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Ultra high performance liquid chromatography tandem mass spectrometric detection in clinical analysis of simvastatin and atorvastatin

Lucie Nováková[a,*], Hana Vlčková[a], Dalibor Šatínský[a], Petr Sadílek[a], Dagmar Solichová[b], Milan Bláha[c], Vladimír Bláha[c], Petr Solich[a]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Metabolic Care and Gerontology, Charles University, Faculty of Medicine and University Hospital in Hradec Králové, Sokolská 581, 500 05 Hradec Králové, Czech Republic
[c] Department of Haematology, Charles University, Faculty of Medicine and University Hospital in Hradec Králové, Sokolská 581, 500 05 Hradec Králové, Czech Republic

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ABSTRACT

Simvastatin and atorvastatin belong to the group of hypolipidemic drugs, more exactly to the second generation of inhibitors of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. They induce a significant reduction in total cholesterol, low-density lipoprotein cholesterol and plasma triglycerides, therefore they are widely used in the treatment of hypercholesterolemia even of its severe form-familiar hypercholesterolemia. Simvastatin and atorvastatin as the most widely used statins in clinical treatment and their hydroxy-acid/lactone forms were determined by means of UPLC in connection with triple quadrupole mass spectrometer. Deuterium labeled reference standard compounds were used as internal standards for the quantitation. Separation was performed on Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm) using gradient elution by mobile phase containing acetonitrile and ammonium acetate pH 4.0, which is convenient in order to prevent interconversion of analytes. ESI in positive mode was used for the ionization of all compounds. Two SRM (selected reaction monitoring) transitions were carefully optimized for each analyte in order to get high sensitivity and selectivity. SPE on Discovery DSC-18 was used as a sample preparation step. Intra-day precision was generally within 10% RSD, while inter-day precision within 15% RSD. Method accuracy expressed as recovery ranged from 75 to 100%. The method was validated with the sensitivity reaching LOQ 0.08–5.46 nmol/l and LOD 0.01–1.80 nmol/l in biological samples. Atorvastatin, simvastatin, its metabolites and hydroxy-acid/lactone forms were monitored in human serum and in lipoprotein fractions (LDL, HDL and VLDL) at patients with end stage renal diseases.

1. Introduction

Statins are drugs widely used for the treatment of severe forms of hypercholesterolemia, such as familiar hypercholesterolemia. They have potent cholesterol-lowering effect and they could significantly reduce morbidity and mortality associated with coronary heart disease as it was proven by many clinical trials [1–4]. They possess high effectiveness in reducing total cholesterol and low-density lipoprotein (LDL) cholesterol levels in human body. HMG-CoA reductase is the key enzyme that catalyzes the conversion of HMG-CoA to mevalonate, which is an early rate-limiting step in the cholesterol biosynthetic pathway. Statins are effective HMG-CoA inhibitors, however some of statins exhibit a number of adverse effects, such as myopathy or rhabdomyolysis [1]. Therapeutic range of statins is typically 10–80 mg/day. Maximum plasma concentration (c_{max}) has been reported to be 27–66 ng/ml for atorvastatin and 10–34 ng/ml for simvastatin [5,6]. High doses could be used with caution in the elderly, in patients with renal or hepatic insufficiency, hypothyroidism or diabetes. Therapeutic drug monitoring is not routinely done in patients treated by statins. They are only advised to report to their doctors if muscle aches, pains or weakness develop. Therefore it would be highly convenient and helpful to monitor the levels of statins in biological materials in order to establish and control appropriate dosage scheme, which would minimize adverse effects and keep the cholesterol lowering effect. Moreover, the method is useful when some extracorporeal elimination procedure (e.g. hemodialysis) is used in order to determine if loses of statins do not occur during the procedure.

Patients with chronic renal disease often suffer from a secondary form of complex dyslipidaemia [7]. The most important abnormalities in the lipid profile are an increase in triglyceride levels, the presence of small, dense LDL particles and low high-density

* Corresponding author. Tel.: +420 495067345; fax: +420 495067164.
E-mail address: nol@email.cz (L. Nováková).

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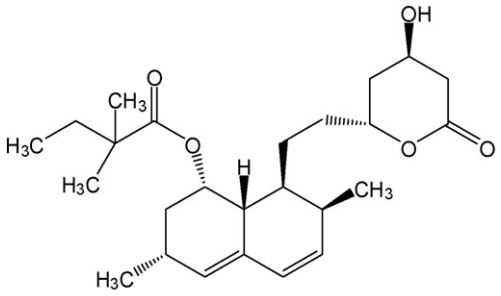
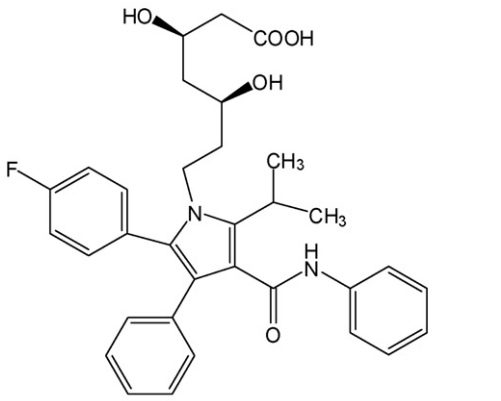


Simvastatin (lactone form)



Atorvastatin (acid form)

Fig. 1. Chemical structures of simvastatin and atorvastatin.

lipoprotein (HDL) cholesterol levels. The increase in triglyceride levels is due to elevated levels of very-low-density lipoprotein (VLDL) remnants and intermediate-density lipoprotein (IDL). Each of these parameters has been associated with increased risk of cardiovascular disease [7].

Atorvastatin and simvastatin are two drugs worldwide the most commonly occurred in commercially available pharmaceutical formulations used in the clinical treatment of hypercholesterolemia. Structures could be seen in Fig. 1. Because of the complex and difficult-to-treat dyslipidaemia in dialysis patients, higher doses of statin might be of value in the treatment of hypercholesterolaemic patients on hemodialysis. Hemodialysis is not expected to enhance significantly the clearance of statin, since the drug is extensively bound to plasma proteins (atorvastatin 80–90%, simvastatin 94–98%). However, it is known that renal dysfunction may hamper the hepatic metabolism of drugs [8,9], which could lead to accumulation of statin and/or its long-lived metabolites, in turn increasing the risk of clinically important adverse events such as rhabdomyolysis. Moreover, as both atorvastatin and simvastatin are lipophilic agents, it can be assumed that not only the changes in liver lipoprotein metabolism, but also the distribution of statin in already abnormally modified lipoprotein fractions can be clinically important. This warrants the performance of supplemental studies on the plasma statin levels and its distribution in lipoprotein fractions, and this is the reason why the study of the presence of atorvastatin in different lipoprotein fractions would be of high significance.

Statin molecules exist in two forms, lactone and open-ring hydroxy acid form [10,11]. In vivo, the hydroxy acid forms are the active drugs to lower plasma cholesterol while the lactone forms are inactive (prodrug). Lactone form of statin can be absorbed from the gastrointestinal tract and transformed to the active drugs in liver and non-hepatic tissues [11]. Simvastatin is a prodrug, which is administered as an inactive lactone form. The lactone is absorbed from gastrointestinal tract and hydrolyzed to the active β-hydroxy acid form in the liver [12,13].

Atorvastatin is administered in the open-ring hydroxy acid form—the active form. It is absorbed from the gastrointestinal tract and it undergoes extensive first-pass metabolism in the liver. Liver metabolism produces two active hydroxy metabolites being ortho-hydroxyatorvastatin and para-hydroxyatorvastatin and three inactive corresponding lactone forms. More than 90% 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 [12–14]. As it figures out from the information above, the levels of statins in biological fluids are very low, probably because only about 5% of dosed statin reaches the systemic circulation. Typical plasma concentrations are in ng/ml levels. The active metabolites of atorvastatin are present at plasma concentration corresponding to pg/ml levels [13], typical concentration range being between 0.1 and 20 ng/ml.

Statins are a typical example of drugs, where the interconversion between lactone and open-ring hydroxy acid occurs [10,11]. When the development of a method for the quantitation of two analytes that can undergo interconversion is performed—the first step is to select the conditions that will eliminate or minimize the interconversion. The second step is to judiciously select the composition of the QC samples and the composition of calibration standards, which should cover the spectrum of the composition of real samples. For the samples of hydroxy acid chemical structure and the corresponding lactone forms it is important to maintain pH between 4 and 5 in order to minimize interconversion. Increasing the pH above 6 facilitates the conversion of the lactone to the acid (in the ionized form), contrariwise, lowering pH facilitates the conversion of the acid to lactone form or the lactone to the acid (in the non-ionized form). The most of assays utilizes pH around 4.5 [10–15].

As it figures out from the different structures of simvastatin and atorvastatin, analytical methods for their quantitative determination were developed individually. Because of the structure properties, there are not many analytical methods which determine these two compounds together in one analytical run or even in combination with other statin molecules. This is also probably due to the fact, that statins are not used with other statins simultaneously during the treatment of hyperlipidemic patients. The methods for the determination of simvastatin and atorvastatin were recently reviewed by our group [15]. In clinical applications HPLC-MS/MS was unequivocally the method of choice in analysis of both simvastatin [16–21] and atorvastatin together with its metabolites [22–25] using typically ESI (electrospray ionization) in positive ion mode.

The aim of this work was to develop fast, reliable, sensitive and selective analytical method for the determination of simvastatin and atorvastatin together with metabolites and lactone/hydroxy-acid interconversion forms using UPLC-MS/MS method. In spite of the fact, that statins are not use simultaneously during the treatment of hypercholesterolemia, such a procedure is useful in daily routine sample handling, when many samples from patients taking either atorvastatin or simvastatin are analyzed in one laboratory. Thus the laboratory does not need to distinguish among samples to be analyzed, to perform two different procedures for individual statins, which increases the sample throughput of the laboratory.

2. Experimental

2.1. Chemicals and reagents

Working standards of simvastatin were obtained from Sigma–Aldrich (Prague, Czech Republic). Working standards of simvastatin acid, atorvastatin lactone and atorvastatin, p-hydroxyatorvastatin, o-hydroxyatorvastatin, simvastatin deuterium labeled (D6-methyl

Table 1
Optimization of specific transitions for all analytes.

	Compound	Precursor	Precursor type	Fragment	Dwell time	Cone voltage	Collision energy	t_R
1	p-Hydroxyatorvastatin	575.0	$[M+H]^+$	440.1 466.2	0.05	30 30	20.0 15.0	2.49
1	o-Hydroxyatorvastatin	575.0	$[M+H]^+$	440.1 466.2	0.05	30 30	20.0 15.0	2.89
2	Atorvastatin	559.0	$[M+H]^+$	440.1 466.1	0.05	30 30	20.0 15.0	2.89
3	Atorvastatin-deuterium labeled	564.0	$[M+H]^+$	445.1 471.0	0.05	30 30	20.0 15.0	2.89
4	Atorvastatin lactone	541.0	$[M+H]^+$	448.0 422.1	0.05	30 30	15.0 20.0	3.19
5	Simvastatin acid	437.0	$[M+H]^+$	303.00 285.3	0.05	15 15	10.0 15.0	3.48
6	Simvastatin	419.0	$[M+H]^+$	199.2 285.3	0.05	20 20	10.0 10.0	4.39
7	Simvastatin-deuterium labeled	425.1	$[M+H]^+$	199.2 285.3	0.05	20 20	10.0 10.0	4.40

groups) and atorvastatin deuterium labeled (D5-phenyl ring) were purchased from Toronto Research Chemicals (Ontario, Canada).

The acetic acid, reagent grade, the ammonium, reagent grade, the formic acid, reagent grade and the acetonitrile, LC-MS grade, were purchased from Sigma–Aldrich. HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it meets European Pharmacopoeia requirements.

2.2. Chromatography

UPLC System Acquity (Waters, Prague, Czech Republic) was used for the purposes of this study. It consists of ACQ-binary solvent manager, ACQ-sample manager and ACQ-tunable UV detector. All UPLC analyses were performed on BEH C_{18} analytical column (100 mm × 2.1 mm, 1.7 μm, Waters, Prague, Czech Republic) based on Bridged Ethyl Hybrid (BEH) particles. Mobile phase was composed of acetonitrile and 0.5 mM ammonium acetate buffer pH 4.0 using gradient elution, initial mobile phase composition being acetonitrile, ammonium acetate buffer (30:70). Thereafter the concentration was changed within 1.5 min to 30% of ammonium acetate buffer and subsequently to 5% of the buffer within 5.25 min. Flow rate was 0.25 ml/min. The analytical column was kept at 35 °C by column oven. The solutions were stored in the autosampler at 4 °C. The full loop injection mode was set up to inject 5 μl using 5 μl injection loop. Acetonitrile was used as a strong wash and 20% acetonitrile in water was used as a weak wash solvent.

2.3. Mass spectrometry

The MS/MS triple quadrupole system was used for the purposes of this study. Quattro Micro (Micromass, Manchester, GB) was equipped with a Multi-Mode Ionization Source (ESCI), which combines high-speed switching between electrospray ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) within one ion source.

Ion source set-up was carefully tuned as follows: capillary voltage: 3500 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 1.0 V. The desolvation gas was nitrogen at flow 500 l/h and at the temperature 375 °C. Cone voltage was set up individually for each analyte (Table 1). Nitrogen was used also as a cone gas (120 l/h) to prevent the contamination of sample cone. Quantitation of all analytes was performed in ESI positive ion mode using SRM (selected reaction monitoring) experiment. Two specific transitions were optimized for each molecule to increase selectivity of the method. Argon was used as collision gas and collision energy was optimized for each analyte individually (Table 1).

The MassLynx 4.1 Data System was used for data MS control and data gathering. QuanLynx software was used for data processing and quantitation–regression analysis of standard curves and calculation of concentrations.

2.4. Preparation of standard solutions

The stock solutions of standards were prepared by dissolving of the amount corresponding to 1.0 mmol/l concentration of appropriate working standard into 1.0 ml of solution media according to the solubility properties, because the molecules differ significantly in solubility. The stock solutions of simvastatin, simvastatin D6 and atorvastatin lactone were prepared in pure acetonitrile. The stock solutions of atorvastatin, atorvastatin D5, atorvastatin hydroxy-metabolites and simvastatin acid were prepared in mobile phase used at initial step of gradient elution—acetonitrile, ammonium acetate buffer 0.5 mM, pH 4.0 (30:70).

Stock solutions were further diluted by mobile phase (from stability reason to keep pH of solution between 4.0 and 5.0 to prevent interconversion) to achieve a concentration 10 nmol/l for SST (System suitability test) measurements, and to get individual points of calibration curve in the range 0.1–100 nmol/l, using seven calibration points (100, 50, 10, 5.0, 1.0, 0.5 and 0.1 nmol/l).

2.5. Sample preparation

Serum and lipoprotein fraction samples were prepared using SPE (solid phase extraction) procedure. These following sorbents were tested: ZORBAX SPE C-18 (100 mg, 1 ml) (Agilent Technologies), Oasis HLB (hydrophilic–lipophilic balance) SPE (60 mg, 3 ml) (Waters), and Discovery DSC-18 (100 mg, 1 ml) (Supelco). SPE columns Discovery DSC-18 were chosen as optimal for final validation of the method.

I.S. (100 μl) was added to 900 μl of the serum samples containing the analytes. This sample was diluted with 1 ml of ammonium acetate buffer and mixed. The mixture was loaded on Discovery DSC-18 sorbent previously activated with 1 ml of acetonitrile and conditioned with 1 ml of 0.1 M ammonium acetate buffer pH 4.5.

The SPE cartridge with loaded sample was washed with 1 ml of mixture acetonitrile:0.01 M ammonium acetate buffer pH 4.5 (15:85, v/v), and subsequently the analytes were eluted with 1 ml of acetonitrile:0.1 M ammonium acetate buffer pH 4.5 (95:5, v/v). The eluate was filtered via 0.20 μm PTFE filter and sample was injected onto HPLC system.

2.6. System suitability test and validation

An important part of method validation is the SST, details of which are usually given in Pharmacopoeias [26,27]. The SST was performed under optimized chromatographic conditions. In mass spectrometric methods only repeatability of retention times and peak area is checked.

Calibration curves of all analytes in the concentration range 0.1–100 nmol/l were measured. Method precision and accuracy were established. For the precision, spiked blank serum at three different concentration levels were measured in three replicates to calculate RSD, which describes the closeness of agreement between series of measurements. Accuracy was determined as a method recovery using spiked blank serum, again at three different levels in three replicates to establish the closeness of agreement between the true and measured value as it corresponds to ICH (International Conference on Harmonization) requirements [28]. QC samples were prepared at the same concentrations as were the concentration levels prepared for precision and accuracy experiments. Lyophilized standard serum samples were used for the purposes of method validation.

Selectivity and matrix effects were also verified. For the determination of selectivity the injection of blank serum treated by the same sample preparation step was used. Matrix effects were established using direct inlet by Hamilton syringe, where standard mix solution was introduced to the mass spectrometer by direct infusion and the blank serum was injected by the autosampler to observe matrix suppressions or enhancements as positive or negative peaks influencing data plot of analytes.

Limit of detection and quantitation was established based on signal-to-noise (S/N) ratio approach. Limit of detection was expressed as S/N = 3, limit of quantitation was expressed as S/N = 10.

2.7. Patients

Plasma levels of statins have been already analyzed in healthy individuals [5]. However the presence of disease or concomitant therapy are important variables modifying the plasma statin levels [6,29]. Because the aim of this work was to study potential benefit from lipid-lowering treatment by statins in a group of high-risk patients on chronic hemodialysis, and because data on statin levels in hemodialysis patients are incomplete, following patients were randomly selected.

Ten end stage renal disease (ESRD) patients (8 females, 2 males, median age 68 years (range 55–83 years)) on chronic hemodialysis (median duration 30 months, range 4–63 months) were randomly selected. All the patients were recruited at the hemodialysis center in Hradec Králové, Czech Republic. Hemodialysis was performed for three times a week, using bicarbonate buffer and polysulfone dialysis membranes. Dialysis adequacy was estimated by Kt/V according to Daugirdas formula [30]. All patients were on a stable anticoagulation regimen using heparin. None of the patients showed clinical evidence of any acute disease, had malignancies, took corticosteroids, or immunosuppressive therapy at the beginning of the study. All of the patients were informed and Local Ethics Committee of our hospital approved the study.

The following concomitant drugs were not permitted during this study: (i) other lipid-lowering drugs or preparations (acipimox, niacin, fibrates, bile sequestrants, other statins, soluble fibre preparations like psyllium and Metamucil); (ii) other drugs known to modulate lipid parameters (corticosteroids, isotretinoin); (iii) antioxidant vitamins; (iv) immunosuppressive drugs; (v) drugs known to be associated with myopathy in combination with HMG-CoA reductase inhibitors, due to competition for metabolic pathways (cyclosporin, macrolide antibiotics, azole antifungals). Permitted medications, e.g. antihypertensive drugs and phosphate-binding drugs, were to be kept constant throughout the study, both in dosage and time of intake. The occasional use of antacids was permitted. Any concurrent medications were to be taken at least 30 min after the study medication. Patients were asked not to change their eating habits during the course of the study.

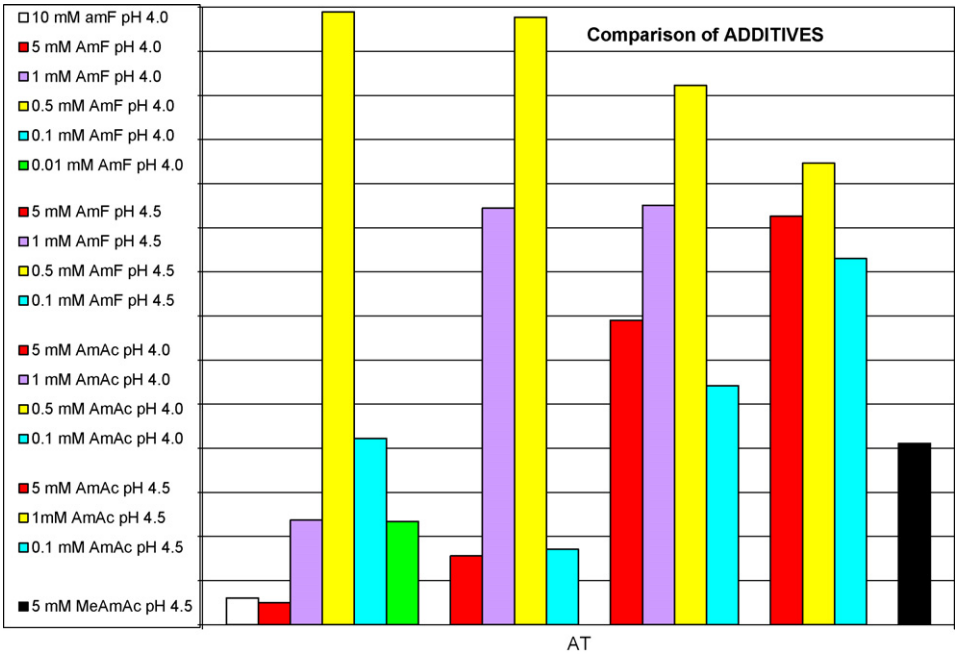


Fig. 2. Optimization of mobile phase additives—the influence of ammonium formate and ammonium acetate at various pH and concentrations.

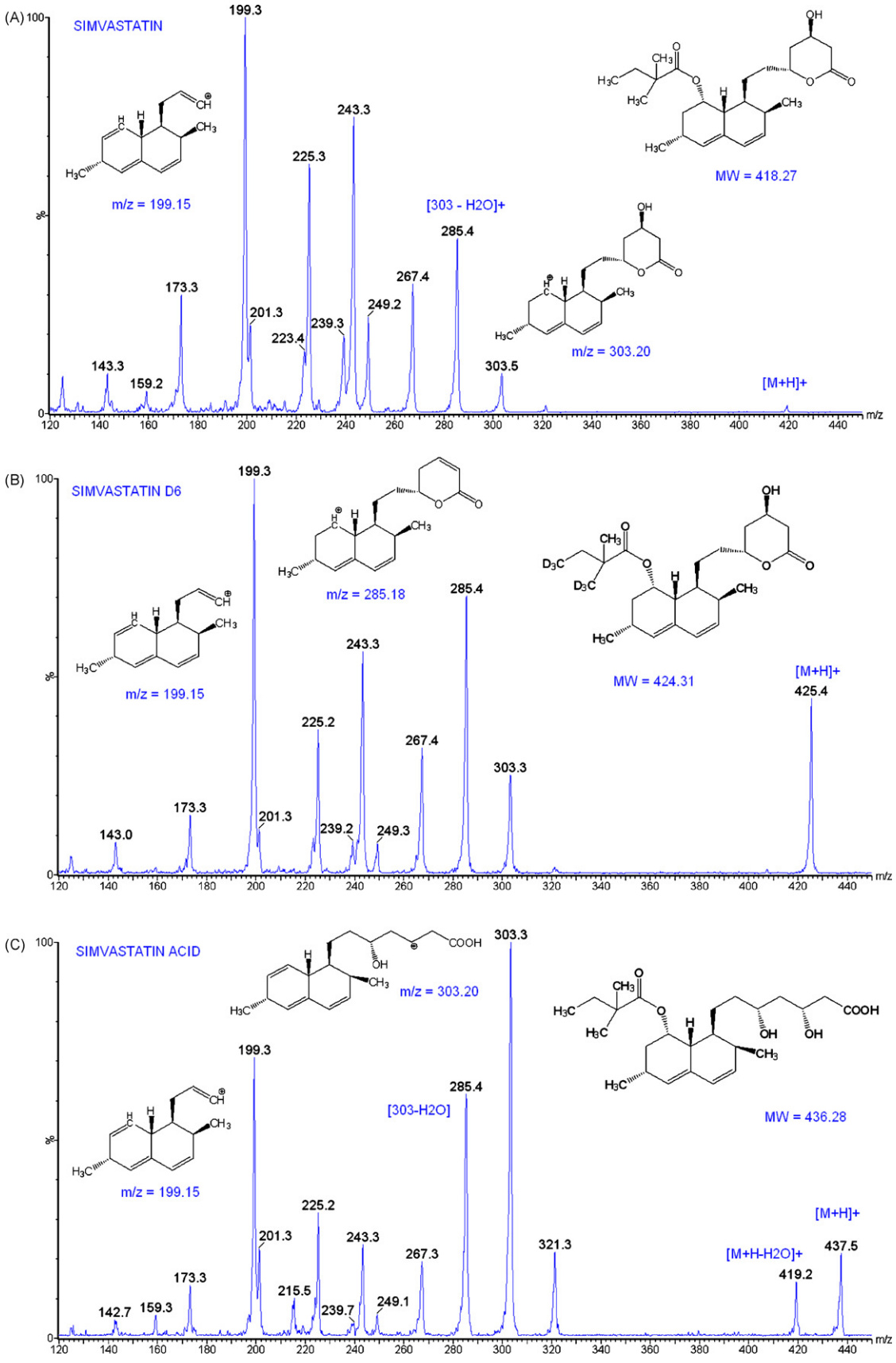


Fig. 3. Product ion spectra of simvastatin (A), simvastatin D6 (B) and simvastatin acid (C).

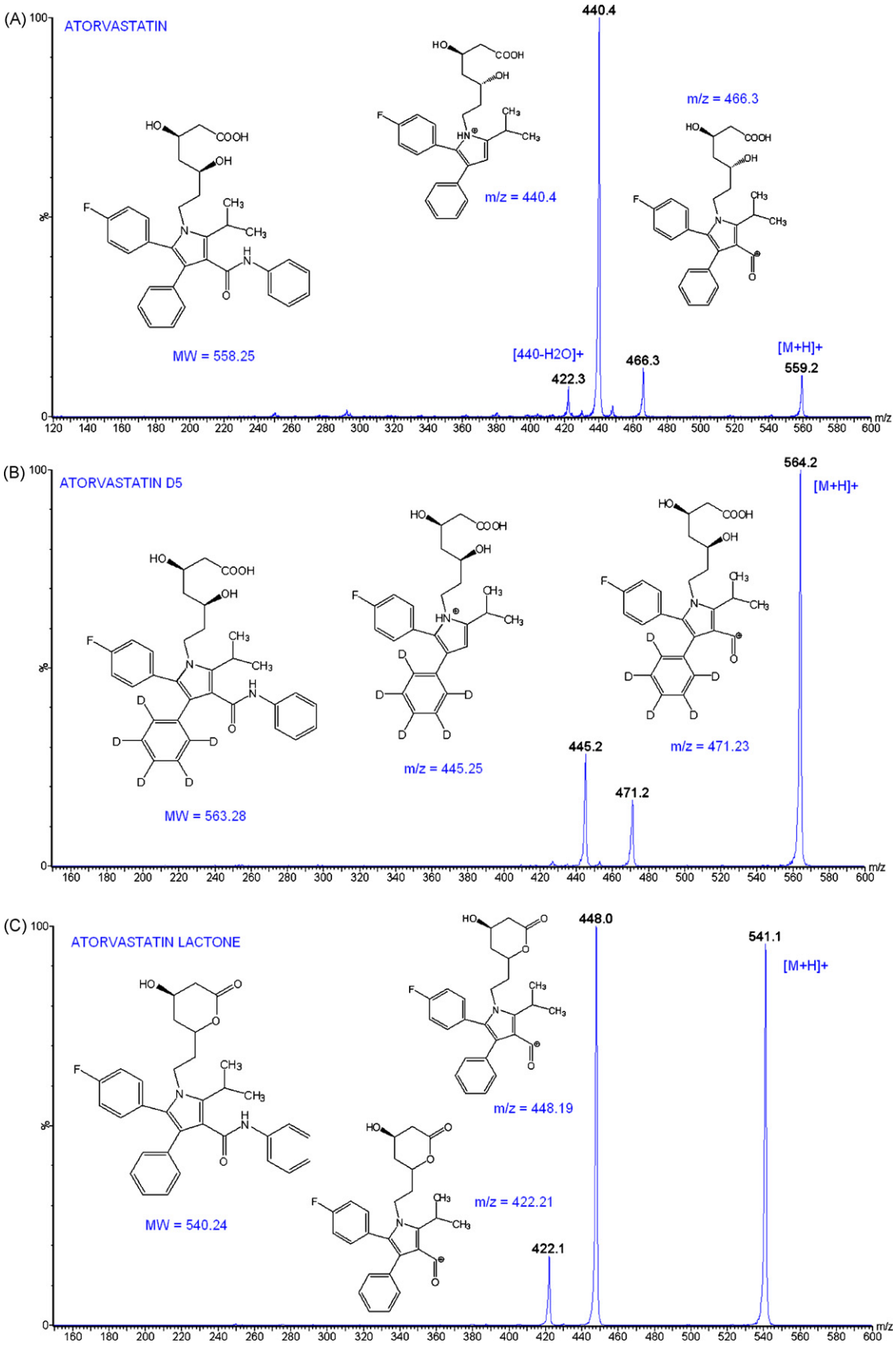


Fig. 4. Product ion spectra of atorvastatin (A), atorvastatin D5 (B) and atorvastatin lactone (C).

2.7.1. Protocol of drug administration and blood sampling

Participants were treated by 40 mg of atorvastatin or 20 mg of simvastatin daily. Drug intake had to be performed at 9.00 p.m. and started at least 4 weeks prior the study.

2.8. Biochemical analyses

For the evaluation of statin levels and its metabolites, two blood samples were taken: one just before the start of the dialysis session, the second just after dialysis. Blood samples were collected at the beginning of the study. The blood was drawn from needle inserted in vascular access for dialysis in fasting state before the start of hemodialysis at 7.00 a.m., and the second blood sample was drawn just after dialysis i.e. after 4 h of hemodialysis. After separation, serum aliquots were stored at −80 °C until analysis. The samples were assayed in random order. All samples were analyzed by personnel who had no knowledge of the subjects' clinical data. Serum lipoprotein fractions were prepared using NaCl density gradient ultracentrifugation (Beckman TL 100, Palo Alto, USA). The lipoprotein fractions were distinguished in the following density ranges: VLDL < 1.006 g/ml; LDL < 1.063 g/ml; HDL > 1.063 g/ml

3. Results and discussion

3.1. Ultra performance liquid chromatography and mass spectrometry

UPLC was used as separation method for the analysis of statins, their interconversion products and metabolites. In early experiments isocratic elution was applied. Minimally 70% of acetonitrile were necessary to elute statins in reasonable retention times. The separation was developed with the regard to the stability of analytes and mass spectrometric detection, which is quite limited in terms of solvents that could be used. Only few additives could enable

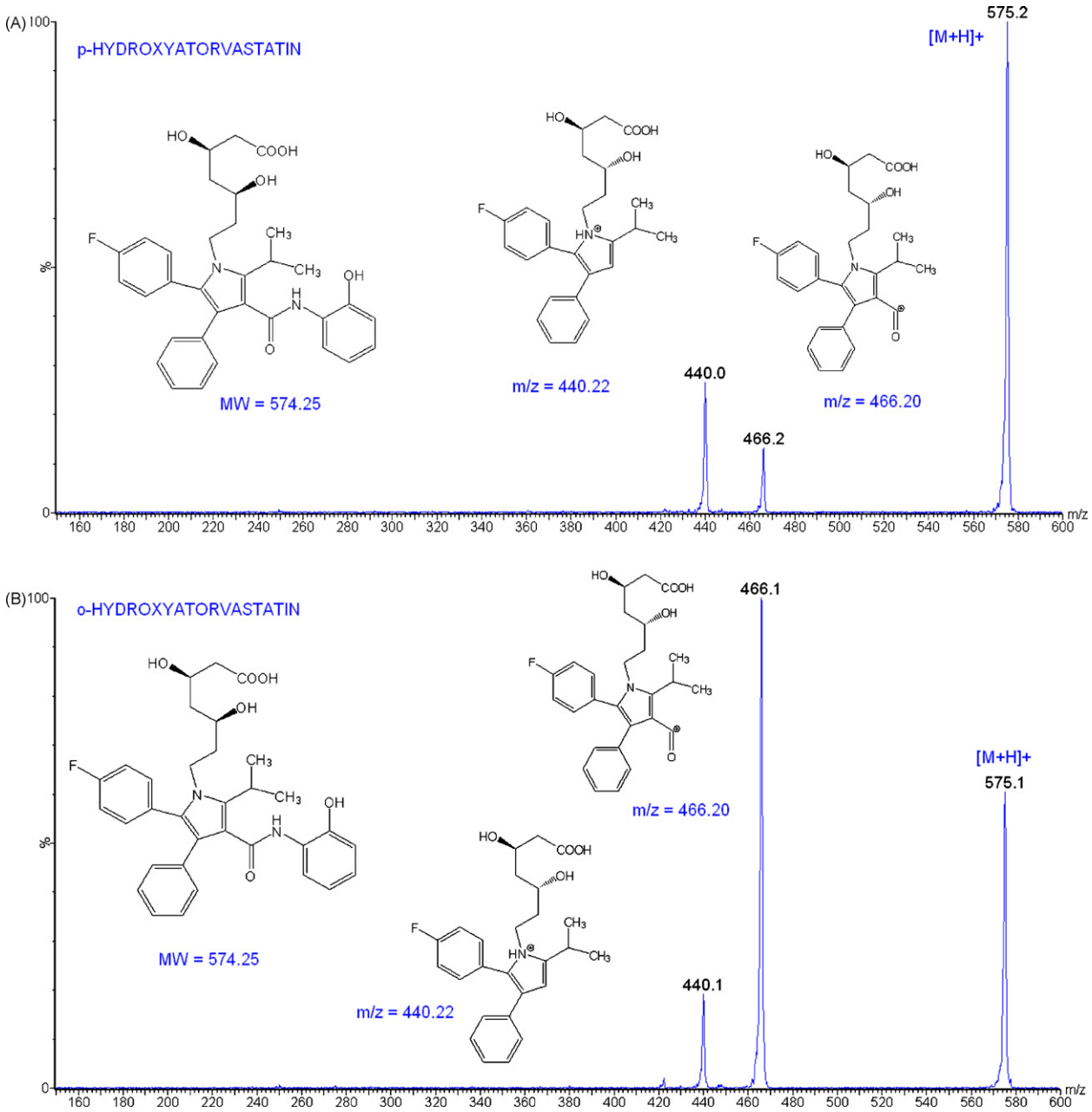


Fig. 5. Product ion spectra of p-hydroxyatorvastatin (A) and o-hydroxyatorvastatin (B).

Table 2
The results of SST, linearity and sensitivity test.

compound	t_R	Repeatability t_R [% RSD]	Repeatability A [% RSD]	Linearity [r^2]	LOQ [nmol/l]	LOD [nmol/l]
p-Hydroxyatorvastatin	2.49	0.27	6.29	0.9997	0.57	0.19
o-Hydroxyatorvastatin	2.89	0.18	1.49	0.9996	0.33	0.11
Atorvastatin	2.89	0.11	1.69	0.9999	0.15	0.05
Atorvastatin-deuterium labeled	2.89	0.18	2.06	0.9996	0.26	0.08
Atorvastatin lactone	3.19	0.21	1.44	0.9993	0.09	0.03
Simvastatin acid	3.48	0.15	4.67	0.9986	4.38	1.46
Simvastatin	4.39	0.12	1.76	0.9997	0.16	0.03
Simvastatin-deuterium labeled	4.40	0.16	1.11	0.9995	0.20	0.05

good stability at pH range 4–5 and volatility together with sensitive mass spectrometric response. In Fig. 2 there is an example of optimization given for atorvastatin, where the buffer pH and concentration in order to get the best S/N ratio of MS detector is performed. Other compounds gave similar response profile. Ammonium formate and ammonium acetate at pH 4.0 and 4.5 were tested at the concentration range 0.01–10 mmol/l. The best response was observed at 0.5 mmol/l buffers, which is in agreement with previously published works concerning the influence of additives—the concentrations higher than 5 mmol/l can significantly decrease the response of mass spectrometer [31]. On the other hand, the concentrations lower than 0.5 mmol/l were not sufficient to keep buffering capacity and they had negative influence to the response of mass spectrometer. Ammonium acetate was preferred before ammonium formate because of better peak shapes. Finally, the mobile phase composition was 70% of acetonitrile and 30% of ammonium acetate buffer 0.5 mmol/l pH 4.0.

In all cases protonated molecule $[M+H]^+$ was monitored in electrospray positive ionization mode. For atorvastatin and its metabolites it was the most intensive ion in mass spectra as published in many papers [22–25] before, however, concerning simvastatin there were strong discussion about the choice of precursor ion [15]. As proposed by Miao and Metcalfe [32], the addition of methylammonium acetate could enhance the formation of methylammonium adduct and that way highly enhance the sensitivity for quantitation using this adduct. In our experiment we did not observe any methylammonium adduct at all using this additive, thus protonated molecule was chosen for quantitation of simvastatin as well. Monitoring of adducts, such as $[M+Na]^+$ or $[M+CH_3CN+Na]^+$ is not correct in quantitative approach even if it was previously published [16–18].

Subsequently, all the parameters of mass spectrometer were finely tuned in order to get good sensitivity of precursor ion $[M+H]^+$ for all analytes—see Section 2.3. Cone voltage was set up individually for each analyte—the results could be seen in Table 1.

Quantitation of all analytes was performed in ESI positive ion mode using SRM mode. Two specific transitions were optimized for each molecule to increase selectivity and identification value of the method. Product ions were chosen according to the fragmentation pathways in Product ion scan mode—see Figs. 3–5. Argon was used as collision gas and collision energy was optimized for each analyte and for each of its two transitions individually in order to get high sensitivity—see Table 1.

3.2. Sample preparation

The sample preparation procedure was optimized using three different SPE sorbents—ZORBAX SPE C-18 (100 mg, 1 ml) (Agilent Technologies), Oasis HLB SPE (60 mg, 3 ml) (Waters), and Discovery DSC-18 (100 mg, 1 ml) (Supelco). Good results of recovery suitable for validation of the method were obtained with using Discovery DSC-18 SPE sorbents. Oasis HLB sorbent showed very different recovery values for atorvastatin (about 56%) and its metabolites (48–140%), thus it could not be used for their simultaneous determination. Sufficient and repeatable recoveries were observed for ZORBAX SPE C-18 but they were withdrawn from commercial market circulation. Serum sample preparation procedure was performed according to the procedure described in Section 2.5.

3.3. System suitability test and validation

The SST was performed by 10 subsequent injections of mixed solutions of standard mixture of statins at the concentration 10 nmol/l. Parameters such as the repeatability of reference standard solution injection were established (retentions times and peak areas were checked, the repeatability was expressed as RSD in %). SST results could be seen in Table 2.

3.3.1. Linearity–calibration range

Calibration curves of all analytes were measured in the concentration range 0.1–100 nmol/l, using seven calibration points (100,

Table 3
The results of validation—accuracy and precision.

Method validation		p-OH-AT	o-OH-AT	AT	ATL	SVA	SV
Accuracy [%] recovery	L1	84.9	65.3	86.0	93.2	78.1	75.6
	L2	89.3	74.4	84.6	91.8	90.5	98.8
	L3	78.8	86.0	86.4	78.5	100.0	89.6
Precision [% RSD] Intra-day	L1	1.5	1.2	1.0	0.8	0.6	9.8
	L2	3.8	3.3	1.6	2.8	4.5	8.6
	L3	8.4	4.7	6.7	11.8	1.8	9.1
Precision [% RSD] Inter-day	L1	2.5	3.9	10.6	6.6	3.5	6.9
	L2	5.5	11.2	10.4	3.2	15.3	6.6
	L3	9.2	4.3	13.7	8.9	11.9	9.6
Repeatability of calibration curve slope [% RSD]		6.0	16.9	14.4	8.4	7.1	9.9
Method selectivity		No interference	No interference	No interference	No interference	No interference	No interference

p-OH-AT = p-hydroxyatorvastatin, o-OH-AT = o-hydroxyatorvastatin, AT-atorvastatin, ATL = atorvastatin lactone, SVA = simvastatin acid, SV = simvastatin, OK = no matrix effect observed, L1, L2, L3 = concentration level 1, 2, 3 (10^{-7} to 10^{-9} mol/l).

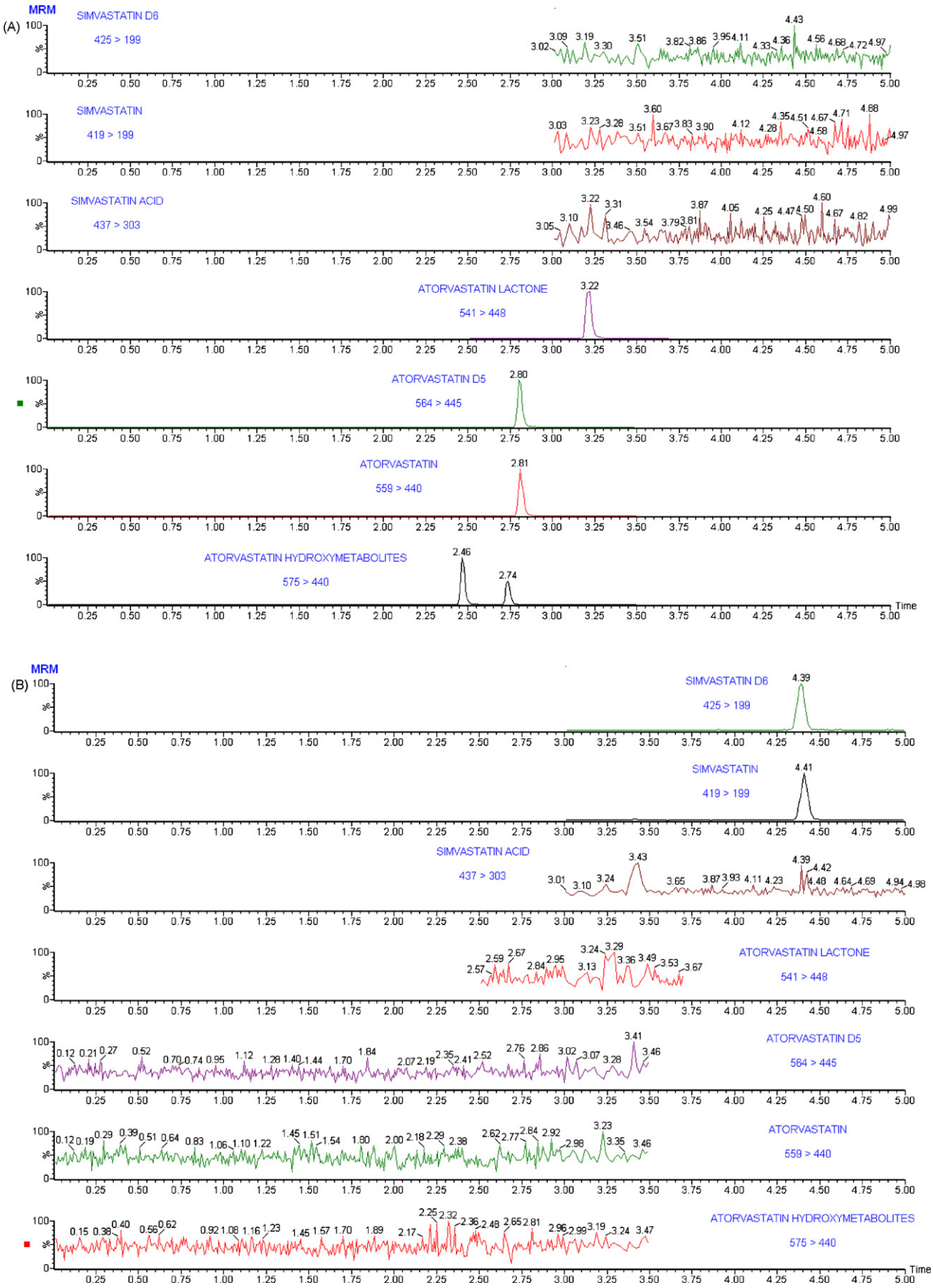


Fig. 6. Chromatogram of analysis of serum samples—patient on atorvastatin (A) and patient on simvastatin (B).

Table 4
Atorvastatin and metabolites—in serum and lipoprotein fractions (VLDL, LDL, HDL) before and after hemodialysis displayed for patients 1 and 2. VLDL = very-low-density lipoprotein, LDL = low-density lipoprotein, HDL = high-density lipoprotein.

Atorvastatin and metabolites in serum and lipoprotein fractions (40 mg dosed)							
compound [nmol/l]		p-OH-AT	o-OH-AT	AT	ATL	SV	SVA
Patient no. 1							
Before hemodialysis	serum	3.82	8.28	20.46	14.34	0	0
	VLDL	0.48	3.26	7.43	5.18	0	0
	LDL	0	2.04	3.4	3.63	0	0
	HDL	2.1	4.43	10.44	9.36	0	0
After hemodialysis	serum	0.49	2.56	3.03	5.64	0	0
	VLDL	0	1.27	0.13	1.01	0	0
	LDL	LOD	0.94	LOD	1.41	0	0
	HDL	LOD	1.69	1.63	4.49	0	0
Patient no. 2							
Before hemodialysis	serum	0.29	3.21	2.19	3.19	0	0
	VLDL	0.05	1.04	0.58	0.58	0	0
	LDL	0	0.74	0.19	0.24	0	0
	HDL	0	1.17	1.11	0.83	0	0
After hemodialysis	serum	0.24	1.95	1.38	0.92	0	0
	VLDL	0	0.8	1.07	0.27	0	0
	LDL	0	0.16	0.65	0.31	0	0
	HDL	0	1.18	1.83	0.89	0	0

50, 10, 5.0, 1.0, 0.5 and 0.1 nmol/l). Results concerning linearity can be seen in Table 2. Matrix calibration curves were prepared using the same calibration points by spiking blank serum samples with standard solutions and subsequent treatment by SPE preparation step. The linearity was found to be satisfactory for all compounds. Such calibration curves were used for quantitation purposes.

3.3.2. Accuracy and precision

Accuracy and precision were established by spiking blank serum samples at three levels of calibration curve—at high (10^{-7} mol/l), medium and low (10^{-9} mol/l) concentration using SPE step described in Section 3.2. Method precision was determined as intra-day and inter-day variability of three determinations at three different levels expressed as % RSD, see Table 3. Intra-day precision was generally within 10% RSD, while inter-day precision within 15% RSD. QC samples were prepared at the same concentration levels.

Method accuracy was determined as % of recovery using blank serum samples spiked with standard solutions treated by SPE extraction and blank serum samples treated by SPE extraction and subsequently spiked by the standard solution at three concentration levels—results could be seen in Table 3. Recovery typically ranged from 75 to 100%.

3.3.3. Method selectivity–matrix effects

For the determination of selectivity and measurement of matrix effects the injection of blank serum treated by the same sample preparation step was used. Matrix effects were established using direct inlet by Hamilton syringe, where standard mix solution was introduced to the mass spectrometer by direct infusion and the blank serum was injected by the autosampler to observe matrix suppressions or enhancements as positive or negative peaks influencing data plot of analytes. First, strong matrix effect was observed at retention time of atorvastatin and its metabolites. It was eliminated by the change of chromatographic conditions. Isocratic elution was changed to gradient profile, starting from 30% of acetonitrile, where statins are not eluted yet to allow interfering compounds from the matrix to be eluted. Thereafter the gradient was run up to 95% of acetonitrile to wash out all other possible interfering compounds—details in Section 2.2. Matrix effects were tested again using the same procedure. Neither negative nor positive peaks were observed at retention times of all analytes.

3.3.4. Limits of detection and quantitation

Limits of detection and quantitation were calculated based on S/N ratio. They were established first using standard solutions in mobile phase by the injection of the smallest amounts which provide S/N = 3. Subsequently this was confirmed by measurements in real matrix, which gave similar values. The results are displayed in Table 2. The method had excellent sensitivity to be able to perform the determination of statins in biological samples reaching LOQ 0.08–5.46 nmol/l and LOD 0.01–1.80 nmol/l.

3.4. Application to real samples

The samples of serum and lipoprotein fractions—HDL, LDL and VLDL were measured using developed UPLC-MS/MS method. A typical chromatogram could be seen in Fig. 6A and B, first transition, which was used for quantitation purpose is displayed. In patients using atorvastatin as a treatment both metabolites and also lactone form of atorvastatin were determined in all samples (Fig. 6A). In total, eight patients taking atorvastatin were included in our study. Atorvastatin levels typically found ranged from 1.33 to 20.46 nmol/l with 6.63 being a mean value. At patients taking simvastatin only simvastatin and simvastatin acid was determined in serum and lipoprotein fractions of patients (Fig. 6B). Only two patients taking simvastatin were included into the study. The levels of simvastatin were substantially lower, probably due to lower biological half-time, they were in the range 0.54–1.74 nmol/l.

The data from the first patient, who was treated by 40 mg of atorvastatin daily, are shown in detail in Table 4. High levels of atorvastatin and its metabolites were found in serum and lipoprotein fractions. The dose of 40 mg atorvastatin was administered at 9.00 p.m. The blood sampling was done the next morning before the start of hemodialysis at 7.00 a.m. The data from the second patient, who was treated by 40 mg of atorvastatin daily, are shown in Table 4 as well. Low levels of atorvastatin and its metabolites were found in serum and lipoprotein fractions. The dose of 40 mg atorvastatin was administered at 9.00 p.m. The blood sampling was done the next morning before the start of hemodialysis at 7.00 a.m.

A high inter-subject variability in pharmacokinetic parameters seen in this study is noteworthy. A high variability in statin kinetic parameters has also been observed in subjects without renal disease. Age, gender, food intake, and level of CYP3A4 expression and

activity all influence the body's handling of atorvastatin [33]. An important characteristic of CYP3A4 is the large inter-individual variability in activity (about 5-fold), which reflects genetic polymorphism combined with modulation by environmental factors [34]. Intake of known strong inhibitors or inducers of CYP3A4 did not occur in this study. However, in hemodialysis patients, who are polymedicated and have complex metabolic disturbances, uncharacterized interactions with concomitant drugs and endogenous substances may have contributed to the large variation in atorvastatin pharmacokinetic parameters.

4. Conclusions

Fast, sensitive and selective method for the determination of simvastatin, atorvastatin, its metabolites and interconversion products of both statins was developed. The method employed UPLC-MS/MS technique as a tool enabling high separation efficiency, speed of analysis and low solvent consumption. MS/MS detection utilized two SRM transitions for each compound to ensure high selectivity and reliability of the method. Deuterium labeled internal standards were used for the purposes of accurate and precise quantitation. Sample pretreatment of serum samples and lipoprotein fractions included stabilization by ammonium acetate buffer pH 4.0 during SPE sample preparation step. This was necessary to prevent the interconversion of analytes. Therefore ammonium acetate was also an inherent part of mobile phase during chromatographic separation. Its concentration was crucial in terms of the support of the ionization of statin molecules. While the concentration higher than 1 mmol decreased significantly ionization of statin molecules, the concentration lower than 0.5 mmol/l was not sufficient to ensure sufficient ionization, buffering capacity and the stability of analytes and therefore the response of mass spectrometer decreased.

The method was validated with good results for linearity (>0.9990, except of SVA), precision (RSD < 15% for all analytes), accuracy (recovery 75–100%) and selectivity showing no interferences with measured compounds. Analytes could be quantified at nmol/l concentrations with typical LOQ 0.09–0.57 nmol/l, except of SVA LOQ being 4.38 nmol/l. The method is applicable to analysis of serum samples and lipoprotein fractions containing atorvastatin or simvastatin. The advantage of the method was simultaneous determination of two clinically widely used statins—one chromatographic run and one sample preparation. It was not necessary to distinguish among the samples of patients and all samples could be analyzed using one procedure, which was very convenient for routine purposes.

This study revealed differences in the inter-individual processing of statins in hemodialysis patients with hyperlipidemia and is addressing the clinically relevant information with respect to achieve adequate levels of exposure to active compound in this group of patients.

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