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Liquid Chromatographic-MS/ MS Determination of Atorvastatin and Metabolites in Human Plasma

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Abstract

The aim of the present study was to develop a chromatographic method for the analysis of atorvastatin, Ortho- and Para -hydroxyatorvastatin in human plasma after administration of atorvastatin at the dose of 40 mg in clinical studies. Sample preparation was performed by solid phase extraction and was followed by separation of the analytes on an HPLC system with a linear gradient and a mobile phase consisting of acetonitrile, water and formic acid. Detection was achieved by tandem mass spectrometry operated in the electrospray positive ion mode. Validation of the method for the compounds for which reference compounds were available (acid forms of atorvastatin, o- and p-hydroxyatorvastatin) showed linearity within the concentration range for atorvastatin acid 0.2 - 40 ng mL^{-1}, Para-hydroxyatorvastatin acid 0.250-50 ng mL^{-1}, and ortho-Hydroxyatorvastatin acid 0.25 - 50 ng mL^{-1} ($r2 \geq 0.99$, n=3 for all analytes). Accuracy and precision (evaluated at 0.5, 17 and 31 ng mL^{-1} for atorvastatin, 0.66, 22, 40 ng mL^{-1} for Para--hydroxyatorvastatin and ortho-hydroxyatorvastatin) were both satisfactory. The detection limit was 0.06 ng mL^{-1} for atorvastatin and para - hydroxyatorvastatin, and 0.15 ng mL^{-1} for ortho -hydroxyatorvastatin. The method has been successfully applied in a clinical study where atorvastatin, o- and para - hydroxyatorvastatin could be detected in a 24-h sampling interval after administration of registered dose of atorvastatin (40 mg) for one week.

Keywords:

Atorvastatin, HMG-CoA reductase inhibitor; Para--hydroxyatorvastatin and ortho-hydroxyatorvastatin; LC- MS/MS

1. Introduction

Atorvastatin is a HMG-CoA reductase inhibitor, which has a widespread use in the prevention of cardiovascular events. Atorvastatin is administered as the calcium salt of its active acid form, (Fig.1). It is metabolised by CYP3A4 to two hydroxylated metabolites, o-hydroxyatorvastatin and p-hydroxyatorvastatin, (Fig.1). About 70% of the total plasma HMG CoA reductase inhibitory activity is accounted for by active metabolites [1]. In several clinical studies regarding statins in general, including atorvastatin, determination of the statins has been performed using alternative methods, such as enzyme inhibition assays, other than determination of the actual plasma concentrations of the compounds [2-5]. Such indirect measurements are relevant when determining the HMG-CoA reductase inhibitory activity of the statin in plasma, but they do not give any further information on metabolites. Information

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about the actual plasma concentration of both parent compound and metabolites is of interest in pharmacokinetic studies, especially with regard to possible side effects and toxicity of metabolites, and also in investigations of the mechanisms of drug–drug interactions. Specific chromatographic methods are therefore required in order to achieve specific determinations of the parent compound and both active and inactive metabolites. Two high-performance liquid chromatographic (HPLC) methods for the determination of atorvastatin in human plasma and serum have previously been published [6,7]. Both methods use liquid–liquid extraction (LLE) with ether for sample preparation. Both of these methods have been applied (with minor modifications) in clinical studies, with atorvastatin doses ranging between 40 and 80 mg [8,9]. A method for specifically detecting atorvastatin and its hydroxyacid metabolites after the administration atorvastatin was needed. Sample preparation by solid phase extraction (SPE) was desirable in order to reduce the amount of organic solvent used, get cleaner extracts, higher recoveries and make the process easily automated compared to the LLE methods [6,7]. Thus, the aim of this study was to develop a chromatographic method for determining the acid forms of atorvastatin, o- and p-hydroxyatorvastatin, in human plasma following administration of atorvastatin, using SPE for sample preparation.

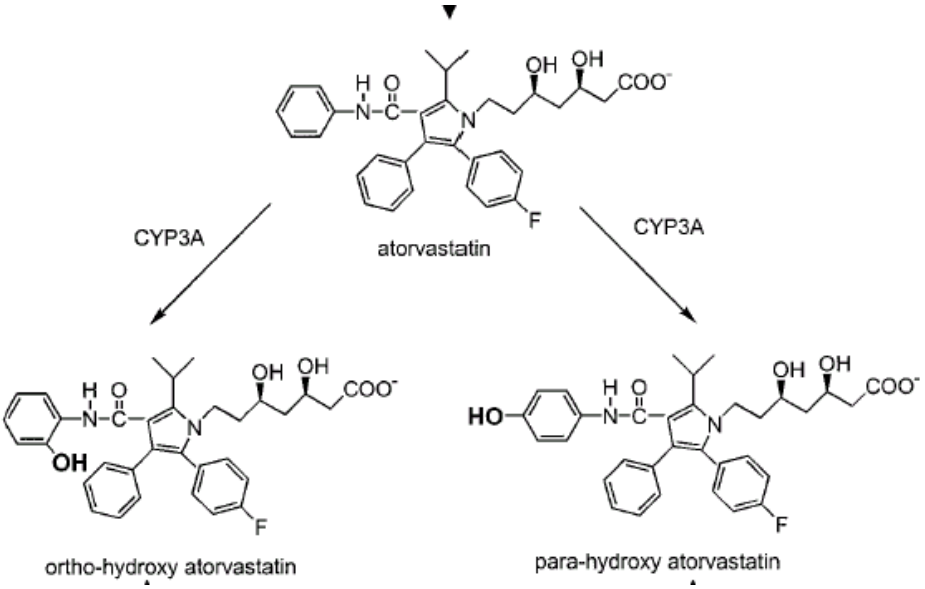


Fig. 1. Atorvastatin metabolized to Ortho hydroxy and Para hydroxy atrovastatin by CYP3A4.

2. Experimental

2.1. Chemicals and reagents

Atorvastatin, o-hydroxyatorvastatin and p-hydroxyatorvastatin were purchased from Synfine Chemicals (Canada) and Pravastatin (Sigma-Aldrich, Norway) was used as internal standard. Pravastatin is also an HMG-CoA reductase inhibitor and concomitant use of both the drugs are not possible and it was therefore unlikely to be used by patients, in subsequent pharmacokinetic studies applying this method. All chemicals used for chromatographic purposes were of analytical grade.

2.2. Preparation of standard samples

2.2.1. Stock solutions

Stock solutions of atorvastatin acid, o- and p-hydroxyatorvastatin acids were prepared in methanol–water (50:50 v/v), Stock solution of the internal standard, Pravastatin, was prepared in HPLC grade water. All stock solutions were stored at -20°C. Drug-free pooled plasma containing K_3EDTA was obtained from five healthy volunteers. Plasma samples for method validation were spiked with aliquots of the stock solutions.

2.2.3. Biological samples

Biological samples were obtained from healthy volunteers serving as controls in a clinical study. Healthy volunteers were given 40 mg atorvastatin and plasma samples were drawn on fixed times from 0 to 24 h post-dose on day 7. Plasma samples were frozen immediately after sample withdrawal to minimize acid–lactone interconversion and stored at -80°C. Individuals participating in this study signed an informed consent, and the study was recommended by the Institutional Ethics Committee of Synchron Research Pvt Ltd.

2.2. Instrumentation

The HPLC equipment consisted of a Alliance HT with intergrated system of quarternary pump and autosampler. For detection, Mass spectrometry was performed using a Quattro LC tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ESI source. Mass Lynx version 4 software was used for system operation and data handling. A Universal centrifuge (Remi equiptments, India) was used for all centrifugation steps in the sample preparation procedure.

2.3. Sample preparation

Preparation of samples from healthy volunteers and plasma samples spiked with aliquots of the analytes was performed by SPE, and the samples were kept on ice at all times during sample preparation. The plasma samples (0.5 mL) were spiked with 5µg of the internal standard pravastatin and 0.5 mL of 2% formic acid in water and were vortexed for 30 seconds. The content was subsequently transferred to 1 mLC18 (100 mg) SPE cartridges (Oasis, Waters Corporations, Milford, Mass, USA) pre-conditioned with 2 mL methanol followed by 1 mL water. The cartridges were washed with 2 mL 2% formic acid in water, and the analytes were eluted with 1 mL 0.1 % formic acid in methanol after the extraction of samples, the extracts were concentrated and evaporated to dryness under a stream of N2 at around 60° C and the residues were reconstituted in 200 µl mobile phase to replace methanol as soon as possible in order to eliminate the ester formation of acidic statins by reaction with methanol. Resulting solution was vortexed for 30 seconds, prior to transferral to HPLC vials and injection of 25 µl in the HPLC system .In addition, LC–MS–MS analysis was conducted as soon as possible to reduce the interconversion of statins between acid and lactone forms.

2.4. Chromatographic conditions

An Cyno 125 X 4 mm, 5µm analytical column for the chromatographic separation of the compounds. The analytes were eluted by mobile phase consisting of acetonitrile–methanol–0.1% formic acid in water (50–30–20, v/v). The flow rate was 0.5 mL/min. The autosampler tray temperature was set at 10 °C. The column oven temperature was set at 45 °C.

2.5. Mass spectrometric conditions

The mass spectrometer was operated in negative ion mode and connected to the chromatographic system using an ESI electrospray interface. The capillary voltage was 3.2 kV, and the voltages of extractor and RF lens were 5.0 and 0V, respectively. The entrance and

exit energies of the collision cell were set at 20.0 V. The cone voltage was operated at an optimal value for each analyte in negative-ion mode. Nitrogen was used as the drying and nebulizing gas at flow rates of 350 and 70 L / h, respectively. Tuning the MS detector for both optimal operating conditions and the optimal collision energy for dissociation was achieved through infusion of atorvastatin, o- and p-hydroxyatorvastatin through a T-connector. Argon was used as collision gas. N2 gas was used for nebulization and dissolution at 10 AU, source voltage at 5 kV and capillary temperature at 120ºC. In order to obtain maximum sensitivity, four segments were used. The MS detector was operated in the Multiple reaction monitoring (MRM) mode (four channel) within each of the four segments. All transitions and relative collision energies are listed in Table 1.

Table .1 Optimized LC–ESI-MS–MS parameters used for statin analysis in positive-ion mode

Segment Compound	Atorvastatin acid	o-Hydroxy atorvastatin acid	p-hydroxy atorvastatin acid	Internal standard (Pravastatin)
Precursor ion (m/z)	557.319	573.361	573.361	423.366
Product ion (m/z)	397.165	278.108	413.143	303.163
Collision energy(%)	30	28	46	40

2.6. Method validation

The concentrations of the compounds in the plasma samples were determined from their peak area, using pravastatin as an internal standard. Linearity was tested in the range for atorvastatin acid 0.2–40 ng mL^{-1}, p-hydroxyatorvastatin acid 0.250-50 ng mL^{-1}, and o-hydroxyatorvastatin acid 0.25–50 ng mL^{-1}. Separate batches of drug-free pooled plasma was spiked with atorvastatin (0.5, 17 and 31 ng mL^{-1}), p-hydroxyatorvastatin (0.66, 22 and 40 ng mL^{-1}) and o-hydroxyatorvastatin (0.66, 22 and 40 ng mL^{-1}) (n=5) to determine the accuracy and precision of within-day and between-day runs. Recovery was measured at above levels by comparing the ratio of response of extracted samples in Plasma: aqueous (n=3 at each concentration). Limit of detection (LOD) was determined at a signal-to-noise ratio of 3/1. Long-term, bench-top and freeze – thaw stability was tested, and was comparable to that by Jemal and Xia [7]. Autosampler stability of atorvastatin, o-hydroxyatorvastatin and p-hydroxyatorvastatin at 10°C was tested on extracted plasma samples in the injection matrix (mobile phase).

3. Results and Discussion

3.1. Sample preparation, chromatography and MS detection

In the present study, a SPE extraction procedure for the preparation of human plasma samples prior to LC– tandem MS analysis was developed. Plasma samples were mixed with 2% formic acid in order to ensure that the analytes were in the unionized form prior to transferral to the SPE cartridges. Wash steps with solutions containing amounts of organic solvent greater than 50% acetonitrile or 30% methanol in water resulted in a dramatic decrease in the extraction recoveries of the metabolites. A wash step with 30% methanol in water resulted in cleaner samples and less variable recovery than wash solutions containing 50% acetonitrile, and was therefore preferred. Acetonitrile–Methanol- Formic acid (50-30-20) was used for elution of the analytes from HPLC column. A volume of 1mL was needed in order to achieve maximum recovery for this eluent. A representative chromatogram from the analysis of a biological sample taken after daily administration of 40 mg atorvastatin for one

week is presented in Fig. 3. Separation of the compounds needed to be good in order to enable the use of MRM, which resulted in lower limits of detection and better signal: noise ratio. Due to the high signal intensity of [M-H], pravastatin and atorvastatin were normally been analyzed in negative-ion mode. Based on achieving a balance between sensitivity, separation efficiency and analysis time, a flow-rate of 0.2 mL/min was selected for the separation of Atorvastatin and Pravastatin (Internal standard). A variety of SPE cartridges, including HLB, Bond Elute C8 and DSC-18, were investigated to find most efficient extraction method for atorvastatin. Elute C8 and DSC-18 could recover very less % of atorvastatin and therefore, HLB cartridges were used to develop the extraction method. The total time of analysis in the chromatographic system was therefore 3.5 min. The retention times of atorvastatin, metabolites and internal standard were at around 2.4 min.

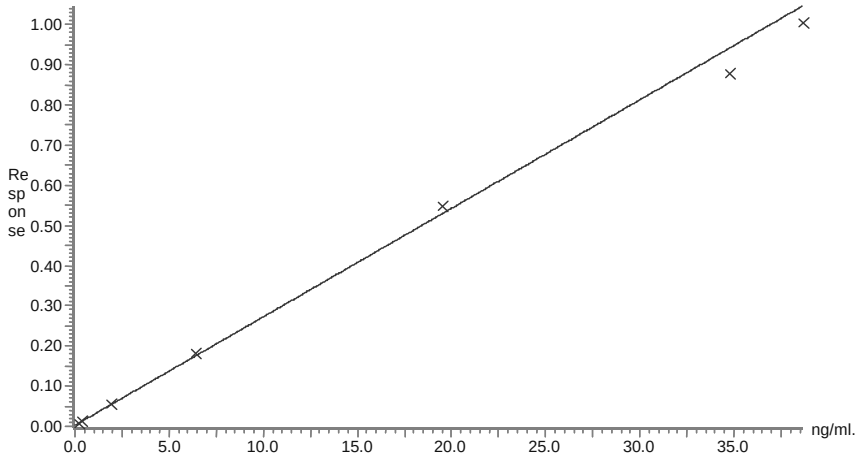
3.2. Validation of the method

Validation of the method showed linearity (Fig 2) within the concentration range 0.2–40 ng mL^{-1} for atorvastatin acid and 0.25-50 ng mL^{-1} for p-hydroxyatorvastatin acid and o-hydroxyatorvastatin acid ($r2 \geq 0.99$, n=3 for all analytes). Relative standard deviations (RSDs) of the estimated slopes were less than 15% over the whole concentration range. The intercepts of the calibration curves were shown to be not statistically different from zero for any of the analytes. LOD was 0.06 ng mL^{-1} for atorvastatin and p-hydroxyatorvastatin, and 0.15 ng mL^{-1} for o-hydroxyatorvastatin. Recoveries for all analytes ranged between 50 and 68% on average. The specificity of the method was tested using extracted drug-free plasma from six individuals. No interfering peaks were found. Also, no interfering ion suppression or matrix effect on signal enhancement was observed. No significant concentration changes in any of the analytes were observed upon autosampler storage at 10°C for a period of up to 24 h. Intra/ Interday precision and accuracy for the study were performed and were found within limit. (Table 2).

The method has been successfully applied in a clinical study, where atorvastatin, o- and p hydroxy atorvastatin could be detected in a 24-h sampling interval on steady state after the administration of the registered dose (40 mg) of atorvastatin to healthy volunteers.

Table 2. Precision and accuracy intra/interday

Intra-day Precision	CV%
Atorvastatin	2.0% to 9.8%
Orthohydroxyatorvaststin	3.5% to 9.7%
Parahydroxyatorvastatin	3.6% to 14.4%
Inter-day Precision	
Atorvastatin	5.5% to 11.1%
Orthohydroxyatorvaststin	6.3% to 9.1%
Parahydroxyatorvastatin	6.3% to 12%
Intra-day accuracy	
Atorvastatin	86.6% to 108.1%
Orthohydroxyatorvaststin	93.3% to 105.0%
Parahydroxyatorvastatin	92.9% to 108.9%
Inter-day accuracy	
Atorvastatin	92.9% to 105.6%
Orthohydroxyatorvaststin	98.5% to 102.6%
Parahydroxyatorvastatin	100.2%to102.7%



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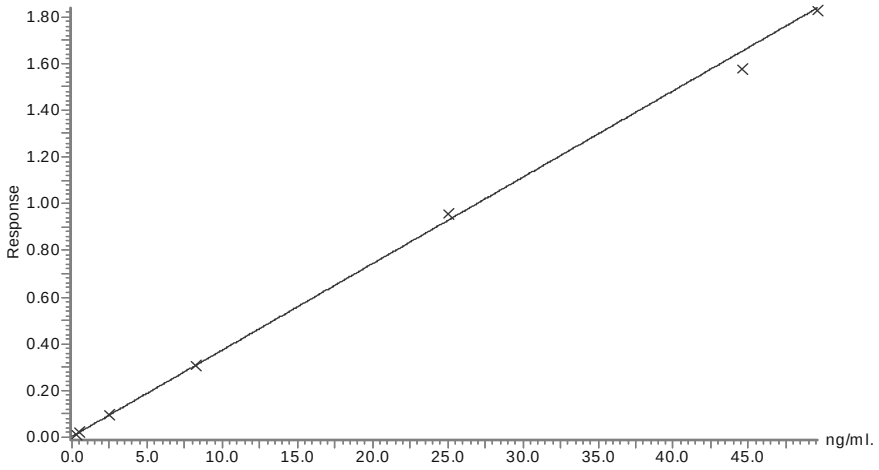

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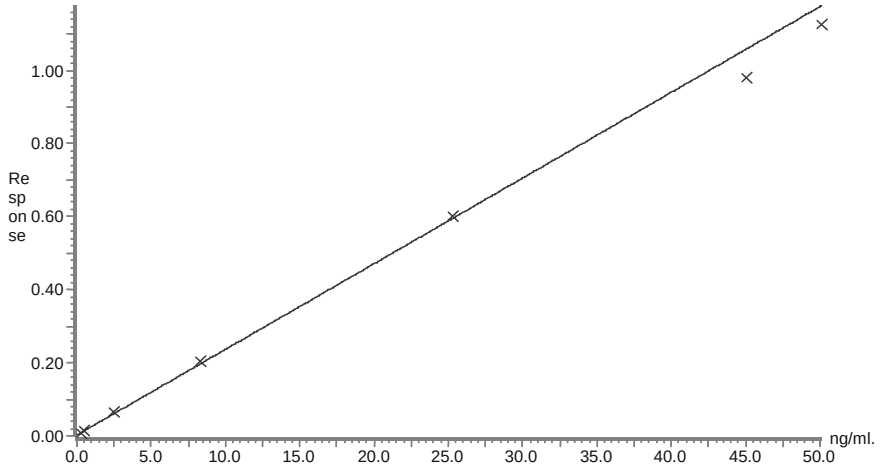

(c)

Fig. 2. Calibration curves of Atrovastatin (a), ortho hydroxyatrovastatin (b) and para hydroxyatrovastatin (c).

4. Conclusion

In the present work, sample preparation of human plasma samples was performed by SPE, in contrast to previous studies using LLE. Sample preparation was followed by HPLC with tandem MS detection. Validation results for atorvastatin acid, o- and p-hydroxyatorvastatin acids were satisfactory. Thus an LC–ESI-MS–MS method coupled with SPE was developed and validated for the quantitative determination of cholesterol-lowering Atrovastatin drug in Plasma samples.

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