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Determination of atorvastatin in human serum by salting out assisted solvent extraction and reversed-phase high-performance liquid chromatography–UV detection

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Abstract A simple and rapid technique based on salting out assisted solvent extraction was developed for extraction of atorvastatin from serum sample and high performance liquid chromatography–UV was used for its detection. In the present study, 1.0 mL serum was extracted by 0.5 mL of acetonitrile and some parameters that can affect extraction such as type and volume of extraction solvent, type of salt, and pH were optimized. Under optimized experimental conditions, the calibration curve was found to be linear in the range of 0.001–10 ng mL^{-1} in human serum and the correlation coefficient (R^2) and the limits of detection were >0.99 and 0.0005 ng mL^{-1}, respectively. The accuracy of the method in terms of average recovery of the compound in spiked serum and water samples was better than 90%.

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1. Introduction

Atorvastatin, [(R-(R*, R*)]-2-(4-fluorophenyl)-b,d, dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenyl-amino)-carbonyl]-1H-pyrrole-1-heptanoic acid calcium salt (Fig. 1.) belongs to the group of statins and it is a second generation HMG-CoA reductase inhibitor recently approved for clinical use as a cholesterol lowering agent (Bullen et al., 1999). These drugs inhibit the rate limiting key enzyme known as 3-hydroxy-3-methyl-glutarylcoenzyme A (HMG-CoA) reductase involved in cholesterol biosynthesis (Janardhanan et al., 2012).
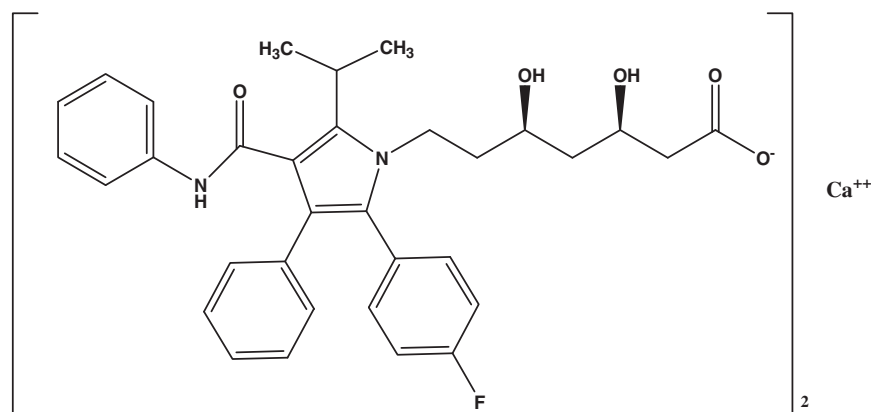
More than 90% of atorvastatin is bound to plasma proteins. About 70% of the total plasma HMG-CoA activity is attributed to active metabolites of atorvastatin, even if their concentrations are very low. Information about the actual plasma concentration of atorvastatin is of interest in pharmacokinetic studies and investigations of the mechanisms of drug–drug interactions (Hermann et al., 2005). To date several HPLC-UV and LC-MS methods have been developed for the

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Figure 1 Structure of atorvastatin calcium salt (2:1).

quantification of atorvastatin in different matrices (Shah et al., 2011; Farahani et al., 2009; Novakova et al., 2008). A convenient sample-preparation procedure should isolate the analytes from the complex matrix while removing endogenous interfering substances. This is often the most time-consuming, critical step of analysis. Sample preparation can include clean-up procedures for very complex (dirty) samples. The analytes should be pre-concentrated in order to increase the sensitivity and the selectivity of the method. Drug analysis in human urine and blood plasma is often complicated by low analyte concentrations, by complex sample matrices, and by limited sample volumes available for the determinations (normally 0.5–1.0 ml for plasma). Because of this, sample preparation is crucial in drug analysis and includes both analyte preconcentration and sample clean-up. The procedures for sample preparation of atorvastatin have in most cases included LLE (liquid–liquid extraction), and SPE (solid-phase extraction). However, the conventional LLE procedures are time consuming, generally labor intensive and require large quantities of expensive, toxic and environmentally unfriendly organic solvents. SPE often suffered from the plugging of cartridge and consumption of appreciable amount of toxic solvents at the elution step. In order to overcome these problems, simple, inexpensive liquid-phase microextraction (LPME) was introduced recently. LPME is a solvent-minimized sample pretreatment procedure of LLE, in which only several μL of solvent are required to concentrate analytes from various samples rather than hundreds of mL needed in traditional LLE. Salting-out is a process of addition of electrolytes to an aqueous phase in order to increase the distribution ratio of a particular solute. Salting-out assisted liquid/liquid extraction (SALLE) with water miscible organic solvent has shown its distinctive advantages in bioanalytical research (Hassan and Farahani, 2011). SALLE is comparable with LLE for extract cleanness (Lovin et al., 2003; Nagaosa and Sakata, 1998; Jia et al., 2006; Myasein et al., 2009). SALLE is a more green technique compared with common sample preparation techniques used for bioanalysis. Acetonitrile was found as a promising extracting solvent owing to its compatibility with reversed-phase HPLC. However, there are a few methods available utilizing sample preparation by salting out phase separation and HPLC.

The aim of the work was to develop a fast, easy and low-volume (for both sample and organic solvent volume) sample preparation technique convenient for routine preparation of biological samples containing atorvastatin. Advantages of

salting-out with acetonitrile have been demonstrated, including the ease of biological sample clean-up as well as analyte enrichment.

2. Experimental

2.1. Chemicals and reagents

Pure reference standard of atorvastatin with chemical purity of > 99.3% kindly supplied from Farayand Chemi Hackim Company (Qazvin, Iran) was used without any further purification. HPLC grade acetonitrile, ammonium acetate, and methanol were of analytical grade supplied by Merck (Darmstadt, Germany). Stock solution of atorvastatin (1000.0 mg L^{-1}) was prepared in methanol. The working standard solutions of atorvastatin were prepared by diluting an appropriate volume of stock solution. All the standard solutions were stored at 4 °C in the refrigerator.

2.2. Instrumentation

The analytical chromatographic system consisted of an Agilent 1200 series vacuum degasser (G1322A), an automatic sample injector, a quaternary pump (1200 series G1311-90011 Quat-Pump), a variable wavelength detector (VWD-G1314A), a column oven (G1313A), (all from Agilent Technologies, Palo Alto, USA), and a Symmetric C18 column 5 μm,4.6 mm i.d., 250 mm column length with guard column (Waters Corporation, Massachusetts, U.S.A.,) and controlled by a computer running Chem Station software (Agilent Technologies). Mobile phases were filtered through a Millipore 0.22-μm membrane filter before use. The column was stabilized at 25 ± 2 °C (room temperature) for 1 h before chromatography. A flow-rate of 1.0 mL min^{-1} was applied in laboratory temperature of 25 (±2) °C. The mobile phase was CH3COONH$_4$ buffer in water (Adjusted pH = 3.0 ± 0.05 with orto phosphoric acid)-acetonitrile (50: 50, v/v) and the detection wavelength was 246 nm.

2.3. Extraction method

Atorvastatin was extracted from 1.0 mL of sample by adding 0.5 mL of acetonitrile, 0.5 mL of ammonium acetate buffer (pH = 4) into a 2 mL polypropylene micro-centrifuge tube,
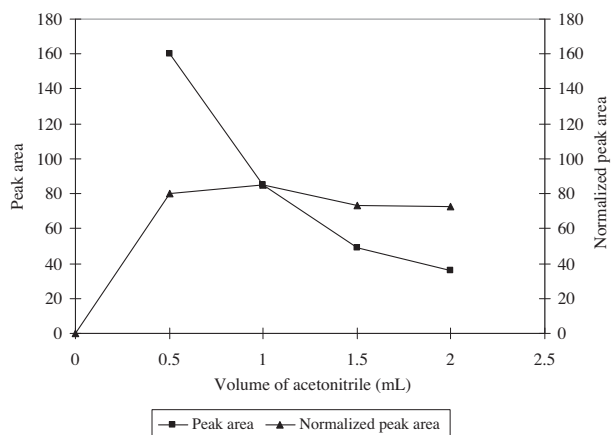
Figure 2 Effect of the volume of acetonitrile on the extraction of atorvastatin from serum sample. Extraction conditions: sample volume, 1.0 mL; pH = 4; concentration of atorvastatin, 1000 ng mL^{-1}, 0.30 g of magnesium chloride.

and vortexed for 30 s. Finally, 0.3 g magnesium chloride was added and mixed for phase separation. The supernatant fluid was transferred to another 1.5 ml polypropylene micro centrifuge tube and 20 µL of the solution was injected into the HPLC column.

3. Result and discussion

The theory of SALLE is similar to that of LLE. The final concentration of the analyte in the sample solution (C_f) is as follows:

$$C_f(\mu g\ mL^{-1}) = \frac{C_{eq}^{ex} V_{ex}}{V_{sam} E_r} \tag{1}$$

The extraction recovery (E_r) was defined as the total analyte amount which was extracted to the extracting phase:

$$E_r = \frac{C_{eq}^{ex} V_{ex}}{V_{sam} C_{ini}} \tag{2}$$

The preconcentration factor (P_f), defined as the ratio of concentrations (signals) after and before extraction:

$$P_f = \frac{C_{eq}^{ex}}{C_{ini}} \tag{3}$$

The enrichment factor (E_f), defined as the ratio of volume of sample and volume of extraction solvent:

$$E_f = \frac{V_{sam}}{V_{ex}} \tag{4}$$

The enhancement factor (E_n), defined as the ratio of slope of calibration curves after and before (direct) microextraction:

$$E_n = \frac{S_{aft}}{S_{dir}} \tag{5}$$

where, C_{eq}^{ex} (µg mL^{-1}) is the final concentration of analyte in the extracting phase, C_{ini} is the initial concentration of analyte in the sample, and V_{ex} (mL) is the final (separated) volume of the extracting phase, E_r is the extraction recovery and V_{sam} (mL) is the volume of the sample, respectively.

3.1. Effect of extraction solvent

The miscibility of solvent and aqueous sample solution is the main point of selection for extraction solvent. Therefore, acetone, acetonitrile and methanol were introduced for this purpose. Acetone cannot deprontonise serum, and methanol was not separate after the addition of salt, thus acetonitrile was selected as the solvent in the subsequent extractions.

3.2. Effect of phase volume ratio

To investigate of volume of acetonitrile on extraction efficiency a series sample solution (serum) containing 1000.0 ng mL^{-1} of atorvastatin was extracted by 0.5, 1.0, 1.5 and 2.0 mL of acetonitrile, separately. The results show the peak area (concentration) of the analytes decreased with the increasing volume of acetonitrile, but extraction efficiency (peak area * volume of acetonitrile) was nearly constant. (Fig. 2.).

3.3. Effect of salting-out on phase separation

There have been limited reports on SALLE with water miscible organic solvents and inorganic salts as the salting-out agent, for the separation, isolation and preparation of biological samples. Although it is well known that acetonitrile is miscible with water in any proportion at room temperature, addition of salt significantly reduced the mutual miscibility, even resulting in phase separation of acetonitrile from the aqueous phase. Three inorganic salts that included sodium sulfate, sodium chloride and magnesium chloride were examined in SALLE. Here magnesium chloride is used as a salting-out reagent due to its high ionic strength per unit concentration in the aqueous phase and good extraction efficiency.

3.4. Effect of pH of sample solution

The effect of sample pH was evaluated in the range of 1–8 and the results are shown in Fig. 3. The pH of the sample solution had a minor effect on the extraction efficiency. At pH lower than 2, it is expected that the amine groups are ionized to the ammonium form, and also at pH > 4 extraction was decreased.
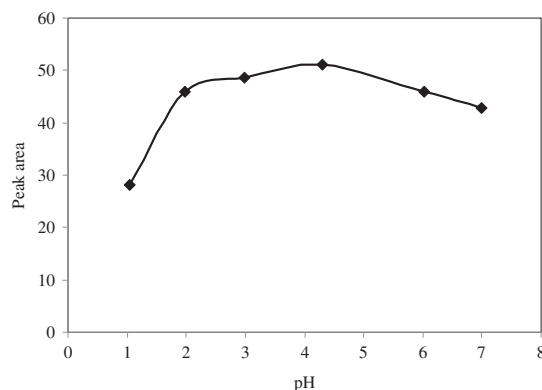
Figure 3 Effect of the type of pH on extraction of atorvastatin from serum sample. Extraction conditions: sample volume, 1.0 mL; concentration of atorvastatin, 1000 ng mL^{-1}, 0.30 g of salt.

Table 1 Figures of merit obtained for direct calibration and after the SALLE–HPLC-UV determination of atorvastatin in human plasma.

Matrix	Regression equation	R^2	Dynamic linear range ($\mu g\ mL^{-1}$)	LOD ($\mu g\ mL^{-1}$)	Enhancement factor
Acetonitrile	Area = 5.1 C + 0.03	0.9997	0.01–100	0.004	1
Water	Area = 57.1 C + 2	0.9989	0.001–10	0.0005	11
Serum	Area = 57.5 C + 0.7	0.9980	0.001–10	0.0005	11

Table 2 Accuracy, precision and analytical recovery obtained for atorvastatin by SALLE method.

Compound	Spiked level ($ng\ mL^{-1}$)	Tap water		Serum	
		Found ± SD[a] ($ng\ mL^{-1}$)	Recovery ± RSD[b] (%)	Found ± SD ($ng\ mL^{-1}$)	Recovery ± RSD (%)
Atorvastatin	–	<LOD	–	<LOD	–
	4.0	3.90 (±0.3)	98.5 (±4)	4.05 (±0.4)	101 (±10)
	10.0	9.95 (±0.6)	99.5 (±6)	9.90 (±0.3)	99 (±3)

[a] SD = Standard deviation.
[b] RSD = Relative standard deviation; $n = 3$.

4. Analytical performance

In order to validate the developed SALLE method, linearity, correlation coefficient, detection limits, and repeatability were tested using spiked samples. Standard calibration curve of atorvastatin in acetonitrile, blank serum sample, and in blank water sample was prepared by the external standard method and the obtained calibration curves were compared with each other. As can be seen in Table 1, there are no differences between slope of calibration curves in serum and water sample matrices. Peak identification was made by comparing the retention times and spectra of samples with those of standards and the correlation coefficients (R^2) of >0.99 were obtained. The limit of detection (LOD) based on signal to noise of three limits of detection obtained was 0.5 ng mL^{-1} for serum matrices. The limit of quantification (LOQ) was defined as the lowest concentration of an analyte that can be determined with acceptable precision and accuracy under the stated conditions of the test (Table 1). The inter-day relative errors and the inter-day relative standard deviations and the recoveries obtained by spiking blank samples at three different levels plus a zero level are reported in Table 2.

5. Conclusion

This procedure (SALLE) afforded a convenient, selective, fast and cost-saving operation with good cleanup ability for the model analyte. The method involved a simple one-step solvent extraction of atorvastatin from serum followed by salting out the organic solvent using magnesium chloride. Salting out assisted liquid–liquid extraction with acetonitrile is a greener method and several samples can be extracted in parallel (high-throughout sample preparation technique).

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