Talanta 90 (2012) 22–29

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Determination of pravastatin and pravastatin lactone in rat plasma and urine using UHPLC–MS/MS and microextraction by packed sorbent

Hana Vlčková [a], Martina Rabatinová [a], Alena Mikšová [a], Gabriela Kolouchová [b], Stanislav Mičuda [b], Petr Solich [a], Lucie Nováková [a,*]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Pharmacology, Faculty of Medicine in Hradec Králové, Charles University in Prague, Šimkova 870, 500 38 Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 13 October 2011
Received in revised form 7 December 2011
Accepted 15 December 2011
Available online 9 January 2012

Keywords:
Pravastatin
Pravastatin lactone
UHPLC–MS/MS
MEPS
Rat plasma and urine

ABSTRACT

A simple and reproducible method for the determination of pravastatin and pravastatin lactone in rat plasma and urine by means of ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) using deuterium labeled internal standards for quantification is reported. Separation of analytes was performed on BEH C_{18} analytical column (50 mm × 2.1 mm, 1.7 μm), using gradient elution by mobile phase consisting of acetonitrile and 1 mM ammonium acetate at pH 4.0. Run time was 2 min. Quantification of analytes was performed using the SRM (selected reaction monitoring) experiment in ESI negative ion mode for pravastatin and in ESI positive ion mode for pravastatin lactone. Sample treatment consisted of a protein precipitation by ACN and microextraction by packed sorbent (MEPS) for rat plasma. Simple MEPS procedure was sufficient for rat urine. MEPS was implemented using the C8 sorbent inserted into a microvolume syringe, eVol hand-held automated analytical syringe and a small volume of sample (50 μl). The analytes were eluted by 100 μl of the mixture of acetonitrile: 0.01 M ammonium acetate pH 4.5 (90:10, v:v). The method was validated and demonstrated good linearity in range 5–500 nmol/l (r^2 > 0.9990) for plasma and urine samples. Method recovery was ranged within 97–109% for plasma samples and 92–101% for the urine samples. Intra-day precision expressed as the % of RSD was lower than 8% for the plasma samples and lower than 7% for the urine samples. The method was validated with sensitivity reaching LOD 1.5 nmol/l and LOQ 5 nmol/l in plasma and urine samples. The method was applied for the measurement of pharmacokinetic plots of pravastatin and pravastatin lactone in rat plasma and urine samples.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Statins represent the most efficient drugs for the treatment of the severe forms of hypercholesterolemia and reduce the morbidity and mortality associated with cardiovascular diseases [1,2]. Pravastatin is a competitive hydrophilic liver-specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme of biosynthesis of cholesterol [3–5]. Pravastatin as well as the other statines exists in two forms, lactone and open-ring hydroxy acid form [6]. Hydroxy acid form is the active drug with cholesterol-lowering effect while the lactone form is inactive (prodrug). Pravastatin is administered as the sodium salt of the active hydroxy acid form [7,8].

A number of statins have been introduced into clinical practice. The first substance was lovastatin followed by atorvastatin and simvastatin. Many pharmacokinetic studies comparing different HMG-CoA reductase inhibitors have been performed. Pravastatin was characterized as one of the most efficient for its greater hydrophilicity [4] and the unique pharmacokinetic properties compared the other HMG-CoA reductase inhibitors. The pharmacokinetics of pravastatin is characterized by low absorption and bioavailability, a fast absorption rate limiting elimination and a relatively low protein binding [9]. The peak plasma concentration of pravastatin after the oral administration is attained within 1–1.5 h. Plasma elimination half-life ranges from 1.3 to 2.6 h [10]. Several high performance liquid chromatography methods with UV or MS/MS detection have been developed for the determination of pravastatin in human plasma or serum [3–5,8,11–14]. The determination of pravastatin in urine has not been described so far. Only one method using UV, which is not selective and sensitive enough towards biological samples, was published [11]. Most of LC–MS/MS assays currently available focus only on pravastatin, which substantially decreases method value. Pravastatin is one of the drugs which is subjected to interconversion between lactone and open-ring hydroxy acid. For this reason the analysis of lactone and acid forms and their chromatographic separation is the key for their

* Corresponding author. Tel.: +420 495067381; fax: +420 495067164.
E-mail address: nol@email.cz (L. Nováková).

0039-9140/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.12.043

Table 1
Optimization of specific transitions for all analytes.

	Compounds	Precursor	Precursor type	Fragment	Cone V	Collision E	Dwell time	t_R
1	PV	423.5	[M-H]⁻	321.5	35	15	0.1	1.72
				303.5	35	15		
2	PV-D3	426.5	[M-H]⁻	321.5	35	15	0.1	1.72
				303.5	35	15		
3	PVL	407.5	[M + H]⁺	183.5	20	15	0.1	1.92
				269.5	20	10		
4	PVL-D3	410.5	[M + H]⁺	183.5	20	15	0.1	1.92
				269.5	20	10		

PV, pravastatin; PV-D3, pravastatin deuterium labeled; PVL, pravastatin lactone; PVL-D 3, pravastatin lactone deuterium labeled.

accurate quantification [6]. The important step for the minimized interconversion is also maintaining of pH between 4 and 5 [1].

All reported LC–MS/MS methods used protein precipitation or solid phase extraction (SPE) on the reverse phase sorbent as sample preparation step. Any LC–MS/MS methods have not used microextraction technique as the sample preparation technique so far. In clinical laboratories the main requirements for sample preparation are rapidity, simplicity and miniaturization, especially when using small volume of samples and organic solvents, while maintaining sufficient selectivity, precision and accuracy. MEPS as miniaturized SPE is logical extension of SPE for the analysis of biological fluids [15]. It can handle small sample volumes (10 μl of plasma, urine or water) as well as relatively large volumes (1000 μl). Small sample volumes allow for analysis of not only human but also the animal samples for example rat plasma where volume availability is very limited [16,17]. Sample preparation takes place on the packed bed. Many sorbent materials for example silica-based (C2, C8, C18), strong cation exchanger (SCX), restricted access material (RAM), HILIC, molecularly imprinted polymers (MIPs) are available [16]. MEPS enable on-line connection to some of GC, HPLC or UHPLC system [15,16,18]. The disadvantages of manual approach of MEPS include irrepeatable speed of plunger movement and non-accurate manual injection of very small volumes of sample (<50 μl) [1]. A compromise between manual and automatic approaches is usage of the eVol hand-held automated analytical syringe which removes the influence of operator and the above mentioned disadvantages of manual approach. Unlike SPE cartridge sorbent MEPS can be reused several times, more than 100 extractions without any loss in its performance for water, urine and centrifuged plasma samples and 30–40 times for non-centrifuged plasma [15,18]. MEPS technique has been used to extract a wide range of analytes from biological samples such as urine, plasma and serum [1,19–24].

The aim of this work was to develop and validate a fast and simple UHPLC-MS/MS method for the determination of pravastatin and pravastatin lactone in rat plasma and urine using MEPS and PP as sample preparation methods. The suitability of the method for the detection of the drug and its lactone metabolite in samples with limited volume availability was verified using rat plasma and urine obtained after intravenous administration of the drug. The novelty of method consists in simultaneous quantification of pravastatin and pravastatin lactone using two deuterium labeled standards for both analytes individually and miniaturized sample preparation step. This UHPLC–MS/MS method was applied not only to the real rat plasma but also to urine samples.

2. Experimental

2.1. Chemicals and reagents

Working standards of pravastatin, pravastatin lactone, pravastatin deuterium labeled (D3) and pravastatin lactone deuterium labeled (D3) were purchased from Toronto Research Chemicals (Ontario, Canada).

The acetic acid, LC–MS grade (>99%), the ammonium hydroxide, LC–MS grade (>25%) 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 and mass spectrometry

UHPLC system Acquity UPLC (Waters, Prague, Czech Republic) was used for the purpose of this study. It consisted of ACQ-binary solvent manager and ACQ-sample manager. All UHPLC–MS/MS analyses were performed on BEH C_{18} analytical column (50 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 1 mM ammonium acetate pH 4.0 using gradient elution with initial mobile phase composition acetonitrile:1 mM ammonium acetate pH 4.0 (05:95). Thereafter the concentration was changed within 1.0 min to 70% of acetonitrile and subsequently to 95% of acetonitrile within 2.00 min. Mobile phase flow rate was 0.2 ml/min. The analytical column was kept at 35 °C by column oven. The solutions were stored in the autosampler at 4 °C.

The MS/MS triple quadrupole system was used in this study. Quattro Micro (Micromass, Manchester, GB) was equipped with a multi-mode ionization source (ESCI). Ion source was set-up in ESI polarity-switching mode as follows: capillary voltage: 3000 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 1.0 V. The desolvation gas was nitrogen at flow 550 l/h and at the temperature 450 °C. Nitrogen was used also as a cone gas (50 l/h). Cone voltage (CV) was set up individually for each analyte (Table 1). Quantification of all analytes was performed using SRM (selected reaction monitoring) experiment. Product ions were chosen according to the fragmentation pathways in Product ion scan mode. Argon was used as a collision gas and collision energy (CE) was optimized for each analyte individually (Table 1). The MassLynx 4.1 Data System was used for MS system control and data gathering. QuanLynx software was used for data processing and quantitation – regression analysis of standard, matrix calibration curves and calculation of concentrations.

2.3. Preparation of standard solutions

The stock solutions of standards were prepared by dissolving of the amount corresponding to 1.0 mmol/l of appropriate working standard into 1.0 ml of dissolution media, due to significant differences in solubility. The stock solutions of pravastatin lactone and pravastatin lactone D3 were prepared in pure acetonitrile. The stock solutions of pravastatin and pravastatin D3 were prepared in mixture acetonitrile:1 mM ammonium acetate pH 4.0 (90:10, v:v). Working solutions of all analytes were further diluted by mixture of ACN:1 mM ammonium acetate pH 4.0, 05:95 (initial mobile phase) to achieve individual points of calibration curve in the range 5–500 nmol/l, using five calibration points (500, 100, 50, 10,

5.0 nmol/l). Working solution of ISs at concentration of 200 nmol/l was prepared by diluting with 0.5 M ammonium acetate pH 3.0 for the stability reason (see Section 2.4). Stock solutions of all tested compounds in mixture of ACN:1 mM ammonium acetate pH 4.0 (90:10) and solutions of analytes in 0.5 M ammonium acetate pH 3.0 were tested for stability at 20, 4 and −18 °C.

2.4. Sample preparation

Protein precipitation (PP) and MEPS were used as the sample preparation techniques. MEPS was utilized for the cleaning up of urine samples and the combination of PP and MEPS for the plasma samples. The eVol hand-held automated analytical syringe was used for MEPS extraction. 25 μl of the working solution of ISs and 25 μl of 0.5 M ammonium acetate pH 3.0 were added to 50 μl of plasma or urine samples containing the analytes. Addition of this buffer was necessary for the stability reason, to adjust and keep pH of sample between 4.0 and 5.0. Such pre-treated sample solutions were applied to the PP or MEPS extraction. The adjustment of pH was curtail, because the pH of sample without buffer addition was about 7, which facilitates the conversion of lactone to acid form.

Plasma and urine samples were stored at −80 °C and after the thaw cycle they were processed immediately by MEPS procedure and analyzed by UHPLC–MS/MS.

2.4.1. Sample preparation for rat plasma

The first step was PP with ACN as the deproteinization agent. 100 μl of ACN was added to 100 μl pre-treated sample solution. This mixture was shaken and incubated for 15 min. The precipitated sample was centrifuged at 4200 RPM for 10 min. The supernatant was withdrawn and diluted by 1.80 ml of 0.01 M ammonium acetate buffer pH 4.5. The C8 MEPS sorbents packed in bin, which is inserted into a needle assembly connected to 500 μl syringe (SGE Analytical science, Germany) was chosen. The whole volume of supernatant was aspirated through MEPS previously activated three times with 250 μl of acetonitrile and conditioned three times with 250 μl of 0.01 M ammonium acetate buffer pH 4.5. The sorbent was washed two times with 250 μl of 0.01 M ammonium acetate buffer pH 4.5 and 250 μl of mixture acetonitrile:0.01 M ammonium acetate buffer pH 4.5 (05:95, v:v). Analytes were eluted with 100 μl of mixture of acetonitrile:0.01 M ammonium acetate buffer pH 4.5 (90:10, v: v). The eluate was filtrated via PTFE microfilter (4 mm × 0.20 μm) and the sample was injected onto UHPLC system. To avoid the carry-over the MEPS cartridge was washed three times by acetonitrile before the application of following sample. The sorbent was re-used about 60 times for urine samples and at least 30 times for the plasma samples without loss of extraction efficiency.

2.4.2. Sample preparation for rat urine

Only MEPS without PP was used for the cleaning up of urine samples. The type of sorbent and solvents and the whole procedure of MEPS was identical as in Section 2.4.1.

2.5. Method validation

SST (system suitability test) is an important part of method validation, details of which are usually given in Pharmacopoeias. The SST was performed under the optimized chromatographic conditions. In LC–MS only repeatability of retention times and peak areas are checked.

Newly developed MEPS-UHPLC–MS/MS was validated in terms of linearity, selectivity, sensitivity (limits of detection and quantitation), method accuracy, precision and matrix effects according to the requirements of ICH (International Conference on Harmonization) [25]. For the determination of linearity, two calibration curves of all analytes were prepared (1) matrix calibration curve using blank rat plasma or urine samples, that were spiked and then treated by MEPS procedure in the concentration range 5–500 nmol/l and (1) standard calibration curve, where stock standard solutions were diluted by mobile phase in the concentration range 1–500 nmol/l.

For method precision, spiked blank rat plasma and urine samples treated by MEPS at three different concentration levels were measured in three replicates in order to calculate % of RSD, which describes the closeness of agreement between series of measurements.

Method accuracy was described as the recovery experiment. Recovery was determined via a comparison of the response of plasma and urine samples spiked prior to MEPS extraction with the response of rat blank plasma and urine samples that were first treated by MEPS procedure and then spiked with the analytes. It was complemented at three concentration levels in three replicates to establish the closeness of agreement between the true and measured value as it corresponds to ICH requirements [25]. QC samples were prepared at the same concentrations as were the concentration levels prepared for precision and accuracy experiments. Matrix effects were evaluated using blank rat plasma and urine samples, which were first treated by MEPS procedure and then spiked by standard solution at three concentration levels within the calibration range. The results were compared with the measurement of standard calibration curves and matrix effects were calculated.

Limits of detection and quantification were established based on signal-to-noise (S/N) ratio approach. Limit of detection was expressed as S/N = 3, limit of quantification was expressed as S/N = 10. The stability of samples in standard stock solutions was evaluated at 20, 4 and −18 °C in a short-term and long-term period.

3. Results and discussion

3.1. Ultra high performance liquid chromatography tandem mass spectrometry detection

UHPLC was used for separation of pravastatin and pravastatin lactone. Incorporation of chromatographic separation of two compounds is necessary because of the potential in-source interconversion between a lactone and its corresponding hydroxy acid form [6]. A selection of analytical column Acquity BEH C18 (50 mm × 2.1 mm, 1.7 μm) and development of method was carried out with the regard to physical–chemical properties, the stability of the analytes and mass spectrometry detection, which is limited in terms of solvents that could be used. The crucial aspect was maintaining the pH in the range 4–5 [1]. Ammonium acetate and ammonium formate at different concentration and pH were tested in the study. In compromise of response of mass spectrometer, analysis time and resolution, acetonitrile in combination with 1 mM ammonium acetate pH 4.0 was finally chosen as the mobile phase. Flow rate was 0.20 ml/min. The gradient elution was applied. Firstly the composition of the mobile phase was optimized on the standard solution. The initial ratio of mobile phase was 70:30 (1 mM AmAc pH 4.0: ACN). When these conditions were applied to the matrix samples, very intensive matrix enhancement was observed because analytes were not enough separated from matrix components. At this point, the profile of gradient elution with different initial conditions was tested. 95% of 1 mM ammonium acetate pH 4.0 was selected as a suitable initial composition of mobile phase. Time of equilibration have been prolonged therefore the total time increased from 2 to 6 min.

An MS/MS triple quadrupole system with electrospray ionization was used for quantification. Both positive and negative ionization modes were examined. The precursor and product ion

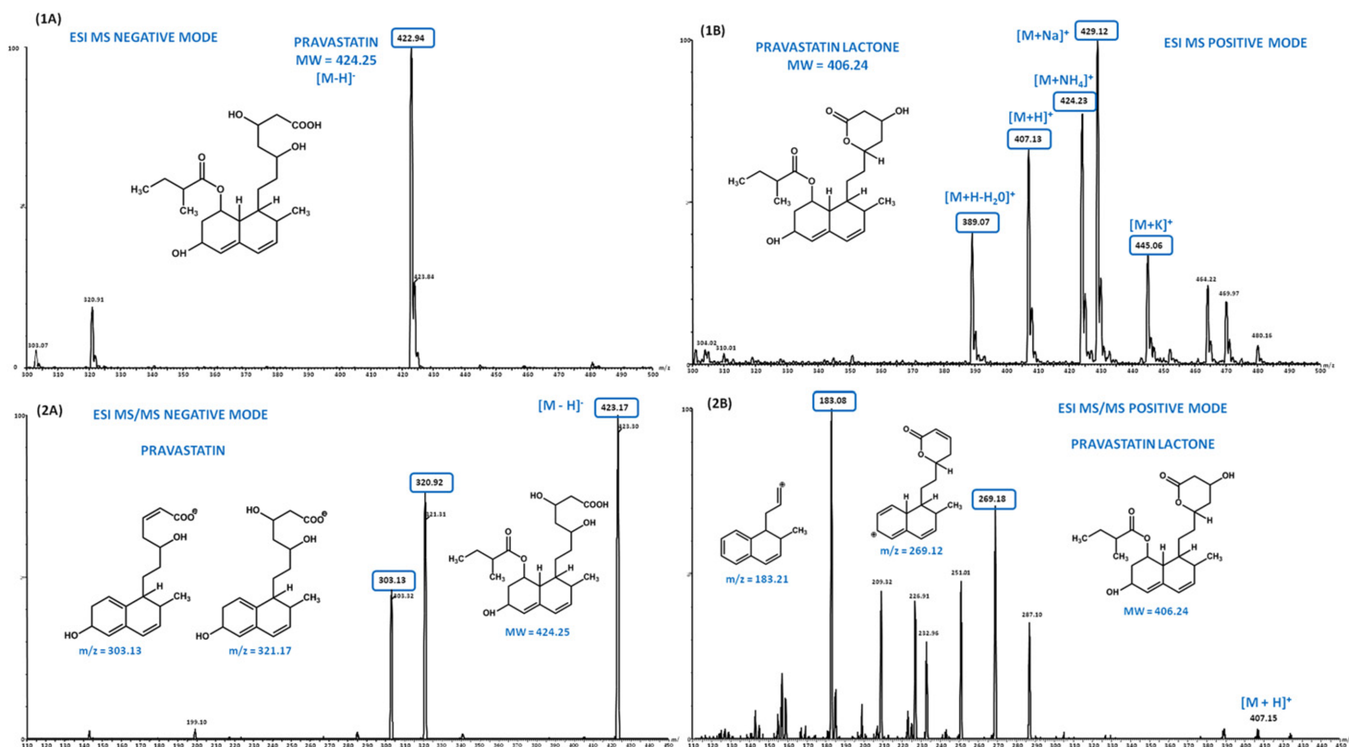

Fig. 1. Precursor (1) and product (2) ion spectra of pravastatin (A) and pravastatin lactone (B).

spectra of pravastatin and pravastatin lactone are shown in Fig. 1. In full scan spectra pravastatin provided a precursor ion $[M-H]^-$ in negative ion mode and $[M+H]^+$ in positive ion mode. In positive ion mode pravastatin lactone offered the protonated molecule $[M+H]^+$ and an ammonium adduct $[M+NH_4]^+$ together with the other adducts inconvenient for quantification, such as $[M+Na]^+$ and $[M+K]^+$. In negative ion mode pravastatin lactone provided acetate adduct $[M+CH_3COO]^-$. The best response was obtained in ESI negative ion mode for the pravastatin $[M-H]^-$ and in ESI positive ion mode for of pravastatin lactone $[M+H]^+$, which provided more reproducible results during quantification using SRM experiment. The ammonium adduct of pravastatin lactone $[M+NH_4]^+$ offered better S/N ratio than protonated molecule, however it demonstrated low repeatability of peak area and linear response. Therefore only a precursor ion $[M+H]^+$ for pravastatin lactone and $[M-H]^-$ for pravastatin were further used for the quantification and fine tuning of all parameters of mass spectometer (see Section 2.2, Table 1).

Two specific transitions were optimized for each molecule to increase selectivity and reliability of the method. The first transition was used for the quantification of analytes, the second one was confirmatory.

While this UHPLC–MS/MS method determined pravastatin and pravastatin lactone together with their deuterium labeled standards, most of LC–MS/MS assays currently available focus only on pravastatin, which substantially decreases method value. Pravastatin is one of the drugs which is subjected to interconversion between a lactone and open-ring hydroxy acid. For this reason the analysis of lactone and acid forms and their chromatographic separation is the key issue for their accurate quantification [6]. Only two reported methods enabled simultaneous quantification of pravastatin and pravastatin lactone in biological fluids. They employed internal standard method for the quantification of analytes but only one of them with deuterium labeled analogues. Because the separation of pravastatin and pravastatin lactone is necessary, use of internal standard for each analyte individually is preferable in order to eliminate matrix effects and to improve accuracy of LC–MS quantification [5,9]. An overview of recently published analytical methods for the determination of pravastatin and its related substances in biological samples is shown in Table 2.

3.2. Sample preparation

MEPS was selected as a sample preparation method because it is fast and simple miniaturized technique. The main reason was the need for very small volume of sample. Accessible volume of biological sample was less than 200 μl. The pH of sample without buffer was about 7, therefore a selection of suitable buffer was the key step in order to keep the pH in the range 4–5. To ensure this 50 μl of 0.5 M ammonium acetate pH 3.0 was added to 50 μl of sample. While urine is relatively simple matrix, plasma is more complex containing many contaminants and a proteins simultaneously. To prolong the life-time of MEPS sorbent PP was used before MEPS extraction of plasma. PP was used mainly to remove proteins from plasma and MEPS to eliminate large amounts of contaminant and salts.

Several types of deproteinization agents and their volume were tested. Precipitation using acidic agents was impossible because shifted pH towards low values induces interconversion of pravastatin to pravastatin lactone. Therefore ACN was finally the most convenient deproteinization agent. The volume of ACN was chosen with regard to compatibility of MEPS extraction and sufficient precipitation. Large volume of ACN in sample caused low recovery the MEPS extraction, because analytes were not captured on the sorbent. 100 μl ACN was chosen as the lowest volume providing sufficient precipitation and good recovery for the deproteinization of 50 μl of plasma sample. After the centrifugation removed supernatant was diluted by 1.8 ml of 0.01 M ammonium acetate buffer pH 4.5, thus the volume of sample for aspiration through the MEPS sorbent increased considerably. Nevertheless the sample volume was still much smaller than for the SPE extraction. Lower volume of sample and solvents are one of the main advantages of MEPS

Table 2
An overview of LC–MS/MS methods used for the determination of pravastatin in biological samples.

Analytes	Internal standard	Sample volume	Sample preparation			Liquid chromatography and mass spectrometry detection			Matrix	Ref.
			Extraction technique	SPE column	Elution solvent	Analytical column	Mobile phase and flow rate	Ionization mode and analyzer		
Pravastatin	Hydrochlorthiazide	0.5 ml	SPE	HLB	MeOH	Betabasic C8 (100 mm × 4.6 mm, 5 μm)	0.1% Ammonium:ACN (20:80) 0.5 ml/min, isocratic elution	ESI negative QQQ	Human plasma	[3]
Pravastatin	Hydroxy-lovastatin	1 ml	SPE	C_{18} Bond Elut	–	Zorbax XDB C8 (50 mm × 2.1 mm, 5 μm)	ACN:1 mM AmF pH 3.3 (2:1) 0.25 ml/min, isocratic elution	ESI negative QQQ	Human plasma	[4]
Pravastatin pravastatin lactone	Pravastatin-D_5 and SQ-1906-D_5	0.50 ml	SPE	Isolute C8 cartridges	ACN:H_2O (70:30)	Keystone Betasil ODS (100 mm × 2.1 mm, 5 μm)	ACN:MeOH:5 mM AmAc pH 4.5 (30:30:40), 0.2 ml/min	ESI Positive QQQ	Human serum	[5]
Pravastatin 3α-isopravastatin 6α-epipravastatin	Pravastatin-D_3	0.020 ml	PP (ACN)	–	–	Atlantis dC18 (2.1 mm × 150 mm, 3 μm)	ACN:0.1% HCOOC in water (29:71), 0.5 ml/min isocratic elution	ESI negative QQQ	Mouse plasma tissue homogenate	[9]
Pravastatin Pravastatin lactone 3-OH metabolites Fenofibric acid	Triamcinolone	1.4 ml	Automatic SPE	DECs	MeOH	Synergi Max-RP (150 mm × 2.0 mm, 4 μm.)	ACN:MeOH:5 mM AmAc pH 4.5 (30:30:40), 0.2 ml/min	ESI positive QQQ	Human plasma	[10]
Pravastatin pitavastatin	Fluvastatin	0.30 ml	SPE	Waters C18 Sep-Pak	MeOH	Luna C18 (50 mm × 2 mm, 3 μm)	ACN:0.1% HCOOC (90:10), 0.2 ml/min, isocratic elution	ESI negative QTrap	Human plasma	[11]
Pravastatin R-416	R-122798 (pravastatin analog)	1 ml	SPE	C8vBond Elut	ACN	Inertsil ODS-3 C18 (150 mm × 4.6 mm, 5 μm)	ACN:H_2O:AmAc:HCOOC:TEA (400:600:0.77:0.2:0.6, v/v/g/v/v), 1 ml/min	APCI negative QQQ	Human plasma	[13]
Pravastatin SQ-31906	Pravastatin-D3	50 μl	–	–	–	Symmetry C18 (50 mm × 3.9 mm, 5 μm)	ACN:1 mM FAc (100–0% HCOOH) 0.5 ml/min gradient elution	ESI negative QQQ	Rat plasma rat serum	[14]

SPE, solid phase extraction; HLB, hydrophilic–lipophilic balance; QQQ, triple quadrupole analyzer; DECs, disposable extraction cartridges; AmAc, ammonium acetate; ESI, electrospray ionization; TEA, triethylamin; APCI, atmospheric pressure chemical ionization; AmF, ammonium formate; PP, protein precipitation.

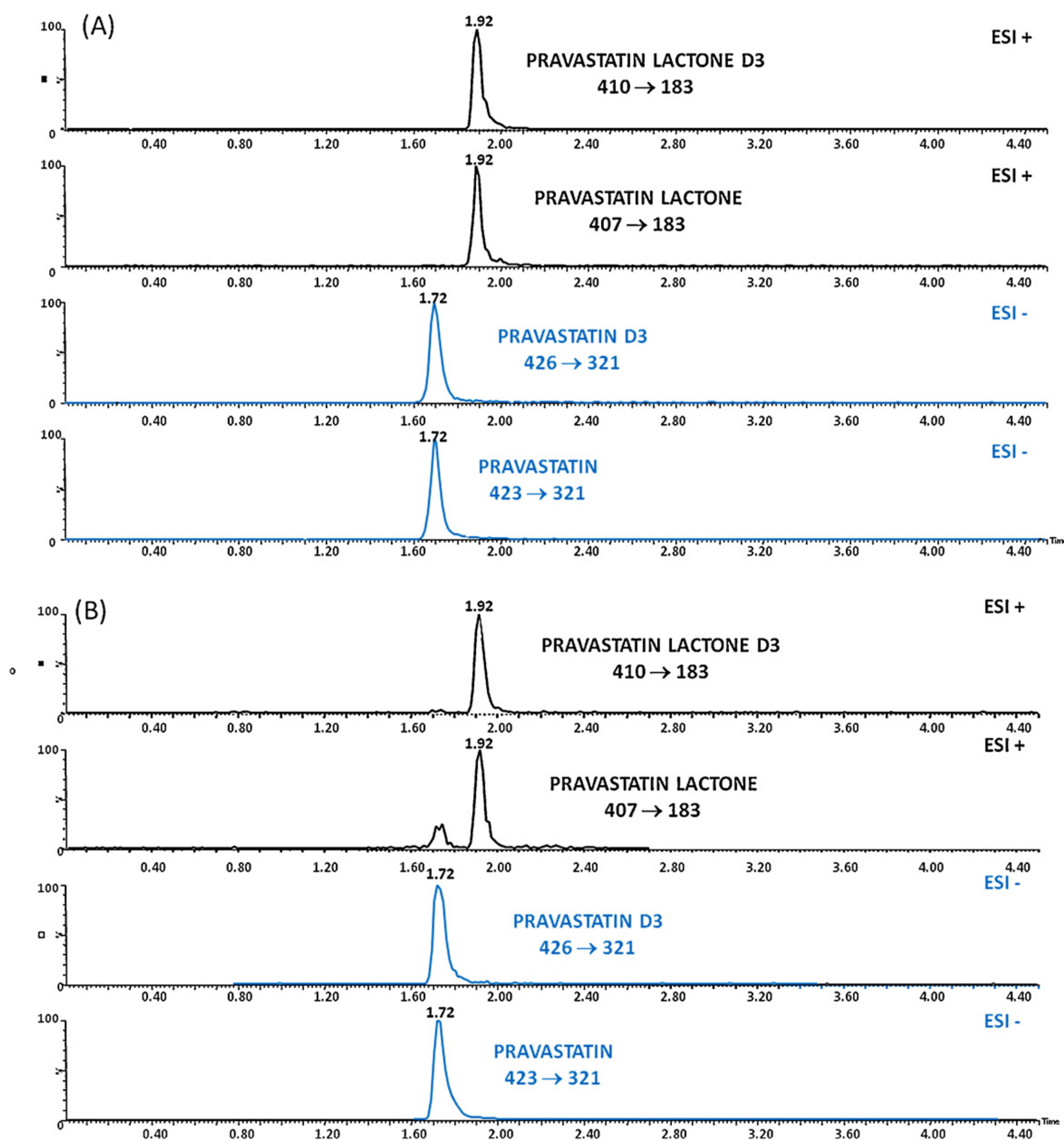

Fig. 2. UHPLC–MS/MS chromatogram of standard mixture of measured analytes (calibration level 5×10^{-7} mol/l) (A) and chromatogram of plasma samples (B) – rat treated by pravastatin.

extraction. Another key step was a choice of the suitable buffer for the dilution of supernatant after PP to make it convenient for load step. In order to prevent the interconversion, 0.01 M ammonium acetate pH 4.5 was selected.

Off-line semiautomatic MEPS technique using the eVol hand-held automated analytical syringe was employed. Two different MEPS cartridges–C18 and C8 were tested during the optimization of sample preparation procedure. Good results of recovery and precision during the method validation were obtained with C8 sorbent. C18 sorbent showed very different recovery values for pravastatin and pravastatin lactone. Adsorption on sorbent and the choice of elution and wash solvents was problematic probably due to greatly different physical- chemical properties. For the pravastatin lactone 100% of ACN was the most suitable as an elution solvent, while the reduction of the % of ACN in elution solvent decreased recovery of pravastatin lactone and increased that of pravastatin. As compromise between recovery of pravastatin and pravastatin lactone 90% of ACN was finally selected. The same problem concerned the wash solvent. Only 5% of ACN could have been used to prevent washing out of pravastatin from sorbent during this cleaning step.

The critical point of MEPS procedure and whole step of sample preparation was the interconversion of pravastatin and pravastatin lactone each other. The maintenance of pH in the range 4–5 in all sample solutions, supernatants and extracts was absolutely essential for the prevention of interconversion. Plasma and serum samples were finally prepared according to the procedure described in Section 2.4.

3.3. Method validation

The chromatogram demonstrating good separation of the analytes is shown in Fig. 2 for both standard solution and spiked rat plasma. The SST and validation parameters included the repeatability of reference standard solution injection, linearity, method accuracy, precision, matrix effects and limits of detection and quantification. An excellent repeatability of injection was obtained for

Table 3
The results of SST and validation: linearity, method accuracy, precision and matrix effects for urine and plasma samples, linearity and sensitivity test.

		PV	PV D3	PVL	PVL D3
t_R		0.72	0.72	0.92	0.92
LINEARITY (r^2) – standard calibration curve		0.9994	0.9996	0.9998	0.9995
Repeatability of calibration curve (%RSD) – inter- day		4.6	–	2.6	–
Repeatability t_R (%RSD) – intra-day		0.3	0.3	0.4	0.3
Repeatability A (%RSD) – intra-day		3.4	4.1	5.7	4.2

Method validation		Plasma samples		Urine samples	
		PV	PVL	PV	PVL
Linearity (r^2) – matrix calibration curve		0.9999	0.9992	0.9994	0.9990
Method accuracy [%]	L1	103.4	96.7	99.4	99.5
	L2	102.7	109.3	98.2	101.1
	L3	98.3	102.6	92.4	97.1
Method precision [RSD%] – intra-day	L1	0.8	4.8	0.7	2.0
	L2	2.4	7.3	1.6	3.6
	L3	6.5	6.6	6.9	4.1
Method precision [RSD%] – inter-day	L1	6.6	3.7	0.7	6.3
	L2	5.0	6.0	4.1	12.4
	L3	2.8	15.0	8.9	15.9
Matrix effect [%]	L1	102.7	108.0	107.0	100.9
	L2	107.6	82.2	109.9	86.3
	L3	118.3	103.5	97.5	83.9
LOD [nmol/l]		1.5	1.5	1.5	1.5
LOQ [nmol/l]		5	5	5	5

PV, pravastatin; PV-D3, pravastatin deuterium labeled; PVL, pravastatin lactone; PVL-D3, pravastatin lactone deuterium labeled; L1, L2, L3, concentration 500, 50 and 5 nmol/l; LOD, LOQ, values for the matrix calibration curve; L1, L2, L3, concentration level 1, 2, 3 (500, 50 and 5 nmol/l).

the retention time (RSD < 0.5%) and for peak area (RSD < 6%) with standard solution. The results of SST and validation are shown in Table 3.

Method linearity and sensitivity – two types of calibration curves using stable isotopically labeled internal standards were measured, matrix and standard calibration curves. The response was linear in the calibration range 5–500 nmol/l for both analytes (r^2 > 0.9990), therefore the calibration curves could be used for quantitative purposes. Inter-day reproducibility of calibration curve was estimated as % RSD and was lower than 5%. Limits of detection and quantification for all analytes in real matrix were LOD = 1.5 nmol/l and LOQ = 5 nmol/l respectively.

Method accuracy and precision were established at three concentration levels of calibration curve, at high (500 nmol/l), medium (50 nmol/l) and low (5 nmol/l). Method accuracy expressed as recovery was within the range of 97–109% for the plasma samples and of 92–101% for urine samples. Method precision was measured as intra- and inter-day variability at three different concentration levels expressed as % RSD (see Table 3). Intra-day precision for pravastatin and pravastatin lactone in plasma samples was lower than 8% and in urine samples it was lower than 7%. Inter-day precision was lower than 15% for both analytes in plasma and urine samples.

The matrix effects were evaluated as the comparison of standard solution and spiked blank plasma samples, which was first treated by MEPS and subsequently spiked by standard solution. Matrix effect values ranged from 82 to 118% for plasma samples and 84 to 110% for urine samples (Table 3). The matrix effects were up to 10% in most cases. Only three values were worse. The matrix effects (<18%) are acceptable for biological sample and the method was found to be selective enough using UHPLC–MS/MS in connection with MEPS sample preparation step.

The carry-over between individual samples was verified by MEPS extraction of blank plasma and urine. To eliminate the memory effect the MEPS sorbent was washed three times by acetonitrile after every extraction. The carry-over was negligible (<0.01%).

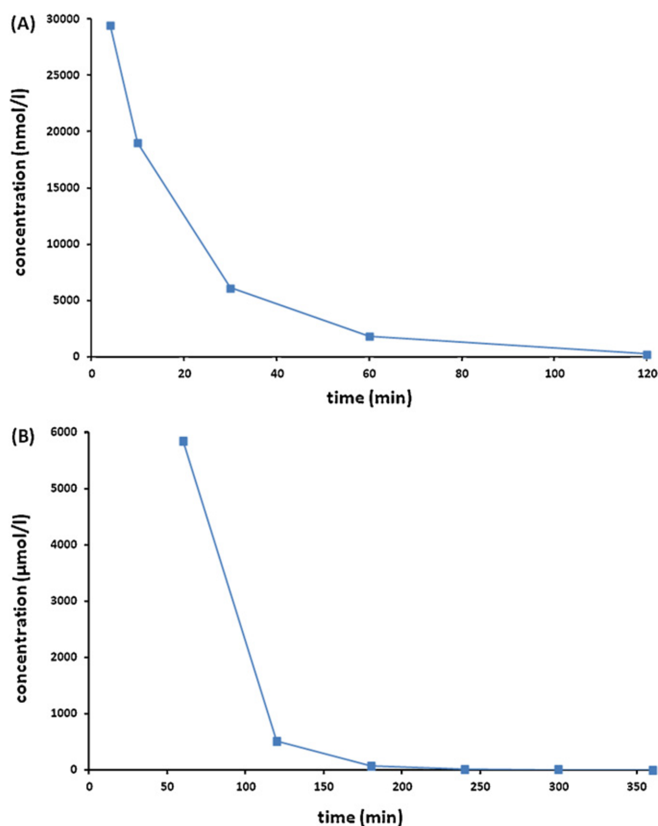

Fig. 3. Pharmacokinetic profile of rat pravastatin measured in rat plasma samples (A) and urine samples (B).

Short-term stability of analytes was assessed at pH 3, and at pH 4 using 0.5 M ammonium acetate buffer (dilution media). Long-term stability was assessed in stock solvent (mixture of ACN and 1 mM

ammonium acetate pH 4.0 (90:10) at 20, 4 and −18 °C. After short term storage at 4 °C, at pH 3 and pH 4 pravastatin and pravastatin lactone were stable for at least 13 h with concentration deviation lower than 5%. During long-term stability both analytes were stable in stock solution at 20, 4 and −18 °C at least 4 weeks with concentration deviation lower than 5%. The results demonstrated that pravastatin had better stability at pH 4. Pravastatin lactone on the other hand was stable at pH 3. These results demonstrated that suitable storage condition in stock solvent at appropriate pH and temperature can significantly increase the stability.

3.4. Application to real samples

Newly developed UHPLC–MS/MS method with MEPS as the sample preparation for the determination of pravastatin and pravastatin lactone was applied to the plasma and urine samples of rats administered intravenously by pravastatin. Sequential blood samples during pharmacokinetic study allowed only minimal volume to be taken. Usage of the microextraction method was therefore essential step for the drug analysis because only about 50 μl of plasma was required, which allowed repeated experiments.

Seven plasma and urine samples (samples at regular intervals) were obtained from one rat. The concentration of pravastatin and pravastatin lactone were determined. This approach enabled detailed characterization of pravastatin and its lactone metabolite concentration-time profiles during initial periods after drug administration including kinetics of their renal excretion (Fig. 3).

4. Conclusions

A fast, sensitive and selective method was developed for the determination of pravastatin and pravastatin lactone by UHPLC–MS/MS in rat urine and plasma samples. MS/MS detection utilized two SRM transitions for each compound to ensure high selectivity and reliability of the method. Deuterium labeled internal standards pravastatin D3 and pravastatin lactone D3 were used for precise and accurate quantification.

A sample pretreatment by means of MEPS was applied for these analytes. Maintaining of pH between 4 and 5 was important to prevent the interconversion of analytes. Therefore ammonium acetate was used for the stabilization of samples during sample preparation and as the part of the mobile phase. The analyses were carried out using small sample volume (50 μl) and in a short time period compared to the methods included in Table 2. The MEPS cartridges could be used more than 60 times (probably more, but more samples were not tested), with urine and precipitated plasma sample. Using the eVol hand-held automated analytical syringe removed the influence of operator on the speed of plunger movement and manual injection of very small volumes of samples (<50 μl) which is very critical for the repeatability and recovery of analytes. In comparison with the other published methods MEPS-UHPLC–MS/MS method provided similar sensitivity, but offers many advantages. It enables fast and simple sample preparation using small volume of sample, washing and elution solvent therefore it is regardful, environmentally friendly and suitable for the samples with limited volume availability such as plasma from small laboratory animals.

The MEPS-UHPLC–MS/MS method for the determination of pravastatin and pravastatin lactone was validated according to the requirements of ICH with good results of linearity, precision, accuracy and matrix effect. Thus the proposed method could be used for the determination of analytes in the rat urine and plasma samples and to evaluate pharmacokinetic profiles.

Acknowledgement

The authors gratefully acknowledge the financial support of research projects UNCE 17/2012 and SVV/2011/263002.

References

[1] H. Vlčková, D. Solichová, M. Bláha, P. Solich, L. Nováková, J. Pharm. Biomed. Anal. 55 (2011) 301.
[2] R. Nirogi, K. Mudigonda, V. Kandikere, J. Pharm. Biomed. Anal. 44 (2007) 379.
[3] D.S. Jain, G. Subbaiah, M. Sanyal, V.K. Jain, P. Shrivastav, Biomed. Chromatogr. 21 (2007) 67.
[4] Z. Zhu, L. Neirinck, J. Chromatogr. B 783 (2003) 133.
[5] D. Mulvana, M. Jemal, S.C. Pulver, J. Pharm. Biomed. Anal. 23 (2000) 851.
[6] M. Jemal, Z. Quayang, Rapid Commmun. Mass Spectrom. 14 (2000) 1757.
[7] Y. Koitabashi, T. Kumai, N. Matsumoto, M. Watanabe, S. Sekine, Y. Yanagida, S. Kobayashi, Life Sci. 78 (2006) 2852.
[8] K. Otter, Ch Mignat, J. Chromatogr. B 708 (1998) 235.
[9] R.W. Sparidans, D. Iusuf, A.H. Schinkel, J.H.M. Schellens, J.H. Beijnen, J. Chromatogr. B 878 (2010) 2751.
[10] B. Mertens, B. Cahay, R. Klinkenberg, B. Streel, J. Chrometogr. A 1189 (2008) 493.
[11] S. Bauer, J. Mwinyi, A. Stoeckle, T. Gerloff, I. Roots, J. Chromatogr. B 818 (2005) 257.
[12] J.W. Deng, K.B. Kim, I.S. Song, J.H. Shon, H.H. Zhou, K.H. Liu, J.G Shin, Biomed. Chromatogr. 22 (2008) 131.
[13] K. Kawabata, N. Samata, Y. Urasaki, J. Chromatogr. B 816 (2005) 73.
[14] M. Jemal, Y. Qing, D.B. Whigan, Rapid Commun. Mass Spectrom. 12 (1998) 1389.
[15] L. Nováková, H. Vlčková, Anal. Chim. Acta 656 (2009) 8.
[16] M. Abdel-Rehim, Anal. Chim. Acta 701 (2011) 119.
[17] P.L. Kole, G. Venkatesh, J. Kotecha, R. Sheshala, Biomed. Chromatogr. 25 (2011) 199.
[18] M. Abdel-Rehim, J. Chromatogr. A 1217 (2010) 2569.
[19] A.R. Chaves, F.Z. Leandro, J.A. Carris, M.E.C. Queiroz, J. Chromatogr. B 878 (2010) 2123.
[20] M.A. Saracino, K. Tallarico, M.A. Raggi, Anal. Chim. Acta 661 (2010) 222.
[21] R. Said, M. Kamel, A. El-Beqqali, M. Admel-Rehim, Bianalysis 2 (2010) 197.
[22] A. El-Beqqali, A. Kussak, L. Blomberg, M. Abdel-Rehim, J. Liq. Chromatogr. R.T. 30 (2007) 575.
[23] A. El-Beqqali, A. Kussak, M. Abdel-Rehim, J. Sep. Sci. 30 (2007) 421.
[24] M. Abdel-Rehim, M. Dahlgren, L. Blomberg, J. Sep. Sci. 29 (2006) 1658.
[25] International Conference on Harmonization (ICH). Q2 (R1): Text on Validation of Analytical Procedures, US FDA Federal Register, 2005.