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Research Article

Pharmacokinetics and Bioequivalence Study of Amlodipine and Atorvastatin in Healthy Male Volunteers by LC-MS

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ABSTRACT

Background: A quick and thoughtful liquid chromatography–tandem mass spectrometry (LC-MS) method has been established and authorized for the estimation of amlodipine and atorvastatin in human plasma. ***Methods:*** LC-MS with electrospray ionization (ESI) interface in positive ion mode was functioned under the multiple-reaction monitoring (MRM) mode was used for detection of analytes. Ethyl acetate was secondhand for extraction of analytes from plasma by simple liquid–liquid extraction technique. The re-formed samples with a C_{18} column by pumping acetonitrile-ammonium acetate buffer (10 mM, pH = 3.0), 70:30 (v/v) at a flow rate of 0.15 mL/min were chromatographed. The standard curves were established to be linear in the range of 0.2–20 ng/mL for atorvastatin and 0.1–10 ng/mL for amlodipine with mean correlation coefficient of ≥0.999 for each analyte. ***Results:*** The lower limit of quantification for amlodipine and atorvastatin were demonstrated to be 0.1 ng/ml and 0.2 ng/ml respectively. The mean (SD) Cmax and Tmax values of amlodipine later supervision of the test and reference were: 6.58 (0.22) versus 6.64 (0.37) ng/mL, 6.12(0.86) versus 6.13 (0.73) hours respectively. The mean (SD) Cmax and Tmax values of atorvastatin later government of the test and reference, were 61.66 (3.05) versus 62.16 (0.76) ng/mL, 4.21(0.86) versus 4.22 (0.73) hours respectively. ***Conclusion:*** The results proposed the test formulation of amlodipine and atorvastatin is bioequivalence with reference formulation and the established evaluate method was successfully realistic to a pharmacokinetic and bioavailability trainings in 20 human male volunteers following oral administration of amlodipine and atorvastatin.

Introduction

Amlodipine (Fig. 1A) and atorvastatin (Fig. 1B) was used in mixture as antagonist or low-channel blocker and cholesterol depressing agent respectively. Amlodipine,[1] is a di hydro pyridine derivative with calcium antagonist movement. It is applied in the running of hypertension, chronic stable angina pectoris and prinzmetal variant angina.[2] Amlodipine prevents the trans membrane arrival of calcium ions into vascular smooth muscle and cardiac muscle.[3-5] Atorvastatin is a potent bloker of HMG-CoA(3-hydroxy-3-methylglutaryl-coenzyme A) reductase, the rate restrictive enzyme in cholesterol biosynthesis and has been established to be active in dipping both cholesterol and triglyceride.[6] It suffers general first-pass metabolism and is chiefly digested by Cytochrome P450 3A4 ($CYP3A_4$). Liver metabolism products two vigorous hydroxyl metabolites, ortho –hydroxy atorvastatin and para-hydroxy atorvastatin, and three corresponding sluggish lactone metabolites.[7] Atorvastatin is broadly applied in the handling and inhibition of atherosclerotic disease. Though, it may origin rhabdomyolysis, the risk of which is improved by CYP3A4 inhibitors.[8] There were many exercises standing for the estimating of amlodipine or atorvastatin in human plasma.[9-13] Newly, only two papers on this topic were accessible. Furthermore, the methods of sample training engaged in those revisions were rather trying, which hampers the routine monitor of amlodipine or atorvastatin in plasma.[14-17] As a outcome, a easy method that can simultaneously evaluate amlodipine and atorvastatin in human plasma was needed. The pervious our work was evaluation of ezetimibe by LC–MS method in human plasma.[18] Our purpose was to progress and authorize a easy and speedy LC–MS method for the termination atorvastatin and amlodipine in human plasma. The method has been charity successfully during a bioequivalence revise on a generic product of the drug with the representative results being accessible in the final part of the article.

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The established assay method was efficaciously functional to a pharmacokinetic training in human male volunteers.

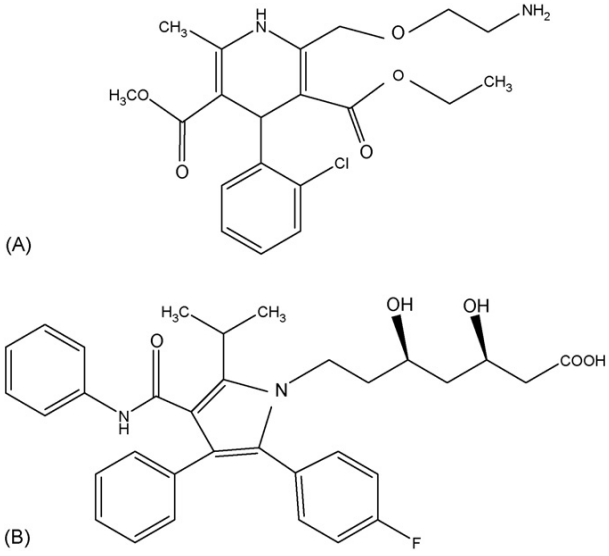

Figure 1. Structure of (A) amlodipine and (B) atorvastatin.

Materials and Methods

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 provided and branded by Pfizer(Irland).Other chemicals and solvents were from 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 achieved using a Agilent LC-1200 HPLC system consisting of an auto sampler (agilent). The column was a Zorbax XDB-ODS C18 column (2.1mm×30mm, 3.5 micron) and was functioned 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 accomplished using an agilentLC-MS-6410 quadrupole mass spectrometer with an electrospray ionization (ESI) interface. The ESI source was conventional at positive ionization mode. The mass selective detector was applied in the multiple reaction monitoring (MRM) mode for the highest possible electivity 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, and 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 for LCMS-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 ready with the appropriate amount of mobile phase and plasma.

Sample preparation and extraction procedure

A 150 µl plasma sample from a human volunteer was purred into a 10 ml centrifuge tube. N-Hexane-isopropanol (95:5, v/v), ethyl acetate and methylene chloride-ethyl acetate (20:80, v/v) were all tried and ethyl acetate was lastly approved because of its high extraction efficiency and less interference. 2.5 ml ethyl acetate was added and then was vortexed for 3 min. After centrifugation of the sample at 15400×g for 10 min, the organic layer was transferred to another 10 ml centrifuge tube and vanished to dryness. The filtrate was liquefied in 150 µl mobile phase. 10 µl was injected into the LC–MS system.

Method Validation

The method was authenticated for discrimination, linearity, precision, recovery, stability, detection limit and quantitation limit steady with the principles of the FDA industry guidance.[19]

Linearity

The plasma 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 approved 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 were determined.

Detection Limit (Limits of detection and quantitation)

Limit of detection (LOD) of the method was evaluated as the lowermost amlodipine and atorvastatin concentration producing a signal-to-noise (S/N) ratio of about 3, 4 respectively. Limit of quantitation (LOQ) was assigned as the lowest amlodipine and atorvastatin concentration capable of being quantitated with sufficient accuracy and precision.

Extraction Recovery

Within- and between-run variations for each sample

verified, the complete recovery of the method was assigned as the percent ratio of the dignified concentration (determined using standard curve) to the corresponding nominal further concentration.

Relative recovery (matrix effect)

Samples with concentrations 0.1, 5, and 10 ng/ml (from high, middle, and low regions of the standard curve) for amlodipine and samples with concentrations of 0.2, 10, and 20 ng/ml (from high, middle, and low sections of the standard curve) for atorvastatin were organized in triplicate and analyzed by LC-Mass method. Then, the ratio of the verified peak heights to the peak heights caused from the direct injection of the aqueous solutions of amlodipine and atorvastatin with the same concentrations were firm as percentage in each case.

Accuracy and Precision

Within-run variations

Samples with concentrations of 0.1, 5, and 10 ng/ml (from high, middle, and low regions of the standard curve) in one run, three 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 organized in triplicate and evaluated by LC-Mass. Then, the coefficient of variations (CV %) of the corresponding strongminded concentrations were assignment in each case.

Between-run variations

Samples from high, middle, and minor concentration sections used for structure of standard curve (the same as within-run variations test) on three different runs, were equipped and examined by LC-Mass method. Then, the corresponding CV% values were designed.

Stability

Freeze and thaw stability

Three concentration levels of Quality control (QC) plasma samples were kept at the stowage temperature (−20° C) for 24 h and thawed unassisted at room temperature.

Short-term temperature stability

Three concentration levels of Quality control plasma samples were reserved at room temperature for 6 h.

Long-term stability

Three concentration levels of Quality control plasma samples retained at low temperature (−20 ◦C) were measured for a period of 4 weeks.

Post-preparative stability

The auto sampler steadiness was steered reanalyzing extracted QC samples retained under the auto sampler situations (4 °C) for 12 h.

Clinical study design

The developed method was recycled efficaciously for assignment of amlodipine and atorvastatin concentrations in plasma samples reserved from 24 volunteers during a double-blind cross over bioequivalence training. For amlodipine and atorvastatin test and reference groups, the 10 mg amlodipine and 40 mg atorvastatin was ordered and blood samples were completed prior to dose administration (time 0) and at 1, 2, 2.5, 3, 3.5, 4, 5, 7, 9, 10.0, 12.0, 24.0,36, 48, 60 and 72 h after the dose. The trials were nearly centrifuged at 1600×g for 10 min. The plasma was detached and kept at −20°C until analysis was finished.

Pharmacokinetic study

The pharmacokinetic factors for amlodipine and atorvastatin were designed by means of standard non-compartmental methods. The peak concentration (Cmax) and the time to range it (Tmax) were evaluated from optical examination of the data and charity as criteria of the rate of absorption. The seeming exclusion rate continual (β) was evaluated by linear regression of log-transformed data in the terminal phase of the concentration-time profile.[20] The exclusion half-life ($t_{1/2}$) was considered by the quotient of 0.693/β. In addition the area under plasma concentration time (AUC0-t) curve was assigned by the linear trapezoidal rule from the dignified concentrations from zero to time of the last quantifiable concentration (Ct). The AUC0-∞, the area under the serum concentration-time curve extrapolated to perpetuity, was calculated as said by the succeeding equation [20]: AUC0-∞ = AUC0-t + Ct/ Kel. The pharmacokinetic profile of amlodipine and atorvastatin from two tablet formulations were related and the comparative bioavailability of test/reference product was designed expending the ratio of AUC0-∞ (test)/ AUC0-∞ (reference).

Results and discussion

Sample preparation

Liquid-liquid extraction was necessary and important because this technique can not only purify but also concentrate the sample.

Separation

Finding of analytes was accomplished by LC-MS with ESI interface in positive ion mode was operated under the MRM mode. Figure 2 show the MRM (+) chromatograms pull out from plasma are as seen, the retention times of amlodipine and atorvastatin were 2.5 and 3.8 min respectively.

Method validation

Assay specificity

Fig. 2.A show an HPLC chromatogram for a blank plasma sample signifying no endogenous peaks at the retention positions of amlodipine and atorvastatin and no intrusions of the analytes were discovered.

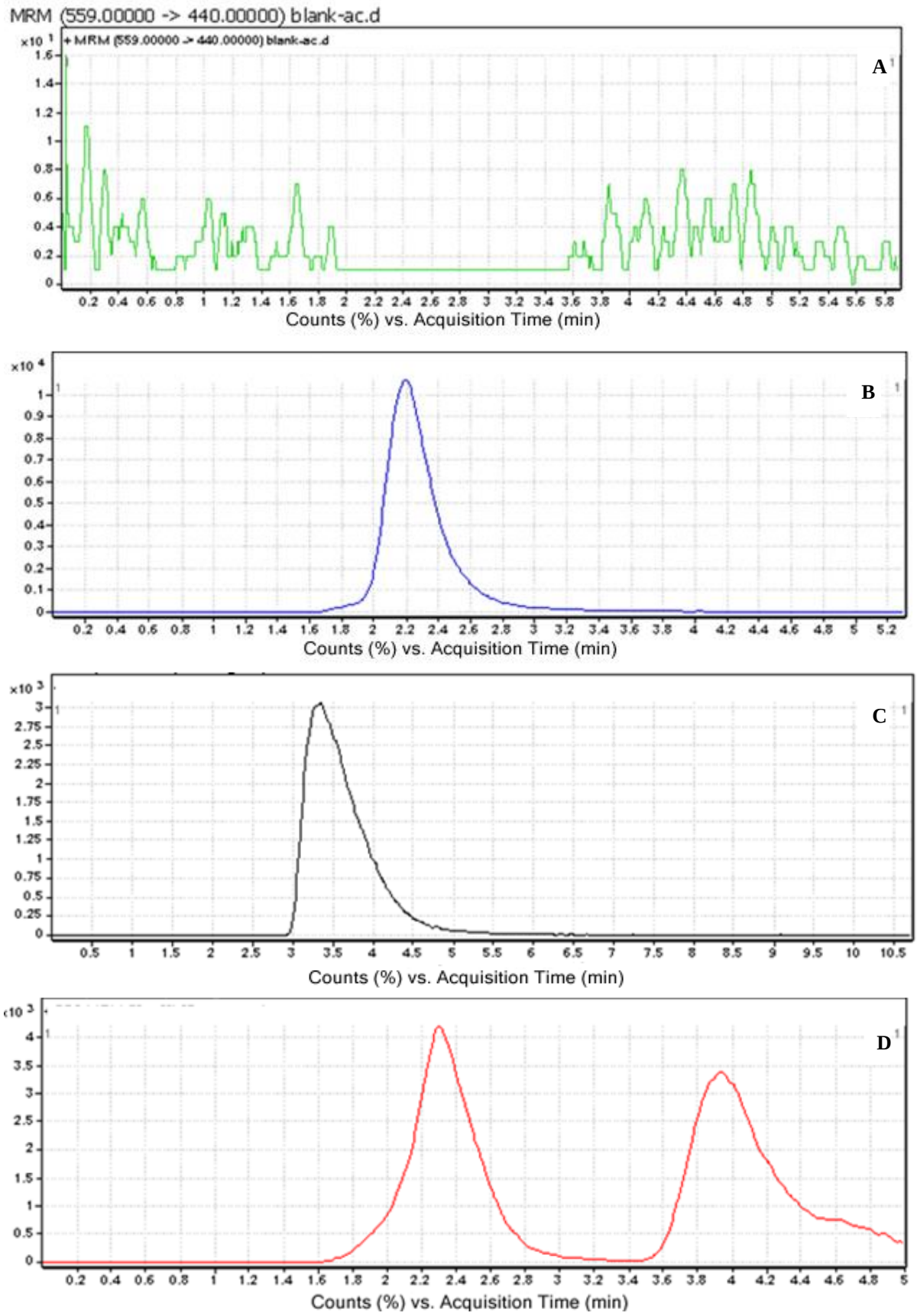

Figure 2. The MRM (+) chromatograms of amlodipine and atorvastatin (A). Blank plasma (B) Supplemented plasma (concentration of amlodipine = 5 ng/ml). (C) Supplemented plasma (concentration of atorvastatin = 5 ng/ml). (D)The MRM (+) chromatograms for plasma sample of a healthy volunteer).The concentration of amlodipine and atorvastatin were 5 ng/ml.

Linearity and LOQ

The method formed linear comebacks through the amlodipine and atorvastatin concentration variety of 0.1-10 ng/ml for amlodipine and concentration range of 0.2-20 ng/ml for atorvastatin, which is safe for suggested purposes. A typical equation of the method was: y = y = 2753 x +1090, for amlodipine and y = 655.8 x +319, for atorvastatin, with x and y showing 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. Figures. 2. B, C show the chromatogram of a pull out sample that confined of amlodipine and atorvastatin with concentrations of 5ng/ml. Fig. 2. D show the chromatogram of an extracted sample that controlled of amlodipine and atorvastatin with concentrations of 3 ng/ml.

Within-run variations and accuracy

The within-run variations of the established LC-MS method as well as the resultant complete recoveries are shown in Table 1, 2.

Table 1. Within–run, between–run variations and accuracy of method for quantitation of amlodipine (n=3) .

Nominal Added Concentration (ng/ml)	Sample Number	Measured Concentration (ng/ml)Within–run	Mean (SD) Within–run	CV% Within–run	Measured Concentration (ng/ml) Between–run	Mean (SD) Between–run	CV% Between–run
0.1	1	0.099	0.098	3.55	0.098	0.098	2.67
	2	0.095	(0.0035)		0.102	(0.0026)	
	3	0.102			0.097		
5	1	4.89	5.00	2.29	4.87	5.10	3.96
	2	5.12	(0.11)		5.23	(0.2)	
	3	5.01			5.21		
10	1	10.11	9.92	1.64	10.21	10.13	2.31
	2	9.81	(0.16)		9.87	(0.23)	
	3	9.85			10.32		

Table 2. Within–run and between–run variations and accuracy method for quantitation of atorvastatin (n=3).

Nominal Added Concentration (ng/ml)	Sample Number	Measured Concentration (ng/ml)Within–run	Mean (SD) Within–run	CV% Within–run	Measured Concentration (ng/ml) Between–run	Mean (SD) Between–run	CV% Between–run
0.2	1	0.19	0.21	9.52	0.18	0.19 (0.02)	10.58
	2	0.23	(0.02)		0.19		
	3	0.21			0.22		
10	1	10.25	10.04	1.86	10.31	10.01	2.56
	2	9.89	(0.18)		9.87	(0.26)	
	3	9.98			9.86		
20	1	19.51	20.38	3.73	20.38	20.07	1.29
	2	21.02	(0.76)		19.98	(0.26)	
	3	20.58			19.89		

Table 3. Qualified recovery of Amlodipine (N=3).

Nominal Added Concentration (ng/ml)	Sample Number	Recovery (%)	Mean (SD)	CV%
0.1	1	90.11	95.17 (4.40)	4.62
	2	97.32		
	3	98.09		
5	1	97.20	94.08 (4.33)	4.59
	2	89.14		
	3	95.89		
10	1	101.00	96.47 (6.46)	6.69
	2	99.34		
	3	89.08		

Between-run variations and accuracy

Table 1, 2 show the between-run variations of the established LC-MS method along with the resultant complete recoveries.

Extraction recovery

Table 3, 4, show the extraction recovery dogged for amlodipine and atorvastatin to be consistent, precise and reproducible.

Table 4. Qualified recovery of Atorvastatin (N=3).

Nominal Added Concentration (ng/ml)	Sample Number	Recovery (%)	Mean (SD)	CV%
0.2	1	93.19	93.62 (3.18)	3.4
	2	90.67		
	3	97.00		
10	1	89.09	95.34 (6.93)	7.27
	2	94.14		
	3	102.81		
20	1	91.00	94.71 (7.53)	7.95
	2	103.39		
	3	89.76		

Stability

Stability data of amlodipine and atorvastatin was showed in Table 5, 6. All the findings revealed the stability of this 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.

Table 5. Recovery values of stability amlodipine in human plasma at different.
QC (Quality control) levels (n=5).

	0.1(ng/ml)	5 (ng/ml)	10(ng/ml)
Short-term stability	91.18	91.2	90.18
Freeze and thaw stability	92.3	94.01	95.21
Long-term stability	96.15	93.65	95.58
Post-preparative stability	97.14	91.87	91.14

Table 6. Recovery values of stability atorvastatin in human plasma at different.
QC levels (n=5).

	0.2(ng/ml)	10 (ng/ml)	20(ng/ml)
Short-term stability	95.57	90.65	95.65
Freeze and thaw stability	96.56	93.25	94.73
Long-term stability	93.61	94.52	95.94
Post-preparative stability	91.65	92.31	91.57

Applicability test

The mean concentration-time curve of reference and test of amlodipine and atorvastatin are exposed in Fig.3 and Fig.4 respectively. By way of it is shown, the mean concentration-time curves from both the test and reference products are about super imposable. Furthermore, there was no important distinction between amlodipine and atorvastatin serum concentrations at each time point subsequent oral administration of the both product. At the first case time (0.5 h), the drug was computable in all subjects following the administration of both arrangements.

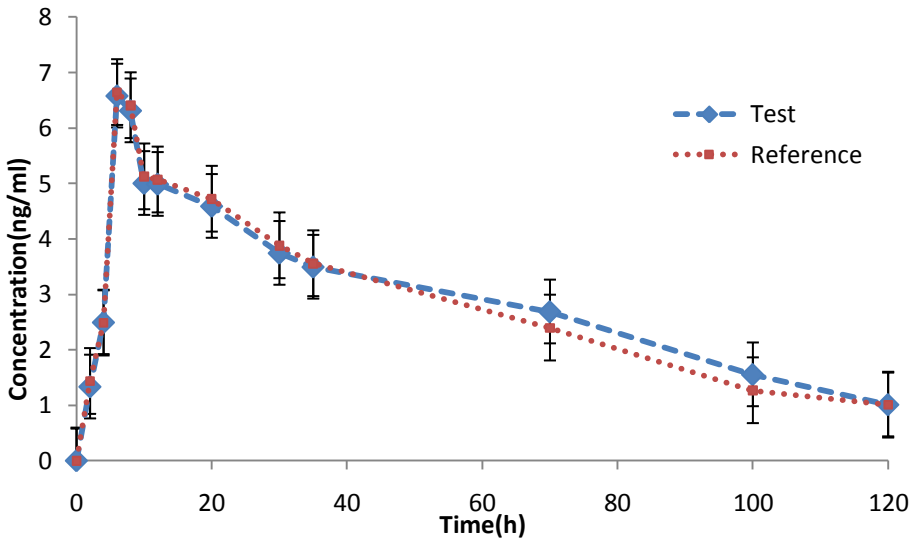

Figure 3. Pharmacokinetic profile of amlodipine of test and reference product of amlodipine.

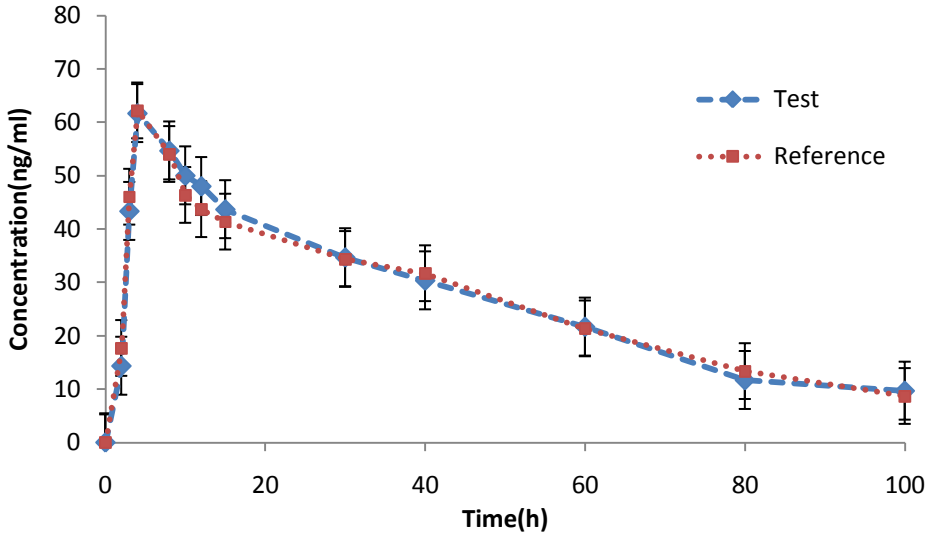

Figure 4. Pharmacokinetic profile of atorvastatin of test and reference product of atorvastatin.

Table 7, 8 show the finding pharmacokinetic parameters amlodipine and atorvastatin. Mean maximum concentrations of 6.58 ± 0.22 ng/ml and 61.66 ± 0.86 ng/ml were obtained for the amlodipine and atorvastatin, respectively. Tmax was 6.12 ± 0.86 h and 4.21 ± 0.76 h, respectively. Additionally to Cmax and Tmax, the ratio of Cmax /AUC0-∞also can be used as a parameter for determination of the absorption rates in bioequivalence trainings.[21-22] these planned ratios were 1.87 % and 6.03 % for the amlodipine and atorvastatin. The parameters used as procedures of the amount of absorption are AUC0-t, AUC0-∞. The AUC0-t and AUC0-∞ for the amlodipine were 290.37 ± 1.13 ng·h/ml and 3500.17 ± 1.98 ng·h/ml, respectively. The considered values for the reference were 821.37± 1.13 ng·h/ml and 1021.98 ± 1.98 ng· h/ml in the order mentioned.

Table 7. Pharmacokinetic factors of amlodipine of the test and reference product (Mean ± SD).

Pharmacokinetics Parameters	Test	Reference
C_{max} (ng/ml)	6.58± 0.22	6.64±0.37
t_{max}	6.12±0.86	6.13± 0.73
AUC_{0-t} (ng/L.hr)	290.37 ± 1.13	2933.39± 1.22
$AUC_{0-\infty}$ (ng/L.hr)	350.17 ±1.98	352.96±1.67
T 1/2	35.12 ±1.90	35.96±1.54

Table 8. Pharmacokinetic factors of atorvastatin of the test and reference product (Mean ± SD).

Pharmacokinetics Parameters	Test	Reference
C_{max} (ng/ml)	61.66± 3.05	62.16±0.76
t_{max}	4.21±0.76	4.22± 0.86
AUC_{0-t} (ng/L.hr)	821.37 ± 1.13	825.39± 1.22
$AUC_{0-\infty}$ (ng/L.hr)	1021 ±1.98	1023±1.67
T 1/2	40.02±1.07	40.12±1.23

Conclusion

For assignment of amlodipine and atorvastatin in human plasma was used of correct and specific LC-MS method with particular ion monitoring by single quadrupole mass spectrometer with ESI interface in positive ion mode with MRM mode was established and authorized. Advantages of this method consist of a quick and easy extraction scheme and a little chromatographic run time, which sorts the method appropriate for the analysis of great sample batches subsequent from the pharmacokinetic, bioavailability or bioequivalent training of amlodipine and atorvastatin.

Acknowledgement

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Conflict of Interest

The authors report no conflicts of interest.

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