Development and Validation of Atorvastatin by LC-ESI-MS and Application in Bioequivalence Research in Healthy Chinese Volunteers

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Abstract

The aim of this research was to develop a sensitive liquid chromatographic-electrospray ionization-mass spectrometric (LC-MS) method for direct measurement of the concentration of Atorvastatin in human plasma. Plasma samples (1 mL) were extracted with 3 mL ethyl acetate, and by a simple reversed-phase chromatography. Pitavastatin was used as internal standard (IS). The LOQ was 0.25 ng mL⁻¹ (RSD 4.24%). The assay was linear from 0.25-20 ng mL⁻¹. The correlation coefficient for the calibration regression line was 0.9996 or better. Intra-day and inter-day accuracy were better than 15%. The method has been successfully used for a pharmacokinetic study with human subjects. A two-period crossover designed bioequivalence research was also performed in healthy Chinese volunteers. Among the pharmacokinetic data obtained, \( t_{1/2} \) was 1.36 ± 0.68 h for reference formulation and 0.81 ± 0.54 h for test formulation. \( C_{\text{max}} \) was 8.54 ± 5.06 ng mL⁻¹ for reference formulation and 9.54 ± 3.68 ng mL⁻¹ for test formulation. \( h_{1/2} \) was 5.80 ± 2.74 h for reference formulation and 9.24 ± 3.17 h for test formulation. \( AUC_{0-\text{tmax}} \) was 54.77 ± 21.82 h ng mL⁻¹ for reference formulation and 55.66 ± 20.91 h ng mL⁻¹ for test formulation. The method was successfully applied to the study of pharmacokinetics of Atorvastatin in healthy Chinese volunteers.

Keywords

Column liquid chromatography
LC-ESI-MS
Bioequivalence
Atorvastatin

Introduction

Atorvastatin (\( [R*(R^*,R^*)]-2-(4-fluorophenyl)-5-(1-methylcyclohexyl)-3-phenyl-4-[phenylamino]carbonyl] \( H \)-pyrrole-1-leptanoic acid, AT) is a potent inhibitor of HMG-CoA reductase, the rate limiting enzyme in cholesterol biosynthesis and has been demonstrated to be effective in reducing both cholesterol and triglyceride. In recent reports, AT is also used to prevent stroke [1-3].

AT is rapidly absorbed after oral administration, due to pre-systematic clearance in the gastro-intestinal mucosa and metabolism in the liver. Absolute bioavailability of AT is approximately 12% and the low concentration [4] in the plasma increases difficulty to determine AT after oral administration. A few chromatographic methods have been reported for determination of Atorvastatin in biological fluids. However, there are some shortages for those methods described in the literature for the determination of AT in the biological samples. Those methods included enzyme immunoassay [5], high-performance liquid chromatography (HPLC) [6, 7], gas chromatography/mass spectrometry (GC-MS) [8], high-performance liquid chromatography and high-performance liquid chromatography equipped with tandem mass spectrometry (LC-MS-MS) [9, 10]. HPLC methods are not sensitive enough to measure Atorvastatin in human plasma. The LOQ of the methods were all above 6 ng, which was reported. LC-MS-MS methods are sensitive with a low LOQ, but these devices may not be available in many laboratories, and the procedure of GC-MS required two derivatization steps. There is, therefore, still a need to develop a simple, sensitive, and safe analytical method for quantification of Atorvastatin in biological fluids. In recent years, the application of LC-MS rapidly increased. For this reason, the development of an LC-MS method for direct measurement of the concentration of Atorvastatin in human plasma was necessary. This paper presents an LC-MS method, which is suitable for use in pharmacokinetic studies. The method is very safe and simple compared with...
other methods. In addition, the limit of quantification of this method was 0.25 ng mL⁻¹, which was the same as in LC–MS–MS. Because of its good selectivity, high sensitivity, fine precision and accuracy, and simple sample treatment, the method was suitable for the research on the pharmacokinetics of Atorvastatin in biological fluids. There are some articles describing the pharmacokinetics of Atorvastatin in human plasma in other countries, but the pharmacokinetic profile of the drug in Chinese plasma has not been reported. Research [11] shows that individual variation of statin could not be neglected, and may concern pharmacogenetics variation. Thus, the investigation of Atorvastatin pharmacokinetic profile in healthy Chinese volunteers is necessary.

Experimental

Materials and Reagents

Atorvastatin and pitavastatin powder (> 99.9%) was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Atorvastatin tablets (10 mg per tablet) were from Yang Jiang Medicine Company (Guangdong Province, China). Methanol (HPLC grade) used in the mobile phase was purchased from Tedia (Fairfield, USA). Ammonium acetate, methanolic acid and ethyl acetate were of analytical grade. Liquid nitrogen was from the Gas Supplier Center of Nanjing University (Jiangsu Province, China).

Instrument and Conditions

LC–MS was performed with a Shimadzu (Kyoto, Japan) LCMS-2010A liquid chromatograph–mass spectrometer equipped with an SIL-HTC autosampler, two LC-10AVP pumps, and an electrospray ionization (ESI) interface. A 150 mm × 4.6 mm stainless-steel analytical column packed with 5 μm Hypersil ODS₂ C₁₈ was purchased from Dalian Elite Company (LiaoNing Province, China).

The mobile phase was 5 mM ammonium acetate solution–methanol–methanoic acid (30:70:0.1, v/v/v) at a flow rate of 1 mL min⁻¹. The column temperature was maintained at 40 ºC. LC–ESI–MS was carried out using nitrogen to assist nebulization. A quadruple mass spectrometer equipped with an electro-spray ionization source was set with a drying gas (N₂) flow of 1.5 L min⁻¹. During tuning the detector potential was 1.60 kV and the probe potential was 3.50 kV. The heat block temperature was 200 ºC and the curved desolvation line (CDL) temperature was maintained at 250 ºC. LC–ESI–MS was performed in selected-ion monitoring mode and Atorvastatin was detected at [M + H]⁺ m/z 559.25 while pitavastatin at [M + H]⁺ m/z 422.15. LC–MS Solution version 3.20 with Windows XP operating system was used for data processing.

Preparation of Calibration Samples and Quality-Control Samples

The stock solution of Atorvastatin was prepared in methanol at the concentration of 100 μg mL⁻¹. Working solutions of Atorvastatin were prepared at concentrations of 2.5, 5, 10, 20, 50, 100, and 200 ng mL⁻¹ by serial diluting the stock solution with mobile phase. The stock solution of IS was prepared in methanol at the concentration of 100 μg mL⁻¹ and diluted to 10 ng mL⁻¹ with mobile phase before using. All the solutions were stored under refrigeration (2–8 ºC) when not in use.

To prepare the calibration samples, 100 μL working solutions were diluted with 900 μL blank plasma to span the calibration range 0.25–20 ng mL⁻¹ (0.25, 0.5, 1, 2, 5, 10 and 20 ng mL⁻¹). The final concentration of the internal standard in all samples was 0.5 ng mL⁻¹. Quality-control (QC) samples (0.5, 2 and 10 ng mL⁻¹) were prepared in the same way and were stored at −20 ºC until analysis. All processes were protected from light.

Sample Preparation

Human blood samples were centrifuged at 4,000g for 5 min immediately after collection and 1 mL of the plasma was promptly transferred to an Eppendorf tube. These samples were immediately stored at −70 ºC until analysis. Immediately before analysis 50 μL pitavastatin solution was added and the mixture was vortex mixed. The sample was then deproteinated with 3 mL ethyl acetate and the precipitate was removed by centrifugation at 4,000g for 3 min. Following centrifugation and separation, the organic phase was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 150 μL of mobile phase, and a 40-μL aliquot was injected for analysis by LC–ESI–MS after centrifugation at 10,000g for 10 min. All processes were protected from light.

Application

The assay was used to determine Atorvastatin in plasma samples after a single oral dose of 20 mg atorvastatin (2 Atorvastatin tablets) of 18 Chinese healthy volunteers. The clinical study protocol was reviewed and approved by the Human Ethics Committee of the Yitishan Hospital of the Wannan Medicine Academy. All volunteers were given written information consent to participate in the study according to the principles of the Declaration of Helsinki.

A two-period crossover designed bioequivalence research was progressed. Eighteen healthy young Chinese volunteers participated in the study. The subjects were 22 ± 1 (mean ± CV) years old and weighed 71 ± 1 kg (mean ± CV). No medicine was administered by the subjects for 2 weeks before and during the experiment. Water was not limited but 12 h before starting the experiment the subjects were not allowed to eat foods. Each subject was given a single oral dose of 20 mg Atorvastatin (2 Atorvastatin tablets) with 30 mL water. Blood was obtained at t = 0.25, 0.5, 1, 1.5, 2, 3, 4, 8, 12, 24, 36, and 48 h and plasma samples were prepared as described above. Model-independent pharmacokinetic parameters were calculated for Atorvastatin. The maximum plasma concentration (Cmax) and the time to it (Tmax) were noted directly. The elimination rate constant (k) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life (t1/2) was calculated using the formula t1/2 = 0.693/k. The area under the plasma concentration–time curve AUC⁰–∞ was calculated by the linear trapezoidal rule.
Table 1. Validation of precision, recovery and matrix effect

<table>
<thead>
<tr>
<th>Concentration (ng mL(^{-1}))</th>
<th>Recovery (mean ± SD, %)</th>
<th>Matrix effect A1/A2 (mean ± SD, %)</th>
<th>Inter-assay precision (mean ± SD)</th>
<th>Intra-assay precision (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>58.9 ± 1.2</td>
<td>112.9 ± 5.9</td>
<td>0.55 ± 0.03</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>60.0 ± 1.2</td>
<td>98.0 ± 4.9</td>
<td>2.00 ± 0.12</td>
<td>1.90 ± 0.09</td>
</tr>
<tr>
<td>10</td>
<td>55.5 ± 1.9</td>
<td>110.0 ± 3.3</td>
<td>9.79 ± 0.72</td>
<td>10.36 ± 0.45</td>
</tr>
</tbody>
</table>

Results

Validation

The method was validated for selectivity, sensitivity, recovery, linearity, precision and accuracy, and stability.

Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank plasma with the corresponding spiked plasma. Figure 1 shows the typical chromatograms of blank plasma, spiked plasma sample with Atorvastatin and IS, and plasma sample from a healthy volunteer 2 h after an oral administration. It is apparent the retention times of Atorvastatin and the IS were approximately 4.45 and 4.68 min, respectively. The overall chromatographic run time was within 5.50 min. So no interfering endogenous compound peaks were observed at the retention times of Atorvastatin or IS in the run time.

Sensitivity (Limit of Quantification) and Linearity of Calibration

Sensitivity was determined by replicate (\(n = 5\)) analysis of control plasma samples spiked with the analyte at the lowest level of the calibration plot.

The sensitivity was 0.25 ng mL\(^{-1}\). The mean percentage deviation from the nominal concentration was ≤ 15% and the RSD was 4.24%. Interference from endogenous plasma constituents was found to be negligible.

The Atorvastatin calibration curve was constructed by plotting the peak area ratios (y) of Atorvastatin to the IS versus the concentrations (x) of Atorvastatin. Typical calibration curve for Atorvastatin was \(y = 0.3646x - 0.0382\), correlation coefficients for the calibration regression plot were 0.9996 or better. The calibration curve was linear over the concentration range 0.25–20 ng mL\(^{-1}\) for Atorvastatin.

Recovery and Matrix Effect

Results from determination of the absolute recovery of Atorvastatin from human plasma are shown in Table 1 (\(n = 5\) for each concentration). Matrix effects caused by ionization competition occurring between Atorvastatin and endogenous co-eluting components was evaluated at three concentrations, by comparing the peak response for Atorvastatin (A1) in extracted samples of blank plasma, spiked after extraction, with the corresponding response (A2) obtained by direct injection of standard solutions prepared in the mobile phase. The results, shown in Table 1, suggest the matrix effect was negligible in this method.

Precision and Accuracy

The intra-day and inter-day accuracy and precision of the method were determined by analysis of plasma samples containing 0.5, 2, and 10 ng mL\(^{-1}\) Atorvastatin. The intra-day deviation from the nominal concentration was ≤ 15% and the intra-day precision was ≤ 4.9% at each concentration. The inter-day deviation from the nominal concentration was ≤ 15% and the inter-day precision was ≤ 7.4% at each concentration. The results demonstrated that the method was accurate and precise (see Table 1).

Stability

The stability of Atorvastatin in human plasma was evaluated at concentrations of 0.5, 2, and 10 ng mL\(^{-1}\). The study revealed that under these conditions Atorvastatin in plasma was stable for at least 8 h at room temperature with a mean percentage change of ≤ 10.1%. It was confirmed that plasma samples of Atorvastatin at three concentrations were stable to repeated freezing and thawing (2 or 3 cycles), the mean percentage change was ≤ 12.8%. The freeze stability of Atorvastatin in plasma at −20 °C was
Table 2. Pharmacokinetic data for 18 subjects after oral administration of 20 mg Atorvastatin

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Reference formulation (mean ± SD)</th>
<th>Test formulation (mean ± SD)</th>
<th>90% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC(0-48h) (ng mL⁻¹ h)</td>
<td>54.77 ± 21.82</td>
<td>55.66 ± 20.91</td>
<td>98.80–109.64</td>
</tr>
<tr>
<td>AUC(0-∞) (ng mL⁻¹ h)</td>
<td>58.32 ± 23.09</td>
<td>39.44 ± 21.88</td>
<td>97.82–108.35</td>
</tr>
<tr>
<td>Cmax (ng mL⁻¹)</td>
<td>8.54 ± 5.06</td>
<td>9.54 ± 3.68</td>
<td>100.16–134.77</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.36 ± 0.68</td>
<td>0.81 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>K (h⁻¹)</td>
<td>0.089 ± 0.025</td>
<td>0.084 ± 0.029</td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>8.50 ± 2.74</td>
<td>9.24 ± 3.17</td>
<td></td>
</tr>
<tr>
<td>MRT (h)</td>
<td>12.22 ± 3.47</td>
<td>12.48 ± 4.27</td>
<td></td>
</tr>
</tbody>
</table>

determined for 14 and 28 days at three concentrations and the mean percentage change was ≤ 11.5%. During stability validation we also compared results from analysis of the QC samples with results from samples from the auto sampler. It was determined for 4 h and 8 h, and the mean percentage change was ≤ 15%. And we also studied the stability of the analytes in solution during the actual analysis. During 28 days, the stability of Atorvastatin in the stock solution was good with the mean percentage change ≤ 5%. Consequently, the stability of Atorvastatin in human plasma is good enough for analysis and is suitable for clinical pharmacokinetics research.

Application

The method was successfully applied to determine the plasma concentration of Atorvastatin up to 48 h after an oral administration of 20 mg Atorvastatin to 18 healthy Chinese volunteers. The bioavailability research of two Atorvastatin formulations (Atorvastatin dispersible tablet, Yang Jiang Medicine Company, Guangdong Province, China as the test formulation, and Lipitor® , Pfizer Ireland Pharmaceuticals, Dublin, Ireland, as the reference formulation) was processed. The mean plasma concentration-time curve of Atorvastatin is shown in Fig. 2. The pharmacokinetic data of two formulation obtained are calculated and summarized in Table 2. On this basis the limit of quantification of the method, 0.25 ng mL⁻¹, is sufficiently sensitive for pharmacokinetic research on Atorvastatin. Pharmacokinetic data were as following. Tmax was 1.36 ± 0.68 h for reference formulation and 0.81 ± 0.54 h for test formulation. Cmax was 8.54 ± 5.06 ng mL⁻¹ for reference formulation and 9.54 ± 3.68 ng mL⁻¹ for test formulation. t1/2 was 8.50 ± 2.74 h for reference formulation and 9.24 ± 3.17 h for test formulation. AUC(0-48h) was 54.77 ± 21.82 h ng mL⁻¹ for reference formulation and 55.66 ± 20.91 h ng mL⁻¹ for test formulation, which approximately correspond to the literature reported by Koytchev [12] but are different from those reported by Mendoza [13]. According to the current study, the relative bioavailability of the test for formulation was 103.07% (mean AUC(0-48h) and 103.68% (mean AUC(0-∞)) respectively. Statistical analysis of bioequivalence parameters was carried out using the SPSS program (version 11.5) for in-transformed pharmacokinetic parameters AUC(0-48h), AUC(0-∞), Cmax. There were no significant differences between the two formulations on the basis of assessment by a two one-sided t test. The 90% confidence intervals of test-to-reference ratio of the AUC(0-48h) were within the bioequivalence criteria range of 80–125%, and that of Cmax was within 70–143%. Therefore, the two products were bioequivalent.

Conclusion

A sensitive LC–ESI–MS method for the quantification of Atorvastatin in human plasma was developed and validated. No significant interferences caused by endogenous compounds were observed. The method is sensitive and selective with an LOQ of 0.25 ng mL⁻¹. This simple and sensitive assay is suitable for the pharmacokinetic study and bioavailability evaluation of Atorvastatin formulations, and can also be used as a therapeutic drug monitoring method in clinics to check the plasma concentration of Atorvastatin. The statistical analysis result of Tmax shows that there are significant differences between the two formulations. It is mainly because a dispersible tablet disaggregates a little faster than a common formulation and is easier for absorption, so the Tmax of the test formulation is faster than that of the reference formulation. The statistical analysis results based on comparison of three parameters (AUC(0-48h), AUC(0-∞), Cmax) confirmed the bioequivalence of the two formulations.

Acknowledgments

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References


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