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Atorvastatin analysis by fully validated HPLC assay in human plasma

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ABSTRACT

A simple, sensitive high-performance liquid chromatography (HPLC) assay for atorvastatin measurement in human plasma was fully validated, and atorvastatin stability was studied. After one-step extraction of 1ml plasma with 5.0ml of ethyl acetate and reconstitution in mobile phase, glipizide (internal standard, IS) and atorvastatin eluted at 4.6 and 8.7 minutes, respectively, on an Atlantis C₁₈, 5µm steel at room temperature (RT), and were detected using a Water 2690 dual absorbance detector set at 247nm. The mobile phase, 0.05 M dibasic sodium phosphate buffer (pH = 4.0) and acetonitrile (50:50, v:v), was delivered at 1.0ml/min. Calibration curves were linear in the range 0.02-1.0µg/ml, and intra- and inter-run coefficients of variation were ≤ 12 % and ≤13.1 %, respectively. Extraction recovery and intra- and inter-run bias were ≥ 81% (mean 91%), ≤14%, and ≤ 10 %, respectively. Atorvastatin was stable in plasma for 24 hours at RT (≥ 95%), 6 weeks at -20°C (≥ 85%), and after 3 cycles of freeze at -20°C and thaw at RT (≥ 90%). In extracted samples, atorvastatin was stable for 24 hours at RT (≥ 96%) and 48 hours at -20°C (≥ 96%). Atorvastatin (1 mg/ml) in water was stable for 48 hours at RT (88%) and 6 weeks at -20°C (112%).

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KEYWORDS

Atorvastatin calcium;
Glipizide;
HPLC;
Stability;
Validation.

INTRODUCTION

Atorvastatin (CAS; 134523-03-8) is a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is used for the treatment of hypercholesterolemia^[1,2]. It is rapidly absorbed after oral administration, however, due to presystemic clearance in the gastro-intestinal mucosa and metabolism in the liver, its absolute bioavailability is approximately 12% and low plasma concentration is achieved following its administration^[3]. Peak plasma concentration of 452 ± 68µg/ml is achieved at 1.8 ± 0.2 hours after a single oral dose of 80mg^[14].

Several methods for the determination of

atorvastatin in pharmaceuticals^[4-12], and biological fluids have been reported, the later include high performance liquid chromatography (HPLC)^[3,13,14], gas chromatography coupled with mass spectrometry^[15], and liquid chromatography coupled with tandem mass spectrometry^[2,16-19]. However, these methods were limited because of low sensitivity or recovery, or because they required laborious preparation, solid-phase extraction, long run time, equipment that are not readily available, or were not validated in human plasma^[20].

The aims of this study were to 1) establish a simple, fully validated HPLC assay to measure atorvastatin level in human plasma with quantitation limit suitable for bioequivalence studies, and 2) determine the stability

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of atorvastatin under various clinical laboratories.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of Waters Alliance 2690 Separations Module, a 5- μm (particle-size) 4.6 \times 150mm Atlantis C₁₈ steel column, a Nova-Pak C₁₈ 4 μm insert in conjunction with Guard Pak pre-column module, and Waters 2960 dual absorbance detector (Water Associates, Milford, MA, USA) set at 247nm. Data were collected with a Pentium IV computer using Millennium³²¹ Chromatography Manager Software (Water Associates, Milford, MA, USA).

Chemicals and reagents

Atorvastatin calcium (Figure 1a), and the internal standard (IS) glipizide (CAS number; 29094-61-9) (Figure 1b) were analytical grade and obtained from USP (Rockville, MD, USA). Acetonitrile, ethyl acetate, phosphoric acid (HPLC grade), and dibasic sodium phosphate were purchased from Fisher Scientific (Fairlawn, NJ, USA). Water for HPLC was prepared by reverse osmosis and further purified by passing through a Milli-Q System obtained from Millipore Co. (Bedford, MA, USA).

Chromatographic conditions

The mobile phase consisted of 0.05 M dibasic sodium phosphate buffer (pH = 4.0) and acetonitrile (50:50, v:v) and was delivered at a flow rate of 1.0 ml/min at room temperature. The mobile phase was filtered through a 0.45 μm size membrane filter (Millipore Co., Bedford, MA, USA) and degassed before use. The auto sampler was programmed to inject 100 μl into the chromatograph with a run time of 10 minutes.

Preparation of stock and working solutions

Atorvastatin calcium (1mg/ml) stock solution was prepared in water and used for stability studies and to prepare a working solution (2 $\mu\text{g}/\text{ml}$) in plasma. The working solution was prepared weekly to construct calibration curve and quality control (QC) samples. Glipizide (IS) working solution (5 $\mu\text{g}/\text{ml}$) was prepared weekly in mobile phase from a stock solution in methanol (1mg/ml).

Calibration standard/ quality control samples

Calibration standards were prepared by mixing nine

different volumes of atorvastatin working solutions in blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.02, 0.04, 0.08, 0.16, 0.20, 0.40, 0.60, 0.80, and 1.00 $\mu\text{g}/\text{ml}$. QC samples were prepared by mixing four different volumes of atorvastatin working solution in blank human plasma to produce final concentrations of 0.02, 0.06, 0.50, and 0.90 $\mu\text{g}/\text{ml}$. Samples were vortexed for 20 seconds, then aliquots of 1 ml of calibration standards QC samples were transferred into Teflon-lined, screw-capped, borosilicate glass culture tubes and stored at -20°C.

Sample preparation

Aliquots of 1ml of calibration standard or QC, were allowed to equilibrate to room temperature. To each tube, 60 μl of the 5.0 $\mu\text{g}/\text{ml}$ IS working solution was added and vortexed for 10 seconds. After the addition of 5.0 ml of ethyl acetate, samples were vortexed again for 1 minute and centrifuged for 20 minutes at 4200 rpm at room temperature. The organic layer was carefully collected, dried under a gentle stream of nitrogen at room temperature, and the residue was reconstituted in 200 μl mobile phase. After centrifugation at 13000 rpm for 3 minutes, the supernatant was transferred into the auto-sampler vials.

Stability studies

Stability of atorvastatin in plasma: Adequate numbers of aliquots of two QC samples (0.06, and 0.90 $\mu\text{g}/\text{ml}$) were prepared. Aliquots were analyzed in 5 replicates immediately (baseline), after being processed and stored at room temperature for 24 h or at -20°C for 48 h (auto-sampler stability), after being allowed to stand on the bench-top for 8 or 24 h at room temperature before processing (counter stability), after being stored at -20°C for 6 weeks before processing (long term freezer stability), or after being stored at -20°C for 24 h and then left to completely thaw unassisted at room temperature before processing (with the cycle repeated three times, freeze-thaw stability).

Stock solutions stability: Five aliquots of the stock solutions of atorvastatin and the IS were analyzed (after dilution to 10 $\mu\text{g}/\text{ml}$ in mobile phase) at baseline, after storage for 48 h at room temperature, or after storage at -20°C for 6 weeks. Stability of the working solutions of atorvastatin and the IS, were evaluated up to 2 weeks at -20°C.

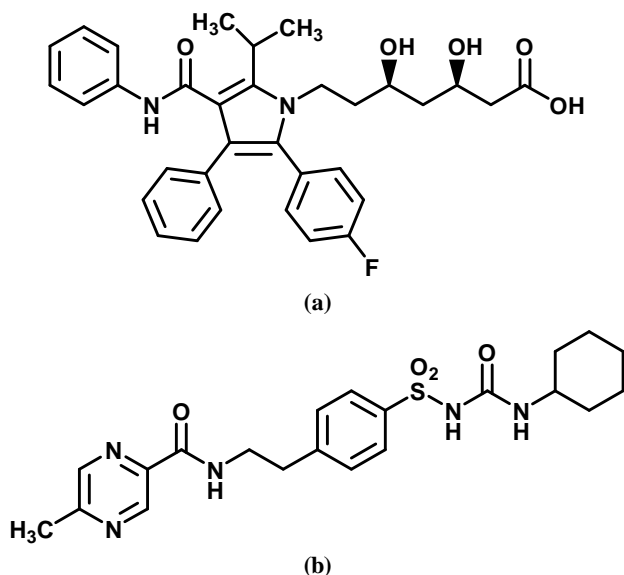


Figure 1 : Chemical structure of atorvastatin (a) and the internal standard glipizide (b)

Assay validation method

The procedures used for validation were as described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance^[21].

RESULTS

Optimization of chromatographic conditions

In order to improve specificity and minimize interference from plasma or solvent system that may occur at lower wavelengths, we optimized the absorbance wavelength based on photodiode array extracted spectra. We performed the analysis at 247nm. Different combinations of one components of the mobile phase composed of dibasic sodium phosphate (pH = 4.0) and acetonitrile were investigated and (50:50, v:v) was found best to achieve separation of atorvastatin from IS and minimize background absorbance. Under the described conditions, the IS and atorvastatin were well resolved within a run time of 10 minutes, and their retention time were 4.6 and 8.7 minutes, respectively.

Linearity

Linearity was determined in the range of 0.02-1.00µg/ml using ten calibration curves. The data were analyzed by linear regression using the formula: $\text{Conc.} = a + b(\text{PAR})$, where Conc. is the concentration of atorvastatin, a is the intercept, b is the slope, and PAR is the peak area of atorvastatin divided by the peak area of the IS. The concentrations of the calibration

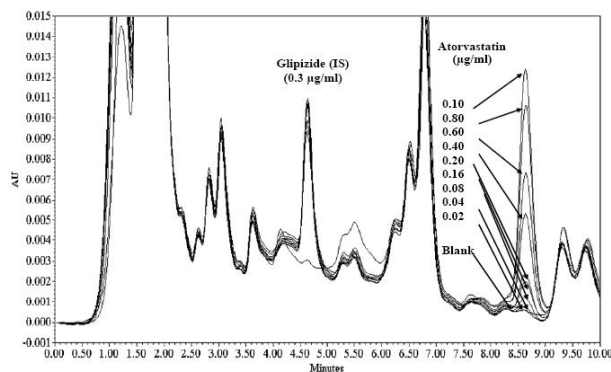


Figure 2 : Overlay of ten chromatograms of blank human plasma spiked with 0 (B), 0.02, 0.04, 0.08, 0.16, 0.20, 0.40, 0.60, 0.80 or 1.00 µg/ml of atorvastatin as well as 0.3 µg/ml of glipizide (IS)

standards of the eight calibration curves were back-calculated using the individual regression lines. Linearity studies (n = 10) showed mean (SD) for R^2 of 0.9942 (0.0037) a slope of 1.3001 (0.3176), and an intercept of 0.0016 (0.0313). Figure 2 depicts an overlay of chromatograms of a representative standard curve.

Limit of detection

The limit of detection (LOD), defined as three times the baseline noise, was 0.01µg/ml.

Specificity

To evaluate specificity, we screened eight frequently used medications (dissolved in methanol: water, 50:50, v:v) and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of eight commonly used drugs co-eluted with atorvastatin or the IS (TABLE 1).

Recovery

The extraction recovery of atorvastatin was determined by dividing mean peak areas of five replicates of four quality control samples (0.02, 0.06, 0.50, and 0.90µg/ml) prepared in plasma (as described under sample preparation), by mean peak areas of five replicates of equivalent concentrations prepared in the mobile phase. The recovery of the IS was determined similarly at a concentration of 0.3µg/ml. The results of the extraction recovery studies of atorvastatin and the IS are presented in TABLE 2. Recovery was $\geq 81\%$ (mean 91%) for atorvastatin and 87% for the IS.

Precision and bias

Precision was calculated as coefficient of variation (standard deviation divided by mean measured con-

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TABLE 1 : Specificity of atorvastatin assay

Drug Name	Retention time
Atorvastatin*	8.7
Glipizide	4.6
Aspirine	2.3
Acetaminophen	2.2
Ranitidine	4.0
Nicotinic Acid*	2.0
Ascorbic Acid*	1.6
Caffeine*	2.3
Ibuprofen	5.0
Diclofenac	7.0

1mg/ml solutions in methanol or water* were diluted in mobile phase to 10 µg/ml and 100 µl were injected

TABLE 2 : Extraction recovery of atorvastatin and glipizide

Nominal concentration (µg/ml)	Plasma		Mobile phase		** Recovery (%)
	*Mean peak area	SD	*Mean peak area	SD	
Atorvastatin					
0.02	3182	286	3918	378	81
0.06	10306	815	11826	332	87
0.5	122906	5339	118414	1407	104
0.9	237549	7407	256578	1847	93
Glipizide					
0.3	160093	10488	183972	1163	87

*Mean peak area of 5 replicates. **Mean peak area of spiked plasma sample divided by mean peak area of spiked mobile phase sample x 100. SD, standard deviation

TABLE 3 : Intra-run and inter-run accuracy and precision of atorvastatin assay

Nominal concentration (µg/ml)	Intra-run (n = 10)				Inter-run (n = 20)			
	Mean measured concentration (µg/ml)	SD	Precision (CV*, %)	** Bias (%)	Mean measured concentration (µg/ml)	SD	Precision (CV*, %)	** Bias (%)
0.02	0.019	0.001	6.1	5	0.021	0.003	13.1	5
0.06	0.065	0.008	11.8	9	0.062	0.005	8.7	3
0.50	0.484	0.058	12	2	0.509	0.062	12.2	2
0.90	1.030	0.055	5.3	14	0.986	0.090	9.1	10

*Coefficient of variation (CV) = Standard Deviation (SD) divided by mean measured concentration×100. **Bias = absolute value of 1 minus mean measured concentration divided by nominal concentration100

TABLE 4 : Stability of atorvastatin in plasma samples and stock solution

Nominal concentration (µg/ml)	*Plasma Samples									
	Unextracted			Extracted		Freeze-thaw			**Stock solution	
	8 h RT	24 h RT	6 wks -20°C	24 h RT	48 h -20°C	One cycle	Two cycles	Three cycles	48 h RT	6 wks -20°C
0.06	93	95	85	96	104	102	84	90	88	112
0.90	109	115	100	100	96	106	115	99		

Stability (%) = mean measured concentration (n = 5) at the indicated time divided by mean measured concentration (n = 5) at baseline×100. *Spiked plasma samples were analyzed immediately (baseline, data not shown), after storing for 8 or 24 hours at room temperature (8 h RT and 24 h RT) or 6 weeks at -20°C (6 weeks -20°C); analyzed after storing the extract for 24 hours at room temperature (24 h RT) or 48 hours at -20°C (48 h -20°C); or analyzed after 1 to 3 cycles of freezing plasma at -20°C and thawing at room temperature (freeze-thaw). ** Atorvastatin, 1 mg/ml in water

centration×100) and inaccuracy (bias) as the absolute value of (1 minus mean measured concentration divided by nominal concentration)×100. The intra-run and inter-run precision and bias were determined by analyzing four QC samples: 0.02, 0.06, 0.50, and 0.90µg/ml over three different days (TABLE 3). Intra-run precision and bias (n = 10) ranged from 5.3 % to 11.8 % and from 2 % to 20 %, respectively. The inter-run precision and bias (n = 20) ranged from 8.7% to 12.2% and from 2 % to 20 %, respectively.

Stability

The stability of atorvastatin in plasma, in processed samples, and under usual storage conditions was investigated. The results of atorvastatin stability studies are presented in TABLE 4. The data indicate that: 1) atorvastatin in plasma is stable for at least 24 hours at room temperature and 6 weeks at -20°C, 2) in extracted samples, atorvastatin is stable for at least 24 hours at room temperature and 48 hours at -20°C, 3) atorvastatin in plasma is stable after at least three

cycles of freeze at -20°C and thaw at room temperature, and 4) atorvastatin in water (1mg/ml), and the IS in methanol (1mg/ml) were stable for at least 6 weeks at -20°C (112 % and 87 %), respectively. Further, the working solutions of atorvastatin and the IS ($2\mu\text{g}/\text{ml}$ in plasma and $5\mu\text{g}/\text{ml}$ in mobile phase, respectively) were stable for at least 2 weeks at -20°C (115% and 85%, respectively).

Robustness

The robustness of the proposed method was evaluated by slightly altering the strength of phosphoric acid and amount of acetonitrile in mobile phase. No significant effects were observed. Further, the chromatographic resolution and peak response were stable over about 700 injections of processed plasma samples using one column.

DISCUSSION

We describe a rapid, simple, accurate, and precise HPLC assay for the determination of therapeutic levels of atorvastatin in human plasma. Previously reported assays were not developed to measure atorvastatin level in biological fluids^[4-12], had relatively high limit of detection^[13], used devices that are not available in many pharmaceutical laboratories or need highly trained persons^[15-18], or were validated using dog plasma^[20].

The advantages of the current assay include: 1) one-step liquid-liquid extraction rather than two steps organic extraction^[3,13,16], protein precipitation followed by solid-phase extraction^[17], or solid-phase extraction^[19], 2) a run time of 10 minutes compared to 23 minutes^[13], and 3) mean extraction recovery for atorvastatin of 91 % compared to 66% and 58% respectively^[17,18].

The comprehensive stability experiments that are reported here extend the known limits of stability of atorvastatin in plasma^[17,18] to 24 hours at room temperature, 6 weeks at -20°C , and three freeze-thaw cycles.

In conclusion, the results of this study expand the information on atorvastatin stability and indicate several advantages of the described assay over previously reported assays, especially for therapeutic drug monitoring and bioequivalence studies.

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