Research Article

Simultaneous and Precise HPLC Method for Quantification of Atorvastatin in Rat Plasma and Intestinal Perfusion Solution

Muznah AlKhani[1*], Antoun Al-Laham[1], Mohammed Amer Al-Mardini[2]
[*1]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Damascus University, Damascus, Syria.
[2]Department of Pharmaceutical Chemistry and Quality control, Faculty of Pharmacy, Damascus University, Syria.
***Corresponding author's E-mail:** mznkhani@gmail.com

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ABSTRACT

A rapid isocratic reversed-phase high performance liquid chromatographic with ultraviolet detector (HPLC-UV) was developed and validated for the bioanalysis of atorvastatin in plasma and intestinal perfusion solution of rats. A simple protein precipitation with acetonitrile was applied for preparation of plasma samples. The separation of atorvastatin was achieved on C18 (4.6-mm × 250-mm) column using acetonitrile- water (with 0.05% formic acid; pH 3.3)- methanol (50:40:10, v/v) as a mobile phase at a temperature of 37°C and a flow rate of 0.9 ml/min. Pump pressure was 78 bar, and the UV wave length of detection was 240 nm. The method was validated according to FDA guidelines for bioanalytical methods. Linear responses had R^2>0.998 in the concentration range of 0.1-10 µg/ml in both studied biological media. Short run time of 15 minutes was achieved with high accuracy and precision. Relative and absolute recoveries were higher than 98%.

Keywords: Atorvastatin, HPLC-UV, Validation, Plasma, Intestinal perfusion solution.

INTRODUCTION

Atorvastatin is a synthetic competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, an early and a rate limiting step in cholesterol biosynthesis. It is used to reduce LDL-cholesterol, apolipoprotein B, and triglycerides, and to increase HDL-cholesterol in the treatment of hyperlipidaemias. 10.82 mg of atorvastatin calcium trihydrate is equivalent to 10 mg of base. The usual initial dose is 10 to 20 mg of atorvastatin once daily; an initial dose of 40 mg daily may be used in patients who require a large reduction in LDL-cholesterol. The dose may be adjusted at intervals of 4 weeks up to a maximum of 80 mg daily.[1]

Atorvastatin calcium is chemically described as 1H-Pyrrole-1-heptanoic acid, 2-(4-fluorophenyl)-ß,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[phenylamino)carbonyl]-,calciumn salt (2:1),trihydrate.[2] Its structural formula is shown in figure 1. Molecular formula is (C33H34 FN2O5)2Ca•3H2O, with a molecular weight of 1209.42 g/mol.

Several analytical methods such as radioimmunoassay[3], HPLC with ultraviolet detection[4,5], and LC-MS/MS methods[6,7] were conducted to estimate atorvastatin in pharmaceutical dosage forms and plasma. The aim of this work was to provide a simultaneous, simple and a cost effective method for quantification of atorvastatin in rat's plasma and intestinal perfusion solution for pharmacokinetic studies. Simple preparation of samples with a short run time were required. Validation method was applied according to FDA, 2014; Analytical Procedures.[8-11]

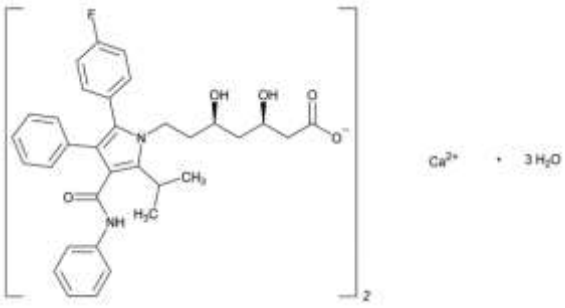


Figure 1: Structural formula of atorvastatin.

MATERIALS AND METHODS

Chemicals and reagents

Atorvastatin calcium (purity, 100.4%) was supplied from Cadila Pharmaceuticals Limited (Ahmedabad, India). Acetonitrile and methanol were HPLC grade. All other salts and reagents were ACS grade from Carl Roth (Karlsruhe, Germany). A UFLC XR Schimadzu HPLC system consisted of two HPLC pumps, and a UV detector, with a vacuum degasser. Chromatograms were recorded by means of a computer and were treated with Realtime Analysis software (Kyoto, Japan). A Knalier Vertex Plus C18, 250X4.6 mm, Eurospher 100-S, 5 µm Column (Berlin, Germany) was used. Siemens Labostar water filteration system, with capsule filter, DNSZ-S 0.2 µm, Type III water (Alpharetta, USA).

Prevalidation

Atorvastatin maximum single oral dose in human is 80 mg once daily. Concerning pharmacokinetic studies the corresponding calculated dose in 300-mg rat was found to be 0.34 mg. Therefore, Intestinal perfusion solution of

30-ml volume would have a concentration of 11.33 µg/ml (9.81 µM).

Samples preparation

Intestinal perfusion solution samples

Experimental rats were obtained from experimental animals facility, Faculty of Pharmacy, Damascus University. Rats were anaesthetized with intramuscular injection of Ketamine, 45-60 mg/Kg and Diazepam, 2.5 mg/Kg combination.[12,13] Abdomen midline incision was performed. Small intestine was canalized starting from duodenum to the end of jejunum (12 cm length). The intestinal lumen was flushed with normal saline warmed to 37°C. Buffer was passed through the lumen at a flow rate of 0.5 ml/min. Krebs-Henseleite Bicarbonate buffer (mM; NaCl, 118; NaHCO3, 24.9; CaCl.6H2O, 2.5; KCl, 4.7; MgSO4.7H2O, 1.2; KH2PO4, 1.2; containing 16.7 mM glucose and 0.01 mM sodium taurocholate, adjusted to pH7.4 using NaOH) was perfused through the intestine. After stabilization period about 20 minutes, intestinal perfusion solution was collected, centrifuged at 3500 rpm for 10 minutes. The supernatant was carefully separated in polypropylene tubes and frozen at -80°C until analysis. Frozen intestinal perfusion samples were thawed at room temperature.[14]

Plasma samples

Blood samples were collected from rats by cardiac puncture, sonicated for 5 minutes to lyse RBCs, centrifuged at 3500 rpm for 10 minutes, and the plasma supernatant was carefully separated and collected in polypropylene centrifuge tubes. To precipitate plasma proteins acetonitrile was added in the ratio 3:1 (acetonitrile:plasma).[15] The matrix was thoroughly mixed and centrifuged for 10 minutes at 6000 rpm. The upper clear solution layer was collected. Plasma aliquot tube was kept on ice until freezing at -80°C. Blank Intestinal perfusion fluid and blank plasma were obtained from six different sources.[16]

Stock solution

Stock solution of atorvastatin was prepared in methanol to obtain drug concentration of 1.0 mg/ml.

Calibration standards (CS) and quality control (QC) samples

Maximum concentrations were determined by calculating the maximum single oral dose in a rat weighing 300 grams, whereas the lower limit of quantitation (LLOQ) was determined through the bioanalytical method. All concentrations of the studied drug should be covered by calibration curve standards. The maximum concentration of atorvastatin in the perfusate would be 11.33 µg/ml. Therefore, the maximum concentration in the calibration curve was 12 µg/ml. During method development it was found that a lower limit of quantitation of 10 ng/ml could be attained. The final concentrations of calibration standards were 10, 50, 100, 250, 500, 1000, 2000, 5000, 10000, 12000 ppm for Atorvastatin.

Standard solutions were prepared by serial dilution in methanol. Precision and accuracy standards were prepared in the same manner. Standard solutions were used to prepare calibration and QC samples in intestinal perfusion solution and plasma, which were spiked after extraction. Samples for relative recovery were prepared by adding standard solutions to blank homogenate before extraction. LLOQ, lower quality control (LQC), medium quality control (MQC), high quality control (HQC) samples were prepared to yield the following concentrations: 10, 30, 90, 4000, 6000, 9000, 12000 ng/ml. Recovery was calculated using the results of QCs used for accuracy and precision on the first day run.

Method validation

The method was validated for selectivity, linearity, precision, accuracy, stability, and recovery. Selectivity was investigated by analysis of blank matrix samples. Lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were calculated according to ICH Q2B guideline on validation. The calculation is based on the standard deviation of the response (σ) and the slope of the calibration curve at levels approaching the limits according to equations below.

$$LLOD = 3.3 \times \left(\frac{\sigma}{S}\right)$$

$$LLOQ = 10 \times \left(\frac{\sigma}{S}\right)$$

The standard deviation of the response was determined based on the standard deviation of the y-intercept of the regression line. Calibration standard samples were prepared for three separate batches. Intra-and inter-batch precision and accuracy were evaluated by measurement of atorvastatin in matrix in five replicates of QC samples at three different concentrations and at LLOQ for three separate batches.

Deviation of the LLOQ from concentration should not exceed 20%, and 15% of standards other than LLOQ. Accuracy was reported as percentage bias or mean % nominal that were calculated from the expressions:

$$\%Bias = \left(\frac{measured\ value - true\ value}{true\ value}\right) \times 100$$

$$Mean\%Nominal = \left(\frac{measured\ value}{true\ value}\right) \times 100$$

Mean % Nominal should be 100 ±15% and 100 ±20% for LLOQ.

Precision was expressed as the percentage coefficient of variance (%CV) or relative standard deviation (RSD) of the replicate measurements

$$\%CV = \left(\frac{standard\ deviation}{Mean}\right) \times 100$$

The stability of atorvastatin in biological matrices was studied at different storage conditions; include stability of stock solution, short-term stability, long-term stability, and freeze-thaw stability.

Recovery was evaluated by replicate analysis of 3 times each at 3 concentration levels (low-, med-, and high levels).

Absolute recovery was determined by comparing the response of spiked matrix with the response of standard solution.

Whereas, relative recovery was the ratio of response to analyte in matrix spiked before extraction to response to analyte in matrix spiked after extraction.

RESULTS AND DISCUSSION

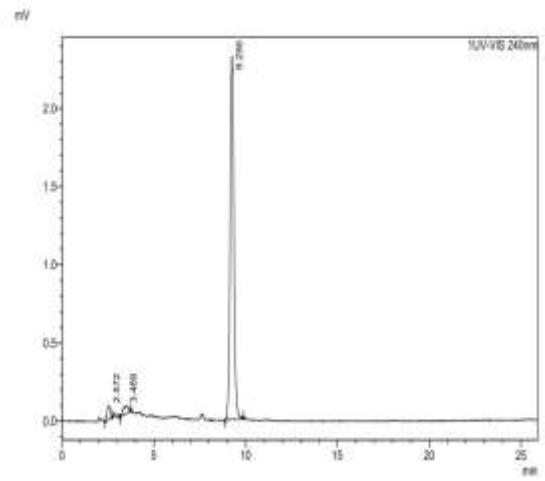


Figure 2: Chromatogram of atorvastatin in solvent under selected chromatographic conditions.

Different chromatographic conditions were tested; column, mobile phase, pH, temperature and flow rate to achieve the desired selectivity, sensitivity and symmetric peak shape in a short retention time.

Chromatographic separation was performed on a 250×4.6 mm, 5 µm Vertex Plus C18 Column maintained at 37°C. Degassing was accomplished by sonicating the mobile phase prior to usage.

Mobile phase solutions were connected to the two inlets of the binary HPLC pump. The separation was under isocratic conditions.

Mobile phase consisted of acetonitrile:water adjusted to pH3.3 using formic acid: methanol (50:40:10, v/v), with a flow rate of 0.9 ml/min.

UV-detector was adjusted on the wave length 240 nm. Pump pressure was 78 bar and the injection volume was 10 µL.

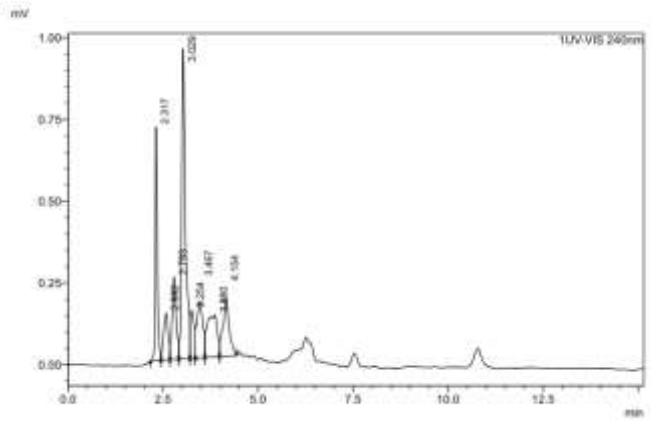
As a result, Short run time of 15 min was achieved. Chromatogram of atorvastatin under the selected chromatographic conditions was shown in Figure 2.

Selectivity and sensitivity

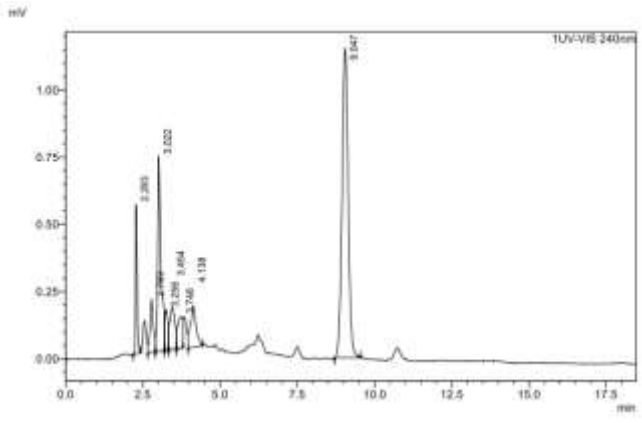
No interfering peaks from endogenous compounds were observed at the retention time of atorvastatin neither in blank plasma matrix (Figure 3) nor in blank perfusate.

Linearity

Linearity was established by analyzing 10 concentrations of atorvastatin ranging between 10 and 12000 ng/ml. Each concentration was measured in triplicate. The curves showed good linear response (R^2>0.998) over the range of 10 to 12000 ng/ml (ppm) for atorvastatin in solvent (methanol), intestinal perfusion solution, and plasma (Figure 4). Microsoft Excel was used to generate linear regression equations for the calibration curves. Calibration curve equation of atorvastatin in solvent was A=0.0012C + 0.1212 with a correlation coefficient (R^2) value of 0.9994 and a standard deviation of intercept equal to 0.0011. In intestinal perfusion solution, the equation was A=0.0012C + 0.1347 with a correlation coefficient (R^2) value of 0.9988 and a standard deviation of intercept equal to 0.0004. In plasma, the equation was A=0.0012C + 0.1357 with a correlation coefficient (R^2) value of 0.9998 and a standard deviation of intercept equal to 0.0003. The regression equation is presented by A=aC+b, where A is the absorbance, a is the slope, b is the intercept and C is the concentration.



(a)



(b)

Figure 3 Blank Plasma matrix (a) and plasma matrix spiked with atorvastatin (b).

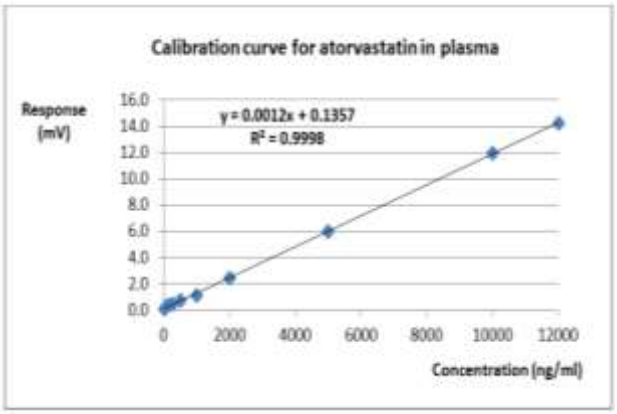


Figure 4: Calibration curve for Atorvastatin in plasma

Determination of limit of quantitation and detection

Lower limits of detection and quantitation were calculated in both perfusate and plasma. In perfusate they were found to be 0.966 and 2.927 ng/ml, respectively. While, In plasma they were found to be 0.728 and 2.205 ng/ml, respectively.

Carry-over in the blank sample following the high concentration standard was not greater than 20% of the LLOQ.

Accuracy and precision

Seven concentration levels covering the calibration curve range; four low concentration levels including the LLOQ and 3× LLOQ (LQC), 3×LQC, two concentration levels within the medium calibration curve range, HQC and ULOQ (upper limit of quantitation). Each concentration level was repeated 5 times within the same day, and in three consecutive days.

The concentrations were calculated from the regression equation. Results for perfusate and plasma samples were shown in Table 1. The within-run %CV values were less than 15% for the QC samples, and less than 20% for LLOQ. Between-run accuracy and precision for atorvastatin in perfusate also showed values within the same acceptance criteria.

Table 1: Intra-and inter-day accuracy and precision in biological matrices

QC spiked conc. (ng/ml)	Precision and accuracy											
	In perfusate						In plasma					
	Intra-day			Inter-day			Intra-day			Inter-day		
	RSD (%)	%RE	Accuracy (%)	RSD (%)	%RE	Accuracy (%)	RSD (%)	%RE	Accuracy (%)	RSD (%)	%RE	Accuracy (%)
10	9.17	-9.17	90.83	8.33	-9.72	90.28	11.36	-2.50	97.50	2.69	-5.28	94.72
30	5.76	-3.61	96.39	3.24	-5.65	94.35	5.10	-2.50	97.50	3.78	-3.17	96.83
90	0.93	-0.46	99.54	3.47	-1.94	98.06	1.70	0.09	100.09	1.75	-1.41	98.59
4000	0.20	-0.15	99.85	0.08	-0.06	99.94	0.29	-0.19	99.81	0.12	-0.33	99.67
6000	0.29	-0.01	99.99	0.06	-0.07	99.93	0.38	-0.25	99.75	0.30	-0.54	99.46
9000	0.01	-0.01	99.99	1.03	-1.02	98.98	1.68	-0.98	99.02	0.41	-0.68	99.32
12000	0.15	-0.21	99.79	1.53	-0.99	99.01	1.53	-1.05	98.95	0.66	-0.33	99.67

Table 2: Stability testing of atorvastatin in intestinal perfusate and plasma (n=3)

Matrix	Stability	QC spiked concentration (ng/ml)	Mean concentration found (ng/ml)	SD	R.S.D. (%)	Relative error (%)	% of initial concentration	% of nominal concentration
Intestinal perfusate	**Short-term stability(6 h)**	30	29.61	0.34	3.596	103.596	98.704	97.111
		4000	3998.31	17.1	0.202	100.202	99.958	106.400
		9000	8993.58	10.83	-0.059	99.941	99.929	97.425
	Three freeze-thaw cycle	30	28.31	1.27	-0.972	99.028	94.352	91.778
		4000	3980.25	19.65	-0.251	99.749	99.506	96.467
		9000	8740.17	375.27	-2.875	97.125	97.113	96.758
	Long-term stability (6 months)	30	28.58	0.83	0.000	100.000	95.278	88.889
		4000	3946.92	51.26	-1.086	98.914	98.673	100.733
		9000	8984.14	90.7	-0.164	99.836	99.824	97.592
Plasma	**Short-term stability (6 h)**	30	29.42	2.20	-0.94	99.06	98.06	92.00
		4000	3988.31	8.67	-0.10	99.90	99.71	101.80
		9000	8983.58	33.21	1.21	101.21	99.82	97.14
	Three freeze-thaw cycle	30	27.19	2.10	-8.42	91.58	90.65	89.111
		4000	3886.64	129.71	-2.65	97.35	97.17	92.600
		9000	8719.14	96.40	-1.77	98.23	96.88	92.892
	Long-term stability(6 months)	30	27.47	2.55	-7.48	92.52	91.57	86.222
		4000	3901.08	118.90	-2.29	97.71	97.53	97.467
		9000	8873.86	98.62	-0.02	99.98	98.60	90.225

Table 3: Recovery of atorvastatin

Added conc (ng/ml)	In perfusate		In plasma	
	Absolute recovery (%)	Relative recovery (%)	Absolute recovery (%)	Relative recovery (%)
30	106.45	99.801	108.33	99.513
4000	99.96	99.718	99.995	98.854
9000	99.64	98.982	99.115	98.179
Mean	102.016	99.500	102.480	99.182

Stability

Stability of atorvastatin was measured by comparing measured low, medium, and high QCs concentrations after experiment with concentrations of freshly prepared QCs. All percent deviation values were <15%, which indicated stability of atorvastatin in the study matrices under the experimental conditions (Table 2).

Stock solution was stable at room temperature for 6 hours (RSD < 1.5%).

Recovery

Absolute and relative recoveries in perfusate and plasma were calculated from the relation of response to atorvastatin spiked in matrices before and after extraction (Table 3).

Robustness

For the evaluation of robustness, minor changes in the pH, mobile phase ratio, temperature of the column did not significantly affect the retention time of the peak.

CONCLUSION

A simple, rapid and cost effective reversed-phase HPLC-UV method for bioanalysis of atorvastatin was developed and validated for pharmacokinetic studies. The method was highly selective, sensitive, accurate and precise, with a high percentage of recovery. A short run time, and small-volume samples were applicable.

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