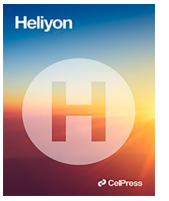
Heliyon 9 (2023) e19993

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



A new stability-indicating HPLC-UV method for determination of amlodipine besylate and its impurities in drug substance

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ARTICLE INFO

Keywords:
High performance liquid chromatography
Single-quadrupole mass spectrometer
Stability indicating
Amlodipine besylate

ABSTRACT

A new fast stability-indication high performance liquid chromatography method was developed and validated for the determination of amlodipine besylate and its organic impurities in drug substance. The separation of amlodipine and its seven impurities was achieved on a core shell C18 column, 100 mm × 4.6 mm; 2.6 μm, within 15 min. The mobile phase comprised of 0.4% ammonium hydroxide in water and methanol delivered in a gradient mode; the method detection wavelength is 237 nm. The selected column is stable at high pH and provided a good peak shape for basic compounds. Amlodipine besylate was subject to acid, base, oxidative, thermal, and photolytic stress conditions. The degradation products were well resolved from the amlodipine peak and its impurities. Major degradants were analyzed by liquid chromatography coupled with single-quadrupole mass detector. Amlodipine peak was shown to be free of co-elution by mass spectral analysis in all stress conditions. The method was validated in terms of specificity, linearity, accuracy, precision, and robustness. The developed method could be applied for routine quality control analysis of amlodipine besylate drug substance.

1. Introduction

Amlodipine besylate {3-ethyl 5-methyl (4RS)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulfonate} is a 1,4-dihydropyridine-3,5-dicarboxylate derivative, its molecular formula is $C_{20}H_{25}ClN_2O_5 \cdot C_6H_6O_3S$, and molecular weight is 567.05 [1]. It is calcium channel blocker that inhibits the influx of calcium ions into the vascular and cardiac muscles [2,3]. World Health Organization's (WHO) lists Amlodipine as an essential antihypertensive medicine and is considered as one of the safest and effective treatment of hypertension, chronic stable angina, and vasospastic angina [4]. Amlodipine besylate was originally marketed in tablet form by Pfizer as a besylate salt under the trade name Norvasc [5] and generic amlodipine besylate tablets have been launched by different manufacturers in Europe over the last twenty years [6].

In the literature, several high-performance liquid chromatography (HPLC) methods for the determination of amlodipine in pharmaceutical preparations as well as in combination with other drugs such atorvastatin, benepril, perindopril, olmesartan, valsartan and hydrochlorthiazide have been described. However, these methods do not address the quantitation of seven organic impurities of amlodipine besylate. Related substances procedure in the European Pharmacopoeia [7] has a run time of 55 min and the method is not stability indicating. The identification and quantification of organic impurities and degradation products in pharmaceuticals is important since impurities may cause the undesirable effects on the patients and may have influence on quality, safety, and efficacy of

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https://doi.org/10.1016/j.heliyon.2023.e19993
Received 3 August 2023; Received in revised form 6 September 2023; Accepted 7 September 2023
Available online 9 September 2023

drug products [8].

Hence, in this work a novel stability-indicating reversed-phase HPLC method was developed for the determination of amlodipine and its organic impurities. The method uses environmentally friendly mobile phase with no pH adjustments, also the run time is short thus reducing waste generation. Altogether, this new method would be of great value to ensure the safety and quality of amlodipine besylate in raw materials.

2. Experimental

2.1. Chemicals and reagents

Amlodipine besylate (purity 99.9%), Amlodipine RC A (purity 97%), Amlodipine RC C (purity 99%) and Amlodipine RC D (purity 100%) were obtained as USP Reference Standards (US Pharmacopoeia, MD, US). Amlodipine RC B (purity 99%), Amlodipine RC E (purity 98%), Amlodipine RC F (purity 97%) were purchased from Toronto Research Chemicals (Toronto, Canada). Hydrogen peroxide (30%, ACS grade) was purchased from Sigma-Aldrich (St. Louis, MI, USA). Acetonitrile (LC/MS grade), hydrochloric acid (12.1 N HCl, ACS grade), and ammonium hydroxide solution (20%, ACS grade) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Sodium hydroxide solution (10 N NaOH, certified) from J. T. Baker (Phillipsburg NJ, USA). Amlodipine Besylate API samples were purchased from different suppliers. All HPLC grade solvents were obtained from distributors. Deionized water (R = 18.2 MΩ cm) was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Instrumentation and analytical parameters

HPLC systems; Agilent 1260 Infinity Quaternary HPLC system with Diode Array Detector (Santa Clara, CA, USA) and Waters Alliance Quaternary HPLC System with Photodiode Array Detector (Milford, MA, USA) were used for analysis. Waters UPLC H-Class PLUS system equipped with Photodiode Array Detector and Acquity QDa (Single-Quadrupole Mass Detector (Milford, MA, USA) in positive ionization mode was used for the analysis of forced degradation samples. Waters Empower 3 data software was used for acquiring data and reporting. The analysis was carried out at 237 nm with Phenomenex Kinetex EVO C18, 4.6 mm × 100 mm, 2.6 μm column and the column temperature was maintained at 35 °C. The mobile phase consisted of 0.4% ammonium hydroxide (mobile phase A) and methanol (mobile phase B). The gradient program was set as 0–2 min, 55% B; 2–5 min, 55%–60% B; 5–12 min, 60%–90% B; 12.1–15 min, 55% B with a flow rate of 1.0 mL/min. The sample injection volume was 20 μL. Caron Photostability Chamber (Marietta, OH, USA) was used for photostability study and thermal and humidity stress was performed on an ESPEC Humidity Chamber (Hudsonville, MI, USA).

2.3. Forced degradation experiments

The forced degradation studies were performed for amlodipine besylate under acid, base, oxidative, thermal, thermal and humidity, hydrolytic, and light stress conditions. Hydrolytic and oxidative stresses were performed by treatment of amlodipine besylate (1 mg/mL) with 0.1 M HCl, 0.1 M NaOH, and 3% hydrogen peroxide at ambient temperature for 3 days. Light stress was performed by exposure of amlodipine besylate to 200 W-h/square meter ultraviolet light (UVA) and then to 1.2 million lux-h white light (Vis). Thermal stress was conducted by storing amlodipine besylate in an oven at 105 °C for 3 days. Thermal and humidity stress was conducted by exposure of amlodipine besylate to 85 °C temperature and 85% relative humidity for 3 days. On the day of analysis, each of the stressed samples was diluted or dissolved to make a sample solution having a concentration of 0.1 mg/mL in diluent, methanol, and water (50:50, v/v).

2.4. Solution and sample preparation

A solution of methanol and water (50:50, v/v) was used as a diluent. Resolution solution (0.2 mg/mL of amlodipine besylate and 2.0 μg/mL of each Amlodipine RC A, Amlodipine RC C, Amlodipine RC D, Amlodipine RC B, Amlodipine RC E, Amlodipine RC F and Deschloro Amlodipine) was prepared by dissolving amlodipine besylate and impurities in diluent. Stock solutions (0.1 mg/mL each of Amlodipine besylate, Amlodipine RC A, Amlodipine RC C, Amlodipine RC D, Amlodipine RC B, Amlodipine RC E and Amlodipine RC F) were prepared by dissolving the materials in the diluent. Impurity stock solutions were prepared by sequential dilution of the stock solutions. The standard solution (0.2 μg/mL each of Amlodipine Besylate, Amlodipine RC C, Amlodipine RC B, 0.6 μg/mL of Amlodipine RC A, 0.3 μg/mL each of Amlodipine RC D, Amlodipine RC E and Amlodipine RC F) was prepared by sequential dilution of the stock solutions. Sensitivity solution (0.1 μg/mL each of Amlodipine Besylate, Amlodipine RC A, Amlodipine RC C, Amlodipine RC D, Amlodipine RC B, Amlodipine RC E and Amlodipine RC F) was prepared by dilution of the impurity stock solution. Linearity solutions were prepared at 0.05%, 0.1%, 0.25%, 0.50, 0.75%, and 1.0% of the sample concentration (0.2 mg/mL) by diluting the impurity stock solution. Sample stock solution used for recovery studies was prepared by dissolving a commercial amlodipine besylate sample to achieve a concentration of 0.4 mg/mL. Sample solutions for commercial drug substances were prepared at a concentration of 0.2 mg/mL. Accuracy solutions were prepared at 0%, 0.05%, 0.5% and 1.0% impurity levels and repeatability solutions at 0.05% level of sample nominal concentration. The standard solution for assay procedure (0.05 mg/mL of amlodipine besylate) was prepared by dissolving amlodipine besylate in diluent. Assay linearity solutions at 50%, 75%, 100%, 125%, and 150% levels were prepared by sequentially diluting a linearity stock solution (0.1 mg/mL of amlodipine besylate), which was prepared by dissolving amlodipine

besylate in diluent.

3. Results and discussion

3.1. Method development

The reversed phase HPLC-UV Related substances procedure described in the European Pharmacopoeia (Ph. Eur.) that includes seven impurities (Imp A, B, D, E, F, G and H) was used as a starting point for method development. An ODS column (250 mm x 4. Mm, 5 μm) and 30 mM ammonium acetate and methanol (30:70 v/v) was used in the official Ph. Eur method. But in our study, a co-elution of impurities A, D and the unknowns present in the amlodipine besylate samples was observed. In the forced degradation studies of amlodipine besylate, a co-elution of unknown degradants with impurities G and D was also observed. As amlodipine besylate is a basic compound, a mobile phase pH higher than the amlodipine besylate pK_a (8.6) was chosen to achieve desired peak shape and separation of amlodipine and its impurities. Different HPLC columns, Waters XBridge BEH C18 and Agilent Zorbax Extend-C18, were tested and found not suitable for the separation of all compounds of interest. The first promising separation was achieved on a Phenomenex Kinetex EVO C18, 100 mm × 4.6 mm, 2.6 μm column using 0.1% ammonium hydroxide in water (mobile phase A) and methanol (mobile phase B) as mobile phase delivered in a gradient mode. The satisfactory peak shape with peak tailing of NMT 1.5 of amlodipine was achieved by increasing the concentration of ammonium hydroxide to 0.4%. The ratios of mobile phase A and B, various gradient programs were also tried to get better resolution and shorter separation time. Final method using 0.4% of ammonium hydroxide and methanol as the mobile phase delivered in a gradient mode with a run time of 15 min on Phenomenex Kinetex EVO C18, 100 mm × 4.6 mm, 2.6 μm was capable of separating amlodipine besylate and its organic impurities (Amlodipine RC A (Imp D), Amlodipine RC B (Imp B), Amlodipine RC C (Imp G), Amlodipine RC D (Imp A), Amlodipine RC E (Imp E), Amlodipine RC F (Imp F), and Deschloro Amlodipine) and unknown degradation products. The API samples were also tested for the separation of the unknown impurities from the amlodipine peak.

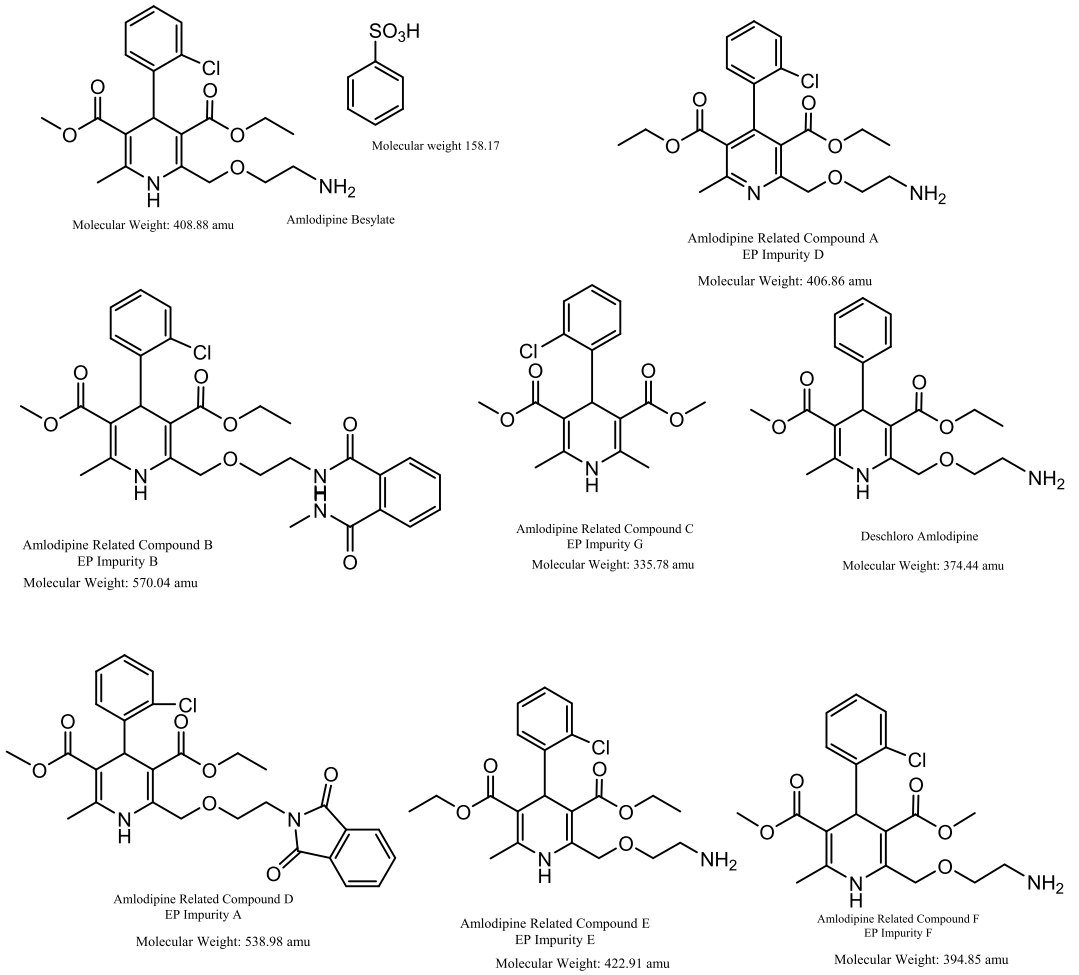

Fig. 1. Chemical structures and molecular weights of amlodipine besylate and its impurities.

A resolution of not less than 2.0 between the amlodipine peak and adjacent peaks was achieved. Also, the impurities peaks were separated from each other by a resolution of not less than 2.0. The chemical structures of amlodipine besylate and related compounds are shown in Fig. 1.

The detection wavelength of 237 nm corresponds to one of the UV maxima of amlodipine and is also specified in both USP Amlodipine Besylate monograph [9] and EP Amlodipine Besilate monograph [7] was retained for the Assay and Organic Impurities procedure. Representative chromatograms are shown in Fig. 2 and the chromatographic performance data is presented in Table 1.

3.2. Forced degradation

The forced degradation study was carried out by treating the drug substance under stress conditions like acid, base hydrolysis, oxidation, heat, heat and humidity and light (Section 2.3). Considerable degradation of amlodipine besylate was observed under base (43%) and photolytic (5%) stress conditions. Under acid and oxidative stress conditions, about 1% of degradation was seen. No degradation was observed under heat and heat/humidity stress conditions (Table 2).

Five prominent ions were detected in the mass spectrum of amlodipine (Fig. 3). The ion at *m/z* 431 corresponds to the sodium adduct [M+Na] of amlodipine. The fragment at *m/z* 392 corresponds to loss of ammonia (NH_3) from the protonated amlodipine molecule. Subsequent fragmentation of the *m/z* 392 ion led to the formation of ions at *m/z* 294 (loss of $C_5H_6O_2$) and *m/z* 238 (loss of C_3H_4O). The typical [M+2] isotope pattern for a molecule containing one chlorine atom was also observed. The ion at *m/z* 238, $[C_{12}H_{13}NO_2Cl]^+$ was found to be the base peak which corresponds to previously reported data [10–12].

The evaluation of the UV chromatogram of base stress sample showed five degradation peaks at retention times of 3.8, 4.5, 5.0 and 5.6 min. The peak at 5.6 min was identified as amlodipine RC F by comparison of the mass spectrum ($[M+H]^+ = 395$, base peak at *m/z* = 238) and retention time match with RCF standard [13]. A direct cleavage of methyl group from the ethyl carboxylate of 1,4-dihydropyridine ring of amlodipine resulted in the formation of RC F. In its subsequent steps loss of methyl but-2-enoate moiety and ammonia resulted in the formation of fragments with *m/z* 280 and *m/z* 238, respectively. The peaks at retention times 3.8, 4.5, and 5.0 have the same mass spectrum and fragmentation pattern as that of amlodipine but distinct UV spectra (Fig. 4) suggesting that these degradation products are positional isomers of amlodipine possibly formed due to methyl shift.

Amlodipine RC A was the only peak seen in the Total Ion Chromatogram (TIC) of oxidative and photolytic stress samples. Oxidative aromatization of dihydropyridine to the pyridine moiety is one of the main degradation pathways of amlodipine [14,15] and occurs both in solution and in solid state and is promoted by light.

The UV chromatogram of acid stressed sample revealed the presence of two degradation products eluting at retention time of 4.5 and 4.9 min. The peak at 4.5 min was identified as amlodipine RC A by comparison of the mass spectrum ($[M+H]^+ = 407$) and retention time match with RC A standard. The peak at 4.9 has the same mass spectrum and fragmentation pattern as that of amlodipine but a distinct UV spectrum (Fig. 5). Summary of forced degradation results are shown in Table 2 and Fig. 6.

Peak purity analysis was performed in the range from 200 to 450 nm by using empower software Analysis confirmed the spectral purity of the amlodipine peak in the control and all stress samples. The peak purity values for amlodipine in sample solutions and stressed solutions (purity angle ≤ purity threshold) indicated that no additional peaks were co-eluting with the API peak (Table 3, Fig. 7). Amlodipine peak purity was also investigated by LC/MS. Mass spectra were compared at the leading, apex and trailing portions of the amlodipine peak in control and stress samples to investigate possible co-elution. No co-elution of the degradation products with the amlodipine in any of the stressed samples was observed thus, confirming the stability-indicating capability of the developed method.

3.3. Method validation

The developed method was validated for specificity, linearity, accuracy, precision, LOQ and robustness as per ICH guidelines [16]. The validation of the method as an organic impurities' procedure was performed for Amlodipine RC A, Amlodipine RC B, Amlodipine RC C, Amlodipine RC D, Amlodipine RC E, Amlodipine RC F, and any unspecified impurities in the range of 0.05%–1.0% of the nominal

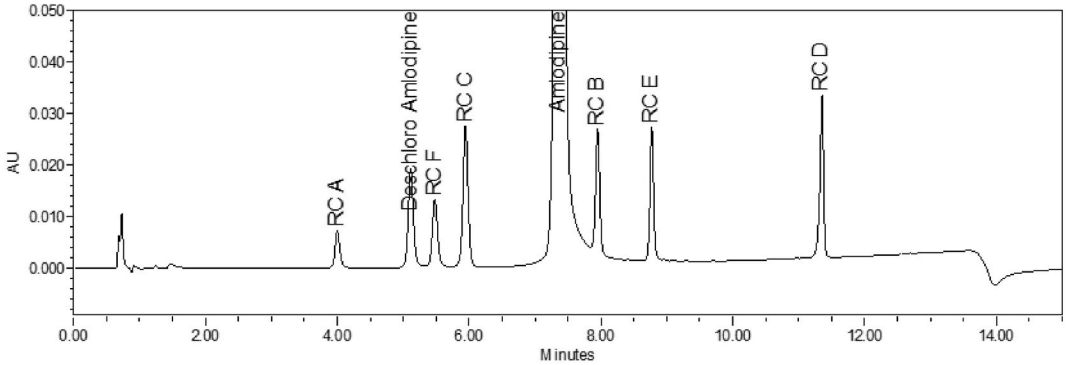

Fig. 2. Chromatogram depicting separation of amlodipine and its impurities.

Table 1
Chromatographic performance data.

Compound	Retention time (min)	Relative retention time[a]	USP Resolution
RC A	4.3	0.59	–
Deschloro amlodipine	5.1	0.70	7.9
RC F	5.6	0.75	2.5
RC C	5.9	0.8	3.2
Amlodipine	7.3	1.0	10.0
RC B	7.9	1.1	4.7
RC E	8.8	1.2	7.6
RC D	11.3	1.5	24.4

Resolutions were calculated between two adjacent peaks.
[a] Relative retention times (RRT) were calculated against the retention time (RT) of Amlodipine.

Table 2
Summary of forced degradation results.

Stress conditions	%Degradants formed			%Recovery
	RC A	RC F	SMU	
Oxidative degradation	0.4	ND	ND	99.6
Acid degradation	0.1	ND	0.4	99.7
Base degradation	ND	3.3	0.6	56.9
Thermal/Humidity degradation	ND	ND	ND	100.3
Thermal degradation	ND	ND	ND	100.7
Photolytic degradation	0.7	ND	ND	94.3

SMU: Single maximum unknown; ND: Not detected.

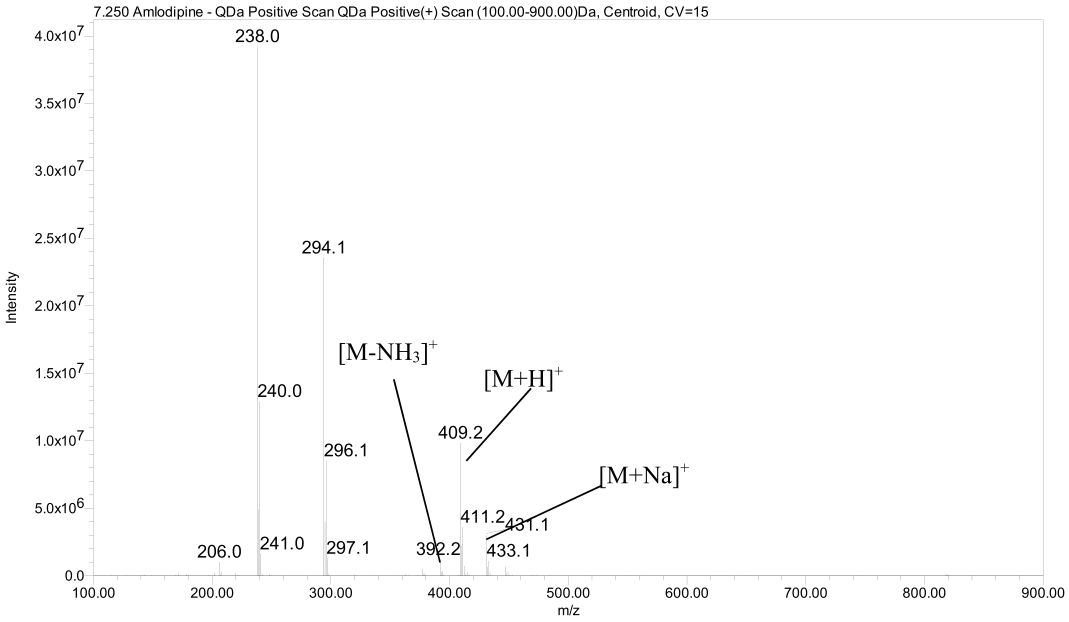

Fig. 3. Electrospray ionization (positive mode) mass spectrum of amlodipine.

sample concentration of 0.2 mg/mL. For an Assay procedure the method was validated in the range of 80%–120% of the nominal sample concentration of 0.05 mg/mL of amlodipine besylate.

3.3.1. Specificity

The specificity of the method was demonstrated by analyzing diluent, standard solution, sample solutions, and spiked sample solutions containing known impurities. No interference to API peak and impurity peaks was detected in sample solutions prepared from commercial API samples. The resolution between each of the adjacent impurity peaks, and between API and adjacent peaks was greater than 2.0.

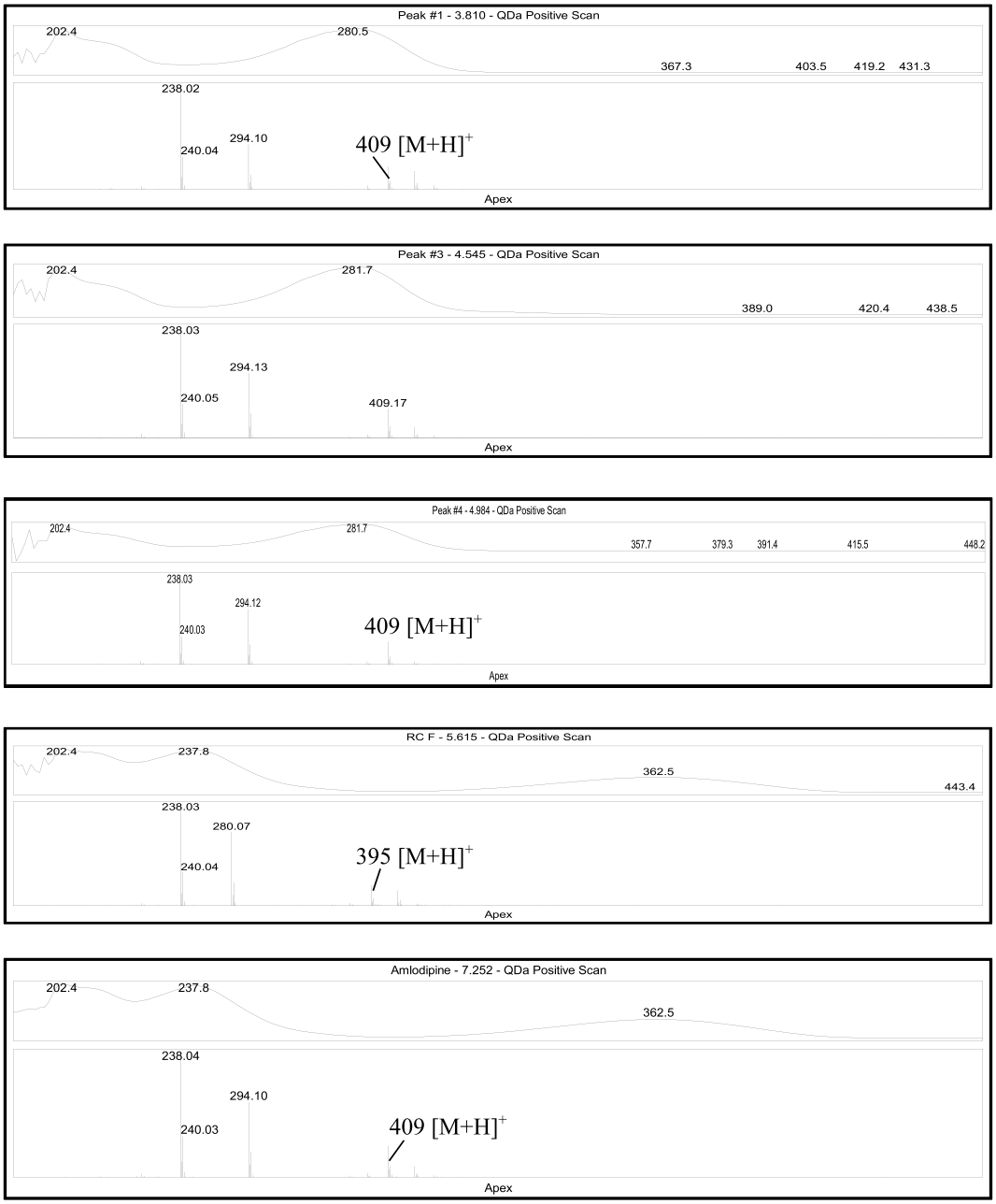

Fig. 4. UV and MS spectra of the degradation products under base stress.

3.3.2. Linearity

The linearity for impurities procedures was studied in the range of 0.05%–1.0% of nominal concentration of the sample solution at 0.2 mg/mL for amlodipine besylate. The correlation coefficient was greater than 0.99. The relative response factors of the impurities were determined as ratio of slope of impurity to the slope of API (Table 4). The linearity for assay procedure was studied in the range of 50%–150% of the normal sample concentration of 0.05 mg/mL. The correlation coefficient was greater than 0.999, and the value of normalized intercept/slope for amlodipine was within ±1%.

3.3.3. Accuracy

The accuracy of the Organic Impurities procedure was established in the range of 0.05%–1.0% for RC A, RC C, RC E, RC F; 0.1%–1.0% for RC B and 0.05%–0.25% for RC D. Recovery was calculated by comparing the theoretical concentration calculated from the calibration curve and the nominal concentration. The corresponding percentage recovery data are summarized in Table 5. The mean of percentage recoveries and the relative standard deviation for each level were calculated.

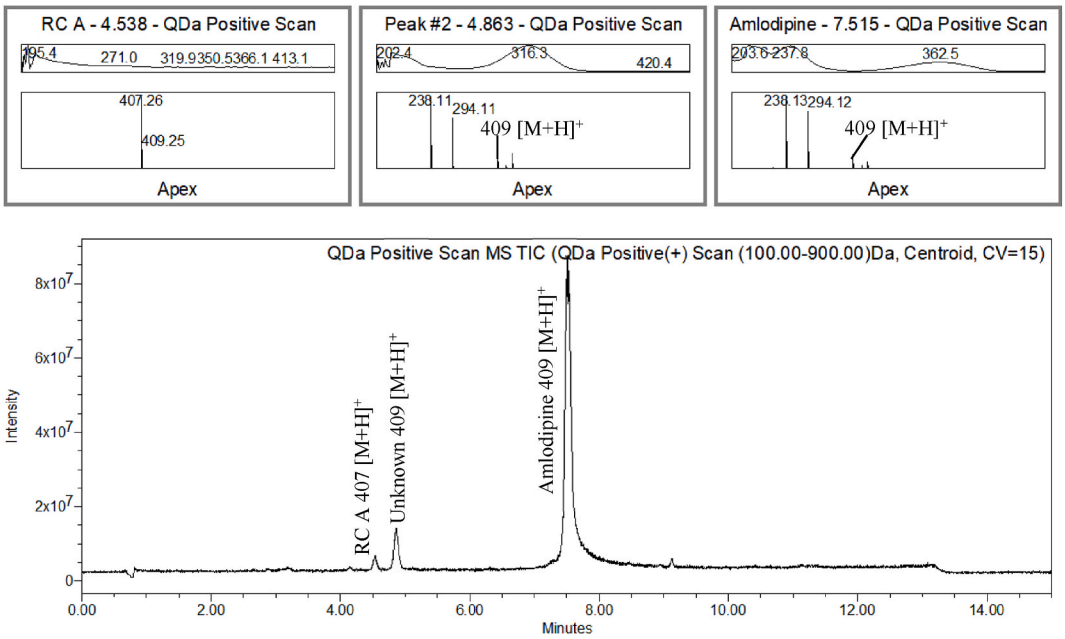

Fig. 5. LC/MS analysis of acid stress sample.

The accuracy of the assay procedure was assessed by comparison % recovery at 80%, 100% and 120% levels (triplicates at each level) with the manufacturer's CoA value. The average assay results at each level were within 2.0% from the CoA value.

3.3.4. Precision

Precision of Organic Impurities procedure was estimated by evaluating six spiked solutions at 0.05% impurity level. The RSD (%) of six recovery values was less than 3% for all impurities. These data indicated that the method is repeatable for simultaneous quantitation of six organic impurities (RC A, RC B, RC C, RC D, RC E and RC F).

Precision of the Assay procedure was evaluated using nine sample solutions, at 80%, 100% and 120% levels (triplicates at each level). The RSD (%) was less than 1.0%. Intermediate precision for both Organic Impurities and Assay method was determined by another scientist on a different brand of HPLC instrument on a different day using a column from a different lot. The results implied that the method is precise and rugged.

3.3.5. Solution stability

Stability study was performed by quantitatively determining the deviation from the freshly prepared sensitivity solution (0.05% level) and 0.05% spiked solution over a period of 24 h. The sensitivity solution and spiked solution at 0.05% level were stable for at least 24 h at room temperature (the change in peak area from the initial time were less than 10%). The standard solution and sample solution for assay were also stable for at least 24 h at room temperature (the change in peak area from the initial time were less than 1.0%).

3.3.6. LOQ

The LOQs of amlodipine and the impurities were based on the signal to noise ratio of greater than 10 for all the peaks in the chromatogram of the sensitivity solution.

3.3.7. Robustness

A resolution solution that contained Amlodipine RC A, Amlodipine RC B, Amlodipine RC C, Amlodipine RC D Amlodipine RC E, Amlodipine RC F, and Deschloro Amlodipine was used for robustness study. Small but deliberate variations in HPLC parameters were made to demonstrate the robustness of the HPLC method. The HPLC parameter variations studied included the composition of mobile phase (Percent of ammonium hydroxide ±10% (0.36% and 0.44%)), column temperature (±3° (32° and 38°), isocratic hold time (±0.5 min (1.5 min and 2.5 min)) and flow rate (±10% (0.9 mL/min and 1.1 mL/min)). The retention times, resolution, and tailing for the peaks of interest were not significantly affected at any of modified chromatographic conditions.

4. Conclusions

A new single HPLC method was developed that offers the separation of amlodipine from related substances, impurities, and degradation products The method is stability indicating and can be effectively used for the determination amlodipine besylate and its

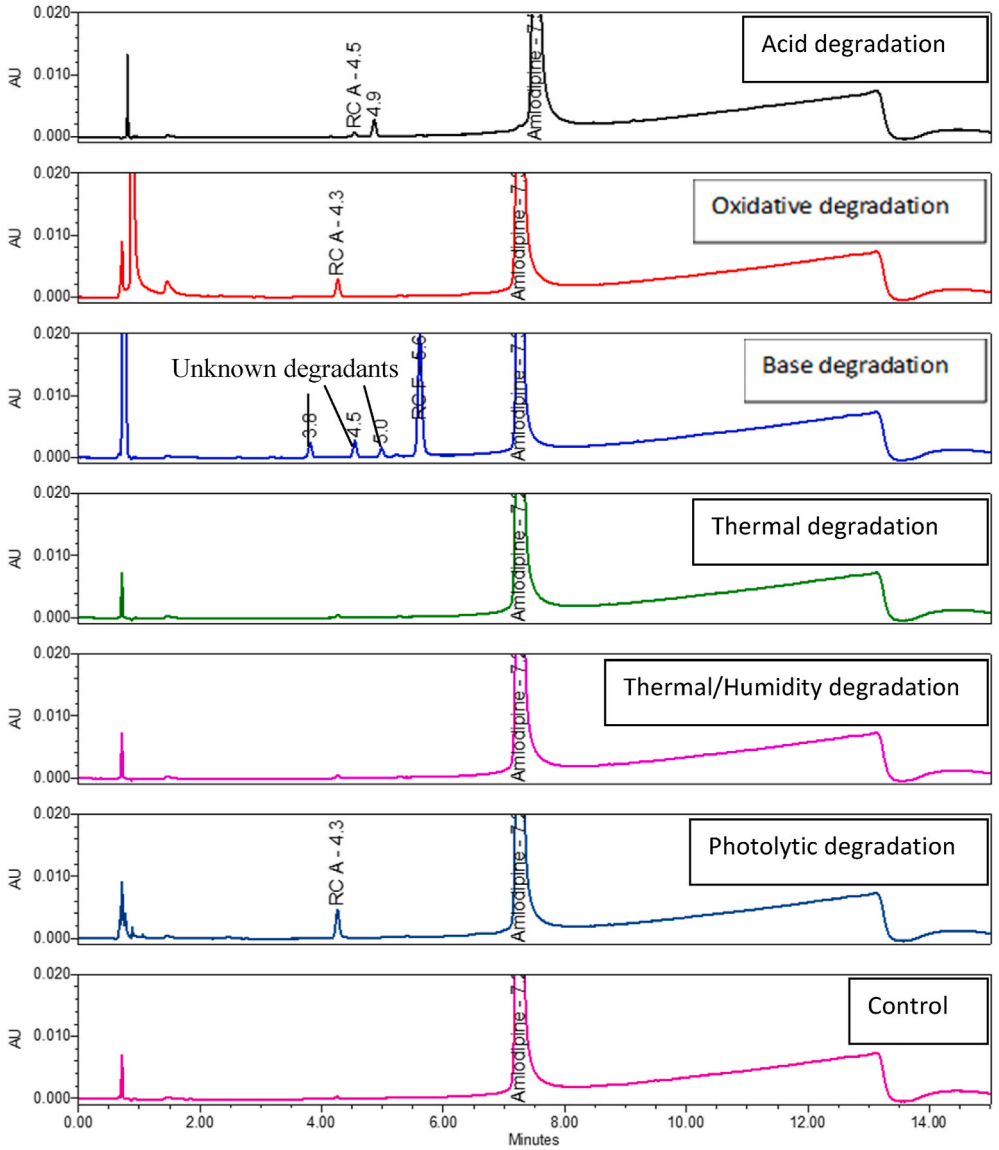

Fig. 6. Chromatograms of forced degradation samples.

Table 3
PDA peak purity analysis of amlodipine in control sample and stress samples.

Sample	Purity angle	Purity Threshold
Control	0.033	0.051
Base stress	0.042	0.081
Oxidative stress	0.034	0.051
Photolytic stress	0.034	0.056
Acid stress	0.032	0.048
Thermal stress	0.035	0.053
Thermal and Humidity stress	0.035	0.052

impurities in drug substance. The results of forced degradation study revealed that the method is specific and selective. Validation results indicated that the method is specific, precise, accurate, and robust. The relatively shorter run time enables rapid routine analysis as well as stability studies of the amlodipine besylate API.

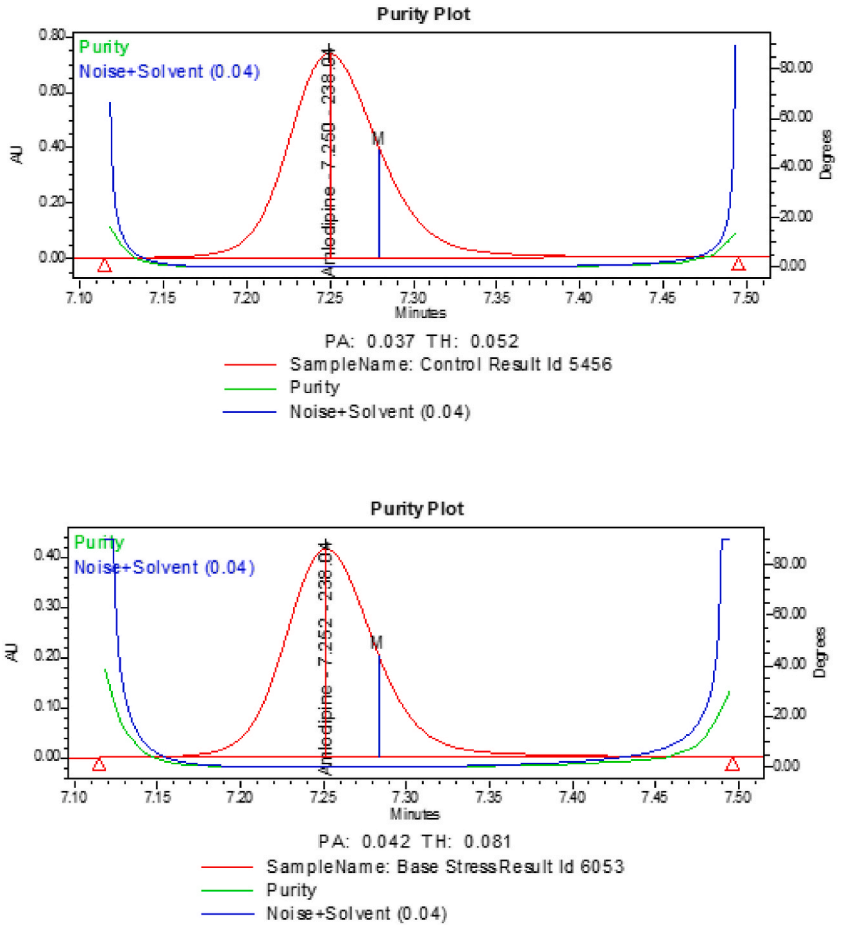

Fig. 7. Purity plots of Control and Base stress samples.

Table 4
Linearity data of amlodipine and related compounds.

Compound	Range (μg/mL)	Linearity Equation	Normalized intercept/slope (%)	r	RRF
Amlodipine	0.1–2.0	y = 32200x - 291	−0.82	1.000	1.0
RC A	0.1–2.0	y = 21300x - 79	−0.35	1.000	0.66
RC B	0.1–2.0	y = 44400x + 217	0.45	1.000	1.38
RC C	0.1–2.0	y = 60600x - 79	−0.12	1.000	1.88
RC D	0.1–0.5	y = 50000x + 1110	7.4	0.991	1.57
RC E	0.1–2.0	y = 42600x - 191	−0.43	1.000	1.32
RC F	0.1–2.0	y = 41100x - 302	−0.69	1.000	1.28

Disclaimer

USP does not endorse or certify any instruments, vendors, or materials identified in this manuscript does not imply that these are the best available products.

Author contribution statement

Salika Jeelani: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Natalia Kouznetsova: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supplementary material/referenced in article. ’.

Table 5
Accuracy results.

Compound	%Amount spiked[a]	% Recovery[b]	% RSD
RC A	0.05	99.9	1.3
	0.5	101.6	0.4
	1.0	100.9	0.2
RC B	0.1	80.6	0.4
	0.5	96.1	0.9
	1.0	97.9	0.6
RC C	0.05	99.4	0.7
	0.5	101.0	0.2
	1.0	100.6	0.2
RC D	0.05	90.4	2.5
	0.15	105.7	1.5
	0.25	107.0	0.9
RC E	0.05	107.4	1.3
	0.5	104.1	0.1
	1.0	104.0	0.1
RC F	0.05	100.9	1.5
	0.5	104.3	0.3
	1.0	104.3	0.1

[a] Amount of impurities spiked with respect to nominal sample concentration of 0.2 mg/mL of amlodipine besylate.
[b] Mean for six determinations for 0.05 or 0.1% and three determinations for other levels.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are thankful to Yang Zhang for performing Intermediate precession and to Nadejda Soukhova and Qun Xu for providing the comments to the manuscript.

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