A stability-indicating high performance liquid chromatographic (HPLC) assay for the simultaneous determination of atorvastatin and amlodipine in commercial tablets

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Abstract

A simple, rapid, precise and accurate isocratic reversed-phase stability-indicating HPLC method was developed and validated for the simultaneous determination of atorvastatin (AT) and amlodipine (AM) in commercial tablets. The method has shown adequate separation for AM, AT from their associated main impurities and their degradation products. Separation was achieved on a Perfectsil® Target ODS-3, 5 μm, 250 mm × 4.6 mm i.d. column using a mobile phase consisting of acetonitrile–0.025 M NaH_2PO_4 buffer (pH 4.5) (55:45, v/v) at a flow rate of 1 ml/min and UV detection at 237 nm. The drugs were subjected to oxidation, hydrolysis, photolysis and heat to apply stress conditions. The linearity of the proposed method was investigated in the range of 2–30 μg/ml (r = 0.9994) for AT and 1–20 μg/ml (r = 0.9993) for AM. The limits of detection were 0.65 μg/ml and 0.35 μg/ml for AT and AM, respectively. The limits of quantitation were 2 μg/ml and 1 μg/ml for AT and AM, respectively. Degradation products produced as a result of stress studies did not interfere with the detection of AT and AM and the assay can thus be considered stability-indicating.

1. Introduction

Amlodipine (AM), 2[(2-aminoethoxy) methyl]-4-(2-chloro-phenyl)-1, 4-dihydro-6-methyl-3, 5-pyridine carboxylic acid, 3-ethyl, 5-methylester (Fig. 1) [1] is a dihydropyridine derivative with calcium antagonist activity. It is used in the management of hypertension, chronic stable angina pectoris and prinzmetal variant angina [2]. AM inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle [3], [4] and [5]. Atorvastatin (AT) is chemically described as [R-(R*, R*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1-heptanoic acid (Fig. 1) [1]. AT is a selective, competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of the sterols, including cholesterol. It is used to reduce LDL-cholesterol, apolipoprotein B, and triglycerides and to increase HDL-cholesterol in the treatment of hyperlipidaemias [2] and [4]. Caduet® is the first commercial product that has been developed and marketed to treat two different conditions, *viz*., high blood pressure and high cholesterol, in one dosage form. Caduet® contains both AM besylate for the treatment of high blood pressure and AT calcium for the treatment of hypercholesterolaemia. Caduet® tablets are intended for oral administration and are available in several different strength combinations including 2.5(AM)/10(AT) mg, 2.5(AM)/20(AT) mg, 2.5(AM)/40(AT) mg, 5(AM)/10(AT) mg, 5(AM)/20(AT) mg, 5(AM)/40(AT) mg, 5(AM)/80(AT) mg, 10(AM)/10(AT) mg, 10(AM)/20(AT) mg, 10(AM)/40(AT) mg and 10(AM)/80(AT) mg. There are many reported methods for the determination of either AM [6], [7], [8], [9], [10], [11], [12], [13], [14], [15], [16], [17], [18], [19], [20], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30], [31], [32], [33], [34], [35], [36], [37], [38], [39], [40], [41], [42] and [43] or AT [44], [45], [46], [47], [48], [49], [50], [51] and [52] alone or in combination with other drugs in pharmaceutical dosage forms or individually in biological fluids. Non of the reported analytical methods describe a stability indicating method for the simultaneous determination of AM and AT in presence of their degradants and their associated main impurities. These methods are not directly applicable for this issue and need more investigation for method development and validation. Only one method [53] has been reported for the simultaneous determination of these compounds, which describes an HPLC method using C18 column, but this method lacks stability indicating nature. To our knowledge, this is the first report of a stability indicating method for the simultaneous determination of both AM and

AT in solid oral dosage forms. The present manuscript describes a simple, rapid, precise and accurate isocratic reversed-phase stability-indicating HPLC method for the simultaneous determination of AT and AM in the same tablet dosage form.

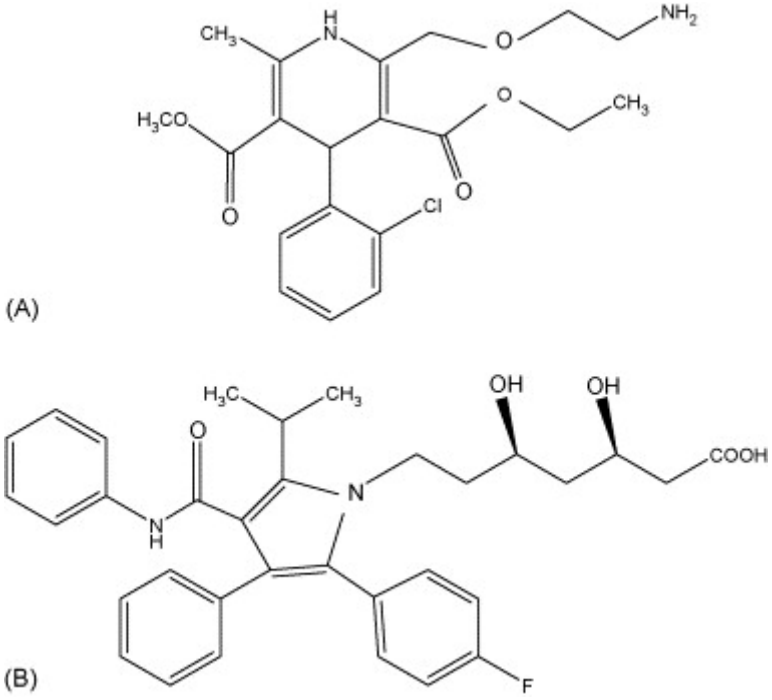

Fig. 1. Structural formulae for (A) amlodipine (MW = 408.9) and (B) atorvastatin (MW = 558.7).

2. Experimental

2.1. Chemicals and reagents

AM besylate (99.94%) and its main impurities including impurity D (98.66%) (3-Ethyl-5-Methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methylpyridine-3, 5-dicarboxylate) and impurity E (98.22%) (3,5-dimethyl-(4RS)-4-(2-chlorophenyl)-2-[2-aminoethoxy) methyl]-6-methyl-1,4-dihydro pyridine-3,5-dicarboxylate), AT calcium (99.87%) and its main impurities including atorvastatin lactone (89.7%), atorvastatin ester (99.1%) ([R-(R*,R*)-2-(4-fluro phenyl)-β,δ-dihydroxy-5-[(1-methyl ethyl)-3-phenyl-4-[(phenyl amino)-carboxyl] 1H-pyrrole-1-heptanoic acid, tert butyl ester) and diastereomer-atorvastatin (83.8%) ([R-(R*, S*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino) carbonyl]-1H-pyrrole-1-heptanoic acid) were kindly supplied by Dr Reddy's, Andhra Pradesh, India and were used without further purification. Caduet® 10(AM)/10(AT) mg tablets were obtained from Pfizer Ireland Pharmaceuticals Dublin, Ireland. Acetonitrile, methanol, sodium dihydrogen phosphate, sodium hydroxide, hydrochloric acid and hydrogen peroxide were obtained from Merck (Darmstadt, Germany). All reagents used, were at least of analytical grade except acetonitrile and methanol which were HPLC grade. HPLC grade water was obtained following distillation in glass and passage through a Milli-Q® system (Millipore, Milford, MA, USA) and was used to prepare all solutions.

2.2. HPLC instrumentation and conditions

The modular HPLC system consisted of a Knauer, Smartline Pump1000, a Knauer injection system and a Knauer solvent degasser (Berlin, Germany), a Waters 486, tunable absorbance detector (Waters Chromatography Division, Milford, MA, USA). A ChromGate® Chromatography Data System Version 3.1 (Berlin, Germany) was used to record and evaluate the

data collected during and following chromatographic analysis. The chromatographic separation was achieved on a Perfectsil® target ODS-3, 5 μm, 250 mm × 4.6 mm i.d. column using a mobile phase consisting of acetonitrile–0.025 M NaH_2PO_4 buffer pH 4.5 (55:45,v/v) at a flow rate of 1 ml/min. The eluent was monitored using UV detection at a wavelength of 237 nm. The column was maintained at ambient temperature (25 °C) and an injection volume of 20 μl was used. The mobile phase was filtered through 0.45 μm Chrom Tech Nylon-66 filter prior to use.

2.3. Preparation of stock and standard solutions

Stock solutions of AT calcium (equivalent to approximately 1 mg/ml of the free base) and AM besylate (equivalent to approximately 1 mg/ml of the free base) were prepared in methanol. The stock solutions were protected from light using aluminium foil and stored for three weeks at 4 °C with no evidence of decomposition. Aliquots of the standard stock solutions of AT and AM were transferred using A-grade bulb pipettes into 10 ml volumetric flasks and the solutions were made up to volume with mobile phase to yield final concentrations of 2, 4, 10, 15, 20, 25 and 30 μg/ml for AT (maintaining the AM concentration at a constant level of 10 μg/ml) and concentrations of 1, 2, 4, 7, 10, 15 and 20 μg/ml for AM (maintaining the AT concentration at a constant level of 10 μg/ml).

2.4. Preparation of tablets for assay

Twenty Caduet® tablets were weighed, crushed and mixed in a mortar and pestle for 20 min. A portion of powder equivalent to the weight of one tablet was accurately weighed into each of nine 100 ml A-grade volumetric flasks and 20 ml of HPLC-grade methanol was added to each flask. The volumetric flasks were sonicated for 20 min to effect complete dissolution of the AM or AT and the solutions were then made up to volume with HPLC grade water. Aliquots of the solution were filtered through a 0.45 μm nylon filter and 1 ml of the filtered solution was transferred to a 10 ml A-grade volumetric flask and made up to volume with mobile phase, to yield concentrations of each of the two drugs in the range of linearity previously described.

2.5. Forced degradation studies of API and tablets

In order to establish whether the analytical method and the assay were stability-indicating, Caduet® tablets and pure active pharmaceutical ingredient (API) of both AT calcium and AM besylate were stressed under various conditions to conduct forced degradation studies [54]. AM besylate is slightly soluble in water and sparingly soluble in ethanol and AT calcium is insoluble in aqueous solutions of below pH 4. In addition, AT calcium is very slightly soluble in distilled water and phosphate buffer (pH 7.4) and is slightly soluble in ethanol. As these drugs are freely soluble and stable in methanol [55] and [56], methanol was used as a co-solvent in all forced degradation studies. All solutions prepared for use in forced degradation studies were prepared by dissolving API or drug product in small volume of methanol and later diluted with either aqueous hydrogen peroxide, distilled water, aqueous hydrochloric acid, or aqueous sodium hydroxide, to achieve a concentration of 100 μg/ml each of AT and AM. After the degradation these solutions were diluted with mobile phase to yield starting concentrations of 10 μg/ml for both AT and AM.

2.5.1. Oxidation studies

Solutions for use in oxidation studies were prepared in methanol and 2.5% H_2O_2 (20:80, v/v) and the resultant solutions analyzed immediately after preparation.

2.5.2. Acid degradation studies

During the initial forced degradation studies it was observed that acid hydrolysis and basic hydrolysis were a fast reaction for both the drugs and almost complete degradation of both drugs occurred when the resultant solution analyzed 20 min after preparation. Thus, in later experiment, the time decreased to 5 min. Solutions for acid degradation studies were prepared in methanol and 0.1 M hydrochloric acid (20:80, v/v) and the resultant solutions analyzed 5 min after preparation.

2.5.3. Alkali degradation studies

Solutions for alkali degradation studies were prepared in methanol and 0.1 M sodium hydroxide (20:80, v/v) and the resultant solutions analyzed 5 min after preparation.

2.5.4. Neutral degradation studies

Solutions for neutral degradation studies were prepared in methanol and water (20:80, v/v) and the resultant solutions heated on a bath water at 90 °C for 20 min prior to analysis.

2.5.5. Temperature stress studies

Caduet® tablets and API were exposed to dry heat of 100 °C in a convection oven for 8 h. The tablets and API powders were removed from the oven and 20 tablets were crushed and mixed and an aliquot of powder equivalent to the weight of one tablet and API powder were then prepared for analysis as previously described.

2.5.6. Photostability studies

Caduet® tablets and API powder and solutions of each drug were prepared and exposed to light to determine the effects of irradiation on the stability of the two drugs in solution and in the solid state. Approximately 50 mg of each API was spread on a glass dish in a layer that was less than 2 mm in thickness. A solution of each API (1 mg/ml) was prepared in methanol and HPLC grade water (20:80, v/v). Tablets were prepared in the same way. All samples for photostability testing were placed in a light cabinet (Suntest CPS/CPS^{+}, Atlas Material Testing Technology, Germany) and exposed to light for 40 h resulting in an overall illumination of ≥200 W h/m^{2} at 25 °C with UV radiation at 320–400 nm. Control samples which were protected from light with aluminium foil were also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

3. Results and discussion

3.1. HPLC method development and optimization

A Perfectsil® target ODS-3, 5 μm, 250 mm × 4.6 mm i.d. column (MZ-Analysentechnik, Mainz, Germany) maintained at ambient temperature(25 °C) was used for the separation and the method validated for the determination of AT and AM in Caduet® tablets. PerfectSil® target is an ultra pure silica gel (>99.999%) based column that is supplied with novel state of the art bonding and end capping technology that is characterized by excellent chemical and mechanical stability and provides excellent peak symmetry for basic analytes. The composition, pH and the flow rate of the mobile phase were

changed to optimize the separation conditions using stressed samples and the main related substances of the two compounds of interest. A mobile phase consisting of 0.025 M NaH_2PO_4 buffer pH 4.5–acetonitrile (45:55, v/v) set at a flow rate of 1 ml/min was selected for use for further studies after several preliminary investigatory chromatographic runs. Under the described experimental conditions, the all peaks were well defined and free from tailing. The effects of small deliberate changes in the mobile phase composition, pH and flow rate were evaluated as a part of testing for method robustness. The reported method for the simultaneous determination of AM and AT in its dosage form [53], dose not give data on specificity and robustness for their estimation in the presence of degradants or impurities.

3.2. Validation of the method

The analytical method was validated with respect to parameters such as linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery and robustness/ruggedness [57], [58] and [59].

3.2.1. Linearity

Linearity was established by least squares linear regression analysis of the calibration curve [60] and [61]. The constructed calibration curves were linear over the concentration range of 2–30 μg/ml and 1–20 μg/ml for AT (n = 7) and AM (n = 7), respectively. Peak areas of AT or AM were plotted versus their respective concentrations and linear regression analysis performed on the resultant curves. Correlation coefficients (n = 3) were found to be more than 0.999 for both the drugs with %RSD values ranging from 0.37–3.85% across the concentration ranges studied. Typically, the regression equations were: $y = 310.5x + 3.84$ (R = 0.9994) for AT and $y = 153.4x + 12.56$ (R = 0.9993) for AM, respectively.

3.2.2. LOQ and LOD

The LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following triplicate injections. The resultant %RSD for these studies was ≤0.65%. The LOQ that produced the requisite precision and accuracy was found to be 2 μg/ml for AT and 1 μg/ml for AM, respectively. The LOD was determined based on signal-to-noise ratios and was determine using an analytical response of three times the background noise [62]. The LOD for both AT and AM were found to be 0.65 μg/ml and 0.35 μg/ml, respectively.

3.2.3. Precision

The intra- and inter-day variability or precision data are summarized in Table 1 and were assessed by using standard solutions prepared to produce solutions of three different concentrations of each drug. AM and AT were used in the same solution for the purposes of these studies. Repeatability or intra-day precision was investigated by injecting nine replicate samples of each of the samples of three different concentrations. Inter-day precision were assessed by injecting the same three samples over three consecutive days.

Table 1
Intra- and inter-assay precision data ($n = 9$)

Actual concentration	Measured concentration (μg/ml), RSD (%)	
	Intra-day	Inter-day
Atorvastatin (μg/ml)		
2	2.05, 2.43	2.00, 3.25
15	15.23, 1.33	15.40, 3.65
30	30.40, 0.35	29.50, 1.65
Amlodipine (μg/ml)		
1	1.02, 2.10	1.00, 3.00
10	10.20, 1.70	10.22, 3.15
20	20.75, 0.40	20.90, 2.50

Data expressed as mean for "measured concentration" values.

3.2.4. Accuracy

Accuracy data for the assay following the determination of each of the compounds of interest are summarized in Table 2. Accuracy was determined by interpolation of replicate (n = 5) peak areas of three accuracy standards of different concentration, from a calibration curve that had been prepared as previously described. In each case, the percent relevant error and accuracy was calculated and found to be less than 3.2% for each of the compounds.

Table 2
Accuracy data ($n = 5$)

	Interpolated concentration (mean ± SD)	RSD (%)	RE (%)
Atorvastatin concentration (μg/ml)			
2	2.06 ± 0.064	3.30	2.90
15	15.36 ± 0.400	2.45	2.35
30	30.50 ± 0.58	1.90	1.70
Amlodipine concentration (μg/ml)			
1	1.022 ± 0.03	2.95	2.0
10	10.100 ± 0.23	2.27	0.9
20	20.650 ± 0.14	0.65	3.1

Data obtained from five replicates at each concentration.

3.2.5. Specificity

The results of stress testing studies in addition to that of monitoring standard solutions of each drug in the presence of their impurities indicated a high degree of specificity of this method for both AT and AM. The degradation product(s) of each of the parent compounds was found to be similar for both the Caduet® tablets and API powders assessed. Typical chromatograms obtained following the assay of untreated tablet powder samples and stressed tablet samples are shown in Fig. 2.

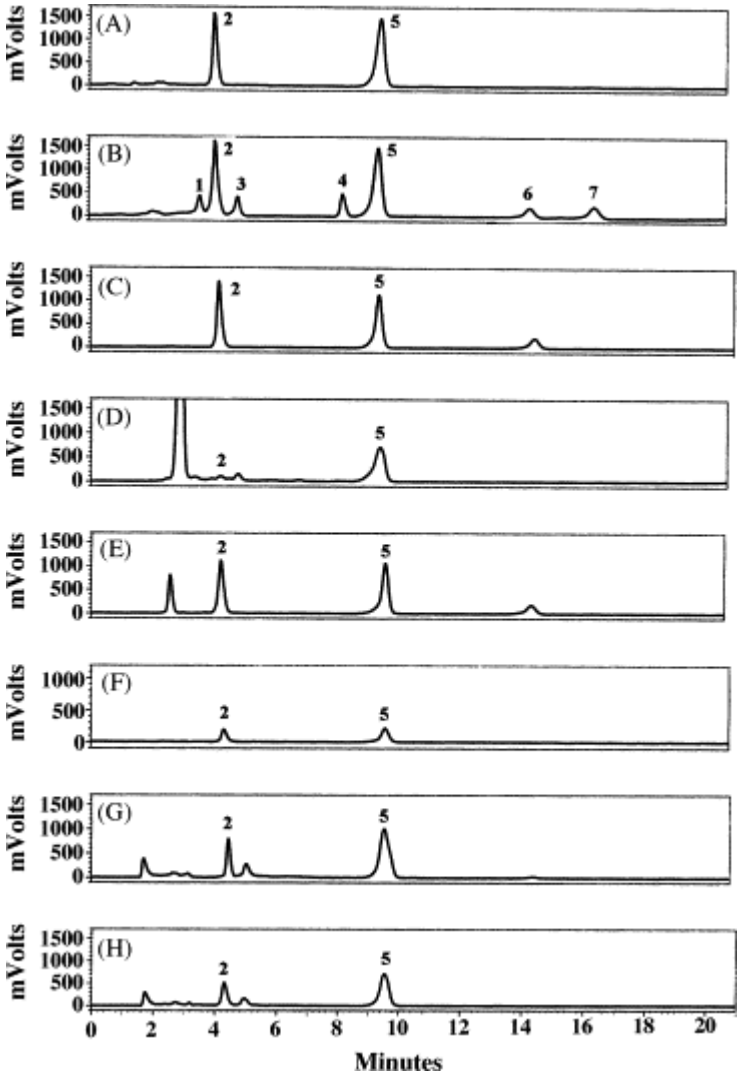

Fig. 2. Typical HPLC chromatograms of: (A) untreated tablet powder; (B) untreated spiked tablet powder; (C) dry-heated tablet powder; (D) oxidative degraded tablet powder; (E) acid hydrolysis-degraded tablet powder; (F) photodegraded tablet powder; (G) neutral-hydrolysis degraded tablet powder; (H) base hydrolysis-degraded tablet powder, showing amlodipine's impurity E (1), amlodipine (2), amlodipine's impurity D (3), diastereomer-atorvastatin (4), atorvastatin (5), atorvastatin lacton (6) and atorvastatin ester(7). The concentration of sample in (B): amlodipine's impurity E (1.5 μg/ml), amlodipine (9.999 μg/ml;observed from tablets assay), amlodipine's impurity D (1.5 μg/ml), diastereomer-atorvastatin (2 μg/ml), atorvastatin (10.003 μg/ml;observed from tablets assay), atorvastatin lacton (1 μg/ml) and atorvastatin ester(1 μg/ml).

3.2.6. Recovery

A known amount of each standard powder was added to samples of tablet powders, which was then mixed, extracted and subsequently diluted to yield a starting concentration of 14 μg/ml for AT and 12 μg/ml for AM. These samples were prepared as described in the Section 2.4 and analyzed as previously described. The assay was repeated over 3 consecutive days (n = 9) to obtain intermediate precision data. The observed concentrations of AT and AM were found to be 14.15 ± 0.25 μg/ml (mean ± SD) and 12.1 ± 0.2 μg/ml, respectively. The resultant %RSD for these studies were found

to be 1.75% for AT and 1.65% for AM with a corresponding percentage recovery value of 99.9% and 100.85%, respectively.

3.2.7. Ruggedness and robustness test

As recommended in the ICH Guidelines and the Dutch Pharmacists Guidelines a robustness assessment was performed during the development of the analytical procedure [63]. The ruggedness [64] of the method is assessed by comparison of the intra- and inter-day assay results for AT and AM that has been performed by two analysts. The %RSD values for intra- and inter-day assays of AT and AM in the Caduet® tablets performed in the same laboratory by two analysts did not exceed 3.8%, indicating the ruggedness of the method. In addition, the robustness of the method was investigated under a variety of conditions including changes of pH of the eluent, flow rate and of buffer composition [65]. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust and the data are summarized in Table 3.

Table 3
Robustness testing of the method

Parameter	Modification	Atorvastatin (%recovery)	Amlodipine (%recovery)
pH	4.4	100.1	98.9
	4.5	100.4	100.2
	4.6	99.8	101.4
Buffer composition (M)	20	98.4	100.0
	25	101.0	99.5
	30	100.6	99.9
Flow rate (ml/min)	0.9	101.7	100.5
	1.0	98.8	100.0
	1.1	99.6	101.0

3.2.8. Stability studies

All stressed samples tested in the solid state and in solution remained colorless following testing and AM and AT were found to be relatively stable following exposure to dry heat conditions, resulting in 5% and 10% decomposition, respectively. Under these conditions the main AT degradation product was atorvastatin lactone. No decomposition was seen on exposure of AT solid drug powder to light in the photostability chamber, whereas photolytic exposure of AT API and in the Caduet® tablets in methanol and water (20:80, v/v) resulted in 40% and 38% decomposition, respectively. Photooxidative degradation of AT in acetonitrile/water solutions have been reported, and its three major by-products isolated and discussed possible mechanisms for the photooxidative processes which lead to them [66]. Photochemical behavior of the drug AT in water has also been reported. Particular attention has been focused on the isolation and characterization of its main photoproducts as well as to the elucidation of the possible mechanistic pathways that lead to the observed products [67].

All compounds of the “1, 4-dihydropyridine” class are susceptible to photolytic decomposition and AM is no exception and undergoes oxidation when exposed to light [68], [69] and [70] resulting in the formation of a pyridine analogue, lacking any therapeutic effect [71]. AM was found to be photosensitive in both the solid state and in solution. In spite of testing in the

solid state, this drug was found to be less stable under photolytic stress conditions for both pure API and Caduet® tablets when stored in methanol and water (20:80%, v/v), resulting in 65% and 70% decomposition for both forms, respectively. AM API and the pharmaceutical tablets resulted sufficiently stable, with a decrease of AM of 10% and 7% after 40 h of light exposure in a photostability chamber, respectively. These drugs were found to be more stable under acidic and neutral degradation conditions rather than under alkali stress conditions. AT API decomposed to 30%, 20% and 50% under acidic, neutral and alkaline stress conditions, respectively. The hydrolysis of the lactone and the lactonization of the β-hydroxy acid can take place at the same time. Reports on such interconversion process have been seen in the literature for AT [51]. On the other hand, AM API decomposed to 25%, 10% and 70% under acidic, neutral and alkaline stress conditions, respectively. The degradation of the molecules is ascribed to the acid or alkaline hydrolyses of the acetyl groups of amlodipine [72] and [73]. These drugs were more unstable under oxidative stress conditions with concentrations decreasing by 70% for AT and 80% for AM, respectively. All potential impurities of AM have been isolated and characterized using LC–MS technique [74]. AM degradation products produced by biotransformation have also been described using LC/MS/MS technique [75]. The stability of stock solutions was determined by quantitation of each drug in solution in comparison to the response obtained for freshly prepared standard solutions. No significant changes (<2%) were observed for the chromatographic responses for the stock solutions analyzed, relative to freshly prepared standards. The stock solutions stability data are summarized in Table 4.

Table 4
Stability of stock solutions stored at 4 °C for three weeks ($n = 5$)

Actual concentration	Interpolated concentration (μg/ml), RSD (%)	
	Standard solution (fresh) (μg/ml)	Stock solution (μg/ml)
Atorvastatin (μg/ml)		
2	2.03, 2.22	2.04, 1.85
15	15.26, 0.45	14.98, 1.00
30	30.30, 1.15	29.80, 2.10
Amlodipine (μg/ml)		
1	1.01, 2.52	1.00, 1.90
10	10.15, 1.64	10.28, 0.66
20	20.56, 0.37	20.21, 2.35

Data expressed as mean for "measured concentration" values.

3.2.9. Assay

The validated method was applied to the determination of AT and AM in commercially available Caduet® tablets. Fig. 3. illustrates two typical HPLC chromatograms obtained following the assay of Caduet® tablets (A) and from a standard solution (B). The result of the assays (n = 9) undertaken yielded 100.03% (%RSD = 1.40%) and 99.99% (%RSD = 2.1%) of label claim for AT and AM, respectively. The observed concentrations of AT and AM were found to be 10.003 ± 0.140 μg/ml (mean ± SD) and 9.999 ± 0.209 μg/ml, respectively. The mean retention times of AM and AT were 4.3 and 9.5 min with associated %RSD values of 0.12% and 0.13%, respectively. The results of the assay indicate that the method is selective for the analysis of both AM and AT without interference from the excipients used to formulate and produce these tablets.

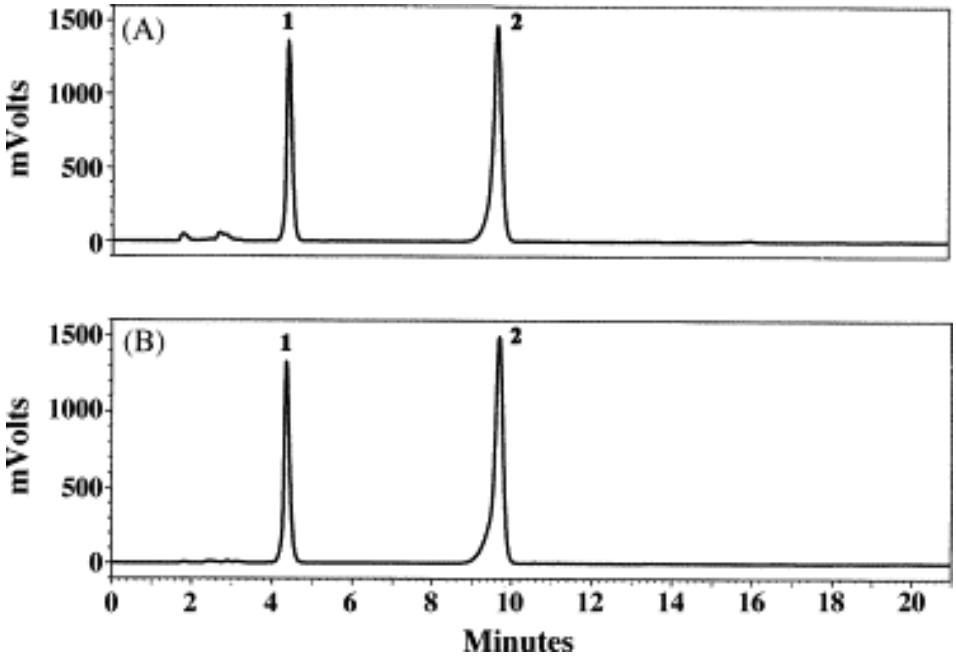

Fig. 3. Resultant HPLC chromatograms obtained following the analysis of Caduet® tablets (A), and a standard solution containing amlodipine (10 μg/ml) and atorvastatin (10 μg/ml) (B), showing amlodipine (1) and atorvastatin (2).

4. Conclusions

A simple, rapid, accurate and precise stability-indicating HPLC analytical method has been developed and validated for the routine analysis of AT and AM in API and tablet dosage forms. The results of stress testing undertaken according to the International Conference on Harmonization (ICH) guidelines reveal that the method is selective and stability-indicating. The proposed method has the ability to separate these drugs from their degradation products, related substances, excipients found in tablet dosage forms and can be applied to the analysis of samples obtained during accelerated stability experiments.

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