Chemical Papers
https://doi.org/10.1007/s11696-019-00792-z

ORIGINAL PAPER

Two validated stability-indicating chromatographic methods for the separation of two anti-hypertensive combinations in the presence of their degradation products or impurities

Noha Samy Mostafa[1] · Ghada AbdElHamid[1] · Hala Elsayed Zaazaa[1] · Sawsan Mohamed Amer[1]

Received: 17 December 2018 / Accepted: 19 April 2019

Abstract
Two RP-HPLC methods were developed, optimized, and validated for the determination of two different anti-hypertensive combinations in the presence of their degradation products or impurities and in their pharmaceutical formulations. The first mixture is Ramipril (RAM) in combination with Amlodipine besylate (AML) [mixture I], while the second one is a combination of Ramipril (RAM), Atorvastatin (ATV), and Aspirin (ASP) [mixture II].The proposed combinations were successfully separated on X-bridge C_{18}column (250×4.6 mm i.d, 5 µm p.s.), using a mobile phase of 0.05 M phosphate buffer-acetonitrile-THF (60:40:0.1% by volume) pH 2.5 and an isocratic mobile phase formed of acetonitrile-0.05 M phosphate buffer-THF (60:40:0.1% by volume) pH 2.5 for mixture (I) and (II) at a flow rate of 1 mL/min and 1.2 mL/min, respectively. The compromising components of the mixtures were detected at 218 nm. For the best separation of the mentioned components different parameters were examined and optimized. The two suggested methods were validated in compliance with the ICH guidelines and were successfully applied for the quantification of the cited components in presence of their obtained degradation products as well as in their commercial pharmaceutical formulations. For both methods the obtained results were statistically analyzed and compared to those of the official and reported methods; using Student's *t* test and *F* test showing no significant difference with high accuracy and good precision.

Keywords Anti-hypertensive combination · Stability-indicating · Liquid chromatography

Introduction

Ramipril (RAM) [2S,3aS,6aS)-1-[(2S)-2-[[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-1-oxopropyl] octahydrocyclopenta[b]pyrrole-2-carboxylic acid)], Fig. 1a, is an angiotensin-converting enzyme (ACE) inhibitor used in the management of mild to severe hypertension(Moffat et al. 2011). It is a prodrug which after absorption undergoes rapid metabolic ester hydrolysis to the active diacidic form ramiprilat (Evoy 2009).

Amlodipine (AML) is chemically known as [3-*O*-ethyl-5-*O*-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate], Fig. 1b. It is a dihydropyridine calcium channel blocker which is used for the management of hypertension and angina (Moffat et al. 2011; Brunton et al. 2006).

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, Fig. 1c, is an HMG-CoA reductase inhibitor used to reduce LDL-cholesterol and triglycerides (Moffat et al. 2011) (Brunton et al. 2006).

Aspirin (ASP) is 2-acetyloxybenzoic acid, Fig. 1d, which is an acetyl derivative of salicylic acid useful in the relief of headache and muscle and joint aches. Aspirin is also effective in reducing fever and swelling (Moffat et al. 2011). It is often used as an analgesic, antipyretic, anti-inflammatory and antiplatelet agent (The Merk index).

Cardace am 5® launched by Sanofi is a combination of two medicines Ramipril (RAM) and Amlodipine (AML) which lowers blood pressure effectively. It acts by relaxing blood vessels so that blood flows more smoothly and the heart can pump blood more efficiently.

✉ Noha Samy Mostafa
noha.ayesh@pharma.cu.edu.eg

[1] Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr Elaini St, P.O. box 11562, Cairo, Egypt

Published online: 30 April 2019

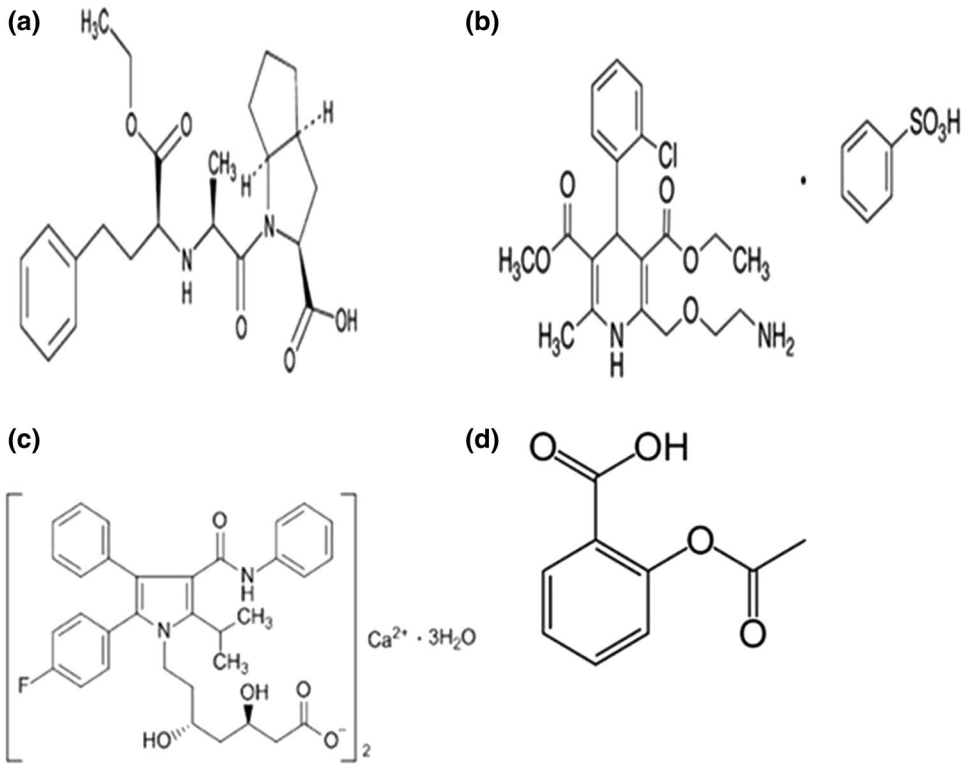

Fig. 1 Structural formula of, **a** RAM, **b** AML, **c** ATV and **d** ASP

Ramipril (RAM), Atorvastatin (ATV), and Aspirin (ASP) were co formulated together in Ramitorva® manufactured by Zydus cardiva. It can be used for the treatment of high cholesterol, high blood pressure, prevention of heart attack, and risk of strokes.

A literature survey revealed that RAM, AML, ATV, and ASP are all official drugs in the British Pharmacopoeia (British Pharmacopoeia, Medicines and Healthcare Products Regulatory Agency (MHRA), London 2013). Up to our recent knowledge different methods have been applied for simultaneous estimation of the binary mixture of RAM and AML (Mixture I) in their bulk powder and pharmaceutical formulation; namely UV spectroscopic method (Patil et al. 2009), thin-layer chromatographic separation (Gupta et al. 2007), high-performance liquid chromatography (Babu et al. 2011; Kumar et al. 2012; Maste et al. 2011; Patel and Patel 2014; Rajput et al. 2012; Ramadevi et al. 2013) and one in the presence of stated impurities which are not the main degradation products (Dai et al. 2013). Also, for the ternary mixture of RAM, ATV, and ASP (Mixture II), different techniques have been applied for their simultaneous determination, including chemometrics-assisted UV-spectroscopic techniques (Sankar et al. 2011a, b) and HPLC (Panchal et al. 2009; Sharma et al. 2012; Patole et al. 2010) in their bulk powder and pharmaceutical formulated capsules. Moreover, they were detected by LC/MS in plasma (Gajula et al. 2012), another stability-indicating UPLC method was reported for a quaternary mixture of RAM, ATV, ASP, and Metoprolol; however, the possible stated degradation products were neither isolated nor characterized (Shetty et al. 2011).

This manuscript aims to establish and validate a stability-indicating HPLC method for the simultaneous determination of RAM either in combination with AML or ATV and ASP in the presence of their hydrolytic degradation products or impurities in their bulk powder and in their pharmaceutical formulations.

The stability-indicating behavior of the proposed drugs was studied by subjecting them to different stress conditions. The stressed samples were analyzed using the proposed (RP)-HPLC methods, which were able to separate the drugs from the compounds produced during the forced degradation studies.

Experimental

Instruments

The liquid chromatography system consisted of an isocratic pump, Model G1310A (Agilent 1100 series liquid chromatography, Germany), an ultraviolet (UV) variable wavelength detector (Model G1314A, Agilent 1100 Series, Germany), a Rheodyne injector, Mode 7725 I (Sigma-Aldrich, Taufkirchen bei Mu¨nchen, Germany) equipped with a 20 mL injector loop (Agilent Technologies, Germany).

The stationary phase was X-bridge RP-C_{18} column (250 × 4.6 mm i.d, 5 µm p.s.).

TLC plates 20 × 20 cm (Sigma-Aldrich, Germany) 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, for degradation tracing.

Jenway 3505 pH meter (Staffordshine, UK), for pH adjustment.

Sonix TVss-series ultrasonicator (USA).

UPLC MS/MS "Waters" 3100 "USA", the sample injected directly on TQ Detector (Acquity ultra performance LC) coupled with Software Mass lynx V4 (programmed on +ve mode).

Materials and reagents

Pure standard

Standard RAM, AML, ATV, and ASP, were supplied by Rameda Pharmaceutical Company (Cairo, Egypt), their purities were assessed according to the official methods (British Pharmacopoeia, Medicines and Healthcare Products Regulatory Agency (MHRA), London 2013) and found to be (99.85% ± 0.71), (100.17% ± 0.99), (99.88% ± 0.97), and (99.68% ± 0.45); respectively. Salicylic acid as aspirin's main impurity was purchased from Sigma Aldrich, where its certified purity is (99.95%).

Phamaceutical formulations

- Cardace am 5® 5/5 mg tablets of RAM and AML were manufactured by Sanofi, India.
- Ramitorva® capsules of 5/10/75 mg RAM, ATV and ASP, respectively, were manufactured by Zydus Cardiva, India.

Both formulations were purchased from the Indian market.

Chemicals and reagents

All chemicals used throughout this work were of high analytical grade, and the solvents were of HPLC grade. These included concentrated hydrochloric acid, 33% ammonia solution (0.88 gm/mL), toluene, ethyl acetate, sodium hydroxide, methanol and chloroform (El-Nasr Pharmaceutical Chemicals, Egypt), tetrahydrofuran (THF), acetonitrile (Sigma-Aldrich, Germany), O-phosphoric acid (85%) (BDH, Poole, England), de-ionized water, and bi-distilled from an Aquatron Automatic Water Still A4000 (Bibby Sterillin Staffordshire, UK).

Degraded samples

RAM was exposed to acidic and alkaline stress conditions. It showed complete degradation upon refluxing with 1 N NaOH and 2 N HCl for 5 h producing two degradation products namely ramiprilat, the active form, and Ramipril DKP (Aschar et al. 2015).

For AML, upon alkaline hydrolysis with 1 N NaOH for 1 h it gives out AML deg, the same degradation product is formed after 2 h of exposure to 2 N HCl. All the hydrolyzed solutions were cooled and neutralized to pH 7.0 and the solutions were further tested for complete degradation by TLC using 33% ethyl acetate–methanol–toluene—33% ammonia solution (6.5:2:1:0.5 by volume) as a developing system (Zaazaa et al. 2012).

ATV showed a complete degradation using 6 N HCl for 3 h and resulting into two degradation products ATV deg_1 (aniline) and ATV deg_2 (Darwish et al. 2016; Hassan et al. 2016).

ATV-degraded solution was then cooled and neutralized to pH 7.0 with NaOH and the solutions were tested for complete degradation by TLC using toluene–methanol (70:30, V/V) as a developing system for ATV (Hassan et al. 2016). All the degradation product solutions were extracted with 10 mL methanol three times to ensure complete extraction and purification, and then the solutions were evaporated.

Standard solutions

Stock standard solutions of Ramipril and Amlodipine (0.1 mg/mL), and their degradation products derived from the complete degradation of RAM and AML (1 mg/mL), were prepared in methanol then further diluted using the mobile phase of 0.05 M phosphate buffer–acetonitrile–tetrahydrofuran (60: 40: 0.1% by volume) and adjusted to a pH of 2.5 ± 0.2 with 85% phosphoric acid for mixture [I].

Meanwhile for mixture [II]; Ramipril, Atorvastain, and Aspirin (0.1 mg/mL) and stock standard solutions of the degradation products derived from the complete degradation of standard solutions of both RAM and ATV (1 mg/mL) and salicylic acid—Aspirin main impurity—were all prepared in methanol then further dilutions were made up using mobile phase acetonitrile-0.05 M phosphate buffer solution—tetrahydrofuran (60: 40: 0.1% by volume) and adjusted to a pH of 2.5 ± 0.2 with 85% phosphoric acid.

All stock standard solutions were freshly prepared and stored in the refrigerator to be used for up to 3 weeks.

Procedures

Chromatographic conditions

HPLC was conducted on an X-bridge RP-C_{18} column (250 × 4.6 mm i.d, 5 µm p.s). The mobile phase for mixture **[I]** consisted of 0.05 M phosphate buffer–acetonitril–tetrahydrofuran (60:40:0.1% by volume) and acetonitrile-0.05 M phosphate buffer solution—tetrahydrofuran (60:40:0.1% by volume) for mixture **[II]** where the final pH of both mobile phases was adjusted to pH 2.5 ± 0.2 using O-phosphoric acid, filtered through a 0.45 mm Millipore membrane filter (Billerica, MA) and were degassed for 30 min in an ultrasonic bath prior to usage. The system was operated at ambient temperature with a flow rate of 1 mL/min and 1.2 mL/min for mixture [I] and [II], respectively, and UV detection at 218 nm. Seeking good equilibrium, the analysis was usually performed after passing 50–60 mL of the mobile phase, for conditioning and pre-washing of the stationary phase.

Linearity

For (Mixture I), standard stock solutions (0.1 mg/mL) of RAM and AML were further diluted with the specific mobile phase to obtain dilutions of RAM and AML in the ranges of (5–50 µg/mL). Triplicates of 20 mL volumes were injected and chromatographed for each solution. The relative peak area ratios, using 30 µg/mL of RAM and AML as a divisor for RAM and AML, respectively, were calculated and plotted against the corresponding concentrations to obtain the calibration graph for each component.

For (Mixture II), standard stock solutions (0.1 mg/mL) of RAM, ATV, and ASP were further diluted with the specified mobile phase to obtain dilutions of RAM in the range of (5–50 µg/mL), while both ATV and ASP working solutions were in the range of (2–16 µg/mL). A volume of 20 mL of each solution was injected in triplicate and chromatographed under the previously mentioned conditions. Using 30 µg/mL of RAM and 10 µg/mL of either ATV or ASP as a divisor, relative peak areas were calculated and plotted against the corresponding concentrations to obtain the calibration graph for each. And the regression equations were computed for each component in both mixtures.

Assay of pharmaceutical formulations

Ten tablets of Cardac am 5® claimed to contain 5 mg of both RAM and AML were weighed, finely powdered and thoroughly mixed. An accurately weighted portion of the powder equivalent to the weight of one tablet was transferred into 100 mL volumetric flask, 50 mL acetonitrile was added, the mixture was sonicated for 30 min. Volumes were completed with the same solvent and then filtered. Suitable dilution was achieved using the appropriate mobile phase to obtain concentrations of (5 µg/mL) of each of RAM and AML for analysis of mixture [I].

The contents of 5 capsules of Ramtrov® claimed to contain (5/10/75 mg of RAM, ATV, and ASP, respectively) was quantitatively transferred into 100 mL volumetric flask, 50 mL acetonitrile was added, the mixture was sonicated for 30 min. Volume was completed and then filtered. Suitable dilution was achieved using the appropriate mobile phase to obtain concentrations of (5, 10, and 7.5 µg/mL) for RAM, ATV, and Aspirin, respectively, for mixture [II]. Then the previously detailed procedures were followed.

Results and discussion

Ramipril was combined in two different formulations, the first combination is with Amlodipine, to manage severe hypertensive cases, and the second one is with Atorvastatin and Aspirin that plays a great role in hypertensive cases with the risk of hyperlipidemia and strokes. It gives a rise to more compliance routes of treatment that the patient instead of taking two or three dosage forms, he takes all in one. From here arises the importance of our work, which gives rise to two simple, rapid, precise and time-saving stability-indicating HPLC methods, that could be applied in everyday quality control assay of the above-mentioned combinations, moreover, it can check for any extent of degradation in their pharmaceutical formulations.

Stability-indicating methods are an integral part of the process of drug product quality control. Thus in the present work a stability study was carried out with complete separation and elucidation of the major degradation products of two commonly used anti-hypertensive combinations as recommended by the ICH guidelines (ICH, Q1A (R2) 2003) and USP (2007).

In this study Ramipril was subjected to alkaline, acidic, and oxidative hydrolysis using 1 N NaOH, 2 N HCl, and 30% H_2O_2, respectively, under reflux for 5 h. All of them resulted in the formation of two degradation products namely ramiprilate (RAM deg_1) which was found to be RAM active metabolite, and ramipril DKP (RAM deg_2) (Aschar et al. 2015).

Both degradates are stated in the British Pharmacopoeia as main impurities; impurity E and impurity D for ramiprilate and RAM DKP, respectively (British Pharmacopoeia, Medicines and Healthcare Products Regulatory Agency (MHRA), London 2013). Although it is one of the most widely used ACE inhibitors, the drug has considerable degree of instability, and the degradation is shown by two major pathways: hydrolysis, forming ramipril-diacid then

cyclization by inter-nucleophilic attack leading to the formation of diketopiperazine (DKP) (Elshanawane et al. 2008; De Diego et al. 2010).

But oxidative degradation resulted only in partial hydrolysis which was confirmed by the appearance of intact RAM peak under the mentioned HPLC condition. The elucidation of Ramipril degradation products was confirmed by LC/MS programmed on +ve mode, showing the disappearance of Ramipril peak at 417.41 m/z and the appearance of the two main degradation products with peak at 389.28 m/z and 399.60 m/z, Fig. 2, for ramiprilate and ramipril DKP, respectively. The suggested pathway of degradation is summarized in Fig. 3a.

Amlodipine was subjected to alkaline hydrolysis by 1 N NaOH for 1 h under reflux and acid hydrolysis using 2 N HCl for 2 h where the complete degradation was confirmed by TLC using ethyl acetate–methanol–toluene–ammonia solution, 33% (6.5:2:1:0.5 by volume) as the developing solvent, where a spot other than the intact AML was observed, which ensures the formation of the same degradation product AML deg. under both hydrolytic conditions. The suggested degradation pathway is presented in Fig. 3b, which indicates hydrolysis of the ester linkages and release of the free alcohols, mass was preformed and compared to the reported one (Zaazaa et al. 2012; Darwish et al. 2016; A.Hassan et al. 2016).

But upon oxidation of AML using 30% H_2O_2 for 5 h, and its application on TLC using the previously mentioned system, no degradation was characterized.

For ATV degradation was carried out under reflux with 6 N NaOH, 6 N HCl for 3 h and oxidative hydrolysis with 30% H_2O_2 for 5 h. Only 6 N HCl showed the complete degradation of ATV which was resulted by the cleavage of the amide bond-producing aniline (ATV deg_1) and (ATV deg_2) with free carboxylic acid group as shown in Fig. 3c, where the degradation process was monitored by spotting on TLC plates using toluene: methanol (7.0: 3.0 v/v) as developing solvent, and it was found that ATV degrades completely after 3 h. The solid ATV deg_2 was extracted from NaCl, after neutralization and evaporation of the solution, by methanol, while ANL was collected with the evaporated solution and extracted after heating at 100 °C to expel the water, confirmation by mass was found identical to the published literature (Darwish et al. 2016). ASP is an ester moiety, which is very susceptible to hydrolysis under different hydrolytic conditions. ASP is known to undergo decomposition by hydrolysis into salicylic acid, when exposed to moisture. It is reported that the decomposition reaction is promoted at high temperature, in alkaline solutions (Zoglio et al. 1986).

Salicylic acid is the precursor and Aspirin main degradation product, it has keratolytic action and bactericidal effect which leads to its usage in topical preparations (Moffat et al. 2011), it is stated in the British pharmacopeia as one of Aspirin main impurities.

Two simple, accurate, and precise RP-HPLC methods were investigated and validated for quantitative analysis of RAM in combination either with AML or with ATV and ASP. The procedures were carried out with a view to develop stability-indicating methods in a reasonable time and with high resolution for the proposed components. Mixture [I] is supposed to contain five components; namely RAM, ramiprilate, ramipril DKP, AML, and AML deg_1. While Mixture [II] is supposed to contain eight components; namely RAM, ramiprilate, ramipril DKP, ATV, ATV deg_2, aniline, ASP and its main impurity salicylic acid.

Parameters affecting the efficiency of the chromatographic separation were tested and optimized in a trial to obtain the best separation for the cited components of both mixtures. The pH of the mobile phase was found to have a great influence on the separation of the components; mainly Ramipril, where pH 2.5 was found to be optimum. Many trials for obtaining the optimum ratio for acetonitrile and phosphate buffer to obtain the best resolution for all mixture components and a reasonable run time were performed. For Mixture [I] the best resolution was achieved upon using a mobile phase consisting of 0.05 M phosphate buffer–acetonitrile–tetrahydrofuran (60:40:0.1% by volume) with pH 2.5 on X-bridge column (250 × 4.6 mm i.d, 5 µm p.s.) under flow rate of 1 mL/min. Scanning was tried at 230, 225, and 218, the scanning at 218 nm which is RAM λ_{max} has showed reasonable sensitivity for the active components. Under these chromatographic conditions, RAM, AML, ramiprilate, ramipril DKP and AML deg. were eluted at 6.23, 7.65, 3.90, 4.85, and 3.40 min ± 0.2, respectively, as shown in Fig. 4.

For Mixture [II] the best resolution was achieved when using a mobile phase consisting of acetonitrile–0.05 M phosphate buffer–tetrahydrofuran (60:40:0.1, by volume) adjusted to pH 2.5, using X-bridge column (250 × 4.6 mm i.d, 5 µm p.s.) under flow rate of 1.2 mL/min at 218 nm. Under the stated chromatographic conditions; peaks for RAM, ATV, ASP, ramiprilate, ramipril DKP, aniline, ATV deg. 2, and salicylic acid appeared at 2.9, 6.29, 3.41, 4.84, 5.28, 2.43, 3.89, and 4.10 min + 0.2, respectively, as shown in Fig. 5.

To validate the performed chromatographic methods, overall system suitability tests (Snyder et al. 2011) were conducted to evaluate the performance of the applied methods; Table 1. ICH guidelines for method validation [ICH, Q1A (R2) 2003] were accomplished for validation of the suggested methods.

Under the previously described experimental conditions, linear relationships were obtained by plotting the drug concentrations versus the relative peak areas for each drug. The corresponding concentration ranges, calibration equations,

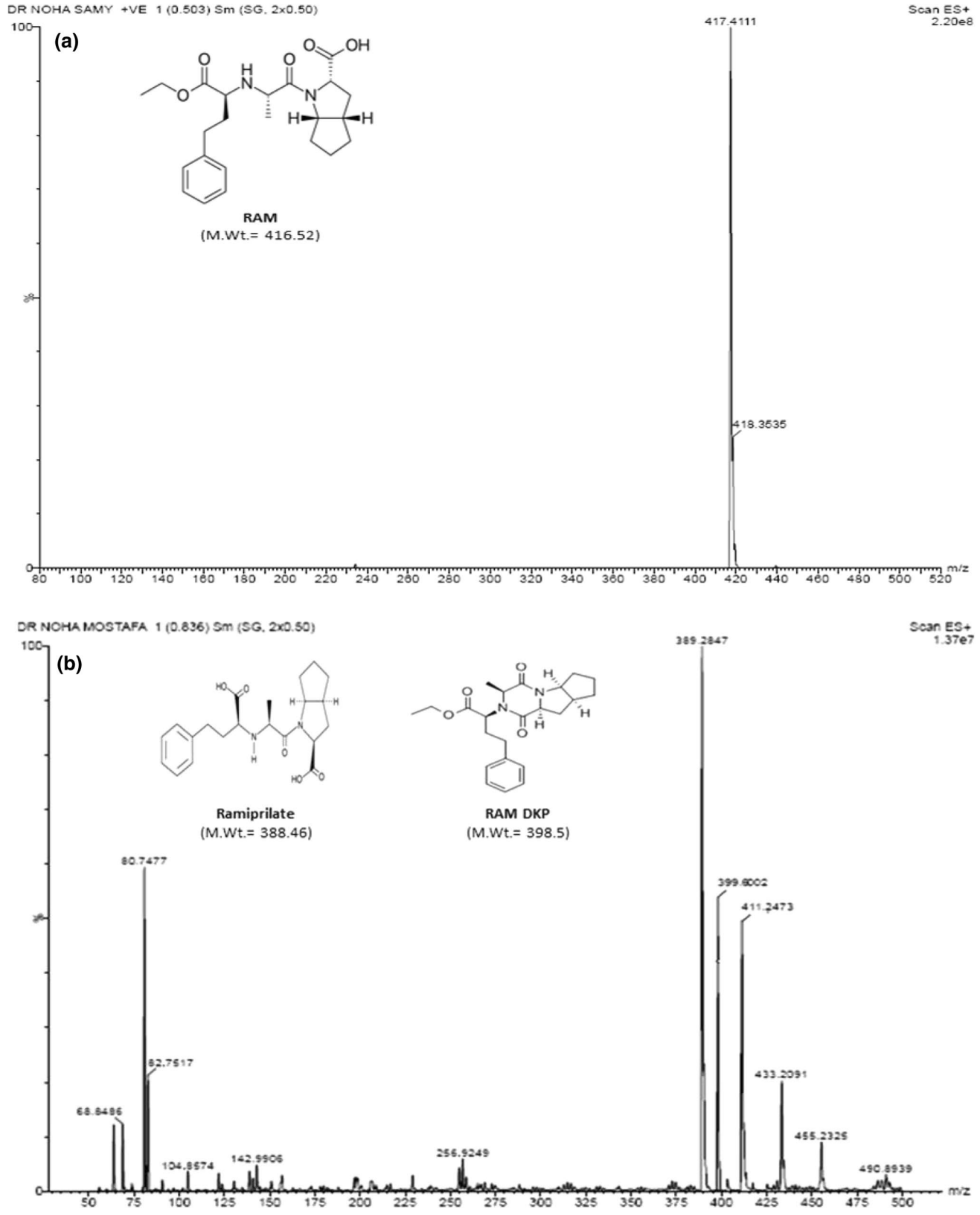

Fig. 2 Mass spectra of, **a** RAM and **b** ramiprilate and ramipril DKP

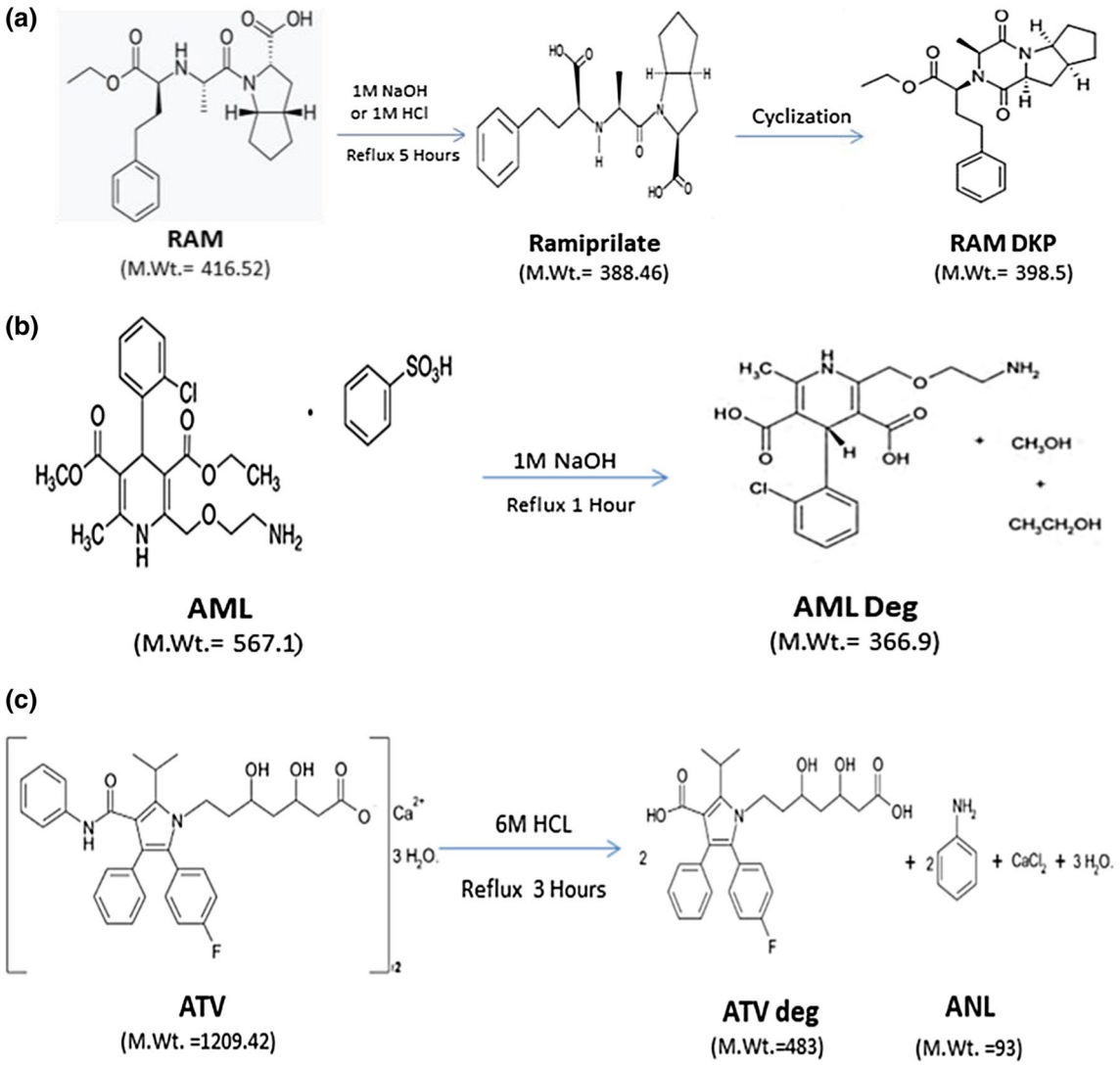

Fig. 3 Suggested degradation pathways for **a** RAM, **b** AML and **c** ATV

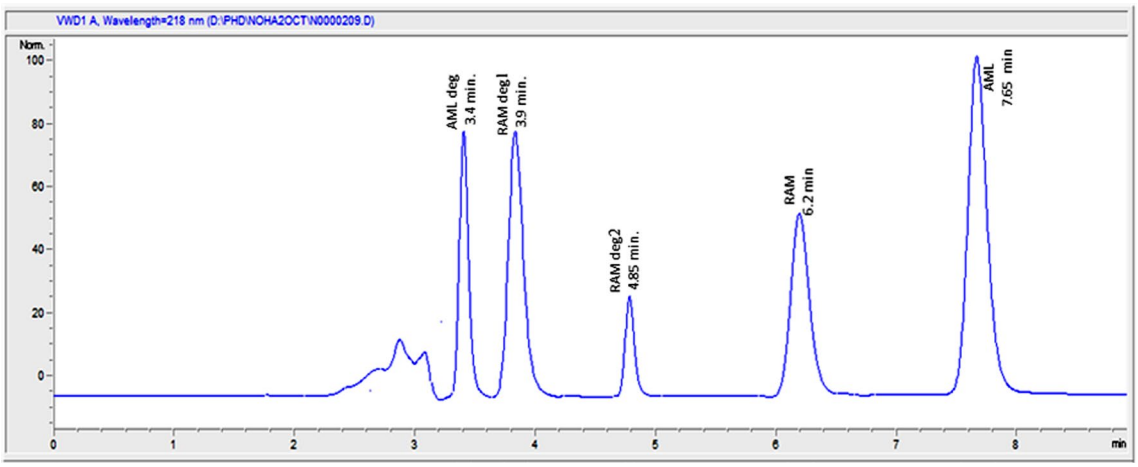

Fig. 4 HPLC chromatogram showing the separation of RAM (6.23 min), AML (7.65 min), RAM deg_1 (3.90 min), RAM deg_2 (4.85 min), and AML deg at (3.40 min), using phosphate buffer (pH 2.5, 0.05 M)-acetonitril-THF (60: 40: 0.1% by volume) as mobile phase

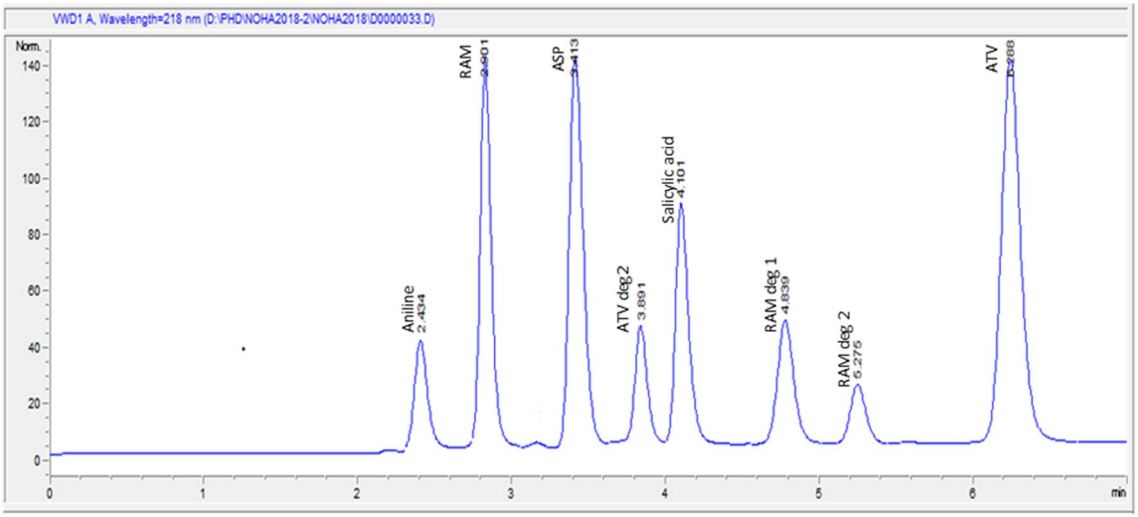

Fig. 5 HPLC chromatogram showing the separation of RAM (2.90 min), ATV (6.30 min), ASP (3.41 min), RAM deg_1 (4.83 min), RAM deg_2 (5.28 min), ATV deg_1 (ANL) at (2.43 min), ATV deg_2 (3.89), and salicylic acid at (4.10 min) using acetonitril-phosphate buffer (pH 2.5, 0.05 M)-THF (60: 40: 0.1% by volume) as mobile phase

limit of detection (LOD) and limit of quantification (LOQ) and other statistical parameters are listed in Table 2.

Achievement of method specificity was taken with analysis of different laboratory prepared mixtures of RAM and AML, RAM, ATV, and ASP for Mixture I and II, respectively, spiked with different levels of degradation products, ranging from 10–70% degradation Table 2.

The accuracy of the investigated methods was validated by analyzing pure samples of RAM and AML for mixture [I], and RAM, ATV, and ASP for mixture [II], while precision was evaluated by calculating intra-day and inter-day precision. Good results are shown in Table 2.

To determine the robustness of the developed HPLC methods, experimental conditions were subjected to minor changes in flow rate, pH value and acetonitrile composition and the tailing factor, capacity factor and resolution between the studied drugs were recorded for mixture I and II, respectively (Tables 3, 4).

The two proposed HPLC methods were successfully applied to determine Ramipril and Amlodipine in Cardac am 5® tablets 5/5, also Ramipril, Atorvastatin and Aspirin in Ramitorva® capsules using the previously mentioned conditions stated for each mixture, Table 5.

The results obtained for the analysis of RAM, AML, ATV, and ASP in their pure form by the proposed HPLC methods were statistically compared to those obtained by applying the official methods [British Pharmacopoeia, Medicines and Healthcare Products Regulatory Agency (MHRA), London 2013]. The calculated t and F values were less than the tabulated ones, which revealed that there is no significant difference between the two methods with respect to accuracy and precision, Table 6.

To investigate the accuracy of the pharmaceutical formulation analysis, the results obtained were compared statistically to those from reported methods (Patel and Patel 2014; Sharma et al. 2012) for Mixtures [I] and [II]; respectively, using Student's *t* test and the variance ratio F-test. The results showed no significant differences between the results obtained from these methods and the published ones Table 7.

Table 1 Statistical analysis of parameters required for system suitability testing of hplc method for Mixture I and II

Parameters	Mixture I				Mixture II						Reference values
	RAM		AML		RAM		ASP		ATV		
$t_{R\,(}$relative retention time)	6.23 ± 0.03		7.65 ± 0.02		2.9 ± 0.03		3.4 ± 0.03		6.3 ± 0.04		> 1
N (column efficiency)	5013.91		6907.2		2592		3317.76		6881.3		$N > 2000$ Increases with efficiency the efficiency of the separation
K' (capacity factor)	3.24		4.20		1.00		1.34		3.34		1–10 acceptable
α (separation factor)	1.29				1.34		2.49				> 1
	RAM	RAM deg2	AML	AML deg	RAM	RAM deg1	ASP	SA	ATV	ATV deg1	
	1.41		3.08		2.33		1.31		2.01		
HETP (height equivalent to theoretical plates)	0.00299		0.00217		0.00579		0.00452		0.00218		The smaller the value, the higher the column efficiency
T (tailing factor)	1.06		1.00		0.88		0.94		1.06		$T < 2$ T = 1 for symmetric peak
Rs (experimental resolution)	4.11				2.48			5.24			Rs > 2
	RAM	RAM deg2	AML	AML deg	RAM	RAM deg1	ASP	SA	ATV	ATV deg1	
	11.36		10.46		9.92		3.33		11.26		

Table 2 Validation parameters for the simultaneous determination of the sited components in mixture I and II by the proposed HPLC methods

Parameters	Mixture (I)		Mixture (II)		
	RAM	AML	RAM	ATV	ASP
Specificity (recovery ± SD, %)[a]	100.45 ± 1.12	100.09 ± 0.73	99.68 ± 0.87	100.29 ± 1.20	99.72 ± 1.14
Accuracy (recovery, %)[b]	100.56	101.33	100.22	100.35	99.98
Precision, RSD (%)					
Repeatability[c]	0.714	0.542	0.687	0.435	0.215
Intermediate precision[d]	0.759	0.580	0.868	0.924	0.439
LOD (µg/mL)[e]	0.54	0.69	0.43	0.3	0.6
LOQ (µg/mL)[e]	1.91	2.05	1.84	1.56	1.41
Regression line					
Slope	0.0286	0.0085	0.0337	0.0043	0.0485
Intercept	0.0328	0.0334	0.0321	0.0995	0.0935
Correlation coefficient (*R*)	0.9999	0.999	0.999	0.999	0.999
Linearity range (µg/mL)	5–50	5–50	5–50	2–16	2–16

[a]Mean ± SD ($n = 3$) of laboratory prepared mixtures of each mixture spiked with different levels of degradation products, ranging from 10 to 70% degradation

[b]Mean ($n = 4$) of three concentrations (15, 25, 35, and 45 µg/mL) for RAM and AML (3. 5, 9, and 15) for ATV and ASP

[c]Intraday ($n = 3$), three concentrations (10, 30, and 50 µg/mL) for RAM and AML, (4, 8, and 16 µg/mL) for ATV and ASP repeated three times within the day

[d]Interday ($n = 3$), three concentrations (10, 30, and 50 µg/mL) for RAM and AML, (4, 8, and 16 µg/mL) for ATV and ASP repeated three times in three days

[e]LOD and LOQ were calculated using the following equations: LOD = 3.3 σ/S and LOQ = 10 σ/S

Table 3 Robustness of the proposed HPLC methods for mixture I

Drug	Robustness parameter		T^a	K'^a	R_s^b	% Assay[c]
RAM	Flow rate	1.0 + 0.1 mL/min	1.05	3.01	–	100.59
		1.0 − 0.1 mL/min	1.07	3.03	–	99.92
	pH values	2.5 + 0.1 units	1.09	3.13	–	99.45
		2.5 − 0.1 units	1.08	3.15	–	100.71
	Acetonitrile composition	40 + 2%	1.09	2.84	–	99.19
		40 − 2%	1.07	3.09	–	100.83
AML	Flow rate	1.0 + 0.1 mL/min	0.90	4.18	4.12	100.24
		1.0 − 0.1 mL/min	1.10	4.22	4.13	99.41
	pH values	2.5 + 0.1 units	1.01	4.21	4.11	99.09
		2.5 − 0.1 units	1.0	4.22	4.12	99.73
	Methanol composition	40 + 2%	1.03	4.23	4.14	100.33
		40 − 2%	1.05	4.22	4.13	100.92

Table 4 Robustness of the proposed HPLC methods for mixture II

Drug	Robustness parameter		T^a	K'^a	R_s^b	% Assay[c]
RAM	Flow rate	1.2 + 0.1 mL/min	0.87	1.01	–	99.38
		1.2 – 0.1 mL/min	0.89	1.02	–	100.12
	pH values	2.5 + 0.1 units	0.88	1.02	–	100.54
		2.5 – 0.1 units	0.87	1.01	–	99.61
	Acetonitrile composition	60 + 2%	0.89	1.05	–	99.79
		60 – 2%	0.87	1.03	–	99.65
ASP	Flow rate	1.2 + 0.1 mL/min	0.92	1.32	2.49	100.32
		1.2 – 0.1 mL/min	0.96	1.31	2.47	100.21
	pH values	2.5 + 0.1 units	0.95	1.33	2.42	99.42
		2.5 – 0.1 units	0.93	1.31	2.43	100.71
	Acetonitrile composition	60 + 2%	0.94	1.35	2.45	99.32
		60 – 2%	0.93	1.34	2.43	99.73
ATV	Flow rate	1.2 + 0.1 mL/min	1.08	3.32	5.26	99.51
		1.2 – 0.1 mL/min	1.04	3.35	5.22	100.72
	pH values	2.5 + 0.1 units	1.09	3.35	5.23	99.49
		2.5 – 0.1 units	1.05	3.36	5.25	100.15
	Acetonitrile composition	60 + 2%	1.10	3.33	5.21	100.31
		60 – 2%	1.05	3.35	5.24	99.82

*Robustness ($n = 3$), three concentrations (10, 30, and 50 µg/mL) for RAM and AML, (4, 8, and 16 µg/mL) for ATV and ASP

[a]Tailing factor and capacity factor determined for individual peak

[b]Resolution factor determined between each drug peak and the previous one

[c]% assay was calculated from the regression equation

Table 5 Quantitative determination of RAM and AML in Cardac® tablets, and RAM, ATV, and ASP in Ramitorv®capsules by the proposed HPLC methods

Pharmaceutical formulation	RAM			AML		
	Claimed taken (µg/mL)	Found% ± SD*		Claimed taken (µg/mL)	Found % ± SD*	
Cardac® tablets	5	99.46 ± 0.99		5	99.64 ± 0.78	
Ramitorv®capsules	RAM		ATV		ASP	
	Claimed taken (µg/mL)	Found % ± SD*	Claimed taken (µg/mL)	Found % ± SD*	Claimed taken (µg/mL)	Found % ± SD*
	5	99.62 ± 0.78	10	100.69 ± 0.81	7.5	100.48 ± 0.95

*Average of five experiments

Table 6 Statistical comparison of the results obtained by the proposed HPLC methods and the official methods for the analysis of the proposed components in their pure forms

Parameters	Proposed HPLC method					Official method**			
	Mixture I		Mixture II						
	RAM	AML	RAM	ATV	ASP	RAM	AML	ATV	ASP
Mean	100.01	99.89	99.75	100.42	100.10	99.85	100.17	99.88	99.68
SD	1.12	1.35	0.80	0.58	0.75	0.71	0.99	0.97	0.45
Variance	1.2544	1.8225	0.6400	0.3364	0.5625	0.5041	0.9801	0.9409	0.2025
n	6	6	6	8	8	5	5	5	5
Student's *t* test	0.063 (2.262)*	0.041 (2.262)*	0.055 (2.262)*	0.344 (2.201)*	0.211 (2.201)*				
F value	2.488 (5.192)*	1.860 (5.192)*	1.2700 (5.192)*	2.800 (4.120)*	2.777 (4.120)*				

RAM, potentiometric titration using 0.1 N NaOH

AML, HPLC method using C18 column, methanol: ammonium acetate (70:30 v/v) as a mobile phase, flow rate 1.5 mL/min, and UV detection at 237 nm

ATV, HPLC method using C18 column, acetonitrile: ammonium acetate: THF (67:21:12 by volume) as a mobile phase, flow rate 1.5 mL/min, and UV detection at 244 nm

ASP, by acid–base titration using 0.5 M HCl for the back titration of the excess unreacted 0.5 M NaOH, using 0.2 ml phenolphthalein as an indicator

*These values represent the corresponding tabulated values of *t* and *F* at $P = 0.05$

**The British pharmacopoeial

Table 7 Statistical analysis of the proposed HPLC methods and the reported methods for determination of the proposed components in their pharmaceutical formulations

Parameter	HPLC method		Reported method**[a]	
	RAM	AML	RAM	AML
Cardac® Tablets				
Mean %	99.46	99.64	101.09	100.82
SD	0.99	0.78	1.06	1.10
Variance	0.9801	0.6084	1.1236	1.2100
n	5	5	5	5
Student's *t* test (2.306)*	0.801	0.721		
F value (6.388)*	1.146	1.988		

	HPLC method			Reported method**[b]		
	RAM	ATV	ASP	RAM	ATV	ASP
Ramitorv® Capsules						
Mean %	99.62	100.69	100.48	98.94	100.63	101.04
SD	0.78	0.81	0.95	0.93	0.86	1.12
Variance	0.6084	0.6561	0.9025	0.8649	0.7396	1.2544
n	5	5	5	5	5	5
Student's *t* test (2.306)*	0.421	0.036	0.289			
F value (6.388)*	1.422	1.127	1.389			

*These values represent the corresponding tabulated values of *t* and *F* at $P = 0.05$

**[a] HPLC method The column used is Hypersil BDS C8 with mobile phase consisting of phosphate buffer (pH 2.5):acetonitrile (70:30). (150 × 4.6 mm, 5 µm) with flow rate of 1.2 mL/min with UV detection at 210 nm

**[b] HPLC method was performed on C18 column the mobile phase is mixture of (A) acetonitrile methanol (65:35) and (B) phosphate buffer (pH 3.0)in the ratio of (60:40 v/v) at a flow rate of 1.5 ml min^{-1}, UV detection was performed at 230 nm

Conclusion

The proposed chromatographic methods were satisfyingly used for the analysis of the two anti-hypertensive formulations under study, especially in quality control laboratories. The method furnished beneficial linearity and being trusted to selective determination of the studied drugs in presence of their degradation products resulted from different stress conditions.

Conflict of interest All the authors working in this manuscript have no conflict of interest.

References

Aschar GD, Oliveira L, Cesar P, Rosa P (2015) The degradations routes of Ramipril and its products. Am J Pharmatech Res 5(2):81–94

Babu KA, Kumar GV, Sivasubramanian L (2011) Simultaneous estimation of ramipril and amlodipine in bulk and tablet dosage form by RP-HPLC method. Int J Pharm Pharm Sci 3(4):196–198

British Pharmacopoeia, Medicines and Healthcare Products Regulatory Agency (MHRA), London 2013

Brunton LL, Lazo JS, Parker KL (2006) Goodman & Gilman's the pharmacological basis of therapeutics, 11th edn. McGraw-Hill, New York

Dai SY, Qiu ST, Wu W, Fu CM (2013) Development and validation of an rp-hplc method for simultaneous determination of Ramipril and Amlodipine in tablets. J Pharm Anal 3(6):440–446. https://doi.org/10.1155/2014/874587

Darwish HW, Hassan SA, Salem MY, El-Zeany BA (2016) Advanced stability indicating chemometric methods for quantitation of amlodipine and atorvastatin in their quinary mixture with acidic degradation products. Spectrochimica Acta—Part A: Mol Biomol Spectrosc 154:58–66. https://doi.org/10.1016/j.saa.2015.10.007

De Diego M, Godoy G, Mennickent S, Olivares M, Godoy R (2010) Stress degradation studies of ramipril by a validated stability-indicating liquid chromatographic method. J Chil Chem Soc 55(4):450–453. https://doi.org/10.4067/S0717-97072010000400008

Elshanawane AA, Mostafa SM, Elgawish MS (2008) Application of a validated, stability-indicating LC method to stress degradation studies of Ramipril and Moexipril. HCl Chromatographia 67(7/8):567–573. https://doi.org/10.1365/s10337-008-0544-3

Evoy GKM (2009) AHFS Drug Information, American Society of Health-System Pharmacists, Bethesda, pp 2016–2019

Gajula R, Pilli NR, Ravi VB, Maddela R, Inamadugu JK, Polagani SR, Busa S (2012) Simultaneous determination of atorvastatin and aspirin in human plasma by LC-MS/MS: its pharmacokinetic application. Sci Pharm 80(4):923–940. https://doi.org/10.3797/scipharm.1206-12

Gupta KR, Wankhede SB, Tajne MR, Wadodkar SG (2007) Simultaneous determination of Amlodipine and Ramipril by high performance thin layer chromatography. Asian J Chem 19(6):4177–4182

Hassan SA, Elzanfaly ES, Salem MY, ElZeany AB (2016) 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. Acta Parm 66:479–490. https://doi.org/10.1515/acph-2016-0040

ICH, Q1A (R2) (2003) Stability testing of new drug substances and products. International Conference on Harmonization, IFPMA, Geneva, Switzerland

Kumar AM, Kumar PV, Nasare M, Rao V, Parasad VVL, Diwan VP (2012) Isocratic RP-HPLC estimation of Ramipril and Amlodipine in pharmaceutical dosage form. J Adv Pharm Educ Res 2(3):137–145

Martindale (2003) The complete drug reference; Pharmaceutical Press: London, 33rd edition. 966

Maste MM, Kalekar MC, Kadian N, Bhat AR (2011) Development and validation of RP-HPLC method for simultaneous estimation of Amlodipine and Ramipril in bulk and tablet dosage form. Asian J Res Chem 4(8):1210–1213

Moffat AC, Osselton MD, Widdop B (2011) Clarke's analysis of drugs and poisons. Pharmaceutical Press, London

Panchal HJ, Suhagia BN, Patel NJ, Rathod IS, Patel BH (2009) Simultaneous estimation of Atorvastatin Calcium, Ramipril and Aspirin in capsule dosage form by RP-LC. Chromatographia 69(1–2):91–95. https://doi.org/10.1365/s10337-008-0831-z

Patel J, Patel M (2014) RP-HPLC method development and validation for the simultaneous estimation of ramipril and amlodipine besylate in capsule dosage form. J Chem Pharm Res 6(6):725–733

Patil PR, Rakesh SU, Dhabale PN, Burade KB (2009) Simultaneous estimation of ramipril and amlodipine by UV spectrophotometric method. Res J Pharm Technol 2(2):304–307. http://www.indianjournals.com/ijor.aspx?target=ijor:rjpt&volume=2&issue=2&article=017

Patole SM, Khodke AS, Potale LV, Damle MC (2010) A validated HPLC method for analysis of atorvastatin calcium, ramipril and asprin as the bulk drug and in combined capsule dosage forms. Int J Pharm Sci Rev Res 4(3):40–45. www.globalresearchonline.net

Rajput PS, Kaur A, Gill NK, Mittal K, Sarma GS (2012) Simultaneous estimation of ramipril and amlodipine in bulk and tablet dosage form by RP-HPLC method. J Appl Pharm Sci 2(7):160–165. https://doi.org/10.7324/japs.2012.2724

Ramadevi K, Saraswathi Z, Maniklal D (2013) RP-HPLC method development for simultaneous determination of the drugs Ramipril and Amlodipine. IJSR 2(2):4–7. https://doi.org/10.15373/22778179/FEB2013/123

Sankar ASK, Vetrichelvan T, Venkappaya D, Nagavalli D (2011) Simultaneous estimation of Ramipril, Aspirin and Atorvastatin Calcium by classical least squares regression in capsule dosage form. Res J Pharm Technol 4(3):398–401. https://www.researchgate.net/publication/286696813_Simultaneous_estimation_of_ramipril_aspirin_and_atorvastatin_calcium_by_classical_least_squares_regression_in_capsule_dosage_form

Sankar ASK, Vetrichelvan T, Venkappaya D (2011b) Simultaneous estimation of ramipril, acetylsalicylic acid and atorvastatin calcium by chemometrics assisted UV-spectrophotometric method in capsules. Acta Pharmaceutica 61(3):283–296. https://doi.org/10.2478/v10007-011-0027-1

Sharma R, Khanna S, Mishra GP (2012) Development and validation of RP-HPLC method for simultaneous estimation of Ramipril, aspirin and Atorvastatin in pharmaceutical preparations. E-J Chem 9(4):2177–2184. https://doi.org/10.1155/2012/891695

Shetty SK, Surendranath KV, Radhakrishnanand P, Borkar RM, Devrukhakar PS, Jogul J, Tripathi UM (2011) Stress degradation behavior of a polypill and development of stability indicating UHPLC method for the simultaneous estimation of aspirin, atorvastatin, ramipril and metoprolol succinate. Am J Anal Chem 2:401–410. https://doi.org/10.4236/ajac.2011.24049

Snyder LR, Kirkland JJ, Dolan JW (2011) Introduction to liquid chromatography, 3rd edn. Wiley, London

United States Pharmacopeia (2007) 30th edition. U.S. Pharmacopeial Convention Rockville, MD

The Merck index, An encyclopedia of chemicals, drugs and biological, 13th Merck Research

Zaazaa HE, Abbas SS, Essam HAM, El-bardicy MG (2012) Validated chromatographic methods for determination of perindopril and amlodipine in pharmaceutical formulation in the presence of their degradation products. J Chromatogr Aphic Sci 51:533–543. https://doi.org/10.1093/chromsci/bms171

Zoglio MA, Maulding HV, Haller RM, Briggen S (1986) Inhibition of stearate lubricant induced degradation of aspirin by the use of certain organic acids. J Pharm Sci 57:1877–1880. https://doi.org/10.1002/jps.2600720914