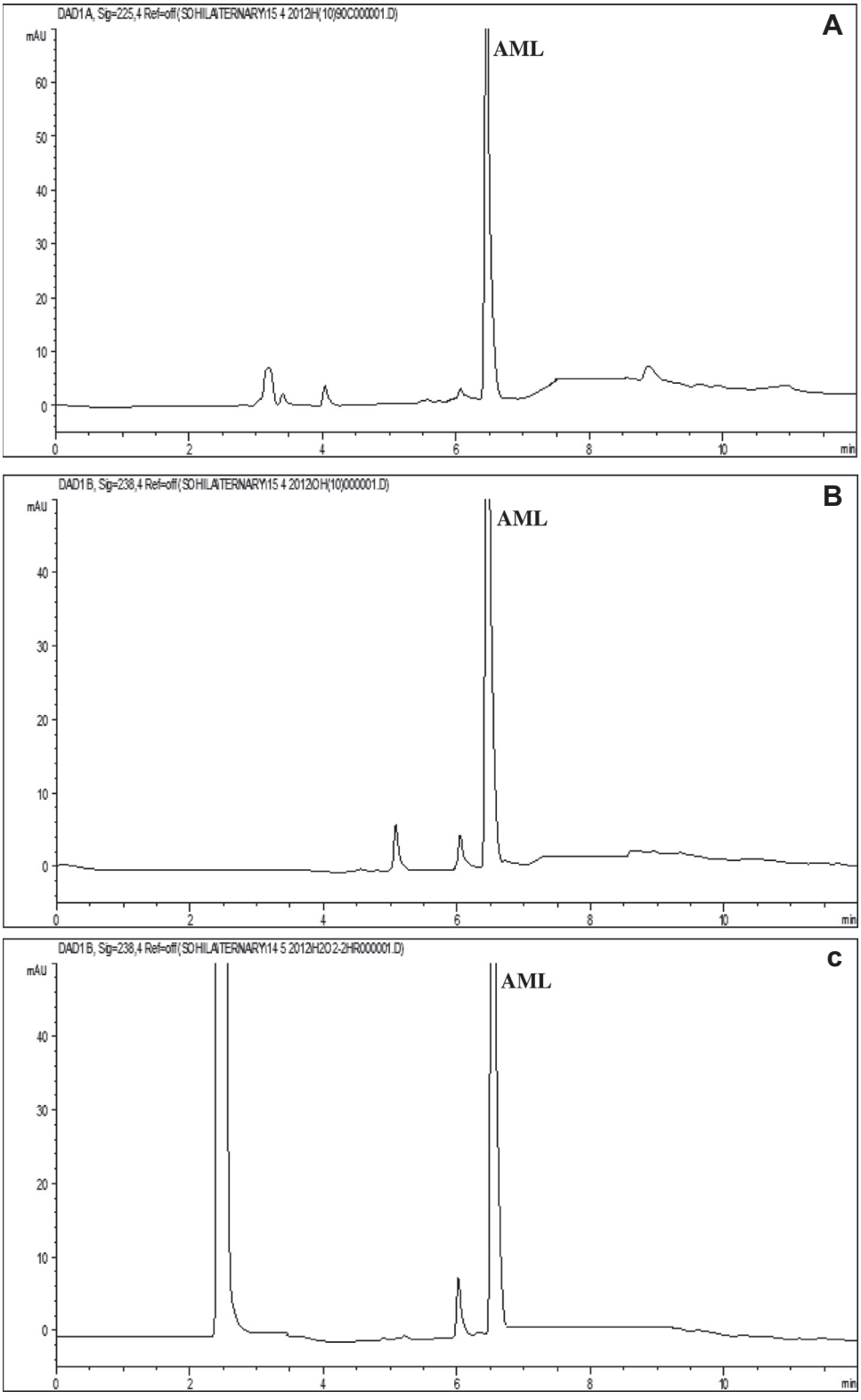

Figure 5 HPLC chromatograms of 20 μg/ml solution of AML after exposure to (A) acid degradation, (B) base degradation, (C) oxidative degradation, (D) photolytic degradation, and (E) thermal dry heat degradation.

has a signal-to-noise ratio of 3:1, while for limit of quantification, the ratio considered is 10:1. The LOD and LOQ values for the three antihypertensives were calculated and presented in Table 4.

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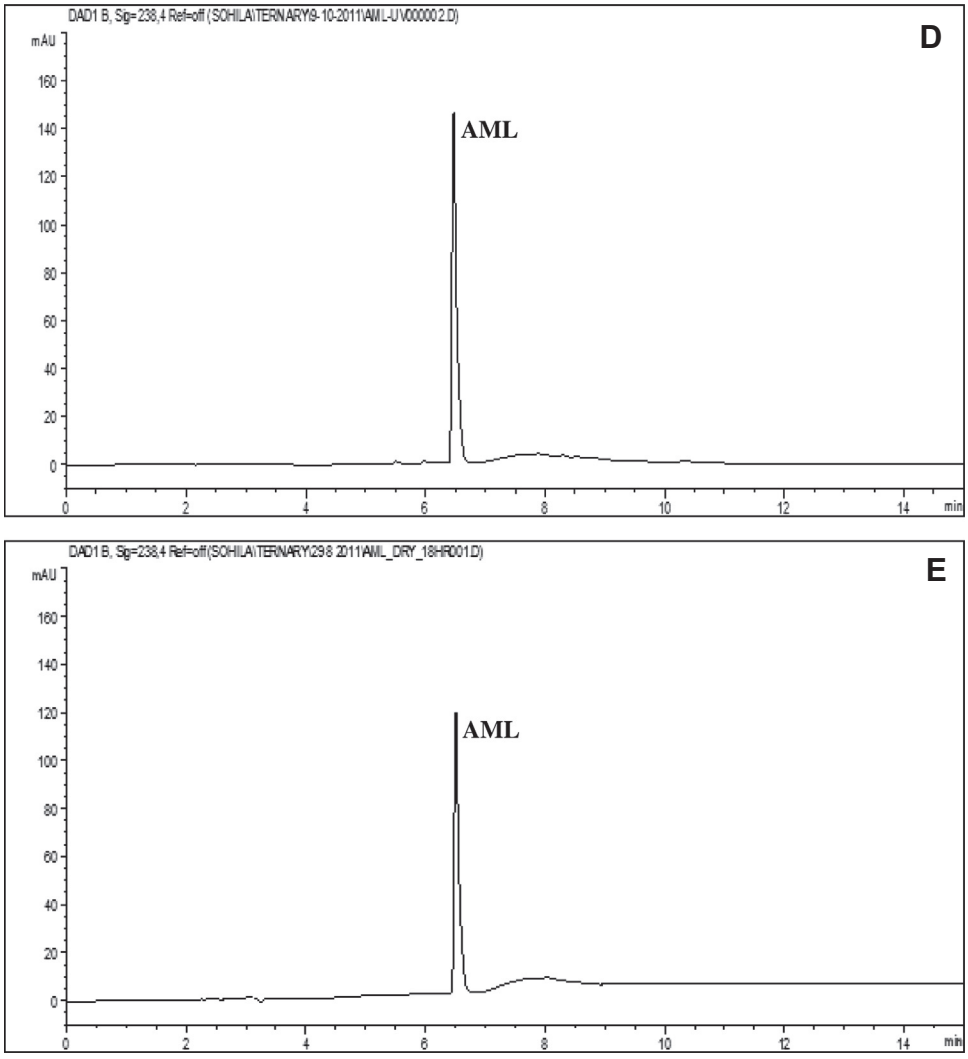

Fig. 5 (*continued*)

Table 4 Analytical parameters for the determination of AML-VAL-HCT mixture using the proposed HPLC-DAD method.

Parameter	AML	VAL	HCT
Wavelength (nm)	238	225	225
Concentration range (μg/mL)	5–200	5–200	10–200
Intercept (*a*)	−137.47	134.36	44.41
S_a[a]	25.14	74.15	55.66
Slope (*b*)	51.26	72.38	117.05
S_b[b]	0.56	0.73	1.78
RSD% of the slope (S_b%)	1.08	1.01	1.52
Correlation coefficient (*r*)	0.99935	0.99959	0.99967
$S_{y/x}$[c]	65.70	172.87	88.98
F[d]	8497.32	9792.09	9537.13
Significance *F*	3.06×10^{-17}	1.21×10^{-13}	2.13×10^{-9}
LOD[e] (μg/mL)	0.26	0.24	0.12
LOQ[f] (μg/mL)	0.85	0.80	0.40

[a] Standard deviation of the intercept.
[b] Standard deviation of the slope.
[c] Standard deviation of residuals.
[d] Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals).
[e] Limit of detection.
[f] Limit of quantification.

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Table 5 Precision and accuracy for the determination of AML, VAL and HCT in bulk form using the proposed HPLC-DAD method.

Analyte	Nominal value (μg/mL)	Within-day			Between-day		
		Found ± SD[a] (μg/mL)	RSD(%)[b]	E_r(%)[c]	Found ± SD[a] (μg/mL)	RSD(%)[b]	E_r(%)[c]
AML	10	9.99 ± 0.07	−0.7	−0.10	10.06 ± 0.08	0.78	0.01
	20	19.99 ± 0.30	1.52	−0.05	19.78 ± 0.11	0.55	−1.10
	150	149.85 ± 0.22	0.15	−0.10	149.73 ± 0.81	0.54	−0.18
VAL	20	20.11 ± 0.15	0.74	0.54	19.69 ± 0.17	0.85	−1.55
	80	79.95 ± 0.50	0.62	−0.05	80.96 ± 0.88	1.09	1.18
	200	199.50 ± 0.20	0.10	−0.004	200.02 ± 1.07	0.54	0.01
HCT	25	25.08 ± 0.22	0.87	0.32	25.02 ± 0.36	1.46	0.08
	100	99.83 ± 0.60	0.60	−0.17	100.61 ± 0.68	0.68	0.61
	150	152.24 ± 1.13	0.75	0.15	152.79 ± 0.63	0.41	1.86

[a] Mean ± standard deviation for three determinations.
[b] % Relative standard deviation.
[c] % Relative error.

3.3.3. Precision and accuracy

The within-day (intra-day) precision and accuracy for the proposed method were studied at three concentration levels for each compound using three replicate determinations for each concentration within one day. Similarly, the between-day (inter-day) precision and accuracy were tested by analyzing the same three concentrations for each compound using three replicate determinations repeated on three days. Recoveries were calculated using the corresponding regression equations and they were satisfactory. The percentage relative standard deviation (RSD%) and percentage relative error ($E_{r\%}$) did not exceed 1.6% proving the high repeatability and accuracy of the developed method for the estimation of the analytes in their bulk form (Table 5).

3.3.4. Selectivity and specificity

Method selectivity was examined by preparing several laboratory-prepared mixtures of the three compounds at various concentrations within the linearity ranges mentioned in Table 4. The laboratory-prepared mixtures were analyzed according to the previously described procedure. The analysis results including percentage relative standard deviation (RSD%) and the percentage relative error ($E_{r\%}$) values shown in Table 6 were satisfactory thus validating selectivity, precision and accuracy of the developed method and demonstrating its capability to resolve and quantify the analytes in different ratios. Specificity is defined as the ability to access unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components (USP, 2011), and this is well demonstrated in detail through the analysis of pharmaceutical dosage form and forced degradation studies. No extra peaks were observed from any of the inactive ingredients in the dosage form. Also, the DAD enables peak purity verification, where no signs of co-elution from any of the inactive components were detected. Selectivity was demonstrated by separation of the three analytes from their relevant degradation products under different stress conditions.

3.3.5. Robustness

Robustness was examined by evaluating the influence of small variations in different conditions such as concentration of phosphoric acid solution (±0.025 M), source of acetonitrile (Scharlau Chemie S.A., Spain or SDS, France or Labscan, Poland), working wavelength (±2 nm), flow rate (±0.1 mL/min) and column temperature (±2 °C). These variations did not have any significant effect on the measured responses or the chromatographic resolution. RSD% for the measured peak areas using these variations did not exceed 3%.

3.4. Stability of solutions

The stability of standard working solutions as well as sample solutions in distilled water was examined and no chromatographic changes were observed within 24 h at room temperature. Also, the stock solutions prepared in HPLC-grade methanol were stable for at least 2 weeks when stored refrigerated at 4 °C. Retention times and peak areas of the drugs

Table 6 Determination of AML-VAL-HCT laboratory-prepared mixtures using the proposed HPLC-DAD method.

Nominal value (μg/mL)			Found ± SD[a] (μg/mL)			RSD(%)[b]			E_r(%)[c]		
AML	VAL	HCT	AML	VAL	HCT	AML	VAL	HCT	AML	VAL	HCT
100	100	100	100.02 ± 0.88	100.37 ± 0.52	100.30 ± 0.33	0.88	0.51	0.33	0.02	0.37	0.30
25	25	150	25.12 ± 0.12	25.13 ± 0.13	149.87 ± 0.57	0.46	0.53	0.38	0.48	0.52	−0.09
25	150	25	24.91 ± 0.12	150.35 ± 0.49	25.07 ± 0.08	0.49	0.33	0.32	−0.36	0.23	0.28
150	25	25	150.20 ± 0.26	25.19 ± 0.23	24.91 ± 0.05	0.18	0.89	0.18	0.13	0.76	−0.36
25	160	25	24.99 ± 0.10	159.67 ± 0.42	25.01 ± 0.28	0.38	0.26	1.13	−0.04	−0.21	0.04

[a] Mean ± standard deviation for five determinations.
[b] % Relative standard deviation.
[c] % Relative error.

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Table 7 Application of the proposed HPLC-DAD method to the analysis of AML-VAL-HCT mixture in pharmaceutical tablets.

	External standard			Reference method			Standard addition		
	AML	VAL	HCT	AML	VAL	HCT	AML	VAL	HCT
% Recovery ± SD[a]	100.55 ± 0.85	100.65 ± 1.23	99.36 ± 1.07	100.28 ± 1.12	100.56 ± 1.10	99.67 ± 0.93	99.88 ± 1.13	100.38 ± 1.31	99.04 ± 0.88
RSD%[b]	0.85	1.22	1.08	1.12	1.09	0.93	1.13	1.31	0.89
t	0.42	0.12	0.49						
F	1.74	1.25	1.32						

Quantification was carried out at the following wavelengths: 238 for AML, 225 for VAL and HCT.
Theoretical values for *t* and *F* at $P = 0.05$ are 2.31 and 6.39, respectively.
[a] Mean ± standard deviation for five determinations.
[b] % Relative standard deviation.

remained unchanged and no significant degradation was observed during these periods.

3.5. Analysis of pharmaceutical dosage form

The optimized HPLC-DAD procedure was applied for the assay of this drug combination in the pharmaceutical formulation (Exforge HCT tablets). The active ingredients were extracted with the same solvent used for the preparation of the standard stock solutions (HPLC-grade methanol) then dilution was made with distilled water to reach concentration levels within the specified ranges. The active ingredients eluted at their specific retention times. No interfering peaks were observed from any of the inactive ingredients or the colored coat of the analyzed tablets. The diode-array detection enables peak purity verification where no signs of co-elution from any of the inactive adjuvants were detected. Recoveries were calculated using both external standard and standard addition methods. The assay results revealed satisfactory accuracy and precision as indicated from % recovery, SD and RSD% values (Table 7). Furthermore, a reference RP-HPLC method (Shaalan and Belal, 2012) was applied for the estimation of the analytes in their combined formulation. Recovery data obtained from the developed HPLC method were statistically compared with those of the reference method using Student's *t*- and the variance ratio *F*-tests. In both tests, the calculated values did not exceed the theoretical ones at the 95% confidence level which indicated that there were no significant differences between the recoveries obtained from the developed method and those of the reference method (Table 7). It is evident from these results that the proposed method is applicable to the assay of this drug combination with a satisfactory level of selectivity, accuracy and precision.

4. Conclusions

In this study, a simple, specific and reliable gradient elution HPLC-DAD procedure was developed for the assay of amlodipine besylate (AML), valsartan (VAL) and hydrochlorothiazide in their pharmaceutical combination. The three analytes were subjected to forced degradation using several stress (hydrolytic, oxidative, photolytic and thermal) conditions, and the proposed method was successfully employed for the resolution of the analytes' peaks from those of the forced degradation products. The most important feature in the proposed method is the specificity and stability-indicating merits. To our present knowledge, no such specific and stability indicating procedure has been reported for the assay of this ternary drug mixture. The developed method made use of DAD as a tool for peak identity and purity confirmation; however, it can be adapted to conventional HPLC with UV detection which is the most popular in quality control laboratories. Finally, the method was thoroughly validated, therefore, it can be recommended for routine analysis and for checking quality during stability studies of the cited drugs.

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