ORIGINAL ARTICLE

Development and validation of an RP-HPLC method for simultaneous determination of Ramipril and Amlodipine in tablets

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Abstract   An RP-HPLC method for the simultaneous determination of Ramipril (RP) and Amlodipine (AL) in tablets was developed and validated by Chinese Pharmacopoeia 2010. The linearity of the proposed method was investigated in the range of 0.01–0.25 mg/mL ($r^2 = 0.9998$) for RP and 0.014–0.36 mg/mL ($r^2 = 0.9997$) for AL. The limits of detection (LOD) were 0.06 μg/mL and 0.02 μg/mL for RP and AL, and the limits of quantitation (LOQ) were 0.2 μg/mL and 0.07 μg/mL, respectively. Some major impurities and degradation products did not disturb the detection of RP and AL and the assay can thus be considered stability-indicating.

1. Introduction

Ramipril (RP), with the chemical name [(2S, 3aS, 6aS)-1-[(S)-2-[[(S)-1-(ethoxycarbonyl)-3-phenylpropyl] amino] propanoyl] octa-hydrocyclopenta [b] pyrrole-2-carboxylic acid (Fig. 1I)], is an angiotensin-converting enzyme inhibitor (ACEI), which is widely used in the treatment of hypertension and congestive heart failure.

RP plays an important role in inhibiting the conversion of the inactive angiotensin I to the active angiotensin II. However, it may cause hypotension, cough and other side effects [1–3].

Amlodipine (AL), [3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dic-arboxylate (Fig. 1VI)], is a long-acting dihydropyridine calcium channel blocker (CCB) with dose-related antihypertensive efficacy. It inhibits calcium ions to be transported into vascular smooth muscle and cardiac muscle to protect the target organs. But it would also cause peripheral edema as a side effect. It is always used in the treatment of hypertension and angina [4–6].

Either RP or AL is a good choice for the treatment of hypertension. But in fact, a large majority of hypertensives ultimately require drug combination to decrease the damage of heart, brain, kidney, etc. Fixed-dose combinations of drugs with complementary properties offer the

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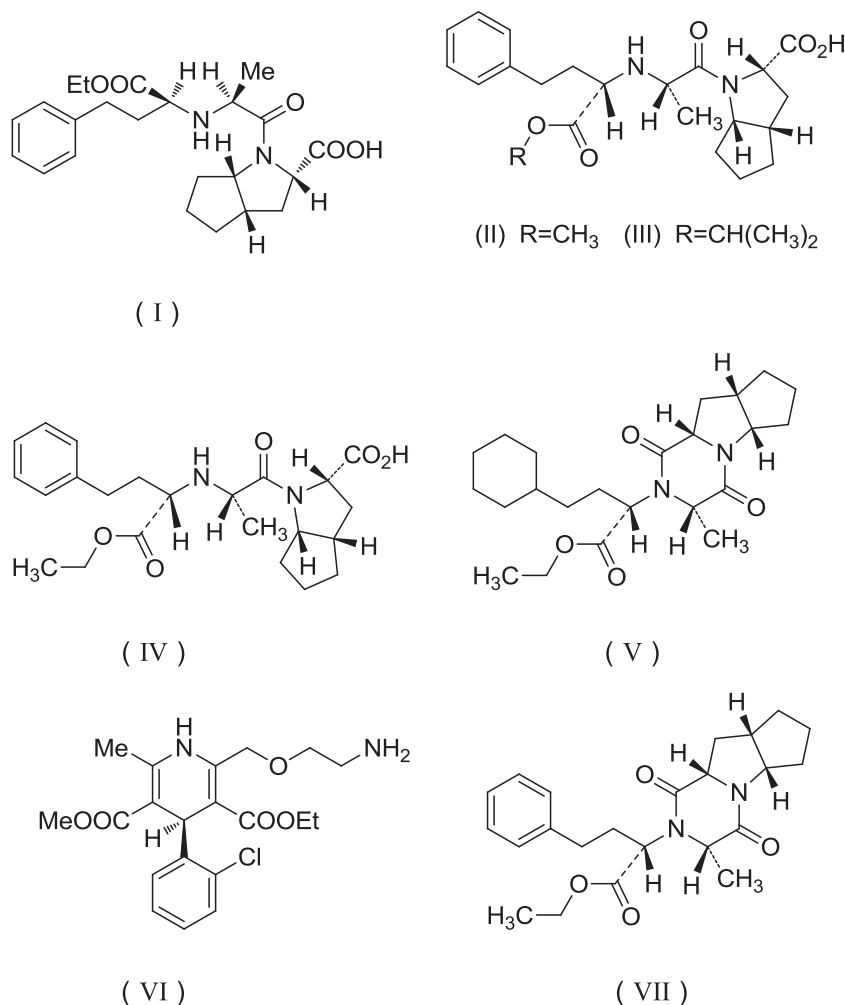Peer review under responsibility of Xi'an Jiaotong University.

Fig. 1 Chemical structures of Ramipril (I), Ramipril impurities A (II), B (III), C (IV), D (V), Amlodipine (VI), and Amlodipine impurity D (VII).

advantages of simplicity, tolerability, convenience, and cost effectiveness, as well as the compliance [7]. The combination therapy of ACEI and CCB has been proved to be effective [8–10]. So the combination of RP and AL would also be a good therapeutic option.

There are many reported methods to determine either RP [11–13] or AL [14–17] alone or in combination with other drugs [18–22] in dosage forms. But to the best of our knowledge, none has been reported the simultaneous determination of RP and AL in the presence of the degradants and the five major impurities (Ramipril impurity A (Fig. 1II), B (Fig. 1III), C (Fig. 1IV), D (Fig. 1V) and Amlodipine impurity D (Fig. 1VII)). This paper aims to describe the development and validation of the HPLC method for the simultaneous determination of RP and AL in the same tablet dosage forms.

2. Materials and methods

2.1. Chemicals and reagents

RP active pharmaceutical ingredient (API) was obtained from the Green Syn Co., Ltd. (Guangzhou, China), and AL besylate API was from the Weihai Disu Pharmc Co., Ltd. (Weihai, China). Compound Ramipril and Amlodipine besylate tablets (each tablet containing 2.5 mg of RP and 5 mg of AL besylate) and the tablet excipients were kindly supplied by Chengdu Haisco Pharmaceutical Co., Ltd. (Chengdu, China).

The reference standard of AL besylate was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The RP reference standard, impurity standards of RP and AL were procured by European Directorate for the Quality of Medicines of European Council.

HPLC-grade acetonitrile was obtained from Honeywell (USA). HPLC-grade triethylamine was obtained from Kermel chemical reagents company (Tianjin, China). Water was prepared by ultra pure water system (UPA, Chongqing, China). All the other used reagents were of analytical grade.

2.2. HPLC instruments and analytical conditions

Chromatographic separation was achieved by using a Shimadzu model 20A liquid chromatographic system (Tokyo, Japan), equipped with a 20AT pump and a PDA detector (SPD-20A). The system was controlled by a system controller (SCL-20A) and a personal computer.

The chromatographic column utilized in these studies was an Inertsil ODS-3 column (250 mm × 4.0 mm, 3 μm). A 10 mm × 4.0 mm (i.d.) guard column packed with 5 μm diameter Inertsil ODS-3 packing was also utilized. The column temperature was maintained at 55 °C.

The mobile phase A consisted of 60 mM sodium perchlorate buffer (containing 7.2 mM triethylamine)-acetonitrile (60:40, v/v) and mobile phase B was 60 mM sodium perchlorate buffer (containing 7.2 mM triethylamine)-acetonitrile (20:80, v/v). The apparent pH of the mobile phases was adjusted to 2.6 with phosphoric acid. The gradient program used is given in Table 1. The flow rate was 1.0 mL/min and the injection volume was 20 μL.

The spectra were obtained from the PDA detector. Peak purity analysis was carried out over a wavelength range of 190–350 nm by using the Shimadzu LC-solution software. The detection wavelength was set at 210 nm because all the components had higher responses.

2.3. Solutions and sample preparation

For the system suitability test, the solution containing 0.1 mg/mL of RP, 0.2 mg/mL of AL besylate and each 0.1 mg/mL of five major impurities was prepared by mobile phase A. For the linearity studies, a standard stock solution containing 0.25 mg/mL of RP and 0.5 mg/mL of AL besylate (equivalent to approximately 0.36 mg/mL of the free base) was prepared by mobile phase A and diluted with the same solvent to yield solutions at different concentrations. These solutions were protected from light using aluminum foil and stored at 4 °C in the refrigerator.

The test sample solution was prepared from 20 pulverized compound RPAL tablets. A 10-tablet equivalent mass corresponding to 25 mg RP and 50 mg AL besylate was weighed into a 250 mL volumetric flask, and mobile phase A was added to the volume. After ultrasonicated for 15 min, the mixture was centrifuged at 10,000 rpm for 10 min. The supernatant was separated and transferred into the HPLC instrument to be analyzed.

Samples were subjected to stress conditions of light, heat, acid, base and oxidation in order to evaluate the ability of the proposed method to separate RP and AL from both known and unknown degradation products. In the stress studies, all the solutions were prepared by weighing 4-tablet equivalent mass of sample powders containing about 10 mg RP and 20 mg AL besylate into 10 mL volumetric flasks, or by weighing 10 mg RP or 20 mg AL besylate pure API. Acid degradation was conducted using 2 mL of 0.1 M hydrochloric acid, and alkali degradation was carried out in 2 mL of 0.1 M sodium hydroxide. The stressed solutions were kept in water bath for 5 min, neutralized and then diluted by mobile phase A. Oxidation degradation was performed by adding 2 mL of 3% H_2O_2 and kept in water bath for 5 min, and then diluted with mobile phase A. For the temperature stress study, compound

Table 1 Gradient program proposed for the analysis of AL (Amlodipine), RP (Ramipril) and their related substances.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Profile
0–10	100	0	Isocratic
10–15	100→60	0→40	Linear ramp to 40% B
15–20	60	40	Isocratic
20–25	60→30	40→70	Linear ramp to 70% B
25–35	30	70	Isocratic
35–40	30→100	70→0	Linear ramp to 100% A
40–55	100	0	Isocratic

RPAL tablets, RP and AL besylate API were exposed to dry heat of 60 °C in a convection oven for 10 days. For photo stability studies, compound RPAL tablets, RP and AL besylate API were exposed to 4500 lx light in a light cabinet for 10 days. After degradation, these stressed tablets were removed, crushed and mixed. Then the powers were dissolved and extracted with 10 mL mobile phase A for further analysis. Ten microgram RP or 20 mg AL besylate API were also resolved in 10 mL mobile phase A to study the origination of related substances.

3. Results and discussion

3.1. Method development

A gradient HPLC method was adopted to get a shorter runtime and higher sensitivity due to the polarity differences among RP, AL and their related impurities. And also considering the stability of the system, the related substances test method of RP described in the European Pharmacopoeia (Ph. Eur.)[23] was used as the starting point for further development. A stainless steel column (250 mm × 4.0 mm, 3 μm) was used in the official Ph. Eur. method. The mobile phases A and B all consisted of acetonitrile, sodium perchlorate buffer and 1 mL triethylamine with different pH values adjusted to 3.6 and 2.6 with phosphoric acid, respectively. But to our study, RP and AL could not be separated well and the peaks with bad symmetries were also observed under this condition.

As RP and AL had N–H groups as basic nitrogen centers, the amount of perchlorate and the pH of mobile phase would affect the peak shape, resolution and symmetry, since perchlorate plays as ion-pair reagent and the pH would affect the protonation reactions. So the amount of perchlorate and the pH of mobile phases should be adapted. The results showed that the retention times of RP and AL were lengthened when the amount of perchlorate was increased. The retention time of RP would be shortened while the retention time of AL had no significant change when the pH was decreased. The amount of triethylamine used as a tailing-suppressing reagent was not investigated as it was unnecessary to change when the amount of perchlorate and the pH of mobile phase were appropriate. The ratios of mobile phase A and B, various gradient programs were also tried to get better resolution and shorter separation time. After a lot of work, the amount of sodium perchlorate, the pH and ratios of mobile phases given in Section 2.2 and the gradient program presented in Table 1 were found to give symmetric peak, higher column performance, shorter running time and good resolution among the main components and the related substances.

The column diameter of 5 μm was also investigated but with a worse column performance. So the column diameter of 3 μm was chosen. Besides above consideration, the column temperature was also studied. The column temperatures of 60 °C, 55 °C and 50 °C were studied and 55 °C was finally chosen by considering the working life of the column as well as the pressure and stability of the system. Typical chromatograms obtained with the final condition are shown in Fig. 2.

As seen from the chromatogram, the method was capable of separating RP, AL and the 12 related substances. Peak numbered 1 was identified as benzene sulfonic acid. Peaks 4, 6, 8 and 11 were identified and correspond to Amlodipine impurity D, Ramipril impurity B, C and D by comparing the relative retention time (RRT) with the available reference impurities. Other peaks that
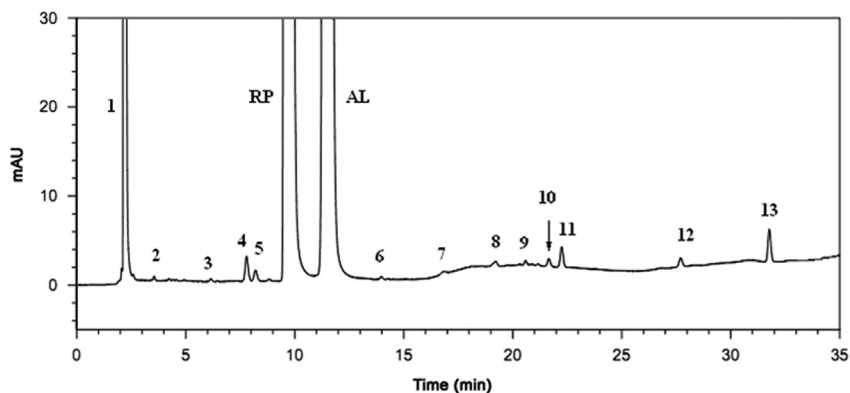
Fig. 2 Chromatograms of sample solution (1 mg/mL RP and 2 mg/mL AL solution using the final optimized mehtod). 1. Benzene sulfonic acid, 2. Unknown 1, 3. Unknown 2, 4. Amlodipine impurity D, 5. Unknown 3, 6. Ramipril impurity B, 7. Unknown 4, 8. Ramipril impurity C, 9. Unknown 5, 10. Unknown 6, 11. Ramipril impurity D, 12. Unknown 7, and 13. Unknown 8.
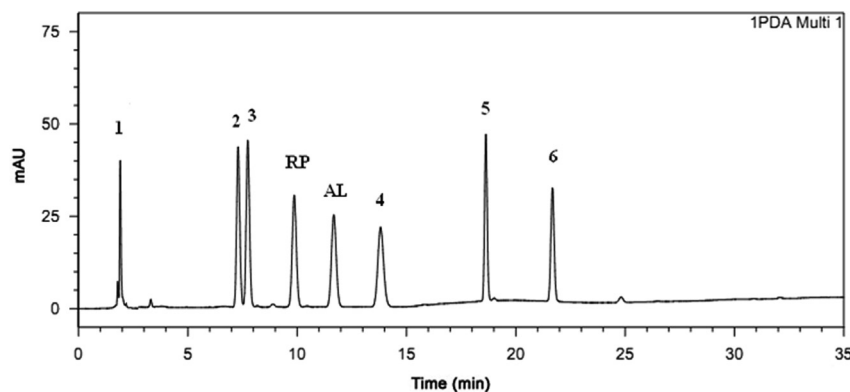

Fig. 3 Chromatogram of the system suitability test solution. 1. Benzene sulfonic acid, 2. Ramipril impurity A, 3. Amlodipine impurity D, 4. Ramipril impurity B, 5. Ramipril impurity C, and 6. Ramipril impurity D.
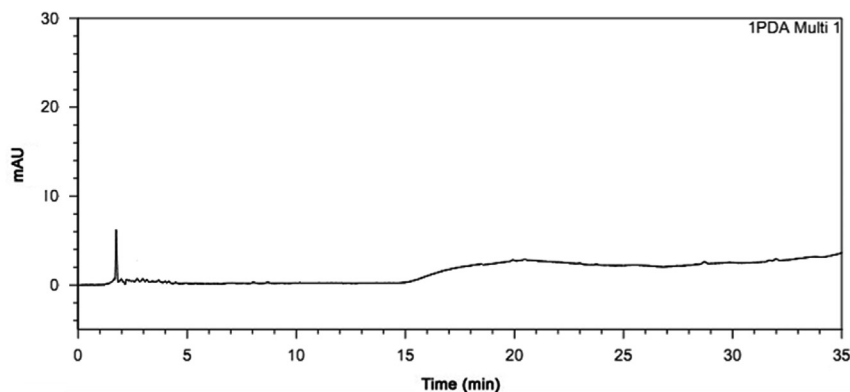

Fig. 4 Chromatogram of the tablet excipients.

were not numbered were below the disregard limit, which was set at 0.1%.

3.2. Method validation

The proposed method was validated with the aspect of system suitability test, specificity, linearity and range, accuracy, precision, LOD, LOQ, stability and robustness according to the Chinese Pharmacopoeia Volume II requirements [24].

3.2.1. System suitability test

System suitability was determined by six replicate injections of the system suitability solution. The acceptance criteria were less than 2% relative standard deviation (RSD) for peak areas, greater than 3000 column plates, less than 1.5 of the USP tailing factor, and greater than 1.5 of the resolution. The results obtained were all within the acceptable limits. The resolutions among RP, AL and the closest eluting peaks were bigger than 2 which indicated that this method was reliable for the quantification of RP and AL. A typical chromatogram for the system suitability test is shown in Fig. 3.

3.2.2. Specificity

The selectivity of the method was confirmed by observing potential interferences caused by excipients of tablet formulations and degradation products under stress conditions as indicated by ICH [25].

The chromatogram of the tablet excipients (Fig. 4) shows that there were no interference of peaks to the determination of RP and AL.

All stressed samples were compared with an un-stressed sample solution. The proposed chromatographic conditions were found to be specific under all applied stress conditions, which was visibly

confirmed in Fig. 5. The peak purity indices for RP and AL in stressed solutions were found to be better (purity angle < purity threshold) indicating that no additional peaks were co-eluting with the analytes. And they also evidenced the ability of the method to assess unequivocally the analytes of interest in the presence of potential interference.

3.2.3. Origination of related substances

The origination of the related substances in tablets was investigated. As can be seen from the chromatograms of RP and AL
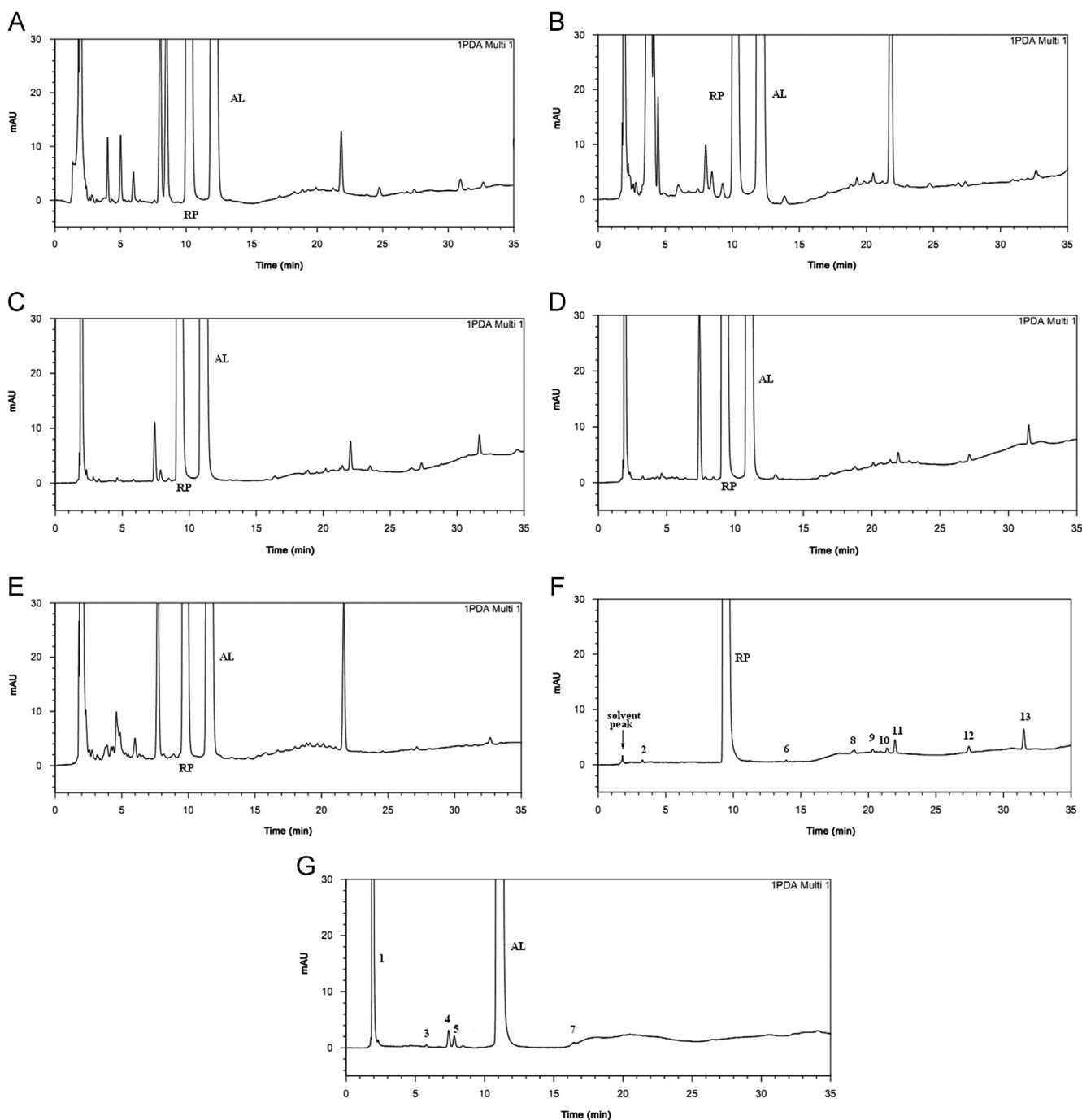

Fig. 5 Chromatograms of stress test. (A) Acid hydrolysis-degraded tablet powder; (B) base hydrolysis-degraded tablet powder; (C) dry-heated tablet powder; (D) photo degraded tablet powder; (E) oxidation degraded tablet powder; and chromatograms of (F) RP (Ramipril) API and (G) AL (Amlodipine) besylate API (the peak number is the same as in Fig. 2).

besylate API in Fig. 5, the peaks numbered 6, 8, 11 as defined in Fig. 2 (Ramipril impurity B, C, D, respectively, according to the RRT) would stem from RP API. In addition to the known impurities, the unknown impurities peaks numbered 2, 9, 10, 12, 13 originated in RP API. And the peaks numbered 1 (benzene sulfonic acid), 3, 4(Amlodipine impurity D), 5 and 7 would originate in AL besylate API. The stress test chromatograms of RP and AL raw materials (Fig. 5) were also corresponding to the stress test chromatogram of the tablet. A hypothesis may be made that the purities of the raw materials of RP and AL may ultimately affect the number and content of the related impurities in tablet dosage forms.

3.2.4. Linearity and range

The linearity was checked by analyzing six working solutions of RP over the concentrations range 0.01–0.25 mg/mL (0.01, 0.025, 0.05, 0.1, 0.125, 0.25 mg/mL) and 0.014–0.36 mg/mL (0.014, 0.036, 0.07, 0.14, 0.18, 0.36 mg/mL) for AL. The following results were obtained: $y=44164x+303.08$ ($r^2=0.9998$) for RP, and $y=37963x-597$ ($r^2=0.9997$) for AL, where $y=$peak area, $x=$concentration of solution; $r^2=$the square of determined correlation coefficient. The results indicated that the method was linear over the concentration range studied.

3.2.5. Accuracy and precision

The accuracy of the method was assessed by recovery test. A known amount of each standard powder was added to blank sample composed of all the excipients equivalent to the ratio of the tablet formulation, which was then mixed, extracted and subsequently diluted to yield three different concentrations of each drug (0.08, 0.10 and 0.12 mg/mL for RP and 0.16, 0.20 and 0.24 mg/mL for AL besylate, $n=3$). These samples were prepared as described in Section 2.3 and analyzed as previously described. The corresponding percentage recovery data are summarized in Table 2.

Repeatability or intra-day precision was investigated by injecting six replicate sample solutions on the same day. Inter-day precision was assessed by analyzing newly prepared sample solutions in triplicate over three consecutive days. Precision was expressed as RSD value of the analyte peaks. RSD values obtained for the peak areas of RP and AL on a single day (day 1, $n=6$) were 0.46% and 0.55%, respectively. RSD values on triplicate injections on three successive days (days 1–3, $n=9$) were 1.6% and 1.8%, respectively. The results implied that the method developed was accurate for the determination.

3.2.6. LOQ and LOD

The LOQs for RP and AL corresponding to a signal-to-noise ratio of 10 were 0.2 µg/mL and 0.07 µg/mL for RP and AL, respectively. And the LODs corresponding to a signal-to-noise ratio of 3 were 0.06 µg/mL and 0.02 µg/mL. The resultant RSD values for these studies were $\leq 3.5\%$ ($n=6$).

3.2.7. Stability of solutions and robustness

The stability of the standard stock solutions was determined by quantitatively determining each drug in different time comparing to the response obtained for freshly prepared standard solutions. No significant changes ($<2\%$) were observed according to the chromatographic responses for the stock solutions stored at 4 °C for two weeks, relative to freshly prepared standards. The stability of sample solution was tested for every 2 h interval up to 12 h. The

Table 2 Accuracy results [Recovery (%)] for the determination of AL (Amlodipine) and RP (Ramipril).

Compound	Quantity (mg/mL)			
	Added	Found*	Recovery (%)	RSD (%)
RP	0.0792	0.0785	99.1	0.62
	0.1008	0.1005	99.7	0.46
	1.2010	1.2082	100.5	0.81
Average recovery			99.7	0.83
AL	0.1605	0.1613	100.5	0.50
	0.1980	0.2005	101.3	0.53
	0.2414	0.2408	99.8	0.57
Average recovery			100.5	0.79

*Mean of three determinations for each concentration.

Table 3 Chromatographic parameter setting applied in the robustness investigation.

Parameter	Modification	Recovery (%)		Number of impurities
		RP	AL	
pH	2.5	101.1	98.5	12
	2.6	100.5	98.7	12
	2.7	100.3	99.2	12
Flow rate (mL/min)	0.9	99.9	99.3	12
	1.0	100.5	98.7	12
	1.1	99.5	99.5	12
Column temperature (°C)	50	100.2	99.0	12
	55	100.5	98.7	12
	60	100.3	99.5	12

RSD values of RP and AL were 1.2% and 1.0%, respectively. This indicated that the sample solution was stable up to 12 h.

The robustness of the method was investigated by a small variety of conditions including changes of pH of the eluent, flow rate and column temperature. The assay results for RP and AL by three different analysts in the same laboratory were also investigated. The RSD values did not exceed 2.5%. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators demonstrated that the method was robust, and the data are summarized in Table 3.

3.3. Assay of AL and RP in tablets

Three batches of compound tablets were analyzed using the developed method. Satisfactory results were obtained that the mean percentage found for RP and AL were in good agreement with the label claimed. The mean percentage found and the RSD values (Table 4) indicated that the proposed method could be adopted for the determination of RP and AL in compound tablets.

Table 4 Assay results of AL (Amlodipine) and RP (Ramipril) in compound tablets.

Batch number	Found* (%)		RSD (%)	
	RP	AL	RP	AL
B1	100.9	103.5	1.1	1.2
B2	98.28	101.1	0.95	0.93
B3	99.53	102.4	1.4	1.4

B1, B2, and B3 refer to three different batches.
 *Mean of three determinations.

4. Conclusions

A gradient LC method has been developed and validated for the analysis of RP and AL in tablet dosage forms. The results of the stress testing revealed that the method was specific and selective. The proposed method has the ability to separate the two main components from their degradation products, related substances found in tablet dosage forms and the tablet excipients. Therefore, the chromatographic method can be used to analyze samples obtained during accelerated stability experiments and routine assay of RP and AL in combined tablet dosage forms. In addition, the procedure can be further applied for the detection and determination of the related substances in tablets.

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