Original Research Paper

Acta Chromatographica 28(2016)3, 281–298
DOI: 10.1556/1326.2016.28.3.1

LC–MS/MS Method for Quantification of Atorvastatin, *o*-Hydroxyatorvastatin, *p*-Hydroxyatorvastatin, and Atorvastatin Lactone in Rat Plasma

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Summary. A simple and sensitive liquid chromatography–tandem mass spectrometry method was developed for the quantification of atorvastatin, *ortho*-hydroxyatorvastatin, *para*-hydroxyatorvastatin, and atorvastatin lactone in rat plasma. Solid-phase extraction was used for preparation of samples. Rosuvastatin was chosen as an internal standard. Chromatographic separation was achieved on ZORBAX Eclipse C_{18} Analytical, 4.6 × 100 mm (3.5 μm) column with a gradient mobile phase composed of acetonitrile and 0.1% acetic acid, at a flow rate of 400 μL min^{-1}. The column was kept at constant temperature (25 °C), and autosampler tray temperature was set at 4 °C. The following selected reaction monitoring (SRM) transitions were selected: (*m/z*, Q1 → Q3, collision energy) atorvastatin (559.47 → 440.03, 22 eV), atorvastatin lactone (541.36 → 448.02, 19 eV), *ortho*-hydroxyatorvastatin (575.20 → 440.18, 20 eV), *para*-hydroxyatorvastatin (575.54 → 440.18, 20 eV), and rosuvastatin (482.25 with selected combination of two fragments 257.77, 31 eV, and 299.81, 35 eV) in positive ion mode. The method was validated over a concentration range of 0.5–20 ng mL^{-1} for *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin and 0.1–20 ng mL^{-1} for atorvastatin and atorvastatin lactone with excellent linearity ($r^2 \geq 0.99$). This method demonstrated acceptable precision and accuracy at four quality control concentration levels. The detection limits were 0.1 and 0.13 ng mL^{-1} for *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin, respectively, and 0.05 ng mL^{-1} for atorvastatin and atorvastatin lactone. All analytes were found to be stable at examined conditions. Validated method was applied for determination of atorvastatin and its metabolites in plasma of experimental animals.

Key Words: atorvastatin, atherosclerosis, metabolites, LC–MS/MS

First published online: June 30, 2015 *ISSN 2083-5736 © The Author(s)*

Introduction

According to published data, cardiovascular diseases are the leading cause of death in developed countries [1]. The major cause of coronary heart disease is increased intake of food rich in cholesterol. Statins (3-hydroxy-3-methylglutaryl-coenzyme A [HMG-CoA] reductase inhibitors) are widely used for the treatment of hypercholesterolemia. They reversibly inhibit *HMG-CoA reductase* which is the rate-limiting enzyme in the endogenous biosynthesis of cholesterol. Statins also have many cholesterol-independent ("pleiotropic") beneficial effects. One of the most important is their antioxidative activity [2–4]. Although the side effects of statins are relatively low, they can cause rhabdomyolysis in rare cases [5]. This fact increases importance of monitoring atorvastatin (AT) levels in biological fluids.

Atorvastatin is administered as the calcium salt of its active acid form and undergoes extensive first-pass metabolism [6]. It is metabolized in liver by CYP3A4 to two active hydroxy metabolites, *ortho*-hydroxyatorvastatin (oOH-AT) and *para*-hydroxyatorvastatin (pOH-AT), and three corresponding lactones *(Fig. 1)* [7]. *HMG-CoA reductase* inhibitory activity is mainly (around 70%) attributed to active metabolites [8]. The plasma concentration of active and inactive metabolites as well as parent drug is very low (nanogram per milliliter levels). Therefore, a bioanalytical method that is going to be used for the quantification of atorvastatin and its metabolites needs low limits of detection and quantitation.

There are published methods for the quantification of AT in biological matrix, pharmaceutical products, and bulk drugs. Some of those methods include enzyme inhibition assay [9], radioimmunoassay [10], electrochemical method [11], high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [12–15], liquid chromatography–mass spectrometry [16], microbore liquid chromatography/electrospray ionization–tandem mass spectrometry [17], and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [18–27]. Immunoassay methods lack specificity and, in some cases, are subject to problems with cross-reactive interferences. Also, enzyme inhibition assay is relevant when determining the *HMG-CoA reductase* inhibitory activity of AT in plasma, but it does not give any further information about metabolites.

Information about plasma concentration of parent drug and active and inactive metabolites is of interest in various types of studies, especially for investigations of possible drug–drug or drug–natural product interactions, pharmacokinetic studies, and side effects and toxicity of metabolites.

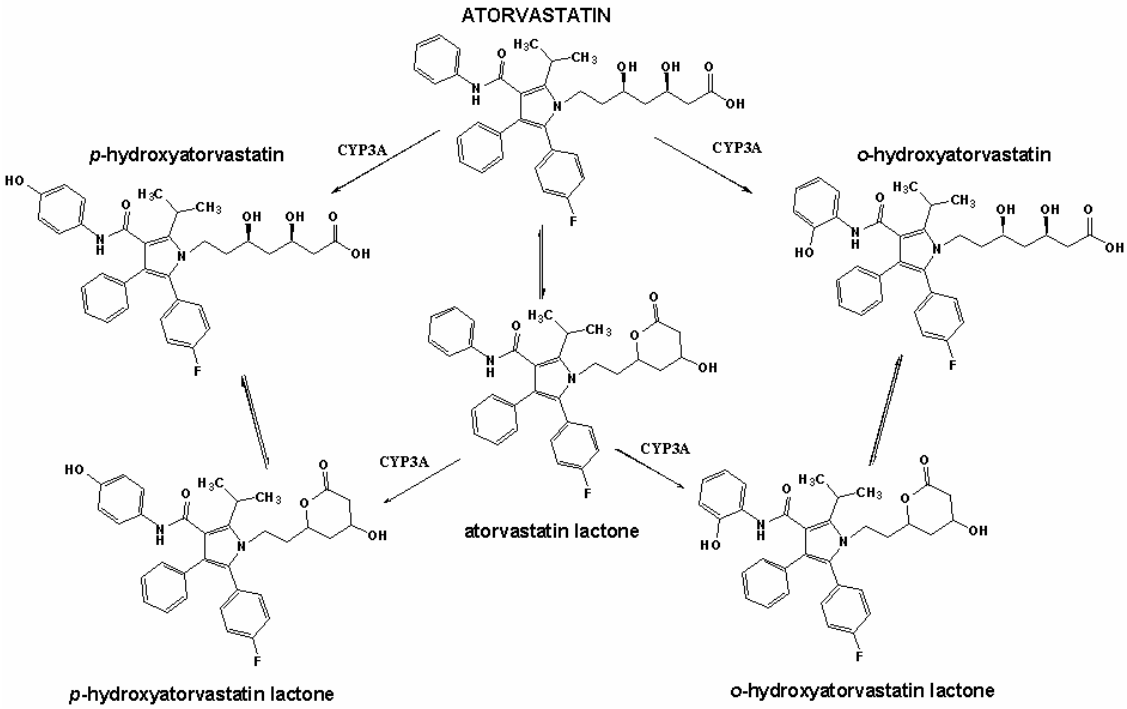

Fig. 1. Metabolism scheme of atorvastatin

The aim of this study was to develop and validate simple and sensitive LC–MS/MS method for quantification of atorvastatin and its metabolites in plasma samples obtained from experimental animals orally treated with atorvastatin for 6 weeks.

Experimental

Reagents and Chemicals

Atorvastatin (99.87%), *o*-hydroxyatorvastatin (99.92%), *p*-hydroxyatorvastatin (99.97%), and atorvastatin lactone (AT-L, 99.89%) were provided by Nobel Ilaç Company (Istanbul, Turkey). Purity of standards was determined by manufacturers by high-performance liquid chromatography (HPLC) method. Unfortunately, we were not able to obtain reference standards of *o*-hydroxyatorvastatin lactone and *p*-hydroxyatorvastatin lactone for method validation, so this method excluded these two metabolites. Deuterated analogs of atorvastatin and its metabolites, which are known to be the most suitable for LC–MS methods, were not available, and rosuvas-

tatin (Medicines and Medical Devices Agency, Belgrade, Serbia) was used as internal standard. HPLC-grade acetonitrile and glacial acetic acid were obtained from J.T.Baker (Deventer, Netherlands). Ammonium acetate used for preparation of buffers was purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by means of a TKA Water purification system (Niederelbert, Germany).

Preparation of Standard Samples

Stock solutions of atorvastatin, *o*-hydroxyatorvastatin, *p*-hydroxyatorvastatin, and rosuvastatin were prepared by separately dissolving 5 mg of each substance in a mixture of acetonitrile and water (10 mL, 90:10, *v*/*v*). Stock solution of atorvastatin lactone was prepared by dissolving 5 mg in acetonitrile (5 mL). These stock solutions were divided into 500-μL portions and stored at −8 °C until further use. For method validation, 500-μL portions of AT, oOH-AT, pOH-AT, and internal standard (IS) stock solutions were thawed and then separately freshly diluted to 50 mL with 100 mM ammonium acetate buffer, pH 4.6 (pH was adjusted with glacial acetic acid). A 500-μL portion of AT-L stock solution was diluted to 50 mL with 100 mM ammonium acetate buffer, pH 4.6. Five milliliters of this solution was further diluted to 10 mL with same buffer. The concentrations of all analytes in prepared solutions were 5 μg mL^{-1}. Method optimization was conducted with solutions prepared by separately diluting 1 mL of 5 μg mL^{-1} solutions with buffer to the concentration of 50 ng mL^{-1}. Calibration standards were prepared by diluting 50 ng mL^{-1} standard solutions to obtain concentrations in range of 0.1–20 ng mL^{-1} for AT and AT-L and 0.5–20 ng mL^{-1} for oOH-AT and pOH-AT. Internal standard working solution was prepared separately by diluting 200 μL of 5 μg mL^{-1} solution to 10 mL with buffer to obtain concentration of 100 ng mL^{-1}. All solutions were kept on ice to minimize interconversion of lactone and acid forms. Heparin drug-free plasma was obtained from six animals. Calibration standards and quality control samples were prepared by spiking drug-free plasma with aliquots of the working standard solution.

Biological Samples

Male Wistar albino rats were used for the experiment. Animals were obtained from the Military Medical Academy Farm (Belgrade, Serbia). Animal blood samples were collected directly from the heart in test tubes containing

heparin and centrifuged 3000 rpm for 15 min. Plasma samples were frozen immediately after sample withdrawal to minimize acid–lactone interconversion and stored at −80 °C. All experimental procedures and protocols conformed to institutional guidelines for the care and use of animals in research no. 5/10 (Ethics Committee of the Faculty of Pharmacy, University of Belgrade).

Instrumentation

Analyses were carried out on an ultra-high performance liquid chromatography (UHPLC)–MS/MS system consisting of a Thermo ACCELA UHPLC (Thermo Scientific, Waltham, MA, USA) coupled to a triple quad Mass Spectrometer Thermo TSQ Quantum Access Max (Thermo Scientific, Waltham, MA, USA) with a heated electrospray ionization (HESI) interface operated in the positive mode. All data were acquired and processed by Xcalibur software (Thermo Fisher, San Jose, CA, USA). EBA 8S (Hettich Zentrifugen, Tuttlingen, Germany) was used in sample preparation. SPE procedure was carried out on Baker spe-12G manifold (J.T.Baker, Deventer, Netherlands). A ZORBAX Eclipse C_{18} Analytical, 4.6 × 100 mm (3.5 μm) column (Agilent, Santa Clara, CA, USA), was used for the chromatographic separation of the compounds. The column was kept at constant temperature at 25 °C, and autosampler tray temperature was set at 4 °C to minimize possible interconversion between acid and lactone forms. The analytes were eluted by a gradient mobile phase system which consisted of acetonitrile (channel A) and 0.1% CH_3COOH (channel B) *(Table I)*. The flow rate was constantly kept on 400 μL min^{-1}. The mass spectrometer was operated in positive ion mode and connected to the chromatographic system using an ESI interface. Tuning the MS detector for optimal operating conditions to get low limit of quantification (LOQ) was achieved through infusion of standard mixture (including internal standard) in 50 ng mL^{-1} concentration through a T-connector. Collision gas was argon. Spray voltage was set on 5500 V; vaporizer temperature at 250 °C; sheath gas pressure, 40 units; ion sweep gas pressure, 0 units; auxiliary gas pressure, 10 units; capillary temperature, 250 °C; capillary offset, 35; tube lens offset, 78; and skimmer offset, −6. The MS detector was operated in the selected reaction monitoring (SRM) mode with the following SRM transitions: (*m/z*, Q1 → Q3, collision energy) of AT (*m/z*, 559.47 → 440.03, 22 eV), AT-L (*m/z*, 541.36 → 448.02, 19 eV), oOH-AT (*m/z*, 575.20 → 440.18, 20 eV), pOH-AT (*m/z*, 575.54 → 440.18, 20 eV), and IS (*m/z*, 482.25 with selected combination of two fragments to improve signal 257.77, 31 eV, and 299.81, 35 eV).

Table I. Gradient mobile phase

t	%A	%B
0	65	35
4	65	35
4.5	80	20
7	80	20
7.05	65	35
8	65	35

A — acetonitrile; B — 0.1% CH_3COOH

Sample Preparation

Samples were prepared by solid-phase extraction and kept on ice at all times. Calibration standards and quality control (QC) samples were prepared by mixing 500 μL of drug-free plasma with 50 μL of internal standard (IS) working solution and adequate amount of 50 ng mL^{-1} working standard solution and diluted with ammonium acetate (0.1 M, pH 4.6) to achieve total volume of 1 mL. Samples were further vortexed and centrifuged at 1600*g* for 5 min. The supernatant was subsequently transferred to 1 mL OASIS® HLB SPE cartridges (Waters, Milford, Massachusetts, USA), preconditioned with 2 mL acetonitrile followed by 2 mL water. The cartridges were washed with 1 mL water and 1 mL acetonitrile–water (5:95, *v*/*v*). The analytes were eluted with 1 mL acetonitrile–ammonium acetate buffer (0.1 M, pH 4.6, 95:5, *v*/*v*). Pure extracts were directly transferred to autosampler vials.

Plasma samples of animals that received atorvastatin were prepared by mixing 500 μL of plasma with 50 μL of internal standard (IS) working solution and diluted with ammonium acetate (0.1 M, pH 4.6) to achieve total volume of 1 mL. Samples were further vortexed, centrifuged, and transferred to SPE cartridges in same manner as calibration standards and quality control (QC) samples.

Method Validation

The method was validated according to the bioanalytical method validation guidelines of the European Medicines Agency [28] and Food and Drug Administration [29]. Validation was performed by analyzing calibration and quality control standards to demonstrate acceptable within run and be-

tween run accuracy and precision over the desired range of concentration. Quantification was based on ratio of peak areas of investigated analytes and peak area of internal standard. A batch included six standard levels spanning the concentration range from 0.1 to 20 ng mL^{-1} for atorvastatin and atorvastatin lactone and 0.5 to 20 ng mL^{-1} for *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin, blank (plasma without analytes and IS) and zero (plasma without analytes, with IS) samples. Quality control samples at four concentration levels (0.1, 0.3, 10, and 15 ng mL^{-1} for AT and AT-L and 0.5, 1, 10, and 15 ng mL^{-1} for oOH-AT and pOH-AT) were used for determination of within-run and between-run accuracy, precision, and recovery.

Selectivity and specificity

The selectivity of the method was determined with six different blank rat plasma samples, which were analyzed to test the potential interferences of endogenous compounds coeluting with analytes and IS. Chromatographic peaks of analytes and IS were identified based on their retention times and SRM responses. The peak area of interferences in blank samples should not be more than 20% of the mean peak area at LOQ levels.

Matrix effect

In order to evaluate possible ion suppression due to the matrix effect, blank rat plasma samples obtained from six different sources were extracted and spiked to the concentration of 5 ng mL^{-1}. These samples were injected with samples containing no matrix components.

Linearity

Calibration curves were obtained by linear regression. Weighted linear regression was previously performed, and best results were achieved with weighting factor of 0, which led to the conclusion that no weighting was necessary. The ratio of analytes peak area to IS peak area was plotted against analytes concentration in nanograms per milliliter. Calibration curve standard samples and quality control samples were prepared in replicates (n = 5) for analysis. The obtained correlation coefficient was higher than 0.9950.

Precision and accuracy

Precision and accuracy should be within ≤15% and ±15% of their nominal values. For lower limit of quantification (LLOQ), the precision and accuracy should be within ≤20% and ±20%.

Stability

Stability testing was evaluated with low (LQC) and high (HQC) level concentration of quality control sample (LQC; 0.3 ng mL^{-1} for AT and AT-L and 1 ng mL^{-1} for *o*OH-AT and *p*OH-AT; HQC: 15 ng mL^{-1} for all compounds). Autosampler stability was determined by keeping samples in autosampler at 4 °C for 24 h. Bench-top stability was evaluated by keeping LQC and HQC at the same conditions as samples from experimental animals for 8 h. Long-term stability was checked after keeping QC samples at −80 °C for 1 month. Freeze and thaw stability was tested in freshly spiked HQC and LQC samples that were stored at −80 °C for 12 h and then were thawed on ice bath.

Extraction efficiency

Determination of the extraction efficiency was performed by comparing the response of extracted samples spiked before extraction and the response of extracted blank samples spiked before injection to LC–MS. A recovery of more than 50% was considered adequate.

LLOQ and LOD

LLOQ was determined at a signal-to-noise (S/N) ratio of 10:1. LOD was determined using S/N ratio of 3:1 by comparing test results from samples with known concentrations of analyte with those from the blank samples.

Results and Discussion

Optimization of Solid-Phase Extraction (SPE) Procedure

Preparation of plasma samples is the most important step in method development. Liquid–liquid extraction is time consuming, and protein precipitation leads to significant ion suppression in mass spectrometry. Solid-phase

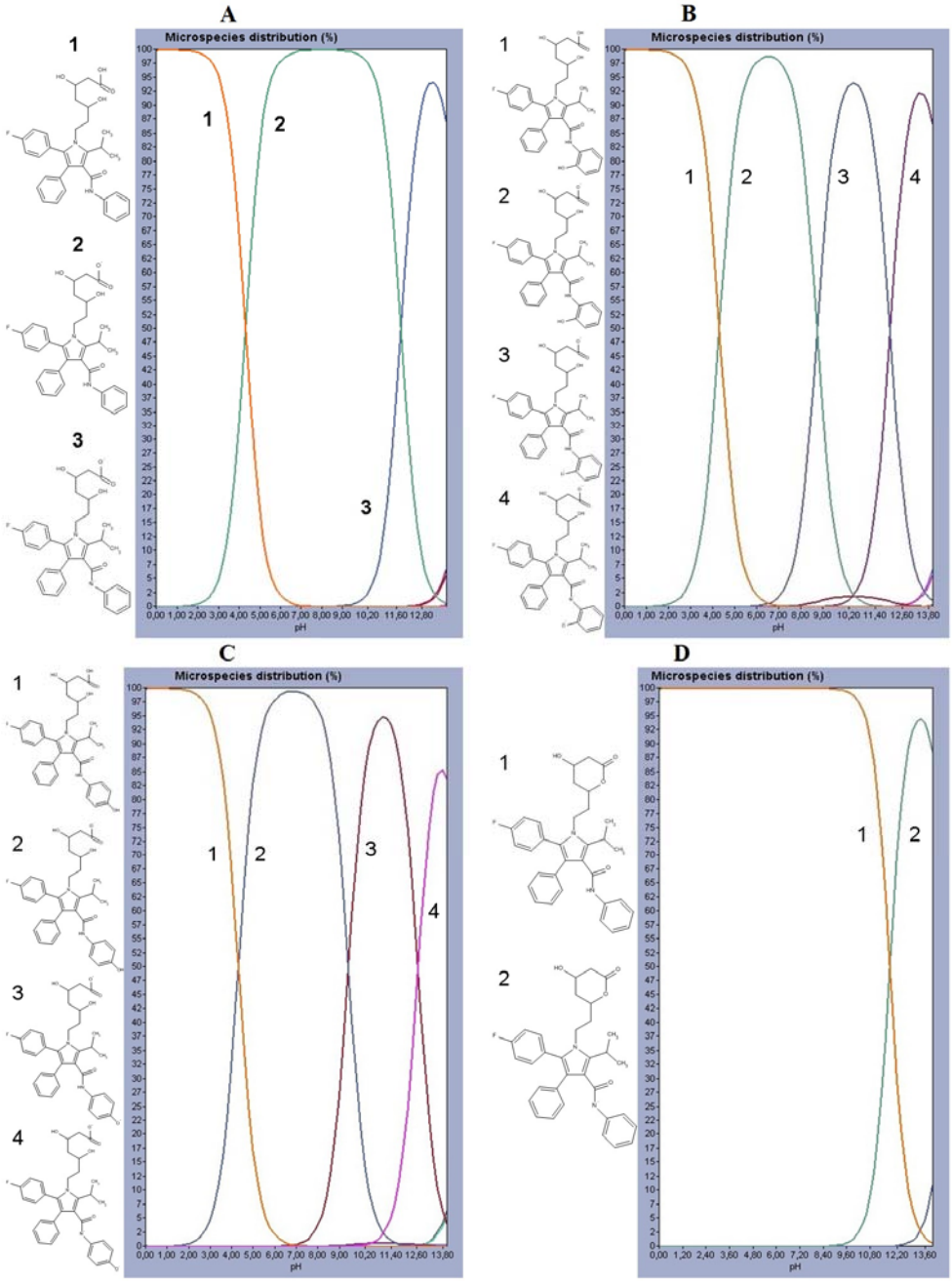

Fig. 2. Ionization profiles of A — atorvastatin, B — *ortho*-hydroxyatorvastatin, C — *para*-hydroxyatorvastatin, and D — atorvastatin lactone; pH range: 1–12

extraction is a simple and fast method which produces clean samples with no significant matrix effect. Plasma samples were mixed with ammonium acetate buffer, pH 4.6 (0.1 M) in order to minimize possible interconversion between acid and lactone forms [30]. Carboxylic group in structure of AT, oOH-AT, and pOH-AT has pKa value of 4.33. At chosen pH, AT, oOH-AT, and pOH-AT are 65% in ionized and 35% in unionized form, and AT-L is 100% in unionized form (does not have acidic group in structure). Distribution of ionized and unionized forms of analyzed compounds at pH range 1–12 is shown in *Fig. 2*.

OASIS® HLB cartridges were chosen since they are acceptable for both hydrophilic and lipophilic compounds. Comparing to mostly used C_{18} cartridges, these cartridges have advantage — they are simpler for use and they can dry out during extraction procedure. First wash step was carried out with water since analytes of interest are not soluble in water. Second wash step with 5% acetonitrile gave cleaner samples and better recoveries than washing with same percentage of methanol. Washing cartridges with higher percentage of acetonitrile would give lower recoveries since all analytes are soluble in this solvent. The procedure of extraction analytes from cartridges was examined with a different mixture: acetonitrile and buffer in different ratios and mixture of methanol and buffer, but mixture of acetonitrile and ammonium acetate buffer, pH 4.6 (0.1 M; 95:5, v/v), gave the highest recoveries.

Elution was completely performed with 1 mL of mixture. Extracts were filtered and transferred to LC–MS vial without further concentration steps.

LC–MS/MS Determination

LC–MS/MS conditions were set in order to get the best chromatographic separations and the best possible sensitivity of the method. Good chromatographic separation was important since pOH-AT and oOH-AT have the same precursor → ion-product transitions and because of possible acid–lactone interconversion. There are some articles describing isocratic separation of some of the investigated compounds, but they do not include investigation of lactone [27], run is 20 min [21], or precipitation of proteins was used instead of SPE [26].

Isocratic elution with mobile phase (0.1% CH_3COOH–acetonitrile; 35:65, v/v) was tested. Good separation was achieved, but run lasted considerably longer. In order to shorten run time and keep good separation, gradient mobile phase was used. The total time of analysis in the chromatographic system was 8 min, including the time for reequilibration. The re-

tention times of investigated compounds were: 2.60 (pOH-AT), 3.88 (oOH-AT), 4.48 (AT), 6.15 (AT-L), and 3.05 for rosuvastatin. Chromatogram at LLOQ levels is shown in *Fig. 3*, and chromatogram of a plasma sample taken from rats after 6 weeks of daily atorvastatin intake is shown in *Fig. 4*.

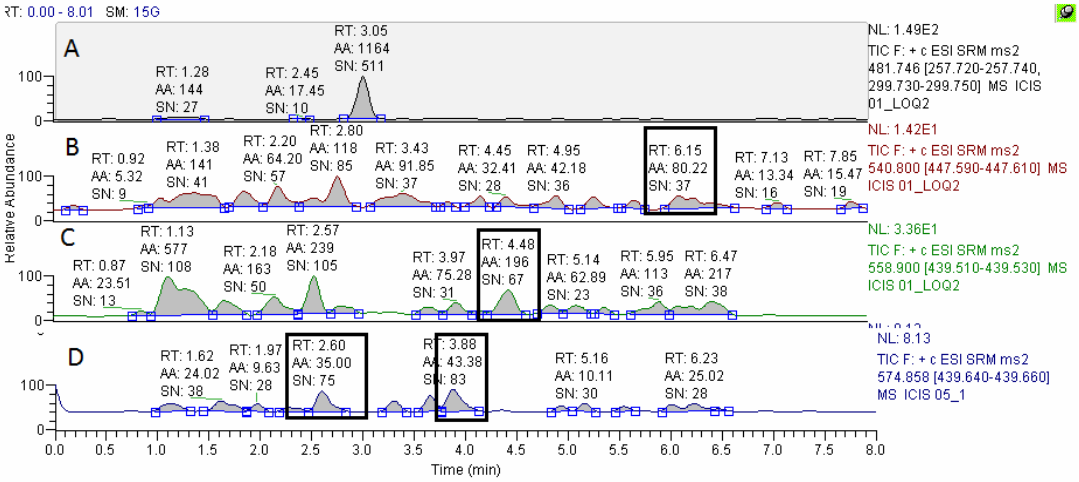

Fig. 3. Chromatogram at LOQ level of: A — internal standard (rosuvastatin), B — atorvastatin lactone, C — atorvastatin, and D — *para*-hydroxyatorvastatin and *ortho*-hydroxyatorvastatin. Peaks of analytes of interest are marked

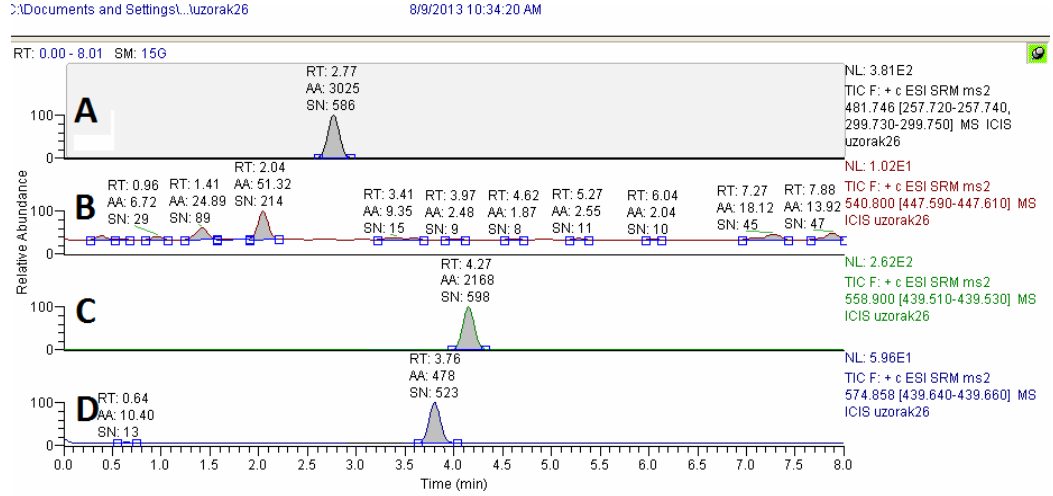

Fig. 4. Chromatogram of a plasma sample taken from rats after 6 weeks of daily atorvastatin intake (A — internal standard (rosuvastatin), B — atorvastatin lactone, C — atorvastatin, and D — *para*-hydroxyatorvastatin and *ortho*-hydroxyatorvastatin)

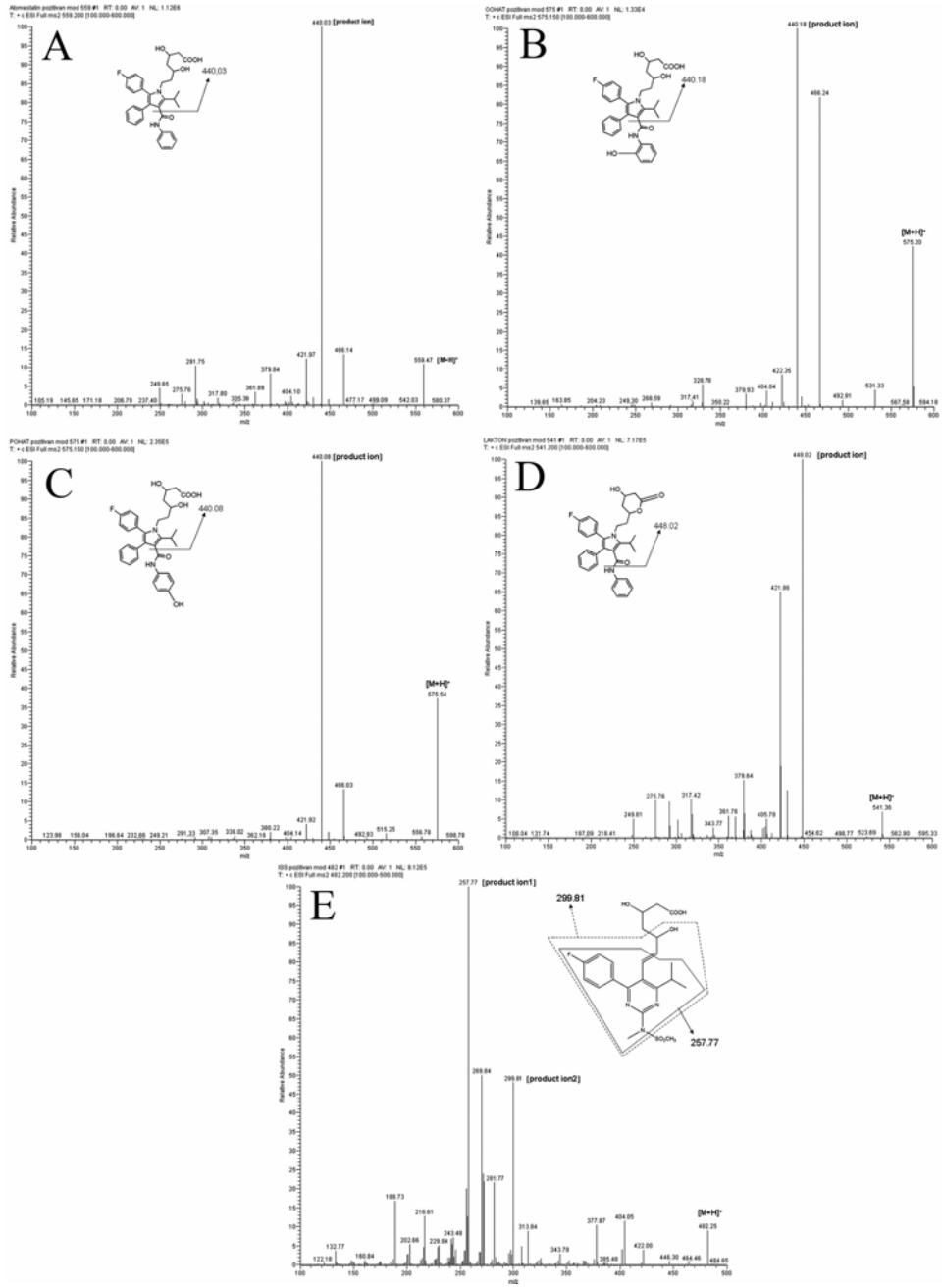

Fig. 5. Fragmentation pattern of: A — atorvastatin, B — *ortho*-hydroxyatorvastatin, C — *para*-hydroxyatorvastatin, D — atorvastatin lactone, and E — rosuvastatin (IS) (chosen fragments are marked)

The MS/MS was operated using electrospray ionization probe in positive ion mode. Protonated molecular ion ($[M+H]^+$) was the major parent ion used to obtain the product ion spectra for all compounds. MS source parameters were optimized based on pOH-AT since its concentration in plasma is lower than other analytes. All parameters were optimized to achieve maximum signal. Fragmentation pattern of all tested compounds is shown in *Fig. 5*.

Method Validation

Validated method showed linearity within the concentration range of 0.5–20 ng mL^{-1} for *ortho*-hydroxyatorvastatin and *para*-hydroxyatorvastatin and 0.1–20 ng mL^{-1} for atorvastatin and atorvastatin lactone ($r^2 \geq 0.99$, $n = 6$ for oOH-AT and pOH-AT and $n = 7$ for AT and AT-L). Calibration curve data are shown in *Table II*. Student *t*-test confirmed that the intercepts of the calibration curves were not statistically different from zero for any of the analytes. Determined LOD was 0.1 and 0.13 ng mL^{-1} for oOH-AT and pOH-AT, respectively, and 0.05 ng mL^{-1} for AT and AT-L. Lowest concentration on calibration curve was LLOQ. Extraction efficiencies were between 57% and 75% for all analytes, which is acceptable for bioanalytical methods.

Table II. Parameters of calibration curve for atorvastatin, atorvastatin lactone, *para*-hydroxyatorvastatin, and *ortho*-hydroxyatorvastatin in rat plasma ($n = 5$)

Analyte	Slope	Intercept	Coefficient of correlation (r)
Atorvastatin	1.9478	−0.0670	0.9982
Atorvastatin lactone	1.6274	−0.1257	0.9993
para-Hydroxyatorvastatin	0.2380	−0.1998	0.9963
ortho-Hydroxyatorvastatin	0.3545	−0.2443	0.9978

Within-run and between-run precision was presented as %CV and was lower than 15% for all analyzed samples. At some points, values were very close to 15, but that was acceptable because of very labile nature of analytes and easily undergoing to interconversion. Within-run and between-run accuracy was calculated as percentage of nominal concentration of controlled samples. Results were within acceptance criteria (±15% for all QC except for

Table III. Precision and accuracy of the LC–MS method for atorvastatin, atorvastatin lactone, *para*-hydroxyatorvastatin, and *ortho*-hydroxyatorvastatin in rat plasma (n = 5)

Analyte	Nominal concentration (ng mL^{-1})	Within run (n = 5)			Between run (n = 3)		
		Found concentration (mean ± SD, ng mL^{-1})	Precision (%CV)	Accuracy (%)	Found concentration (mean ± SD; ng mL^{-1})	Precision (%CV)	Accuracy (%)
Atorvastatin	0.1	0.09 ± 0.01	14.50	90.58	0.10 ± 0.01	11.49	104.30
	0.3	0.27 ± 0.02	9.69	89.27	0.27 ± 0.03	14.56	89.66
	10	11.46 ± 1.86	14.34	114.63	11.35 ± 1.35	12.83	113.46
	15	16.57 ± 1.32	7.56	110.45	16.20 ± 2.01	9.57	108.01
Atorvastatin lactone	0.1	0.12 ± 0.01	11.25	117.99	0.11 ± 0.01	11.13	111.66
	0.3	0.28 ± 0.04	14.29	93.31	0.27 ± 0.03	11.11	90.79
	10	11.58 ± 1.08	9.33	114.77	11.46 ± 1.37	9.89	114.59
	15	16.77 ± 0.08	0.48	111.78	16.35 ± 1.42	8.68	109.00
para-Hydroxyatorvastatin	0.5	0.58 ± 0.01	1.19	116.00	0.59 ± 0.05	8.47	117.00
	1	1.06 ± 0.10	8.75	105.62	1.06 ± 0.09	8.49	106.20
	10	9.57 ± 0.01	0.12	95.71	9.62 ± 0.87	9.04	96.19
	15	14.67 ± 0.46	3.71	97.79	15.33 ± 1.43	9.33	102.20
ortho-Hydroxyatorvastatin	0.5	0.57 ± 0.01	1.03	114.01	0.57 ± 0.01	1.75	115.11
	1	0.87 ± 0.10	11.47	86.56	0.92 ± 0.03	3.26	91.83
	10	8.75 ± 0.88	10.02	87.52	11.23 ± 1.34	11.93	112.32
	15	15.65 ± 2.55	14.88	104.36	15.28 ± 1.73	11.32	101.86

LLOQ, where ±20% is allowed). All accuracy and precision results are shown in *Table III*.

The specificity of the method was tested using drug-free plasma from six animals. No interfering peaks and no matrix effect were found for any of the analytes. Chromatograms of blanco (without analytes and IS) and zero (without analytes but with IS) are shown in *Fig. 6*.

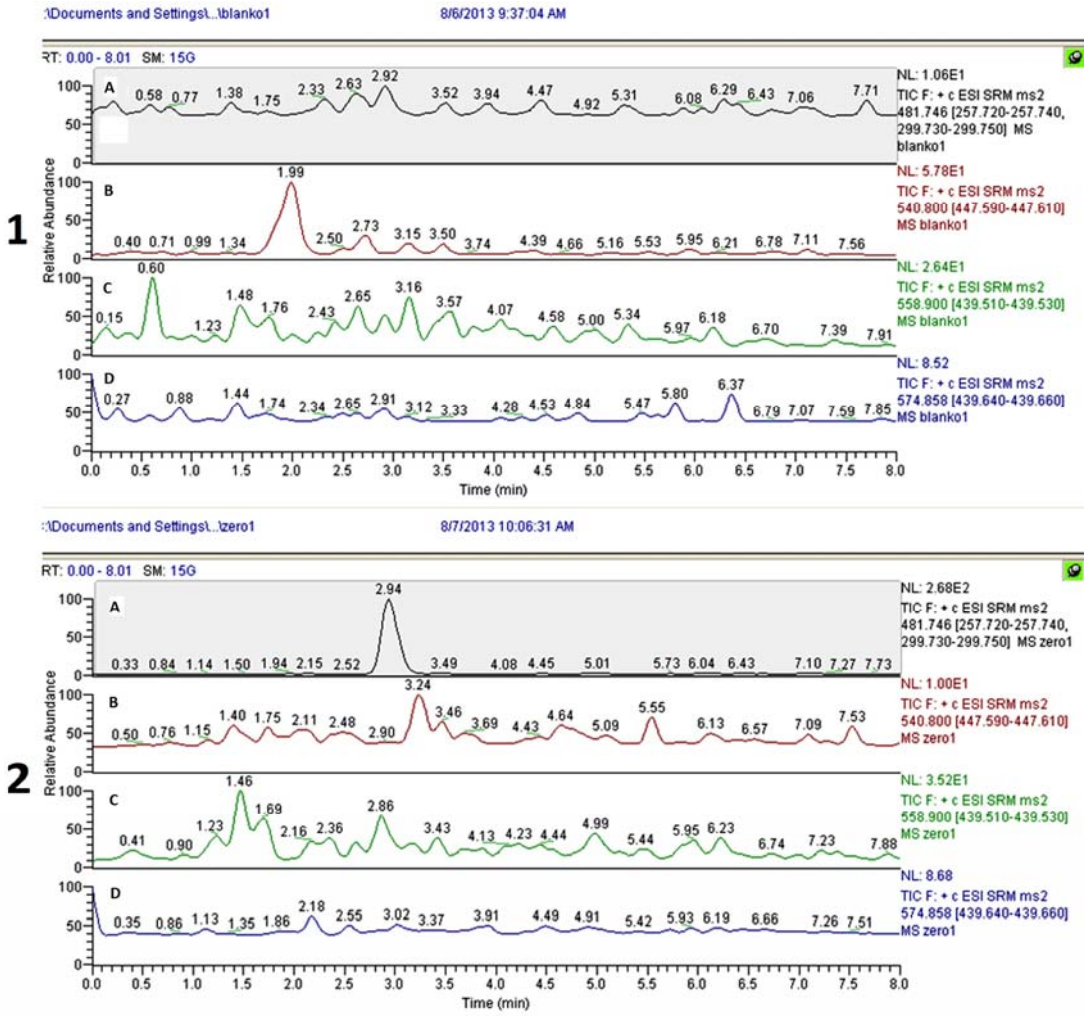

Fig. 6. Chromatograms of: 1 — blanco (without analytes and IS) sample and 2 — zero (without analytes but with IS) sample (A — internal standard [rosuvastatin], B — atorvastatin lactone, C — atorvastatin, and D — *para*-hydroxyatorvastatin and *ortho*-hydroxyatorvastatin)

Stability of analytes was tested through three different levels: short-term stability, freeze–thaw stability, and long-term stability. The obtained results were between 92% and 103% for all tested conditions.

Method Application

This method was successfully applied to determine concentrations of AT, AT-L, oOH-AT, and pOH-AT in rat plasma after administration of atorvastatin for 6 weeks. The aim of this animal study was monitoring changes in plasma concentration of atorvastatin and its active and inactive metabolites as a result of its interaction with other drugs and natural products used for treatment of hyperlipidemia. Results obtained from plasma samples of experimental animals treated with atorvastatin and combination of atorvastatin and artichoke leaf extract (whose effects were investigated in this study) are shown in *Table IV*. Plasma samples were spiked with internal standard and extracted as described in experimental procedure. *Figure 4* shows one of the chromatograms from experimental animal treated with atorvastatin.

Table IV. Concentrations of atorvastatin and its metabolites in plasma samples of rats treated with atorvastatin and combination of atorvastatin and artichoke leaf extract

Analyte	Treatment		*p*
	AT	AT + artichoke leaf extract	
AT (ng mL^{-1})	0.5052 ± 0.3585	0.1455 ± 0.0716	<0.05
oOH-AT (ng mL^{-1})	0.2363 ± 0.3542	0.1025 ± 0.0168	0.377
pOH-AT (ng mL^{-1})	1.0049 ± 0.1182	0.8441 ± 0.0709	<0.05
AT-L (ng mL^{-1})	0.8437 ± 0.0253	0.9527 ± 0.1034	<0.05

Conclusions

The aim of this study was to establish LC–MS/MS method for monitoring changes in plasma concentration of atorvastatin and its active and inactive metabolites.

Method was validated according to bioanalytical method validation guidelines. Comparing to previously published method, the advantage of this method is determination of inactive lactone as well as active hydroxy metabolites, using SPE procedure instead of protein precipitation and short

run time. The described method is sensitive, simple, and reliable and was successfully used for further evaluation of the interaction of atorvastatin with artichoke leaf extract, which is often used for treatment of hyperlipidemia.

Acknowledgments

This work was financially supported by a grant from the Ministry of Science and Environmental Protection, Republic of Serbia (Project number OI 172041).

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