Determination of serum atorvastatin concentrations in lipid-controlling patients with and without myalgia syndrome

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Abstract
The cholesterol-reducing drug atorvastatin is widely used in hyperlipidemic patients and it is crucial to consider its potential myotoxic effect when adjusting the prescription for patients. The aim of this study was to establish a simple high-performance liquid chromatography method that would be applicable for the quantitative determination of high serum atorvastatin concentrations in patients for the assessment of myalgia. The average recovery of atorvastatin from the extraction using ethyl acetate was 85.1%. The extract was dried, re-dissolved in methanol, and subjected to chromatographic separation using a reverse-phase C18 analytical column and a mobile phase consisting of 61.3% methanol in 0.05 M sodium phosphate buffer at pH 3.5. The concentration of atorvastatin was quantitatively determined by measuring the absorbance at 247 nm and β-naphthoflavone was used as an internal standard. The lower limits of detection and quantification of atorvastatin were 1.2 ng/mL and 3.0 ng/mL, respectively. There was a good linear relationship (r = 0.999) of the peak area and atorvastatin within a concentration range of 3.0 to 150 ng/mL in human serum. Among 61 lipid-controlling patients with detectable serum atorvastatin concentrations, 7 patients had myalgia and their serum concentrations were not significantly greater than those in patients without myalgia.

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1. Introduction
Statins are considered to be the most effective drugs for the treatment of hyperlipidemia. Atorvastatin belongs to the group of statins and is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme in cholesterol biosynthesis. Atorvastatin accounted for the second highest drug expense of the National Health Insurance in Taiwan (Annual Report of the Bureau of National Health Insurance 2011, Department of Health, Executive Yuan, Taiwan). Compared to other statins, atorvastatin has a relatively low clearance (0.25 L/h/kg) and its half life is longer (15–30 hours) [1]. The absorption and oral bioavailability of atorvastatin were...
only 30% and 12%, respectively. Myopathy [sore and weak muscles with creatine kinase (CK) ≥ 10 times the upper limit of normal (ULN) range] is the primary cause of statin intolerance, and the development of myopathy has been suggested to be affected by factors resulting in an increase in the blood concentration of statins [2]. However, the induction of myopathy by statins is not common (about 0.1–2.5%). Compared to myopathy, myalgia (sore and weak muscles with normal CK levels) is a milder syndrome and occurs more frequently (5–10%) [3]. Among statins, a meta-analysis of myalgia in a total of 48,138 patients in 21 studies showed that only atorvastatin elicited a significantly higher risk of myalgia than placebo [4]. It has been suggested that the incidence of myopathy increased in patients taking a high dosage (80 mg/d) of simvastatin [5]. Other potential risk factors of statin-induced myopathy include age, sex, drug interaction and polymorphic expression of cytochrome P450s and transporters [1,4–7]. According to the higher myopathy/myalgia risk in the high statin-dosing group [3,5] and that blood statin (including atorvastatin) concentration or pharmacokinetic parameters could be changed through drug interactions [7–9] and polymorphic expression of drug-metabolizing enzyme/transporters [1,4,6,10], it could be important to monitor the high blood concentrations of atorvastatin in patients for the assessment of myalgia risk. However, there are few reports showing the difference in blood atorvastatin concentrations between patients with and without myalgia syndrome.

Although gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) provide methods with high detection sensitivities for the determination of atorvastatin [2,11], the high cost of these instruments, instrument maintenance and sample preparation as well as the limited uses are problems in many research laboratories. Bahrami et al [12] reported a simple and fast high-performance liquid chromatography (HPLC) method using diclofenac as an internal standard and a column oven temperature of 62°C. This method was suggested to be applicable for the determination of the bioequivalence of different atorvastatin preparations in healthy participants. However, diclofenac is currently used as an analgesic, which may be taken together with atorvastatin in hyperlipidemic patients. There is difficulty in the application of this method to monitor atorvastatin concentrations in patients undergoing multitox treatment. In this report, we describe a HPLC method using a non-therapeutic agent, β-naphthoflavone, as an internal standard for the quantitative analysis of atorvastatin in blood samples of lipid-controlling patients. This HPLC analysis was carried out at room temperature and there was no need for a column oven. Serum atorvastatin concentrations were determined in 69 Taiwanese patients and 7 of them had myalgia.

2. Methods

2.1. Chemicals

The calcium salt of atorvastatin was purchased from the U.S. Pharmacopeial Convention (USP, Boston, MA, USA). Methanol and ethyl acetate were purchased from Merck Co. (Darmstadt, Germany). Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide and o-phosphoric acid were purchased from JT Baker (Malinckrodt Baker, Inc., Phillipsburg, NJ, USA). Acetaminophen, diclofenac, α-naphthoflavone, β-naphthoflavone, theophylline and verapamil were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Participants and sample preparation

Studies involving the handling of human blood samples were approved by the institutional review board of Taipei Veterans General Hospital, Taiwan (VGHIRB no.: 201000091A). A total of 69 patients with a mean ± standard deviation (SD) age of 60.1 ± 12.7 years (range, 19–86 years), taking a QD dose of 10 mg (32 patients) or 40 mg atorvastatin (37 patients) for more than 3 months were included. Before blood collection, the patients did not drink grapefruit juice for at least 2 weeks. Neither inducers/inhibitors of cytochrome P450 3A4 nor inhibitors of organic anion transport protein 1B1 was taken by the participants. The mean ± SD low-density lipoprotein cholesterol and CK levels of the 69 patients were 97.5 ± 23.8 mg/dL (range, 40–173 mg/dL) and 97.3 ± 90.5 U/L (range, 29–207 U/L), respectively. In this study, none of the patients had a CK level greater than 10 times the ULN (the normal range of CK is 27–168 U/L in males and 24–120 U/L in females). Three of the 10 mg-dosing group and four of the 40 mg-dosing group had myalgia with complaints of muscle pain/soreness or weakness. Among the participants, seven had CK levels greater than the ULN and only one female patient (40 mg-dosing) had muscle pain/soreness. Her CK level was 148 U/L, whereas the other patients with myalgia had CK levels within the normal range. Blood samples were collected from participants after overnight fasting at their regular (monthly) hospital visits between 9:00 AM and 12:00 PM. Sera were prepared by centrifugation of blood samples at 1800g for 10 minutes at 4°C. After centrifugation, the supernatant (serum) was collected and stored at −75°C until analysis. During the handling of human samples, protective clothes and gloves were put on. The waste left from handling human samples was disinfected by autoclave.

2.3. Extraction and chromatography

Atorvastatin and β-naphthoflavone (internal standard) solutions were prepared using methanol and stored at −20°C. Stock solutions (1 mg/mL atorvastatin and 1 mg/mL β-naphthoflavone) were stable for at least 4 weeks. To a 1-mL aliquot of human serum samples, 200 ng of β-naphthoflavone was added. An equal volume of 0.1 M sodium phosphate buffer (pH 7.0) was added, mixed and subjected to extraction by 5 mL of ethyl acetate [6]. After mixing by vortex for 30 seconds, the samples were centrifuged at 1600g for 10 minutes at room temperature and the organic layer was collected and evaporated to dryness under nitrogen. The residue was re-dissolved in 80 μL of methanol and an aliquot of 60 μL was injected into a HPLC system (Agilent 1100 series; Agilent Technologies, Santa Clara, CA, USA) equipped with a degasser, a pump and a photodiode array detector (G1315B). Chromatographic separation was performed using a C18 analytical column (150 mm × 4.6 mm I.D., 5 μm) (Ascentis; Sigma-Aldrich) and a mobile phase of 10% methanol in 0.05 M sodium phosphate
buffer (adjusted to pH 3.5 with o-phosphoric acid) and methanol (43/57, v/v) at a flow rate of 1.2 mL/min. The extraction efficiencies (recoveries) of atorvastatin were assessed from triplicate determinations by adding 5, 10 and 50 ng/mL of atorvastatin to the blank serum and the percentages of the resulting peak areas compared to the respective peak areas of pure solutions of the same atorvastatin concentration (in methanol) were calculated. The concentration of atorvastatin was quantified by measuring the absorbance at 247 nm, and the peak area of atorvastatin normalized by the relative peak area of β-naphthoflavone (mean peak area of β-naphthoflavone was defined as 1.0) was used for the calculation of concentration.

2.4. Calibration curve and method validation

Calibration curves were established by linear regression analysis of the peak area as a function of atorvastatin concentrations in sera spiked with 3, 4, 5, 10, 25, 50, 100 and 150 ng/mL of atorvastatin. The extraction, chromatography and normalized peak area determination were carried out as described above. The specificity of the method was evaluated by the analysis of blank serum samples from five healthy volunteers. Intra- and inter-day precision and accuracy were determined by analyzing 5.0–150 ng/mL of atorvastatin analytes in triplicate on the same day and on 3 different days, respectively. Determinations were performed using the same stock solutions and a pooled serum sample from healthy volunteers. The accuracy was calculated as the percentage of mean observed concentration compared to the nominal concentration. The precision evaluation shown as the relative standard deviation (RSD) was calculated as the percentage of standard deviation compared to the observed concentration. The limit of detection (LOD) for this method was defined as the concentration of drug giving a signal-to-noise ratio of 3. The limit of quantification (LOQ) was defined as the lowest concentration of atorvastatin in blank serum that could be quantified with the inter-assay SD of less than 20%.

2.5. Data analyses

The data were expressed as mean ± SD. The median value and interquartile range were calculated using SPSS version 17.0 (IBM Corp., New York, NY, USA). The statistical significance in the difference between serum atorvastatin concentrations in patients with and without myalgia was evaluated by logistic regression analysis using SPSS software.

3. Results and discussion

To establish a HPLC method for the quantification of atorvastatin in patients, we compared the retention time of several internal standard candidates including acetaminophen, diclofenac, α-naphthoflavone, β-naphthoflavone, theophylline and verapamil for a qualified separation from atorvastatin (Table 1, Fig. 1A) and background peaks of serum samples. The chromatogram of human blank serum from healthy volunteers is shown in Fig. 1B; several absorbance peaks appear in the chromatogram of the blank serum sample. In the blank serum sample spiked with atorvastatin and β-naphthoflavone, β-naphthoflavone was clearly separated from the endogenous peaks (Figs. 1C–E). No interference peak appeared at the retention time relevant to atorvastatin and β-naphthoflavone, indicating a good selectivity of this method. The retention times of atorvastatin and β-naphthoflavone were 19.8 and 56.1 minutes, respectively. However, compared to β-naphthoflavone, the other candidate chemicals did not show better separation from atorvastatin and the endogenous peaks (Table 1). The spectra (200–400 nm) of selected peaks with retention time equivalent to atorvastatin and β-naphthoflavone in the chromatograms of patients’ sera were comparable to the pure standard solution (data not shown). Thus, β-naphthoflavone was used as an internal standard in the chromatographic analysis.

The extraction recoveries of atorvastatin from sera spiked with 5, 10 and 50 ng/mL of atorvastatin are listed in Table 2. The mean recovery of sample preparations was 85.1%. The LOD and LOQ were 1.2 ng/mL and 3.0 ng/mL, respectively. Using liquid–liquid extraction in the blood sample preparation and HPLC method in the detection of atorvastatin, other reports had LOD and LOQ values of 1.0–1.9 ng/mL and 3.0–5.6 ng/mL, respectively [12–14]. Our method had detection sensitivity comparable to these reported methods. In addition to absorbance measurement, atorvastatin also has detectable fluorescence [15]. However, the quantification of blood atorvastatin concentration by measuring its fluorescence has not been reported. A method using acceptor droplet directly suspended in liquid–liquid–liquid microextraction for sample preparation was reported to decrease the LOD of the HPLC method to 0.4 ng/mL [16]. However, this method has not been commonly used. In our determinations, atorvastatin concentrations at 3.0–150 ng/mL had a good linear relationship with the peak area in the chromatograms of blank sera spiked with atorvastatin (r = 0.999) (Fig. 2). The RSD values of intra- and inter-day analyses were within a range of 2–17% (Table 3). The accuracies of intra-day and inter-day analyses were 92.8–118.7% and 92.2–119.8%, respectively. For the quantification of atorvastatin, LC–MS/MS analysis provides a highly sensitive method with a quantification limit as low as 0.25 ng/mL [2]. In studies using this method in healthy volunteers of different ethnicities, the mean Cmax values after taking a 10–40 mg dose of atorvastatin were 12.7–50.1 ng/mL (Table 4) [2,7–9,16]. In lipid-controlling Caucasian patients, the serum atorvastatin concentrations were in the range of 0–30.4 ng/mL, with median values of 5.4–23.7 ng/mL [16]. Although the HPLC method was not as sensitive as the

**Table 1 – Retention times of candidates for internal controls.**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>2.1</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>18.2</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>41.1</td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>56.1</td>
</tr>
<tr>
<td>Theophylline</td>
<td>2.2</td>
</tr>
<tr>
<td>Verapamil</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Chromatography was carried out as described in the Methods section.
LC–MS/MS method, the LOD and LOQ values of our method and the other HPLC methods were lower than the mean C\text{max} and median values of atorvastatin concentrations shown in Table 4. These results indicate that the HPLC method may provide a lower-cost alternative for the study of the adverse effects potentially induced by high concentration exposure.

In this study, blood samples were collected during the outpatients’ regular hospital visit in the morning. The time period from drug administration to blood sampling could be different among patients and might have affected the serum concentrations.

Table 2 – Recoveries of samples prepared from sera spiked with defined concentrations of atorvastatin.

<table>
<thead>
<tr>
<th>Atorvastatin concentration (ng/mL)</th>
<th>Recovery of atorvastatin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>88.5 ± 3.6</td>
</tr>
<tr>
<td>10</td>
<td>82.4 ± 1.5</td>
</tr>
<tr>
<td>50</td>
<td>84.4 ± 1.6</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD of three determinations.

Fig. 1 – (A) HPLC chromatogram of internal control candidates: 1 = theophylline; 2 = verapamil; 3 = diclofenac; 4 = α-naphthoflavone; 5 = β-naphthoflavone. (B) Representative HPLC chromatogram of blank human serum sample. Representative HPLC chromatograms of blank human serum samples spiked with β-naphthoflavone 200 ng/mL and: (C) atorvastatin 3 ng/mL; (D) atorvastatin 5 ng/mL; (E) atorvastatin 50 ng/mL; (F) atorvastatin 100 ng/mL. Peaks 1 and 2 in panels C–F indicate the appearance of atorvastatin and β-naphthoflavone, respectively. The signal-to-noise ratios for 3, 5, 50 and 100 ng/mL of atorvastatin in the HPLC chromatograms were 5.5 ± 0.4, 7.9 ± 0.4, 45.6 ± 2.2 and 102.8 ± 2.2, respectively (mean ± SD of three determinations).
concentration determination. Since the half life of atorvasta- 

Fig. 2 – The linear relationship between the peak area 
ratios of atorvastatin to β-naphthoflavone and atorvastatin 
concentrations in human sera spiked with increasing 
concentrations of atorvastatin. Various concentrations 
(β-150 ng/mL) of atorvastatin were added to the blank 
serum, extracted, dried under nitrogen, and re-dissolved in 
methanol as described in the Methods section. The areas of 
atorvastatin and β-naphthoflavone peaks in the 
chromatogram with absorbance detection at 247 nm were 
determined. The results represent the mean ± SD of three 
determinations.
concentration determination. Since the half life of atorvasta-
tin is 15–30 hours, the difference in serum concentration 
caused by variations in the time period after dosing may be 
less than 50%. Our determination of serum atorvastatin con-
centrations using the HPLC method showed that the serum 
atorvastatin concentrations in Taiwanese patients were 
within the concentration ranges for different ethnic groups 
(Table 4). Serum atorvastatin concentrations in eight patients 
were below the detection limit. The median value and inter-
quartile range of detectable atorvastatin concentrations in the 
other 61 patients were 4.3 ng/mL and 4.5 ng/mL, respectively. 
The mean ± SD serum concentration was 10.8 ± 22.8 ng/mL. 
The variations between individuals were high with the SD 
being greater than the mean value. This HPLC method may 
not be ideal for the measurement of low serum concentra-
tions, but concentrations greater or equal to median values 
were quantifiable. This method could provide a cheaper, less
technique-intensive and more robot-friendly method than mass spectral determination for the quantification of high serum concentration for risk assessment in patients.

Reddy et al [9] reported that the median values of serum concentrations in 20 mg and 40 mg atorvastatin-dosing groups are 226% and 339% higher than that in the 10 mg-dosing group of patients, respectively (Table 4). Our results showed that the median value and interquartile range of detectable serum concentrations in the 10 mg-dosing group were 3.5 ng/mL and 8.7 ng/mL, respectively (Fig. 3). The median value and interquartile range of serum atorvastatin concentration in the 40 mg-dosing group were 4.4 ng/mL and 3.6 ng/mL, respectively. The median value of serum concentrations in the 40 mg-dosing group was only 26% higher than that in the 10 mg-dosing group. This difference is not as much as that reported by Reddy et al [9]. However, the mean value of serum concentration was not higher in the higher dosing group. Factors including the detection sensitivity of low serum concentration, outliers and high inter-individual difference could interfere with the group comparison when the mean value is used.

In the 10 mg-dosing group with myalgia, the median atorvastatin concentration in patients with and without myalgia were 11.0 (n = 3, the interquartile range could not be calculated) and 3.0 (n = 24, with an interquartile range of 6.7 ng/mL), respectively. The median value in the group of patients with myalgia was 293% greater than in the patients without myalgia (Fig. 3). However, there was no similar observation in the 40 mg-dosing group. In the 40 mg-dosing group, the median values were almost the same in patients with and without myalgia (the difference was less than 5%). A myalgia study of Caucasiens in Norway [17] demonstrated that the atorvastatin concentrations in lipid-controlling patients with muscular side effects were not significantly higher than those in healthy participants taking the same dosing regimen of atorvastatin. Our study compared patients with and without myalgia and found no significant difference either. Logistic regression analysis of serum concentrations in 61 patients showed that there was no significant increase in serum atorvastatin concentration in patients with myalgia (p = 0.293). Among the 61 patients, 27 and 34 patients took a daily dose of 10 mg and 40 mg of atorvastatin, respectively. The difference in concentrations between patients with and without myalgia was not significant in both the 10 mg (p = 0.689) and 40 mg (p = 0.575) dosing groups.

The more lipophilic lactone form (pharmacologically inactive) of atorvastatin has been suggested to cause higher toxicity than the acid form (pharmacologically active) in skeletal muscle cells [18,19]. Due to the significant difference between the portion of correctly predicted positive and negative diagnoses, the ratio of atorvastatin lactone to acid has been suggested to be helpful in the assessment of statin-induced myopathy [19]. However, the ratio of lactone to acid in patients with myalgia is not significantly different from the ratio in patients without myalgia. The cytotoxicity of lactone metabolite in muscle cells occurs at an exposure concentration greater than 100 μM, which is much higher than the plasma Cmax values (~3.6–37.2 nM) of lactone metabolite in patients and healthy participants taking atorvastatin [10,17].

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Fig. 3 – Serum atorvastatin concentrations in hyperlipidemic patients with and without myalgia after taking atorvastatin. Among 61 patients with a detectable serum level of atorvastatin, 27 patients took 10 mg of atorvastatin and 3 of them had myalgia, while 34 patients took 40 mg of atorvastatin and 4 of them had myalgia. The top and bottom lines of the box represent the 75th and 25th percentiles, respectively. The line in the middle represents the median value. The whiskers extending from the box represent the highest and lowest values that are not outliers or extreme values. Circles and asterisks represent the outliers (values that are between 1.5 and 3 times the interquartile range) and extreme values (values that are more than 3 times the interquartile range), respectively.
In this study, we did not determine the serum concentration of atorvastatin lactone. The contributions of lactone metabolite to statin-induced myopathy and its underlying mechanism require further investigation. We could not exclude the involvement of risk factors other than the level of lactone metabolite in atorvastatin-induced myotoxicity. Other factors including alcohol consumption, diabetes or muscle inflammation may increase the risk of muscle injury in patients [20].

4. Conclusion

In this report, a HPLC method was established with the use of \( \beta \)-naphthoflavone as an internal standard for the quantitative determination of serum atorvastatin in lipid-controlling patients. Although this method is time-consuming, the clear separation and low cost make this method applicable for the quantitative determination of high serum concentrations in patients with potential myalgia adverse effect. The results of our study showed that the serum concentrations in lipid-controlling patients with myalgia were not significantly higher than those in patients without myalgia.

Acknowledgments

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