Original research paper

Development and validation of HPLC and CE methods for simultaneous determination of amlodipine and atorvastatin in the presence of their acidic degradation products in tablets

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Two methods were developed for separation and quantitation of amlodipine (AML) and atorvastatin (ATV) in the presence of their acidic degradation products. The first method was a simple isocratic RP-HPLC method while the second was capillary electrophoresis (CE). Degradation products were obtained by acidic hydrolysis of the two drugs and their structures were elucidated for the first time by IR and MS spectra. Degradation products did not interfere with the determination of either drug and the assays were therefore stability-indicating. The linearity of the proposed methods was established over the ranges 1–50 µg mL^{-1} for AML and ATV in the HPLC method and in the range of 3–50 and 4–50 µg mL^{-1} for AML and ATV, respectively, in the CE method. The proposed methods were validated according to ICH guidelines. The methods were successfully applied to estimation of AML and ATV in combined tablets.

Keywords: atorvastatin, amlodipine, capillary zone electrophoresis, HPLC, stability, acidic degradation

Amlodipine [AML, 3-*O*-ethyl-5-*O*-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate] (1) is a dihydropyridine calcium channel blocker, used for the management of hypertension and angina (2) (Fig. 1a). Atorvastatin {ATV, [*R*-(*R**,*R**)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1–methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1*H*-pyrrole-1-heptanoic acid} (1) is an HMG-CoA reductase inhibitor used to reduce LDL-cholesterol and triglycerides (2) (Fig. 1b). Caduet® tablets, launched by Pfizer Ltd. for simultaneous treatment of hypertension and dyslipidaemia, contain both AML and ATV.

AML and ATV are both official in *British Pharmacopoeia* (3) and *United States Pharmacopeia* (4) and different methods were reported for the determination of both drugs in different dosage forms (5–12). Also, different methods have been applied for simultaneous esti-

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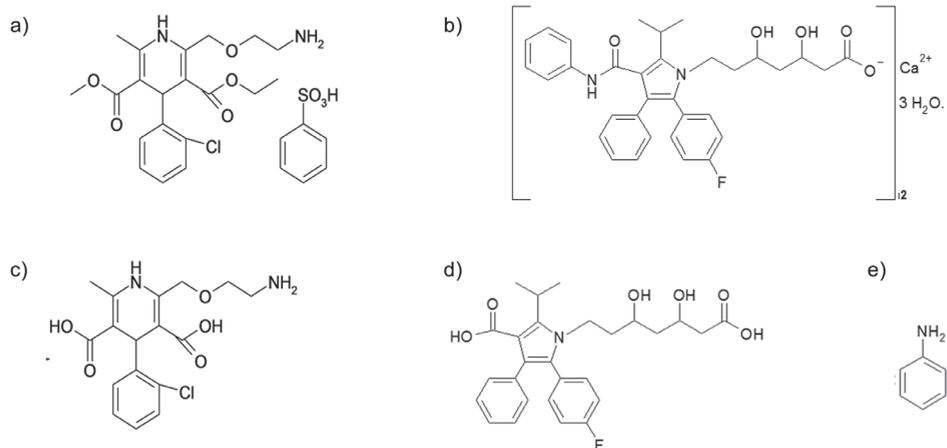* Correspondence; said.hassan@pharma.cu.edu.eg

Fig. 1. Structural formulae of: a) amlodipine (AML), b) atorvastatin (ATV), c) AM_{deg}, d) AT_{deg1} and e) AT_{deg2} (aniline).

mation of AML and ATV in their binary mixtures (13–21). Stability indicating HPLC (22–24) and CE (25) have been used for the analysis of such a mixture. However, they neither isolated nor characterized the possible degradation products of the two drugs. The acidic degradation products of the two drugs were prepared, isolated and their structures were elucidated for the first time using MS and IR spectra in our previous work (26) (Fig. 1), then full (PLS) and full (ANN) methods were applied for determination of the two drugs in the presence of these degradation products.

Table I. Separation parameters for HPLC and CE method

	HPLC			CE	
	AML	ATV		AML	ATV
t_R (min)	3.97	6.52	t_R (min)	3.84	6.28
T^a	0.97	1.03	T^a	1.01	0.97
Rs^b	2.82	2.02	Rs^d	21.38	21.42
TPM $(m^{-1})^c$	5776	15904	TPM ^{c} (m^{-1})	213320	233286
HETP (μm)	173	63			
k'^a	5.06	9.13			
α^b	1.36	1.15			

[a] Tailing factor and capacity factor determined for individual peaks.

[b] Chromatographic resolution and selectivity factor are determined between AML and AM_{deg} peaks and between ATV and AT_{deg1} peaks.

[c] Efficiency expressed as the number of theoretical plates (*N*) per meter for AML and ATV peaks.

[d] Resolution factor determined between AML and AT_{deg2} peaks and between ATV and AT_{deg2} peaks.

This manuscript aims to develop and validate stability indicating HPLC and CE methods for the simultaneous determination of AML and ATV in the presence of their acidic degradation products in laboratory prepared mixtures and in pharmaceutical dosage forms.

EXPERIMENTAL

Materials and reagents

Pure amlodipine besylate was kindly supplied by Al-Hekma Pharmaceutical Company, Egypt; its purity was certified to be 99.89 ± 0.69 %. Pure atorvastatin calcium was kindly supplied by the Al-Delta Pharmaceutical Company, Egypt; its purity was certified to be 99.79 ± 0.46 %. Caduet® 5 mg/10 mg tablets and Caduet® 10 mg/10 mg tablets 10 mg ATV and 5 and 10 mg AML, resp., were manufactured by Pfizer Ltd., Egypt. They were procured from the local market. Acetic acid, chloroform, hydrochloric acid, methanol, sodium hydroxide and toluene, all of analytical grade, were purchased from El-NASR Pharmaceutical Chemicals Co., Egypt. Acetonitrile, methanol and sodium tetraborate decahydrate were purchased from Sigma-Aldrich, Germany.

Instruments and conditions

HPLC, CE and GC instrumental setup comprised products of Agilent/Agilent Technologies (USA, Germany)

HPLC. – All analyses were performed in an air-conditioned laboratory (18 ± 2 °C). An Agilent 1100 series liquid chromatograph consisting also of a dual pneumatic pumping system (model G1310A), an ultra-violet variable wavelength detector (model G1314A) and Rheodyne injector (model 7725 I) equipped with 20-μL injector loop (Agilent, USA) was used. Agilent Zorbax® ODS column (5 μm, 4.6 x 250 mm), flow rate of 1.0 mL min^{-1} and UV detection were performed at 254.0 nm. An Agilent 7100 CE system, with a diode array detector (Agilent Technologies) was controlled by Chemstation software.

The mobile phase was prepared by mixing acetonitrile/methanol/phosphate buffer pH = 3.0 (45:30:25, *V/V/V*); pH was adjusted to 2.5 ± 0.1 with orthophosphoric acid. The mobile phase was degassed by ultrasonic vibrations for 30 min prior to use.

CE. – Separation was carried out at 18 ± 2 °C using a voltage of 15 kV (positive polarity) and detection at 200.0 nm. Peak purity was assessed using DAD. Bare fused silica capillaries used (Agilent Technologies) were of 50 μm id, 325 μm od, with a total/effective length of 48.5/40.0 cm.

Preconditioning of the capillary before each run was carried out by flushing with 0.1 mol L^{-1} NaOH, MilliQ water and finally with a background electrolyte (BGE) for 3 min each. Post-conditioning was carried out by flushing with MilliQ water for 5 min. Samples were injected hydrodynamically at 5 kPa for 5 s and 50 mmol L^{-1} borate buffer at pH 8.0 was used as the BGE.

TLC plates 20 x 20 cm (Sigma-Aldrich, Germany) were coated with a 0.2-mm silica gel 60 F254 layer. The sample was applied to the plates using micro-droppers and a UV lamp at 254 nm was used for visualization of the spots.

The gas chromatograph coupled to a mass spectrophotometer was Shimadzu Qp-2010 (Japan) with a Shimadzu AOC-20i autosampler. A 30 m, HP-5 MS column [(5 %-phenyl)-methylpolysiloxane] was employed for separation, with a 0.253-mm i.d. and 0.50-μm film thickness (Agilent). The IR spectrophotometer was Shimadzu 435 and sampling was undertaken as potassium bromide discs and NaCl plates.

Preparation and separation of acidic degradation products (26)

Amlodipine degradation product. – Fifty milliliters of 1.0 mol L^{-1} HCl were added to 50.0 mg AML in a conical flask, the solution was refluxed for 2 h and then cooled. NaOH (1.0 mol L^{-1}) was added to the degraded solution up to pH 7.0 and the solution was tested for complete degradation by TLC using chloroform/methanol/acetic acid (86.2:11.5:2.3, *V/V/V*) as developing solvent. The solution was then evaporated slowly in a rotary evaporator to dryness. The degradation product was extracted with methanol and then methanol was evaporated. The extraction was repeated three times to ensure complete extraction of the degradation product. Purity of the degradation product was tested by dissolving a small portion in methanol, applying it onto TLC plates and developing using the previously mentioned solvent system and then visualizing under a UV lamp. The structure of the isolated degradation product was elucidated using mass spectrometry and IR (26) (Figs. S1 and S2). The mass spectrum of the AML degradation product showed a peak at *m/z* 367 corresponding to AM$_{deg}$ and the IR spectra showed a shift of the carbonyl band from 1682 to 1579 cm^{-1}, indicating formation of an acid (26).

Atorvastatin degradation products. – Fifty milligrams of ATV were dissolved in the smallest volume of methanol in a conical flask, then 50.0 mL of 6.0 mol L^{-1} HCl solution was added, the solution was refluxed for 3 h and then cooled. NaOH (6.0 mol L^{-1}) was added to the degraded solution up to pH 7.0 and the solution was tested for complete degradation by TLC using toluene/methanol (70:30, *V/V*) as a developing solvent (27). The solution was then evaporated slowly in a rotary evaporator to dryness to obtain the first degradation product (AT$_{deg1}$). The collected evaporated liquid was heated at 100 °C to get rid of solvents and the second degradation product (AT$_{deg2}$) was obtained. The degradation product (AT$_{deg}$) was extracted from solid NaCl (produced from HCl neutralization by NaOH) with methanol and then methanol was evaporated. The extraction was repeated three times to guarantee completeness. Purity of the degradation product was tested by dissolving a small portion in methanol, applying it onto TLC plates and developing using the previously mentioned solvent system and then visualizing under a UV lamp. Structures of the isolated degradation products were elucidated using IR and mass spectrometry (26) (Figs. S3 and S4). The mass spectra showed peaks at *m/z* 483 and 93 corresponding to AT$_{deg1}$ and AT$_{deg2}$. The IR spectra showed disappearance of the NH band at 3421 cm^{-1} and appearance of a forked peak at 3431 and 3356 cm^{-1} corresponding to the NH$_2$ group in AT$_{deg2}$ (26).

Standard solutions

Standard stock solutions of AML and ATV were 1 mg mL^{-1} in methanol. Standard working solutions of AML, ATV, amlodipine degradation product (AM$_{deg}$), atorvastatin degradation products (AT$_{deg1}$ and AT$_{deg2}$, namely, aniline) were 100 μg mL^{-1} in methanol.

Procedures

Calibration curves. – For HPLC, solutions of AML and ATV of 1.0–50.0 µg mL^{-1} were prepared. The volume of 20 µL of each solution was injected in triplicate into the liquid chromatograph under the previously mentioned chromatographic conditions. The average peak area obtained for each AML and ATV concentration was plotted *versus* concentration, and regression equations were computed.

For CE solutions of 3.0–50.0 and 4.0–50.0 µg mL^{-1} of AML and ATV, respectively, were prepared in BGE. Samples were injected hydrodynamically under the previously mentioned electrophoretic conditions. Migration time-corrected peak areas were plotted *versus* AML and ATV concentrations and the corresponding regression equations were calculated.

Validation of HPLC and CE methods

Validation of the proposed methods was performed with respect to specificity, accuracy, precision, *LOD, LOQ,* linearity and range, robustness and stability according to the ICH guidelines (28).

Specificity. – Specificity of the methods was achieved with analyses of different laboratory prepared mixtures of AML and ATV spiked with different levels of degradation products, ranging from 10–70 % degradation.

Accuracy. – Accuracy was confirmed by applying the proposed methods to determination of three concentrations (8, 12 and 16 µg mL^{-1}) of AML and ATV standards ($n = 3$).

Precision. – Repeatability and intermediate precision were assessed using three concentrations (10, 20 and 30 µg mL^{-1}) of standard AML and ATV solutions ($n = 3$).

LOD *and* LOQ. – According to the ICH recommendations (28), the approach based on the residual standard deviation of a regression line and the slope was applied to calculate the detection and quantitation limits.

Linearity and range. – Linearity of the methods was evaluated by analyzing six concentrations of AML and ATV ($n = 3$). The assays were performed according to the experimental conditions previously mentioned. A linear relationship was established by plotting the peak area (for HPLC) or migration time-corrected peak area (for CE) against the drug concentration.

Robustness. – Robustness of the proposed methods was evaluated by the reliability of the analysis with respect to small variations in the experimental conditions. These parameters included the portion of acetonitrile (45 ± 5 %), acidity of mobile phase (pH 2.5 ± 0.3), flow rate (1.0 ± 0.1 mL min^{-1}) and detection wavelength (254.0 ± 2.0 nm) for the HPLC method, while the parameters for the CE method were acidity of the BGE (pH 8.0 ± 0.3) and applied voltage (15 ± 1 kV). Three concentrations (10, 20 and 30 µg mL^{-1}) of standard AML and ATV ($n = 3$) were analyzed and only one parameter was changed at a time.

Application of HPLC and CE for determination of AML and ATV in Caduet® tablets

Ten tablets of both Caduet® 5/10 mg and 10/10 mg were accurately weighed and finely powdered. The amount of the powder equivalent to 2 mg ATV was accurately weighed and

Table II. Validation of the proposed HPLC and CE methods for simultaneous determination of AML and ATV in the presence of acidic degradants

Parameter	HPLC method		CE method	
	AML	ATV	AML	ATV
Specificity (recovery ± SD, %)[a]	100.9 ± 1.0	99.9 ± 1.1	99.7 ± 1.0	100.4 ± 0.7
Accuracy (recovery, %)[b]	100.2	99.6	100.5	100.3
Precision, RSD (%)				
– Repeatability[c]	0.9	0.9	0.8	0.8
– Intermediate precision[d]	1.0	1.2	1.0	1.0
LOD (μg mL^{-1})[e]	0.31	0.29	0.92	1.21
LOQ (μg mL^{-1})[e]	1.00	0.98	3.06	4.03
Regression line				
– Slope ± RSD (%)	69.2673 ± 1.2	59.289 ± 1.2	0.1687 ± 1.1	0.2220 ± 1.5
– Intercept ± RSD (%)	62.8858 ± 1.7	–14.9302 ± 2.0	–0.3046 ± 1.3	–0.7732 ± 1.4
– Correlation coefficient (R)	0.9999	0.9998	0.9996	0.9997
Linearity range (μg mL^{-1})	1.0–50.0	1.0–50.0	3.0–50.0	4.0–50.0
Robustness (recovery ± SD, %)[f]	100.2 ± 0.8 %	100.3 ± 0.9 %	100.1 ± 0.8 %	100.1 ± 0.7 %

[a] Mean ± SD (n = 3) of laboratory prepared mixtures of AML and ATV spiked with different levels of degradation products, ranging from 10–70 % degradation.

[b] Mean (n = 3) of three concentrations (8, 12 and 16 μg mL^{-1}) for AML and ATV.

[c] Intraday (n = 3), three concentrations (10, 20 and 30 μg mL^{-1}) for AML and ATV repeated three times within the day.

[d] Interday (n = 3), three concentrations (10, 20 and 30 μg mL^{-1}) for AML and ATV repeated three times in three days.

[e] LOD and LOQ were calculated using the following equations: $LOD = \dfrac{3.3\sigma}{S}$ and $LOQ = \dfrac{10\sigma}{S}$ (σ is the residual standard deviation, S is the slope).

[f] Robustness (n = 3), three concentrations (10, 20 and 30 μg mL^{-1}) for AML and ATV.

dissolved in methanol by shaking in an ultrasonic bath for about 30 min. The solution was filtered and transferred quantitatively into four separate 100-mL volumetric flasks. The volume was then completed to the mark with the mobile phase or BGE. Suitable aliquot was transferred into four 10-mL measuring flask, the volume was completed with the mobile phase or BGE and analyzed as described above.

RESULTS AND DISCUSSION

Methods development and optimization

HPLC. – Different columns, such as Zorbax Eclipse C$_8$ (5 μm, 4.6 x 150 mm) and Agilent Zorbax® ODS (5 μm, 4.6 x 250 mm) were investigated. Zorbax® ODS produced sufficient separation of AML from ATV, with different retention times and excellent resolution

from the degradation products. Different mobile phases were investigated. Different ratios of acetonitrile or methanol with water were tested [with organic solvent from 40 to 80 %, no separation was observed, and with different ratios of organic modifiers and water (equal volumes of methanol and acetonitrile with different % of water) no resolution was noted]. Phosphate buffer was then introduced in different percentages instead of water and the pH was adjusted from 2.5 to 8.0 using orthophosphoric acid or triethylamine. The mobile phase of choice was found to be acetonitrile/methanol/phosphate buffer, (45:30:25, *V/V/V*); pH was adjusted to 2.5 ± 0.1 with orthophosphoric acid. UV detection was tested at 238.0 and 254.0 nm (Fig. S5) and the highest sensitivity was obtained using the latter (Fig. 2 and Table I).
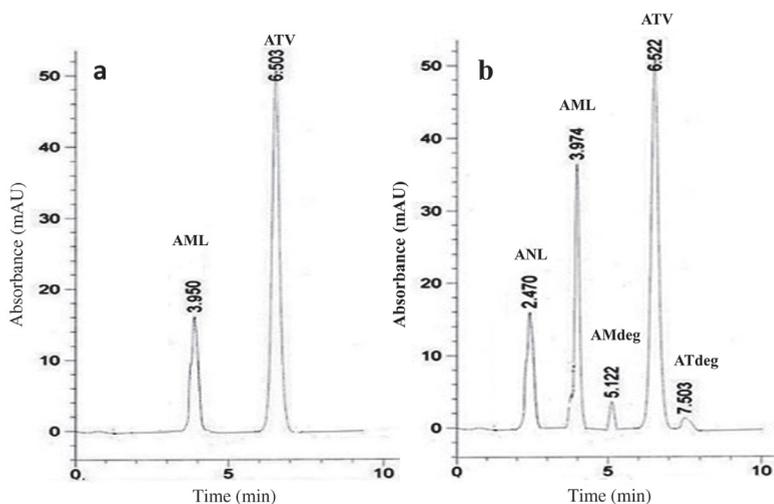

Fig. 2. HPLC chromatogram of: a) Caduet® 5/10 tablets, amlodipine (AML) and atorvastatin (ATV), b) synthetic mixture of the two drugs together with the degradation products, AT_{deg2} (aniline), AML, AM_{deg}, ATV and AT_{deg1}.

CE. – The buffer type, pH and applied voltage are the key factors for optimization of separation. As pK_a of AML is 8.6 while pK_a of ATV is 4.46 and the degradation products were of acidic nature, alkaline pH would be a good choice for applying electric charge to the compounds. Phosphate and borate buffers were tested at pH 8.0 and 10.0. Best results concerning migration time, resolution, peak shape, peak height, baseline noise, and the electric current produced were achieved by borate buffer pH = 8.0. Voltages of 15 and 20 kV were applied and the best separation and migration times were achieved with 15 kV. The detection was tested at 238.0, 244.0 and 200.0 nm (Fig. S5), and the best sensitivity was obtained at 200.0 nm (Fig. 3 and Table I).

Method validation

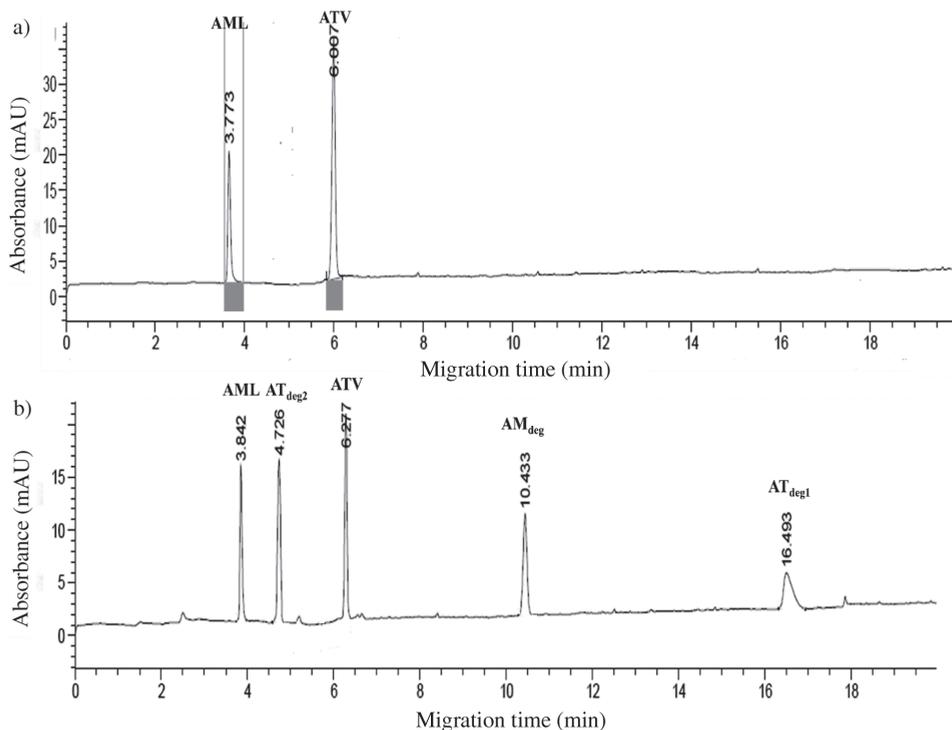The results of method validation are summarized in Table II.

Fig. 3. Electropherogram of: a) Caduet® 5/10 tablets, amlodipine (AML) and atorvastatin (ATV), b) synthetic mixture of the two drugs together with the degradation products, AML, AT_{deg2} (aniline), ATV, AM_{deg} and AT_{deg1}.

Linearity and range. – Concentration ranges were found to be linear in the range of 1–50 μg mL^{-1} for AML and ATV in the HPLC method and 3–50 and 4–50 μg mL^{-1} for AML and ATV, respectively, in the CE method.

Specificity. – Satisfactory recoveries were obtained ranging from 99.4–101.8 % and 98.9–101.8 % for AML and ATV, respectively, in the HPLC method and 98.6–101.6 % and 99.3–101.2 % for AML and ATV, respectively, in the CE method. These results demon-strated that the assay results were unaffected by the presence of the degradation products (see Table SI). Specificity was also assessed by observing the possible interferences from excipients in the analysis of tablets. The obtained chromatograms and electropherograms did not show any additional peaks when compared to those of the synthetic mixtures (see Figs. 2 and 3).

Purity of the peaks was evaluated by DAD in the CE method with the purity factor of 999.876 and 999.583 for AML and ATV, respectively, when their laboratory prepared mix-ture was spiked with the degradation products (see Figs. 3, S6 and S7).

Accuracy. – Satisfactory mean percentage recoveries were obtained, 100.2 and 99.6 % for AML and ATV, respectively, in the HPLC method, and 100.5 and 100.3 % for AML and

ATV, respectively, in the CE method (Table II). The accuracy was confirmed using the standard addition technique (Table III).

Precision. – In the HPLC method, repeatability RSD was 0.9 % for both AML and ATV. In the CE method, it was 0.8 % for both AML and ATV. In HPLC, intermediate RSD was 1.0 and 1.2 % for AML and ATV, respectively, and it was 1.0 % for both in the CE method. The RSD being below 1.5 % for both methods indicated reasonable repeatability and inter-mediate precision.

LOD and LOQ. – In the HPLC method, the *LOD* for AML and ATV was 0.31 and 0.29 µg mL^{-1}, respectively, and it was 0.92 and 1.21 µg mL^{-1} for AML and ATV, respectively, in the CE method. The *LOQ* in the HPLC method was 1.00 and 0.98 µg mL^{-1} for AML and ATV, respectively, and it was 3.06 and 4.03 µg mL^{-1} for AML and ATV, respectively, in CE method.

Robustness. – Small changes, as defined in the Experimental part, did not affect the recovery of the drugs or the system suitability parameters of the proposed methods. Re-coveries ranged from 100.1 to 100.3 % and RSD below 1 % indicating that the methods were robust enough regarding these changes (Tables SII and SIII). (RSD was 0.8 and 0.9 % for AML and ATV, respectively, in HPLC, and 0.8 % for both AML and ATV in the CE method).

Table III. Determination of AML and ATV in Caduet® tablets by the proposed methods and application of the standard addition technique

Product	Drug	HPLC (found, mg mL^{-1})[a]	CE (found, mg mL^{-1})[a]	Taken (µg mL^{-1})	Added (µg mL^{-1})	Standard addition HPLC Recovery ± SD (%)[a]	CE Recovery ± SD (%)[a]
Caduet® 5/10	AML	4.97 ± 0.68	5.02 ± 0.87	10	5.00	99.4 ± 0.9	101.6 ± 0.9
					10.00	100.4 ± 0.6	99.9 ± 0.6
					15.00	99.2 ± 1.0	100.2 ± 1.1
	ATV	9.98 ± 0.57	10.00 ± 1.19	20	10.00	99.2 ± 0.8	99.1 ± 0.9
					20.00	99.3 ± 1.1	98.8 ± 0.8
					30.00	101.0 ± 0.7	100.1 ± 1.0
Caduet® 10/10	AML	10.08 ± 0.59	10.08 ± 0.41	20	10.00	99.6 ± 1.0	99.7 ± 0.7
					20.00	100.9 ± 1.2	101.0 ± 0.9
					30.00	98.7 ± 0.9	101.4 ± 0.8
	ATV	9.98 ± 0.72	10.05 ± 0.89	20	10.00	99.6 ± 1.1	99.7 ± 1.0
					20.00	99.6 ± 0.6	101.0 ± 0.9
					30.00	100.9 ± 1.0	100.6 ± 0.5

[a] Average ± SD (*n* = 3).

Analyses of tablets

Accuracy of the methods was further assured by the comparison of the results of the assay of the studied drugs in the pharmaceutical formulations with those of a reported HPLC method (23). Statistical analysis of the results using Student's *t*-test and variance ratio *F*-test revealed no significant differences between the performance of the new methods and the literature method (Table IV).

Table IV. Statistical analysis of the results obtained by the proposed methods and the reported HPLC method for the analysis of AML and ATV in Caduet® 5/10 tablets

Value	HPLC method		CE method		Reported HPLC method (ref. 23)[a]	
	AML	ATV	AML	ATV	AML	ATV
Mean (%)	100.1	99.8	100.6	100.3	99.9	100.4
RSD (%)	0.9	0.6	0.7	1.0	1.2	1.0
n	6	6	6	6	6	6
Student's *t*-test (2.228)[b]	0.301	1.194	1.255	0.153	–	–
F-value (5.05)[b]	1.635	2.937	3.350	1.038	–	–

[a] Perfectsil® Target ODS-3 column using acetonitrile: 0.025 mol L^{-1} NaH_2PO_4 buffer pH 4.5 (55:45, *V/V*) at 1 mL min^{-1} and UV detection at 237 nm.

[b] Values in the parentheses are the corresponding theoretical values of *t* and *F* at *p* = 0.05.

CONCLUSIONS

Acidic degradation products of AML and ATV were isolated and their structures were elucidated. The main criteria for the development of a successful separation method for determination of AML and ATV in tablets were that the method should be able to determine drugs in a single run and should be accurate, precise, robust, free of interference from excipients or degradation products and simple enough for use in the quality control laboratories. The proposed HPLC and CE methods were selective, precise, accurate and simple- Degradation products did not interfere with the determination of drugs and therefore the assay was considered stability-indicating. The proposed methods can be used for the routine analysis of AML and ATV in pharmaceutical formulations in the presence of their acidic degradation products. Unlike the other stability indicating methods, the degradation products were isolated and identified in this work. The CE method showed better resolution and chromatographic parameters than the HPLC method. In addition, CE shows better environmental acceptability and is considered a green method, since no harmful organic solvents are used. The known disadvantage of CE is its being of lower sensitivity than HPLC, but this was not of great significance in this work as the application was in a pharmaceutical dosage form.

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