

A Quick and Sensitive Liquid Chromatography-Tandem Mass Spectrometry (LC-MS) Method for the Evaluation of Two Formulations of Amlodipine and Atorvastatin in Healthy Male Volunteers

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ABSTRACT: An open-label, single-dose, randomized, 2-way crossover study was conducted in fasted healthy Iranian male volunteers. Eligible participants randomly assigned in a 1:1 ratio to be given one tablet of the test or reference formulation, followed by a 2-week washout period and administration of the exchange formulation. A quick and susceptible Liquid Chromatography-tandem Mass Spectrometry (LC-MS) method for the evaluation of amlodipine and atorvastatin in tablets. Revealing of analysts was achieved by tandem mass spectrometry with Electrospray Ionization (ESI) interface in positive ion mode was operated under the multiple-reaction monitoring mode. The assay results ascertain the presence and compendia quality of amlodipine and atorvastatin in all these products. The validation tests on the developed method indicated acceptable degree of linearity, sensitivity, precision, accuracy and recovery for the method. The intra-day and inter-day precision and accuracy results were well within the acceptable limits. The method is rapid, simple, very stable and specific for the partition, assignment, evaluation of amlodipine and atorvastatin healthy Iranian adult male volunteers.

KEYWORDS: Amlodipine; Atorvastatin; LC-MS; Tablets; Male volunteers.

INTRODUCTION

However, the study for the simultaneous determination of amlodipine and atorvastatin in human plasma was limited. Until Amlodipine (AM), 2[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-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 management of hypertension, chronic stable angina pectoris and prinzmetalvariant angina [2]. AM inhibits the transmembrane influx of calcium ions into vascular

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1021-9986/2017/1/117

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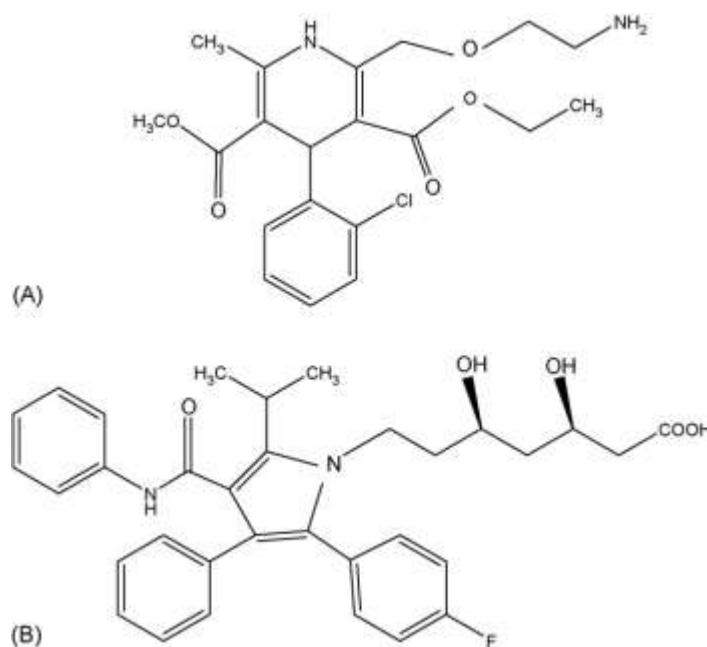


Fig. 1: Structural formulae for (A) amlodipine ($MW= 408.9$) and (B) atorvastatin ($MW= 558.7$).

smooth muscle and cardiac muscle[3–5]. Atorvastatin (AT) is chemically described as [R-(R*, R*)]-2-(4-fluorophenyl)-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid (Fig. 1.B)[1]. Atorvastatin calcium is a potent inhibitor of HMG-CoA(3-hydroxy-3-methylglutaryl-coenzyme A) reductase, the rate-limiting enzyme in cholesterol biosynthesis and has been demonstrated to be effective in reducing both cholesterol and triglyceride [6]. It undergoes extensive first-pass metabolism and is mainly metabolized by Cytochrome P450 3A4(CYP3A4). Liver metabolism produces two active hydroxyl metabolites, ortho-hydroxyatorvastatin and para-hydroxyatorvastatin, and three corresponding inactive lactone metabolites [7]. Atorvastatin is widely used in the treatment and prevention of atherosclerotic disease. However, it may cause rhabdomyolysis, the risk of which is increased by CYP3A4 inhibitors [8]. Amlodipine and atorvastatin fixed dose combinations have been demonstrated in numerous clinical trials to be highly effective in lowering blood pressure and low-density lipoprotein cholesterol. One such combination available in the market is Caduet® (www.caduet.com), which is a combination of amlodipine and atorvastatin in a single

pill. More than one anti-hypertensive agent is indicated for patients with multiple cardiovascular risk factors such as hypertension. Combination therapy with calcium channel blockers and angiotensin-converting enzyme inhibitors provides enhanced antihypertensive activity. There were many studies available for the determination of amlodipine or atorvastatin in human plasma [9–13]. Recently, only two papers on this topic were published. In addition, the methods of sample preparation employed in those studies were rather complicated, which hampers the routine monitor of amlodipine or atorvastatin in plasma [14–17]. The method that can simultaneously determine amlodipine and atorvastatin in human plasma was required. There is an article to this journal for simultaneous determination of sulfamethoxazole and bethalazine by HPLC[18]. The previous our work was the determination of ezetimibe by LC–MS method in human plasma [19–22]. As a result, a simple method that can simultaneously determinate of amlodipine and atorvastatin in volunteers was required. Our aim was to develop and validate a simple and rapid LC–MS method for the quantification of two formulations of amlodipine and atorvastatin in healthy male volunteers.

EXPERIMENTAL SECTION

Materials

Amlodipine and atorvastatin extended release test tablets (batch no. 01, Bakhtar -biochemi), Amlodipine and atorvastatin reference tablets (batch no. 0835019, Pfizer) and amlodipine and atorvastatin reference standard (99.9% purity) were supplied and identified by Pfizer (Ireland). Other chemicals and solvents were from the chemical lab or HPLC purity grades, whenever needed, and were purchased locally. Drug-free human plasma was provided by Iranian Blood Transfusion Organization after routine safety evaluations.

Instrumentation and operating conditions

Liquid chromatography

Liquid chromatography was performed using an Agilent LC-1200 HPLC system consisting of an auto sampler. The column was a Zorbax XDB-ODS C18 column (2.1mm×30mm, 3.5 microns) and was operated at 25°C. The mobile phase consisted of acetonitrile-ammonium acetate buffer (10 mM, pH = 3.0)70:30 (v/v) was set at a flow rate of 0.15 mL/min.

Mass spectrometry

Mass spectrometric detection was performed using Agilent LCMS-6410 quadrupole mass spectrometer with an Electrospray Ionization (ESI) interface. The ESI source was set at positive ionization mode. The mass selective detector was used in the Multiple Reaction Monitoring (MRM) mode for the highest possible selectivity and sensitivity. The MS operating conditions were optimized as follows: Ion spray voltage was set to 4000V, temperature of the ion transfer capillary was 250 °C, Nebulizer gas (NEB) was 30 psi, Dwell time per transition (ms) 200, Gas flow 8 L/min, Collision gas for amlodipine 8 and for atorvastatin 20. Quantitative determinations were performed in multiple reactions monitoring scan mode using the following transitions: m/z 409.1→237.9 for amlodipine, m/z 559.3→440.2 for atorvastatin. The quantification was performed via peak-area. Data acquisition and processing were accomplished using Agilent LC-MS solution Software forLCMS-6410 system.

Standard preparation

A stock solution of 0.2 mg/mL amlodipine and atorvastatin in methanol were prepared, from which

the concentrations of 0.1, 0.5, 0.1, 2.5, 5 and 10 ng/mL for amlodipine and concentrations of 0.2, 2.5, 5, 5, 10, 15 and 20 ng/mL for atorvastatin were prepared by serially diluting this solution with the proper amount of mobile phase.

Estimation of amlodipine and atorvastatin in tablet dosage form

For amlodipine, each tablet contains 5 mg of amlodipine and for atorvastatin 20 mg each tablet contains atorvastatin. Twenty tablets were taken and weighed accurately. The average weight of one tablet was calculated and powdered. Equivalent to 5 mg of amlodipine and 20 mg atorvastatin of powder was taken and transferred to a 100 mL volumetric flask and about 75 mL of phosphate buffer at pH 7.2 was added and sonicated to dissolve. The volume was made up to the mark with phosphate buffer. The solution was filtered through a membrane filter (0.22 µm) and sonicated to degas. Then 5 mL of above solution was pipetted out in 50 mL volumetric flask and volume were made up to the mark with phosphate buffer. The prepared solution was injected into the LC-MS system and the observation was recorded.

Dissolution test

The dissolution test was undertaken using tablet dissolution tester in 6 replicates for each brand. Dissolution media were USP buffer solutions at pH 7.2 (phosphate buffer solution). The medium was maintained at $37 \pm 0.5^\circ\text{C}$. In all the experiments, 5 mL of dissolution sample was withdrawn at 5, 10, 15, 20, 25, 30, 45, 60 and 60 min and replaced with equal volume to maintain sink condition. Samples were filtered and assayed by the LC-MS method. The concentration of each sample was determined from a calibration curve obtained from pure samples of amlodipine and atorvastatin.

Analysis Validation Tests

Standard curve (Linear range)

The samples with a series of known concentrations, prepared as described, were analyzed in three separate runs and, in each case, the linear regression analysis was carried out on known concentrations of amlodipine and atorvastatin against the corresponding peak heights and, then, the regression coefficient (*r*), slope, and *y*-intercept of the resulting calibration curves was determined.

Within-run variations

In one run, three samples with concentrations of 0.1, 5, and 10 ng/mL (from high, middle, and low regions of the standard curve) for amlodipine, three samples with concentrations of 0.2, 10, and 20 ng/mL (from high, middle, and low regions of the standard curve) for atorvastatin were prepared in triplicate and analyzed by developed LC-Mass method. Then, the coefficient of variations (CV %) of the corresponding determined concentrations was calculated in each case.

Between-run variations

On three different runs, samples from upper, intermediate, and lower concentration regions used for construction of standard curve (the same as within-run variations test) were prepared and analyzed by the LC-Mass method. Then, the corresponding CV% values were calculated.

Absolute recovery (Accuracy)

For each sample tested for within- and between-run variations, the absolute recovery of the method was determined as the percent ratio of the measured concentration (determined using standard curve) to the corresponding nominal added the concentration.

Relative recovery (Matrix effect)

Three samples with concentrations 0.1, 5, and 10 ng/mL (from high, middle, and low regions of the standard curve) for amlodipine and three samples with concentrations of 0.2, 10, and 20 ng/mL (from high, middle, and low regions of the standard curve) for atorvastatin were prepared in triplicate and analyzed by developed LC-Mass method. Then, the ratio of the recorded peak heights to the peak heights resulted from the direct injection of the aqueous solutions of amlodipine and atorvastatin with the same concentrations were determined as a percentage in each case.

Limits of detection and quantitation

Limit Of Detection (LOD) of the method was determined as the lowest amlodipine and atorvastatin concentration producing a Signal-to-Noise (S/N) ratio of about 3, 4 respectively. Limit Of Quantitation (LOQ) was determined as the lowest amlodipine and atorvastatin concentration capable of being quantitated with enough accuracy and precision.

Stability

Freeze and thaw stability

Three concentration levels of QC (Quality Control) samples were stored at the storage temperature (-20 °C) for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycle was repeated twice, then the samples were tested after three freeze (-20 °C)-thaw (room temperature) cycles.

Short-term temperature stability

Three concentration levels of QC (Quality Control) samples were kept at room temperature for a period that exceeded the routine preparation time of samples (around 6 h).

Long-term stability

Three concentration levels of QC (Quality Control) samples kept at low temperature (-20°C) were studied for a period of 4 weeks.

Post-preparative stability

The auto sampler stability was conducted reanalyzing extracted QC (Quality control) samples kept under the auto sampler conditions (4 °C) for 12 h.

RESULTS AND DISCUSSION

Separation

Detection of analytes was achieved by tandem mass spectrometry with Electrospray Ionization(ESI) interface in positive ion mode was operated under the Multiple-Reaction Monitoring (MRM) mode. The Multiple-Reaction Monitoring (MRM) mode (+) chromatograms extracted from supplemented are depicted in Fig. 2 As shown, the retention times of amlodipine and atorvastatin were 2.5 and 4.3 min respectively. The total HPLC-MS analysis time was 5 min per sample.

Estimation of amlodipine and atorvastatin in tablet dosage form

The percent content of amlodipine and atorvastatin in tablet dosage form was found to be 105.70% and 98.32 respectively. The USP specifications for the assay are that the amlodipine and atorvastatin content should be less than 90 % and not more than 110 %. Therefore, the assay results ascertain the presence and compendia quality of amlodipine and atorvastatin in all these products.

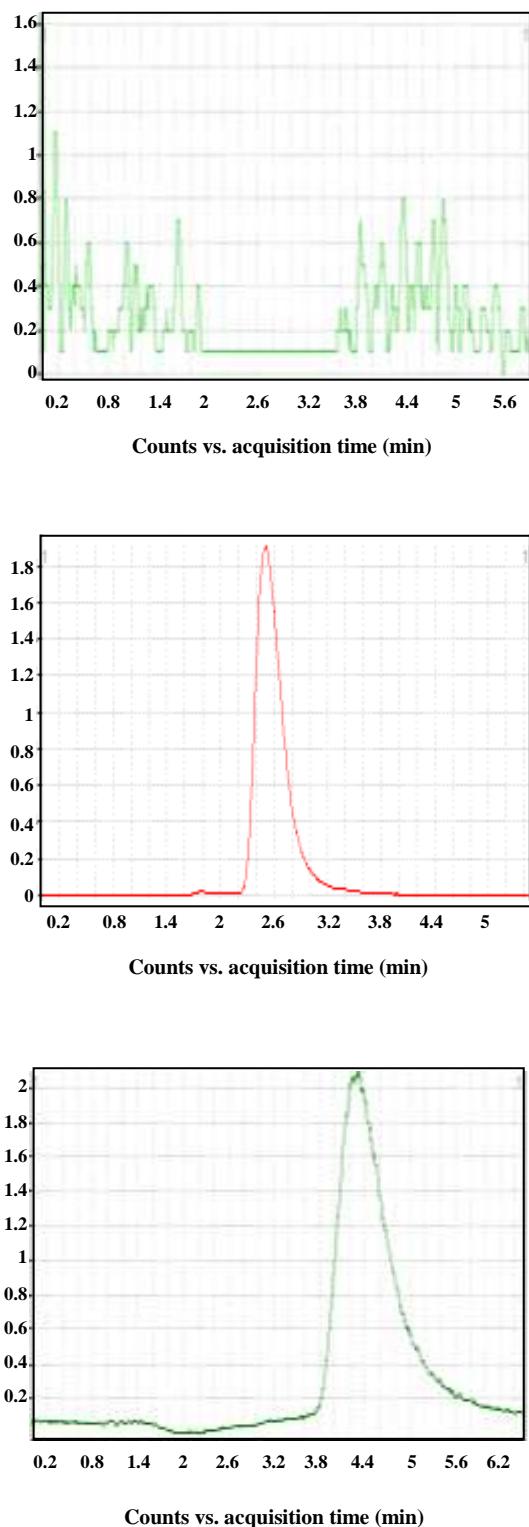


Fig. 2: The MRM (+) chromatograms of amlodipine and atorvastatin (A) Blank (B). Supplemented plasma (concentration of amlodipine = 5 ng/mL). (C) Supplemented plasma (concentration of atorvastatin = 5 ng/mL).

In vitro drug release study

The release of different brands of amlodipine and atorvastatin tablets are shown in Tables 1,2 for atorvastatin and Tables 3,4 for amlodipine. All dissolution data are based on the actual drug content of the test tablets as calculated from the assay results.

Method validation

Assay specificity

No interferences of the analytes were observed. Fig. 2.A shows an HPLC chromatogram of a blank sample indicating no endogenous peaks at the retention positions of amlodipine and atorvastatin.

Linearity and LOQ

The method produced linear responses throughout the amlodipine and atorvastatin. Concentration range of 0.1-10 ng/mL for amlodipine concentration range of 0.2-20 ng/mL for atorvastatin, which is suitable for intended purposes. A typical linear regression equation of the method was: $y = 2753 x + 1090$, for amlodipine and $y = 655.8 x + 319$, for atorvastatin, with x and y representing concentration (in ng/mL) and peak height (in arbitrary units), respectively, and the regression coefficient (r) of 0.999.

The lower limit of quantification for amlodipine and atorvastatin were proved to be 0.1 ng/mL and 0.2 ng/mL respectively. The lower limit of detection for amlodipine and atorvastatin were 0.05 ng/mL and 0.1 ng/mL respectively. Figs. 2. B, C show the chromatogram of a sample that contained amlodipine and atorvastatin with concentrations of 5 ng/mL.

Within-run variations and accuracy

The within-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries for atorvastatin are 0.19, 10.25 and 19.51 for concentrations of 0.2, 10 and 20 ng/mL and for amlodipine are 0.09, 5.1 and 10.07 of 0.1, 5, and 10 ng/mL respectively.

Between-run variations and accuracy

The between-run variations of the developed LC-Mass method as well as the corresponding absolute recoveries for atorvastatin are 0.19, 10.01 and 20.07 for concentrations of 0.2, 10, and 20 ng/mL respectively and for amlodipine are 0.097, 5.13 and 10.21 for concentrations 0.1, 5, and 10 ng/mL.

Table 1: Assay of atorvastatin.

Number	1	2	3	4	5	6	7	8	9	10	M	SD	RSD
Percentage of label claimed	94.99	96.25	102.52	102.25	102.56	96.64	95.16	95.17	95.5	101.72	98.32	3.44	3.5

Table 2: Assay of amlodipine.

Number	1	2	3	4	5	6	7	8	9	10	M	SD	RSD
Percentage of label claimed	101.80	107.08	107.64	107.70	100.13	108.02	106.21	105.05	104.89	108.43	105.7	2.79	2.64

Table 3: The release date of test atorvastatin tablets with dissolution tester.

Time (min)	1	2	3	4	5	6	Mean	SD	RSD
5	56.10	55.64	84.70	57.79	65.94	54.29	54.21	11.68	21.55
10	76.83	83.06	86.83	67.17	74.03	54.32	64.60	11.73	18.16
15	83.87	82.21	91.88	78.39	91.34	61.31	72.00	11.21	15.56
20	83.87	88.31	88.84	80.76	94.52	59.41	73.67	12.30	16.69
25	85.88	96.25	92.28	76.08	97.11	69.85	77.49	11.20	14.45
30	88.44	97.22	97.16	95.95	91.30	91.36	84.49	3.70	4.37
45	88.89	97.21	97.47	100.53	101.73	97.03	89.69	4.49	5.01
60	94.53	100.05	100.52	104.21	107.77	100.54	95.38	4.45	4.66
90	89.78	88.75	92.93	103.29	108.52	102.80	96.58	8.25	8.54

Table 4: The release date of reference atorvastatin tablets with dissolution tester.

Time (min)	1	2	3	4	5	6	Mean	SD	RSD
5	55.56	60.19	56.00	61.65	85.66	61.75	63.47	11.21	17.66
10	77.20	83.05	77.16	61.75	88.94	66.55	75.77	10.12	13.35
15	77.68	95.43	85.42	71.70	94.54	85.49	85.04	9.28	10.91
20	81.86	97.64	88.38	81.04	99.82	89.93	89.78	7.80	8.68
25	86.45	93.47	92.60	91.84	102.32	96.32	93.83	5.26	5.61
30	90.08	94.28	92.60	94.56	102.85	100.53	95.82	4.87	5.09
45	96.43	98.40	92.93	98.21	103.77	101.40	98.52	3.78	3.84
60	100.23	99.02	93.01	101.23	108.56	103.29	100.89	5.11	5.07
90	98.74	93.43	93.60	103.76	103.79	103.76	99.51	5.04	5.06

Extraction recovery

The extraction recovery determined for amlodipine and atorvastatin were shown to be consistent, precise and reproducible. The recovery for amlodipine and atorvastatin are 97.4 and 96.78 respectively.

Stability

The freeze and thaw stability, short term stability, long-term stability and post-preparative stability data amlodipine and atorvastatin are 97.86 and 96.51 respectively. All the results showed the stability behavior during these tests and there were no stability related problems during the samples routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies. The stability of working solutions was tested at room temperature for 6 h. based on the results obtained, these working solutions were stable within 6 h.

CONCLUSIONS

A sensitive, selective, accurate and precise HPLC method with selected ion monitoring by a single quadrupole mass spectrometer with ESI interface in positive ion mode with multiple-reaction monitoring mode was developed and validated for the determination of amlodipine and atorvastatin in healthy male volunteers. The reported method offers several advantages such as a rapid and simple extraction scheme, and a short chromatographic run time, which makes the method suitable for the analysis of large sample batches resulting from the pharmacokinetic, bioavailability or bioequivalent study of amlodipine and atorvastatin.

Acknowledgement

The authors wish to thank Bakhtar Biochemi Pharmaceuticals (Kermanshah, Iran) for kindly providing us with amlodipine and atorvastatin samples. We also would like to thank Zanjan University of Medical Sciences.

Received: Feb. 28, 2015 ; Accepted : May 3, 2016

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