

## A Simple and Sensitive HPLC-UV Method for Quantitation of Lovastatin in Human Plasma: Application to a Bioequivalence Study

Mehrdad HAMIDI,<sup>\*a,b</sup> Najmeh ZAREI,<sup>a</sup> and Mohammad-Ali SHAHBAZI<sup>a</sup>

<sup>a</sup>Department of Pharmaceutics, Faculty of Pharmacy, Shiraz University of Medical Sciences; P. O. Box 71345–1583, Shiraz, Iran; and <sup>b</sup>Department of Pharmaceutics, School of Pharmacy, Zanjan University of Medical Sciences; 45139–56184, Zanjan, Iran. Received June 16, 2008; accepted June 9, 2009

An available, simple, sensitive, and rapid method has been developed for determination of the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor, lovastatin in human plasma. The analytical procedure involves a one-step liquid–liquid extraction method using atorvastatin as internal standard. Chromatographic separation was carried out on a reversed phase C<sub>18</sub> column using a mixture of 0.05 M phosphate buffer (pH 7) and acetonitrile (44.5 : 55.5, v/v) as mobile phase with UV detection set at 238 nm. The total run time of analysis was 6 min with the retention time of lovastatin being 4.3 min. A complete set of analytical method validation tests were carried out on the method. Accordingly, the method was linear in the wide range of 1–100 ng/ml. The limit of detection (LOD) and limit of quantification (LOQ) for lovastatin were 0.5 and 1 ng/ml, respectively. The method was shown to be precise with average within-run and between-run variations of 10.45±6.88 and 8.68±5.13%, respectively. The average within-run and between-run accuracy of the method throughout its linear range was 113.33±3.98 and 105.72±5.07%, respectively. The mean relative recovery of lovastatin from human plasma by the developed method was 88.61±7.00%. The applicability of the method in real pharmacokinetic situations was evaluated successfully during a bioequivalence study in 14 fasting healthy male volunteers.

**Key words** lovastatin; high performance liquid chromatography; HPLC-UV; liquid–liquid extraction; bioequivalence

Lovastatin (structure shown in Fig. 1), a highly effective cholesterol-lowering agent, belongs to a class of the most powerful lipid lowering agents generally called the statins and is a competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase.<sup>1)</sup> Furthermore, it has been reported that lovastatin has a good effect in reducing lethality in coronary heart disease.<sup>2)</sup> After oral administration of lovastatin, the plasma levels of the drug has been reported to be very low, which is mainly attributed to the documented extensive hepatic first-pass effect of the drug.<sup>3,4)</sup> Although a series of sensitive and accurate methods have been described for determination of lovastatin in biological fluids, but all these methods require the use of sophisticated and relatively non-popular instrumentations such as gas chromatography/mass spectrometry<sup>5–7)</sup> or, more recently, high-performance liquid chromatography/mass spectrometry,<sup>8,9)</sup> the availability of a simple, popular and cost-effective method for this purpose remains a limiting factor against pharmacokinetic studies on this drug. The purpose of this investigation was to develop a rapid, popular, and sensitive analytical method, based on HPLC with UV detection of lovastatin in human plasma in order to, primarily, be used throughout a bioequivalence study.

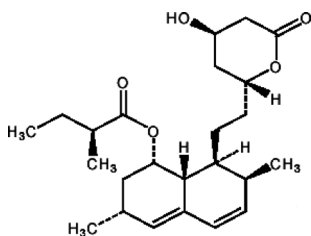


Fig. 1. Chemical Structure of Lovastatin

## MATERIALS AND METHODS

**Chemicals** Lovastatin and atorvastatin were kindly donated by Poursina Pharmaceutical Co. (Tehran, Iran). Test tablets (Lovastatin-Poursina) were manufactured in Poursina Pharmaceutical Co. (Tehran, Iran). Reference tablets (Mevacor®, Merck & Co., NJ, U.S.A.) were purchased locally. All other chemicals and solvents were from chemical laboratory or HPLC purity grades, as needed, and purchased locally.

**Preparation of Calibration Standards and Quality Control Samples** In order to prepare stock standard solution of lovastatin, 100 mg of lovastatin was dissolved in 100 ml methanol. The stock solution was then further diluted with methanol to obtain the different working solutions ranging 40, 100, 200, 400, 1000, 2000, 4000 ng/ml, from which the spiked plasma samples were prepared by appropriate dilution. Quality control (QC) samples were prepared at low (10.0 ng/ml), medium (25.0 ng/ml) and high (50.0 ng/ml) concentrations in the same way as the plasma samples for calibration.

**Samples Preparation** A liquid–liquid extraction procedure was used in this study for isolation of lovastatin from the plasma samples. For this purpose, 20 μl of atorvastatin (internal standard; IS) methanolic solution with concentration of 100 μg/ml was added to 2 ml plasma and after vortex-mixing for 30 s, 4 ml of diethyl ether was added while the mixture was vortex-mixed for another 1 min. The organic and aqueous layers were then separated by centrifugation at 5000×g for 10 min, the organic supernatant was transferred to another tube and evaporated at 40 °C to dryness. Finally, the residue was reconstituted in 200 μl mobile phase and following a brief orbital shaking, 50 μl was injected onto the HPLC system.

**Chromatographic Condition** The analytical column was Symmetry C<sub>18</sub> (Waters, U.S.A.) (particle size 5 μm; 250×4.6 mm) with the corresponding guard column

\* To whom correspondence should be addressed. e-mail: hamidim@zums.ac.ir

(10×4.6 mm, 5  $\mu$ m). A mixture of 0.05 M phosphate buffer (pH 7; adjusted with phosphoric acid) and acetonitrile (44.5 : 55.5, v/v) was used as the mobile phase. The column was kept at room temperature. The mobile-phase flow rate was 1.5 ml/min. A UV detector (Waters, U.S.A., Model 746) was used for analyte detection, set at wavelength of 238 nm.

**Method Validation** Linearity and Range: Linearity was evaluated using freshly prepared spiked plasma samples and the calibration curves were constructed using seven non-zero standard points covering the range of 1–100 ng/ml. In addition, a blank (non-spiked sample) was run to discard the presence of interferences. Plasma samples were spiked at concentrations of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 ng/ml. The samples were extracted and analyzed as described, with the ratio of the corresponding peak heights of lovastatin to atorvastatin (IS) and the nominal lovastatin concentrations of the samples being used as  $y$  and  $x$  variables in a standard regression analysis. The successive decreasing trend of lovastatin concentrations were analyzed as described and a concentration producing the signal-to-noise ratio of about 3 was regarded as limit of detection (LOD) of the method. The limit of quantitation of the method was determined as the minimum concentration capable of being used for drug concentration determination with a maximum relative standard deviation (R.S.D.) of 0.2.

**Precision and Accuracy:** For determination of within-run and between-run precision and accuracy, three different series of samples at concentrations of 10.0, 50.0 and 100.0 ng/ml of lovastatin were analyzed both within a single instrument run and in different runs. The accuracy was calculated from the ratio of measured concentration, based on standard curve, to the nominal added concentration. Precision was evaluated by calculating the within-run and between-run coefficients of variations of the measured concentrations at each level (CV%).

**Relative Recovery (Matrix Effect):** To assess the extraction recovery, three series of samples at concentration levels of 10.0, 25.0 and 50.0 ng/ml of lovastatin, were prepared in two media; one in human plasma and the other replicate in distilled water. All the samples were processed as described and recovery of the analyte from the matrix (plasma) was calculated as the ratio of the corresponding instrument responses (*i.e.*, peak height ratios) in plasma to that in water.

**Bioequivalence Study** In order to evaluate the practical applicability of the developed method, we used it for drug analysis throughout a project designed for bioequivalence analysis of a lovastatin generic product manufactured in Iran with the innovator product Mevacor<sup>®</sup>. For this purpose, fourteen healthy, non-alcoholic, non-smoking, male volunteers, aged within a range 20–24 years (mean  $\pm$  S.D., 21.71  $\pm$  1.16 years), weight 63–91 kg (mean  $\pm$  S.D., 74.51  $\pm$  7.67 kg) were enrolled in this study. The clinical protocol was approved by the local Ethics Committee (the Ethical Committee of Iranian Food and Drug Organization). The volunteers gave written informed consent after they had received detailed instructions about the aims, restrictions and possible adverse effect which could be experienced as a result of taking the drug. Volunteers were healthy and had no history of kidneys and metabolic diseases. Also they had a routine physical examination and the routine laboratory tests found them to be normal. Subjects did not receive any medication during the 2

weeks period prior to the start and also were not undergoing any pharmacological treatment during the study period. The study was an open, randomized, two-period, two-group crossover design with a 7-d washout period between doses. During the first period, volunteers from group A received four test tablets (80 mg) while volunteers from group B received four reference tablets. During the second period, the procedure was repeated on the groups in reverse. The tablets were administered to the volunteers in the next morning after an overnight fast, with 200 ml of water. Volunteers received standard lunch and snack, respectively, 6 and 10 h after drug administration. No other food was permitted for consumption during the sampling period. Blood samples (5 ml) were collected *via* an indwelling catheter into heparin-containing tubes at 0 (baseline), 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0, 10.0 h post-dosing. The blood samples were centrifuged at 2000 $\times g$  for 10 min and the plasma was separated stored at  $-20^{\circ}\text{C}$  until assayed for lovastatin content. Volunteers did not ingest any alcoholic drink, coffee or other xanthine-containing drinks like tea during the trial.

**Pharmacokinetic and Statistical Analyses** Pharmacokinetic parameters were calculated from plasma levels applying a non-compartmental method. The maximum plasma concentration ( $C_{\text{max}}$ ) and time point of maximum plasma concentration ( $T_{\text{max}}$ ) values were obtained directly from plasma lovastatin concentration–time data. The area under the concentration–time curve ( $AUC_{0-10}$ ) was obtained by the trapezoidal rule and the total area under the curve ( $AUC_{0-\infty}$ ) was calculated up to the last measured concentration and extrapolations were obtained using the last measured concentration and the terminal elimination rate constant ( $K_{\text{el}}$ ). The terminal elimination rate constant,  $K_{\text{el}}$ , was estimated from the slope of the terminal exponential phase of the plasma of lovastatin concentration–time curve (by means of the linear regression method). The pharmacokinetic primary variables used to assess bioequivalence in this study were  $AUC_{0-12}$ ,  $AUC_{0-\infty}$ , and  $C_{\text{max}}$ . Bioequivalence of two formulations was assessed by means of an analysis of variance (ANOVA) for crossover design and calculating 90% confidence interval of the ratio of test/reference using log-transformed data. The formulation was considered bioequivalent when the difference between two compared parameters was found statistically insignificant ( $p > 0.05$ ). Furthermore, as a standard requirement, the ratio of averages of log-transformed data should be within 80–125% for  $AUC_{0-10}$ ,  $AUC_{0-\infty}$ , and  $C_{\text{max}}$ .

## RESULTS AND DISCUSSION

**Method Development** In the way to develop a simple and popular method for lovastatin assay in human plasma for pharmacokinetic studies, HPLC with UV detection was selected as the method of choice. Our main challenge was the low concentration of lovastatin even at its peak plasma level, which is resulted from its extensive hepatic first-pass. The simplest way to concentrate the analyte was liquid–liquid extraction. We tested a wide spectrum of organic solvents from different physicochemical categories with different volume fractions as well as combinations. In terms of the analysis condition, various mobile phases, in different proportions, buffered and non-buffered at various pH were attempted to

provide concomitantly the best peak resolution and retention times. Also we tried different column packings, even from normal phase. Finally, after considering all the data, the optimum method condition described earlier was selected to be followed up. The number of theoretical plates, peak symmetry, and analytical retentability of the method all were in the desirable range as can be judged from the chromatograms shown in Fig. 2. Briefly, the notable advantages of the developed method over the history of lovastatin assay can be mentioned as follows:

- Available and popular instrumentation

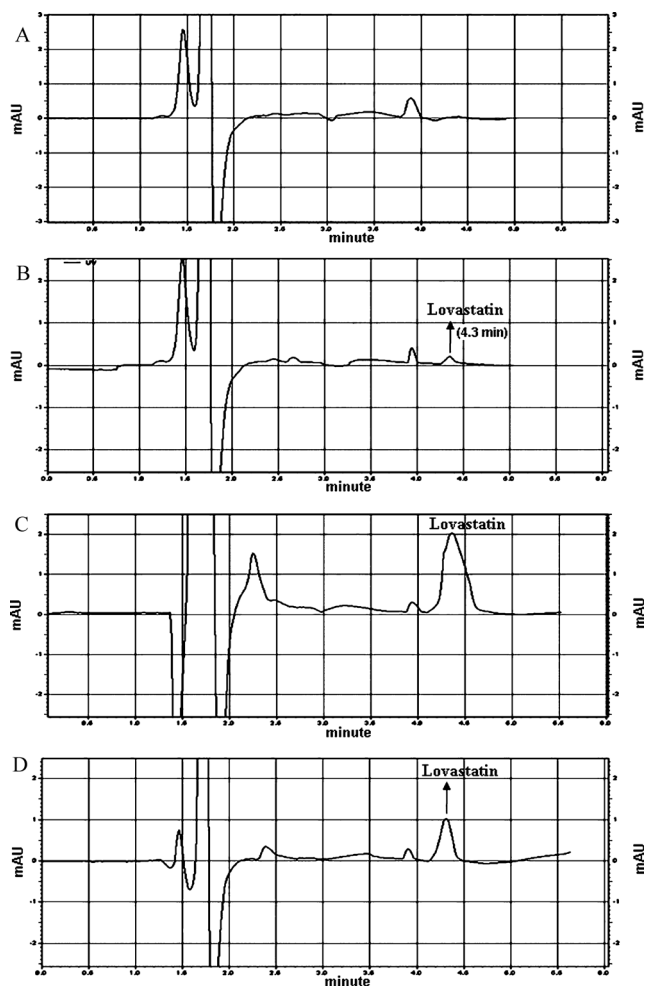


Fig. 2. Chromatograms of Lovastatin in Human Plasma: (A) Blank (Drug Free) Human Plasma; (B) Blank Human Plasma Spiked with 1.0 ng/ml Lovastatin (LOQ); (C) Spiked Human Plasma Containing 10 ng/ml of the Drug; (D) Chromatogram of Lovastatin from a Volunteer 1 h after Administration of a 80 mg Oral Dose

The peaks presented at about 3.8 min and 4.3 min represent atorvastatin (IS) and lovastatin, respectively.

- Available reagents and chemicals
- Simple and fast one-step sample preparation procedure
- Sensitivity high enough for pharmacokinetic studies
- Acceptable accuracy and precision

**Method Validation** Linearity and Range: The method produced highly linear responses within the wide concentrations range of 1.0–100.0 ng/ml, which is desirable for the majority of PK studies on the drug. A typical regression equation of the method response was  $y=0.0185x+0.0318$  ( $r^2=0.9986$ ,  $n=7$ ).

**Specificity and Selectivity:** To investigation of specificity, a series of blank (drug-free) human plasma (total 14 subjects) in addition to the different concentrations spiked were screened and no endogenous interference was observed at the retention time of lovastatin and internal standard. In addition a series of drugs which expected to be administered concurrent to lovastatin in clinical settings, were co-spiked and tested for selectivity. No interferences, even minor, were recorded in any cases.

**Precision and Accuracy:** In Table 1, the CV% values of the measurements made by the method at different levels have been shown along with the corresponding accuracies (absolute recovery). As shown, all the values of variations and accuracies are within the generally acceptable ranges. This, in turn, assures obtaining accurate and precise results from the method.

**Recovery:** A variety of extraction procedures were tested, as described, and the best recovery was achieved for the liquid-liquid extraction with ether. The mean recoveries of lovastatin in different levels on linear range are shown in Table 2. These data indicate an acceptable degree of drug recovery by the extraction method within the whole concentration range tested.

**LOD and LOQ:** The LOD and LOQ of the method for lovastatin were 0.5 and 1.0 ng/ml, respectively. This indices show the remarkable sensitivity of the method for drug analysis.

**Pharmacokinetic Study** The developed analytical method was applied to the determination of lovastatin in plasma samples generated during the bioequivalence study in 14 healthy Iranian male volunteers who were orally adminis-

Table 2. Relative Recovery (Matrix Effect) of the Developed Method for Lovastatin in Human Plasma

QC samples	Concentration added (ng/ml)	Recovery (%) (mean $\pm$ S.D.)	<i>n</i>
Low	10	84.97 $\pm$ (1.25)	3
Mid	25	84.17 $\pm$ (1.61)	3
High	50	96.68 $\pm$ (9.99)	3
Mean		88.61 $\pm$ 7.00	

Table 1. Within-Run and Between-Run Precision and Accuracy of the Developed Method for Lovastatin Assay in Human Plasma ( $n=3$ )

Nominal added concentration (ng/ml)	Precision (CV%)		Accuracy (mean $\pm$ S.D.)	
	Within-run	Between-run	Within-run	Between-run
10	16.11	14.53	109.97 $\pm$ 17.71	110.80 $\pm$ 16.16
50	12.45	4.92	112.30 $\pm$ 13.98	100.66 $\pm$ 4.96
100	2.79	6.60	117.73 $\pm$ 3.29	105.71 $\pm$ 6.98
Mean	10.45 $\pm$ 6.88	8.68 $\pm$ 5.13	113.33 $\pm$ 3.98	105.72 $\pm$ 5.07

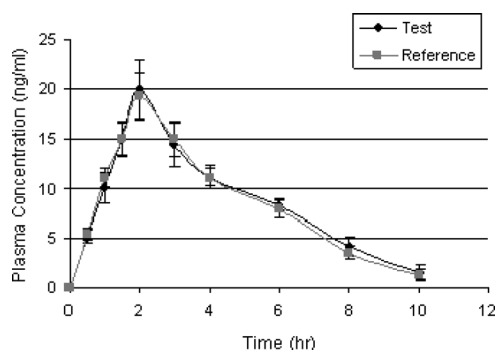


Fig. 3. The Mean Plasma Concentration–Time Profile of Lovastatin Following Administration of a Single Oral Dose of Test and Reference Formulations to 14 Healthy Male Volunteers

tered four tablets of lovastatin. The mean concentration–time profiles after administration of the 80 mg test and reference formulations in 14 subjects are depicted in Fig. 3, which clearly show the applicability of the method, judged by comparing the data generated by the pharmacokinetic profile indexed for the drug.<sup>1,10</sup> The pharmacokinetic parameters derived from these data, including maximum plasma concentration ( $C_{\max}$ ), area under the plasma concentration–time curve from 0 h to the last measured data ( $AUC_{0-10}$ ) and area under the plasma concentration–time curve from 0 h to infinity ( $AUC_{0-\infty}$ ) showed that there is no statistically significant difference between the two products (Tables 3, 4).

## CONCLUSION

A popular, rapid, simple, sensitive and selective HPLC-UV method with liquid–liquid extraction was developed for quantification of lovastatin in human plasma. Benefit of this method was sensitivity and simplicity that makes it suitable for routine as well as specific pharmacokinetic studies. In other words, because of the low cost and short sample preparation and run time, this method is suitable for routine analysis. The method was validated and showed acceptable linearity, precision, accuracy and recovery. Our validated method was successfully applied to a bioequivalence study to determine lovastatin concentration in human plasma samples. The results of the bioequivalence study indicated that the locally

Table 3. Mean Pharmacokinetic Parameters of Lovastatin in 14 Healthy Volunteers after Oral Administration of 80 mg Test and Reference Products

Pharmacokinetic parameter	Lovastatin	
	Reference	Test
$AUC_{0-\infty}$ (ng·h/ml)	91.60 ± 6.52	94.76 ± 10.19
$AUC_{0-10}$ (ng·h/ml)	87.60 ± 5.26	89.63 ± 7.87
$T_{\max}$ (h)	2.96 ± 0.13	3.00 ± 0.00
$C_{\max}$ (ng/ml)	19.33 ± 2.27	20.03 ± 2.85
$K_{el}$ (h <sup>-1</sup> )	0.370 ± 0.064	0.355 ± 0.093

$AUC_{0-\infty}$ : area under the curve extrapolated to infinity;  $AUC_{0-t}$ : area under the curve up to the last sampling time;  $C_{\max}$ : the maximum plasma concentration;  $T_{\max}$ : the time to reach peak concentration;  $K_{el}$ : the apparent elimination rate constant.

Table 4. 90% Confidence Intervals for Log-Transformed Pharmacokinetic Parameters of Lovastatin after Administration of 80 mg of Test and Reference Products in 14 Healthy Male Volunteers

Pharmacokinetic parameters	$AUC_{0-10}$	$AUC_{0-\infty}$	$C_{\max}$
90% CI	0.97–1.07	0.96–1.10	0.95–1.12

manufactured tablet was bioequivalent to the innovator brand.

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