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# Microextraction by packed sorbent as sample preparation step for atorvastatin and its metabolites in biological samples—Critical evaluation

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ABSTRACT

Atorvastatin belongs to the group of lipid-lowering drugs known as statins. They significantly reduce the levels of total cholesterol, low-density cholesterol and plasma triglycerides therefore they are widely used in the treatment of hypercholesterolemia. Recently developed methods for the determination of atorvastatin and its metabolites in plasma used SPE (solid phase extraction) or LLE (liquid–liquid extraction) as the sample preparation step. However, both procedures are quite time-consuming and need relatively high volume of solvent/sample, which is impractical for the routine analyses of many biological samples.

The aim of this work was to develop and validate more suitable sample preparation method for the determination of atorvastatin and its metabolites in biological samples using MEPS (microextraction by packed sorbent). The optimal conditions of MEPS extraction were using C8 sorbent and only 50 μl of the sample. The analytes were eluted by 100 μl of the mixture of acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). The analytical method was validated and demonstrated good linearity ($r^2 > 0.9990$), recovery (89–115%) and intra-day precision (RSD < 10%). Total time of the sample preparation was three times shorter (7 min) compared to SPE. The volume of sample was twenty times lower and the volume of solvents about ten times lower compared to SPE. Combination of fast MEPS method together with quick UHPLC–MS/MS was used for the determination of atorvastatin and its two metabolites in serum obtained from patients with familiar hypercholesterolemia.



## 1. Introduction

Recently, UHPLC–MS/MS becomes a leading trend in modern bio-analytical methods [1]. There is however a great contrast between ultra-fast chromatographic analysis and conventional sample preparation, which remains highly labour-intensive and time-consuming. Conventional sample preparation techniques such as SPE (solid phase extraction), LLE (liquid–liquid extraction) and PP (protein precipitation) are still dominating in sample preparation area even though many modern approaches including MIP (molecularly imprinted polymers, a method with enhanced selectivity), various microextractions, such as SPME (solid phase microextraction), LLME (liquid–liquid microextraction), MEPS (microextraction by packed sorbent), and an on-line sample preparation techniques using RAM (restricted access material) or in-tube SPME and many others have been developed and introduced in practical use [1]. All these modern sample preparation approaches have gained an attention however, for the moment they have not replaced conventional sample preparation techniques.

Microextraction by packed sorbent is one of the quite new sample preparation techniques developed in the laboratories of Astra Zeneca in 2004 [2,3]. Actually, MEPS is a miniaturization of conventional SPE. Sample preparation there takes place on the packed bed. MEPS can thus be described as a short "LC column in a syringe". The bed dimensions are scaled from a conventional SPE bed and in this way MEPS can be adapted to the most existing SPE methods by simply scaling the reagents and sample volumes from the conventional device to MEPS.

In MEPS, approximately 1–2 mg of solid packing material is either inserted into the barrel of a syringe (100–250 μl) as a plug with polyethylene filters on both sides, or between the syringe barrel and the injection needle as a cartridge (Fig. 1). The bed can be packed or coated to provide selective and suitable sampling conditions. In MEPS, any sorbent material can be used either as packing bed or as a coating. Commercially available sorbents include silica based C2, C8, C18 and M1 (mixed C8 and SCX) [2]. The key factor in MEPS is that the volume of solvent used to elute the analytes from the extraction process is of a suitable order of magnitude to be injected directly on-line into an LC [3–5] or GC [2]

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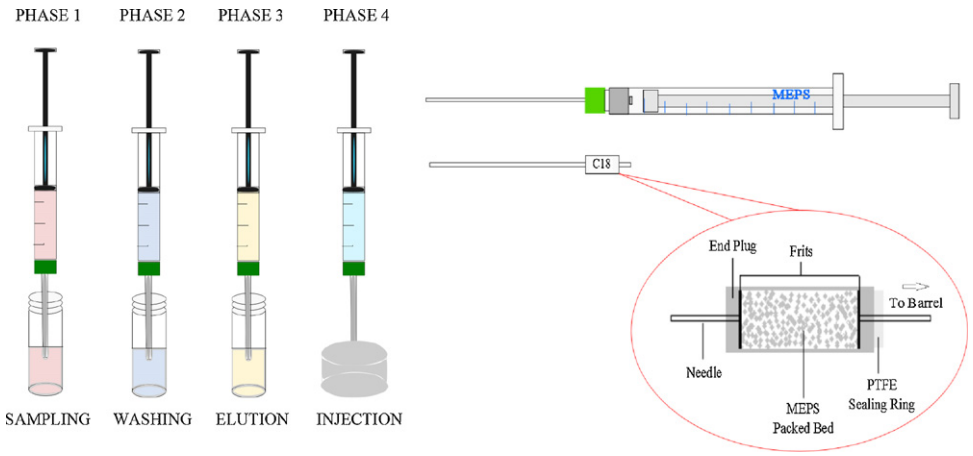


**Fig. 1.** Schematic of microextraction by packed sorbent [1].

system without any modification of the instrument. Connection to a robot makes the method fully automated. MEPS can handle small sample volumes (10 μl of plasma, urine or water) as well as relatively large volumes (1000 μl). MEPS technique differs from commercial SPE in that the packing is inserted directly into the syringe, not into a separate column. Thus, there is no need for a separate robot to apply the sample on the solid phase, which is on the other hand necessary in case of conventional SPE if automation is required.

The MEPS technique has been used to extract various analytes from biological samples. Several drugs such as local anaesthetics and their metabolites [2,3]; the anticancer drugs roscovitine [4], olomoucine [5], cyclophosphamide [6] and busulfan [7]; the β-blocker drugs acebutolol and metoprolol [8]; the anti-depressant drugs dopamine and serotonine [9] as well as anti-addictive methadone [10] have been successfully extracted by MEPS from biological samples such as plasma, urine or blood.

Statins are drugs widely used for the treatment of hypercholesterolemia and also of its severe forms such as familiar hypercholesterolemia. They have potent cholesterol-lowering effect and they significantly reduce morbidity and mortality associated with coronary heart disease as it was proved by many clinical trials [11–13]. Therapeutic range of statins is typically 10–80 mg/day. High doses might 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. In order to establish and control appropriate dosage scheme, which would minimize adverse effects and keep the cholesterol lowering effect, it would be very helpful to monitor the levels of statins in biological materials. Moreover, the method would be very useful for the determination of possible loses of statins during extracorporeal elimination procedures such as hemodialysis, LDL-apheresis.

Statin molecules exist in two forms, lactone and open-ring hydroxy acid form [14,15]. In vivo, the hydroxy acid forms are the active drugs to lower plasma cholesterol while the lactone forms are inactive prodrugs. Atorvastatin is one of the drugs worldwide the most commonly occurring in commercially available pharmaceutical formulations used in the clinical treatment of hypercholesterolemia. It is administered in the open-ring hydroxy acid form – the active form. It is absorbed from the gastrointestinal tract and it undergoes an extensive first-pass metabolism in the liver, which produces two active hydroxy metabolites, ortho-hydroxyatorvastatin and para-hydroxyatorvastatin and three inactive lactones (Fig. 2). More than 90% of atorvastatin 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 [16–18]. 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 typical plasma concentration range of active metabolites of atorvastatin is between 0.1 and 20 ng/ml.

The methods for the determination of simvastatin and atorvastatin were recently reviewed by our group [19]. In clinical applications HPLC–MS/MS was unequivocally the method of choice in analysis of atorvastatin together with its metabolites [20–25] using typically ESI (electrospray ionization) in positive ion mode. SRM (selected reaction monitoring) transition 559 › 440 was monitored for atorvastatin molecule. Recently only four new methods for the determination of atorvastatin were published. Developed HPLC-UV method for the determination of atorvastatin in human plasma however did not determine metabolites together with atorvastatin and used liquid–liquid–liquid microextraction (LLLME) as the sample preparation technique [26]. Another new method for the determination of atorvastatin together with fenofibrate used the UHPLC with UV detection. It was developed and validated for the analysis of atorvastatin, fenofibrate and their degradation products in tablets but not in biological samples therefore the sensitivity of UV detection was insufficient for clinical applications [27]. For the determination of atorvastatin, simvastatin and lovastatin HPLC method with CAD (charged aerosol detector) was applied [28] and the sensitivity of UV and CAD was compared. UHPLC–MS/MS method for the determination of atorvastatin and simvastatin, their metabolites and interconversion products in biological samples was developed and validated with good reproducibility, sensitivity and selectivity and it was applied to serum and lipoprotein fractions in our laboratory [29]. Sample preparation step in bio-analytical assays of atorvastatin and its metabolites employed mostly LLE [21–24] or SPE [20,25,29]. Both approaches are multi-step, time-consuming and also the consumption of sample and organic solvent is quite high, especially in case of LLE. That is not suitable for the routine analyses of huge number of samples therefore new preferable sample preparation procedure would be convenient.

Over the last year only one article presented another sample preparation technique than LLE or SPE. Farahani et al. [26] presented a rapid and economical liquid–liquid–liquid microextraction method for the determination of atorvastatin in human plasma for screening purposes, however no metabolites were determined.

The aim of the work was to develop fast, easy and low-volume (for both – sample and organic solvent volume) sample preparation technique convenient for routine preparation of biological samples containing atorvastatin and its metabolites. On-line MEPS extrac-

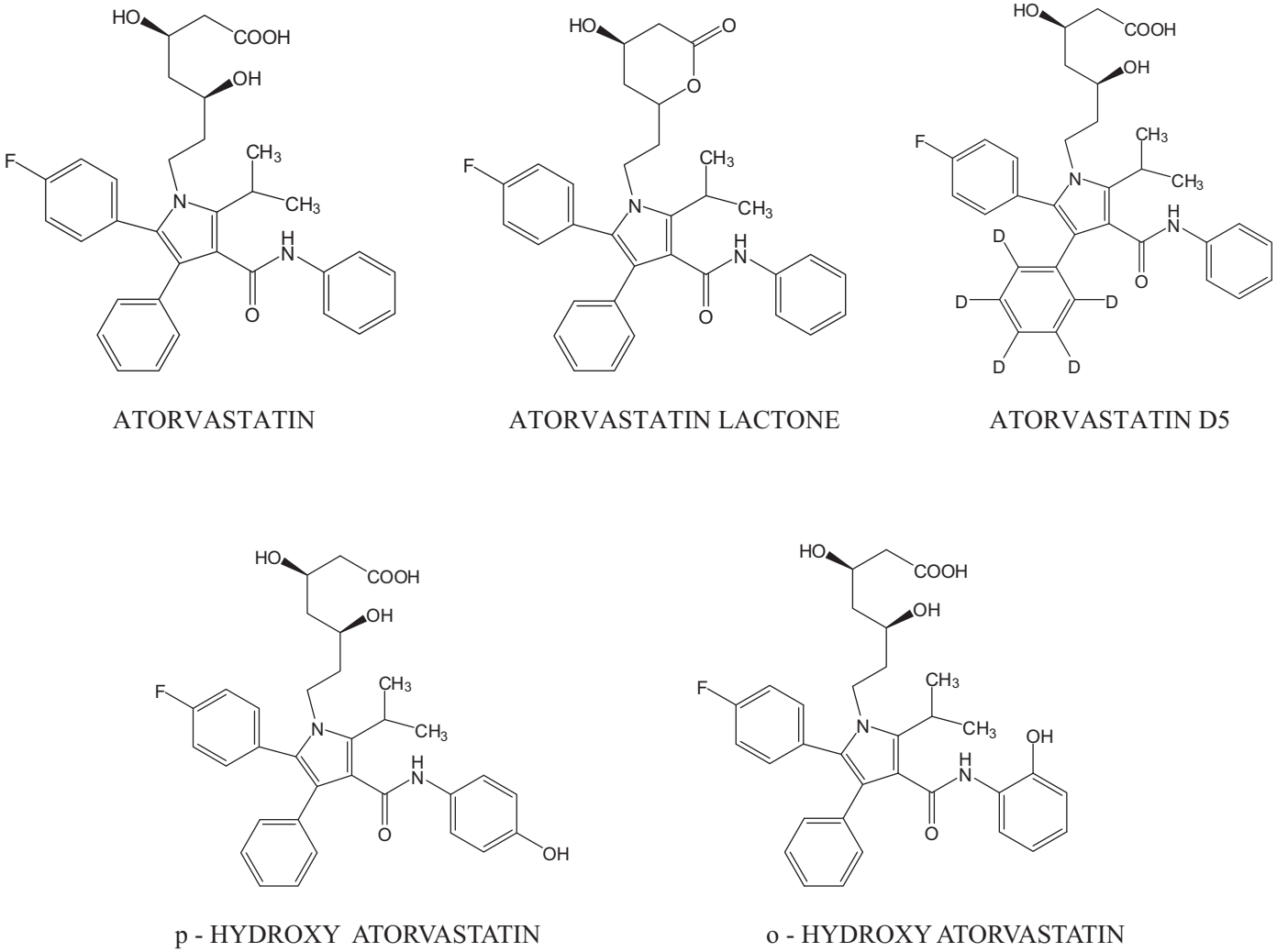


**Fig. 2.** Chemical structure of atorvastatin, atorvastatin hydroxy metabolites and atorvastatin lactone.

tion was previously described as a method convenient for these purposes [2–10] however on-line coupling is not possible with every HPLC or UHPLC system. Therefore the evaluation of MEPS procedure in off-line arrangement is highly valuable.

## 2. Experimental

### 2.1. Chemicals and reagents

Working standards of atorvastatin, atorvastatin lactone, p-hydroxyatorvastatin, o-hydroxyatorvastatin and atorvastatin deuterium labeled (D5 – phenyl ring) were purchased from Toronto Research Chemicals (Ontario, Canada).

Acetic acid, reagent grade, ammonia, reagent grade and 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. UHPLC–MS/MS analysis was performed according to the previously published method [29] with only slight modification. UHPLC 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 pH 4.0 using gradient elution with initial mobile phase composition acetonitrile:ammonium acetate (30:70). Thereafter the concentration was changed within 1.5 min to 70% of acetonitrile and subsequently to 95% of acetonitrile within 5.25 min. Mobile phase flow rate was 0.25 ml/min. The analytical column was kept at 35 °C. 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 positive mode according to [29] as follows: capillary voltage: 3500 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 0.5 V. The desolvation gas was nitrogen at flow 500 l/h and at the temperature 375 °C. Nitrogen was used also as a cone gas (120 l/h). Cone voltage (CV) was set up individually for each analyte. Quantitation of all analytes was performed using SRM (selected reaction monitoring) experiment. Two specific transitions were optimized for each molecule and secondary ion ratio was calculated in order to increase selectivity of the method. Argon was used as collision gas and collision energy (CE) was optimized for each analyte individually – see [29]. The MassLynx 4.1 Data System was used for MS control and data gathering. QuanLynx software was used for data processing and quantitation – regression anal-

ysis of standard and 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 mM of appropriate working standard into 1.0 ml of dissolution media according to the solubility properties, because the molecules differ significantly in solubility. The stock solutions of atorvastatin lactone were prepared in pure acetonitrile. The stock solutions of atorvastatin, atorvastatin D5 and atorvastatin hydroxy-metabolites were prepared in mobile phase used at initial step of gradient elution – acetonitrile:ammonium acetate 0.5 mM, pH 4.0 (30:70, v:v). Stock solutions were further diluted by mobile phase (keeping the pH of solution between 4.0 and 5.0 in order to prevent the interconversion) to achieve individual points of calibration curve in the range 0.1–100 nM, using seven calibration points (100, 50, 10, 5.0, 1.0, 0.5 and 0.1 nM). Stock solutions of all tested compounds were stable for two weeks at 20, 4 and −18 °C with a mean percentage change of <3%. Thereafter, fresh stock solutions were prepared.

### 2.4. Sample preparation – MEPS and SPE

Serum samples were prepared using both methods: newly developed MEPS technique and SPE procedure described previously [29]. Briefly, in SPE internal standard atorvastatin D5 (100 μl) was added to 900 μl of the serum samples containing the analytes. 500 μl of sample 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 pH 4.5. The SPE cartridge with loaded sample was washed with 2 ml of 0.1 M ammonium acetate pH 4.5 and 1 ml of mixture of acetonitrile:0.01 M ammonium acetate pH 4.5 (15:85, v:v), and subsequently the analytes were eluted with 1 ml of acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). The eluate was filtered via 0.20 μm PTFE filter and sample was injected onto UHPLC system.

In MEPS C8 sorbent packed in bin, which is inserted into a needle assembly connected to 100 μl syringe was used (SGE Analytical Science, Germany). The sorbent was activated three times with 100 μl of acetonitrile and conditioned three times with 100 μl of 0.1 M ammonium acetate pH 4.5. 50 μl of sample was aspirated through the syringe. The sorbent was washed two times with 100 μl of 0.1 M ammonium acetate pH 4.5 and consequently with 100 μl of mixture acetonitrile:0.01 M ammonium acetate pH 4.5 (15:85, v:v). The analytes were eluted with 100 μl of mixture of acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). The eluate was filtrated via PTFE microfilter (4 mm × 0.2 μm) and the sample was transferred into the micro insert of vial and injected onto UHPLC system.

Serum samples were kept at −80 °C and after the thaw cycle they were processed immediately by MEPS procedure and analyzed by UHPLC–MS/MS. The change in serum samples concentration at 4 and −18 °C was not greater than 15% within the period of two weeks, which is in agreement with the results of method precision and accuracy.

### 2.5. Method validation

The newly developed MEPS–UHPLC–MS/MS method was validated in terms of linearity, accuracy, precision, selectivity and sensitivity (limits of detection and quantitation) according to the requirements of ICH (International Conference on Harmonization) [30]. For the determination of linearity, two calibration curves of all analytes were prepared: matrix calibration curve using blank serum sample, which was spiked and then treated by MEPS procedure (1) in the concentration range 0.5–100 nM and standard calibration curve (2) where stock standard solutions were diluted by mobile phase in the concentration range 0.1–100 nM.

For method precision, spiked blank serum 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. Inter-day and intra-day precision was measured.

Method accuracy was described as the recovery experiment. Recovery was determined via a comparison of the response of serum samples spiked prior to MEPS extraction with that of blank serum samples that were first treated by MEPS procedure and then it was spiked with the analytes. It was complemented at three different levels in three replicates to establish the closeness of agreement between the true and measured value as it corresponds to ICH requirements [30]. 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. Matrix effect was evaluated using blank serum samples, which were first treated by MEPS procedure and than spiked by standard solution at three concentration levels within the calibration range. The results were compared with the measurement of standard calibration curves (2) and matrix effects were calculated. Limits of detection and quantitation were 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.

The stability of samples in standard stock solutions was evaluated at 20, 4 and −18 °C and in serum samples at 4 and −18 °C in a short-term and long-term measure.

### 2.6. Patients

The long-term patients included 2 men in the age of 44 and 65 years, regularly treated for 7.7 and 9.6 years with extracorporeal elimination procedures – one with LDL-apheresis (Adsorbers Lipopak 400, Pocard, Moscow, Russia) and the second with hemorheopheresis (filters Evaflux 4A, Kuraray, Osaka, Japan). The clinical phenotype of familiar hypercholesterolemia was characterised by an increased level of total plasmatic cholesterol and LDL-cholesterol, and by the occurrence of xanthomas and premature symptoms of ischemic heart disease. The patients were treated with high-doses of statins (maximally tolerated dose – 40 and 80 mg of atorvastatin daily) and with ezetimib (both with 10 mg daily). The use of patient data and samples for the research purposes was approved by an Ethical Committee and patients gave their written consent.

For the evaluation of atorvastatin levels and its metabolites, two blood samples were taken: one just before the start of the extracorporeal elimination procedure, the second just after extracorporeal elimination procedure (LDL-apheresis, hemorheopheresis). The blood was drawn from needle inserted into two peripheral veins before the start of procedures at 9.00 a.m., and the second blood sample was drawn just after procedures i.e. after 4–5 h later. 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.

## 3. Results and discussion

### 3.1. Development of MEPS procedure

The sample preparation procedure was optimized using off-line MEPS arrangement and two different MEPS cartridges – C18 and C8. Although C18 sorbent was used for the SPE extraction, C8 was chosen for the MEPS extraction, because it provided better results for recovery and precision during method validation especially for

**Table 1**
Validation results – linearity, method accuracy, precision, recovery, LOD and LOQ.

| Method validation | p-OH AT | o-OH AT | AT | ATL |
|---|---|---|---|---|
| Linearity ($r^2$) – matrix calibration curve | 0.9992 | 0.9990 | 0.9993 | 0.9993 |
| Linearity ($r^2$) – standard calibration curve | 0.9997 | 0.9998 | 0.9996 | 0.9997 |
| Method accuracy [%] | | | | |
| L1 | 106.38 | 97.09 | 104.55 | 95.01 |
| L2 | 98.54 | 93.32 | 101.45 | 89.10 |
| L3 | 103.99 | 103.80 | 115.81 | 111.24 |
| Method precision [RSD %] | | | | |
| L1 | 6.37 | 2.73 | 1.99 | 4.60 |
| L2 | 1.95 | 9.78 | 1.43 | 4.42 |
| L3 | 7.75 | 7.78 | 1.50 | 2.90 |
| Matrix effect [%] | | | | |
| L1 | 89.30 | 101.38 | 98.39 | 94.69 |
| L2 | 94.30 | 99.00 | 95.58 | 93.29 |
| L3 | 98.88 | 100.55 | 101.64 | 105.63 |
| LOD [nM] | 0.33 | 0.15 | 0.03 | 0.15 |
| LOQ [nM] | 0.66 | 0.49 | 0.08 | 0.18 |

p-OH-AT: p-hydroxyatorvastatin, o-OH-AT: o-hydroxyatorvastatin, AT: atorvastatin, ATL: atorvastatin lactone, L1, L2, L3: concentration 100, 10 and 5 nM, LOD, LOQ: values for the matrix calibration curve.

atorvastatin metabolites. The reason might be the differences in C18 sorbents among individual producers, who consequently offer SPE sorbent of various properties (specific surface area, average pore diameter, surface pH, density of C18 ligand bonding, metal content etc.).

As MEPS and SPE extractions principles are similar the same washing and elution solvents were used [29]. Various elution and sample volumes were tested and finally the washing step was optimized in order to obtain clean extracts. Serum sample MEPS extraction procedure was optimized as following:

An internal standard (25 μl) was added to 225 μl of serum samples containing statins. 50 μl of this sample was drawn by means of syringe through the MEPS sorbent previously activated with 300 μl of acetonitrile and conditioned with 300 μl of 0.1 M ammonium acetate pH 4.5. MEPS sorbent with retained sample was washed with 200 μl of 0.1 M ammonium acetate pH 4.5 and 100 μl of mixture acetonitrile:0.01 M ammonium acetate pH 4.5 (15:85, v:v) and subsequently the analytes were eluted with 100 μl of mixture acetonitrile:0.1 M ammonium acetate pH 4.5 (95:5, v:v). Finally, the eluate was filtered via 0.2 μm PTFE filter and sample was injected onto UHPLC system.

During the development of off-line manual MEPS extraction procedure several problems arose. Although MEPS extraction should enable use of very small volume of elution solvents (until 20–50 μl) and samples (until 10 μl), this is suitable only for the automated MEPS set-up, where the MEPS syringe is connected on-line to an LC. Therefore the most of published articles presented a use of fully automated MEPS system connected on-line to an LC. However, this does not apply for the manual arrangement of MEPS. The main disadvantages of manual arrangement of MEPS are following: non-availability of vial inserts smaller than 100 μl, dependence of analyte recovery on the continuous speed of the movement of the plunger and inaccurate manual manipulation with volumes lower than 50 μl through the 100 μl syringe. In order to solve all above mentioned arising problems, the volumes of sample and solvents must be greater than 50 μl and continuous speed of the movement of plunger must be maintained during the extraction. Both features are very critical for off-line manual MEPS arrangement and are not in agreement with the on-line MEPS procedure.

### 3.2. Validation of UHPLC–MS/MS method using MEPS as sample preparation

A development of UHPLC–MS/MS method used for the determination of atorvastatin and its metabolites in biological samples was described previously [29], including method optimization, SST (system suitability test) measurements and method validation. Validation parameters including linearity, method recovery, accuracy, precision and limits of detection and quantification were evaluated newly because another sample preparation technique employing MEPS was introduced. The results could be seen in Table 1. Method linearity was measured in the calibration range 0.1–100 nM for standard calibration curves (2) and 0.5–100 nM for matrix calibration curves (1). An internal standard (atorvastatin-D5) was used for quantitation. The response was linear in tested concentration range for all analytes ($r^2 > 0.9990$), therefore the calibration curves could be used for quantitative purposes. Method accuracy expressed as recovery was established at the three concentration levels of calibration curve – at high (100 nM), medium (10 nM) and low (5 nN). Method accuracy ranged from 89 to 116%. Method precision was measured using spiked blank serum treated by MEPS at three different concentration levels in three replicates and finally RSD (%) was calculated. Intra-day precision for atorvastatin and atorvastatin lactone was lower than 5% and for hydroxy metabolites of atorvastatin it was lower than 10%. Interday precision values were also lower than 10% for each measured analyte (data not presented). The matrix effect was evaluated as the comparison of standard solution and spiked blank serum sample, which was first treated by MEPS and subsequently spiked by standard solution. Matrix effect values ranged from 93 to 105% therefore no significant matrix effects were observed and the method was found to be selective using UHPLC–MS/MS in connection with MEPS sample preparation step. Limits of detection were expressed as S/N = 3 and limit quantification as S/N = 10. LOD and LOQ for all analytes in real matrix could be seen in Table 1 and their values correspond to the values of LOD and LOQ in standard solution described in previously published article [29].

### 3.3. Application to real samples

Newly developed UHPLC–MS/MS method with MEPS as the sample preparation for the determination of atorvastatin and its metabolites was applied to the serum samples of patients with familiar hypercholesterolemia treated by atorvastatin and simultaneously by extracorporeal elimination procedures (LDL-apheresis, rheopheresis).

Both MEPS and SPE sample preparation methods were used for the treatment of the same real samples of serum, which were split before the procedure. The results of samples treated by MEPS and SPE procedures were compared by means of Student *t*-test (Table 2). The differences of the concentrations of measured

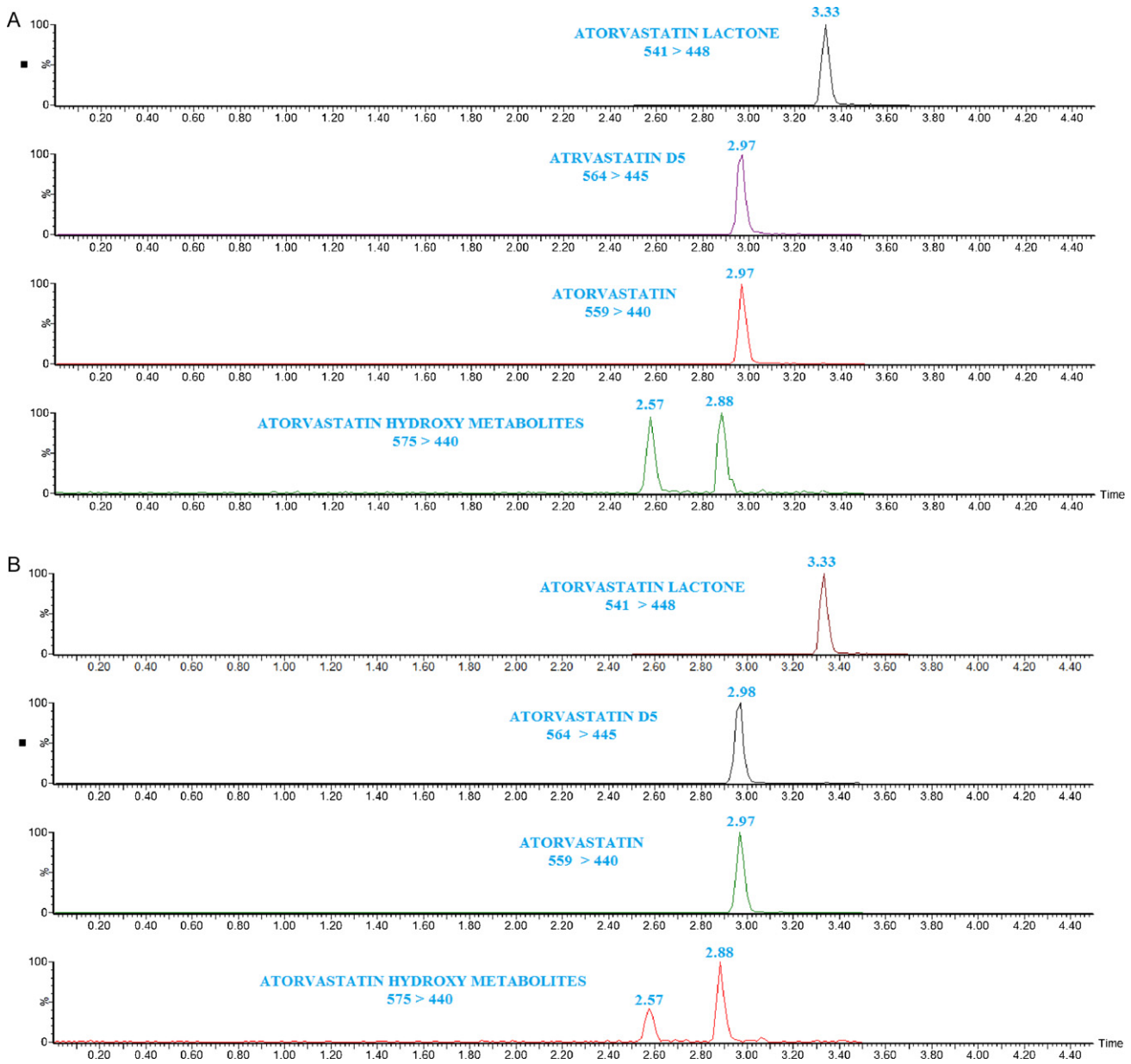


**Fig. 3.** UHPLC–MS/MS chromatogram of standard mixture of measured analytes (calibration level $5 \times 10^{-7}$ M) (A) and of serum samples – patient treated by atorvastatin (B).

analytes obtained by MEPS and by SPE were statistically non-significant.

Two blood samples (before and after extracorporeal elimination procedure) were obtained from one patient. In all samples of serum atorvastatin, atorvastatin lactone and its two metabolites were found (Fig. 3). Total losses of statins during the procedure were calculated. Such monitoring will enable individual adjustment of dosage scheme for each patient.

### 3.4. A comparison of SPE and MEPS extraction procedure

A traditional SPE was directly compared with newly developed MEPS procedure. The patient samples were split and were treated by both formerly developed SPE method [29] and newly developed MEPS. MEPS extraction procedure was found to be fast and simple method with good recovery using very small volume of sample, which is regardful to the patient and using a small volume of solvent, which is environmentally friendly approach. During MEPS extraction the evaporation of extract is not effected. Therefore it was found to be more suitable for the routine analysis of a large number of biological samples.

Moreover, it is possible to use MEPS cartridge repeatedly. We proved about 30 times re-use without any lose of extraction efficiency or sample carry-over (Fig. 4). On the other hand, one of the disadvantages of MEPS technique might be an unavailability of a great variability of sorbent chemistries, which are available for traditional SPE. Another great disadvantage of MEPS is a strong dependence of analyte recovery on continual movement of plunger and on rate of sample passing through the sorbent. This is a critical feature of manual manipulation, which requires skilled operators.

However, much higher speed of MEPS extraction procedure is overwhelming advantage in routine analyses in clinical laboratories. The comparison of data from MEPS and SPE extraction is displayed in Table 3 in detail.

**Table 2**
Atorvastatin and its metabolites in serum – comparison of results obtained using MEPS and SPE method.

| Compounds [nM] | p-OH AT | o-OH AT | AT | ATL |
|---|---|---|---|---|
| Patient no. 1 | | | | |
| Before EEP | | | | |
| MEPS | 3.29 | 5.71 | 14.04 | 9.12 |
| SPE | 3.35 | 6.43 | 14.07 | 9.45 |
| After EEP | | | | |
| MEPS | 1.23 | 2.32 | 4.30 | 3.09 |
| SPE | 1.38 | 1.91 | 4.63 | 4.03 |
| Patient no. 2 | | | | |
| Before EEP | | | | |
| MEPS | 1.53 | 1.59 | 2.80 | 2.67 |
| SPE | 1.80 | 1.55 | 2.78 | 2.47 |
| After EEP | | | | |
| MEPS | 1.25 | 0.93 | 1.71 | 1.85 |
| SPE | 1.46 | 1.17 | 1.67 | 1.70 |
| For the differences of MEPS and SPE | | | | |
| Average | 0.1725 | 0.3375 | 0.0763 | 0.2288 |
| Standard deviation | 0.2704 | 0.5683 | 0.2911 | 0.4971 |
| *t*-Values | 0.1609 | 0.1069 | 0.4412 | 0.3627 |
| Statistical significance | Non-significant | Non-significant | Non-significant | Non-significant |

p-OH-AT: p-hydroxyatorvastatin, o-OH-AT: o-hydroxyatorvastatin, AT: atorvastatin, ATL: atorvastatin lactone, LCL: lower confidence limit, UCL: upper confidence limit, EEP: extracorporeal elimination procedure, MEPS: microextraction by packed sorbent, SPE: solid phase extraction.

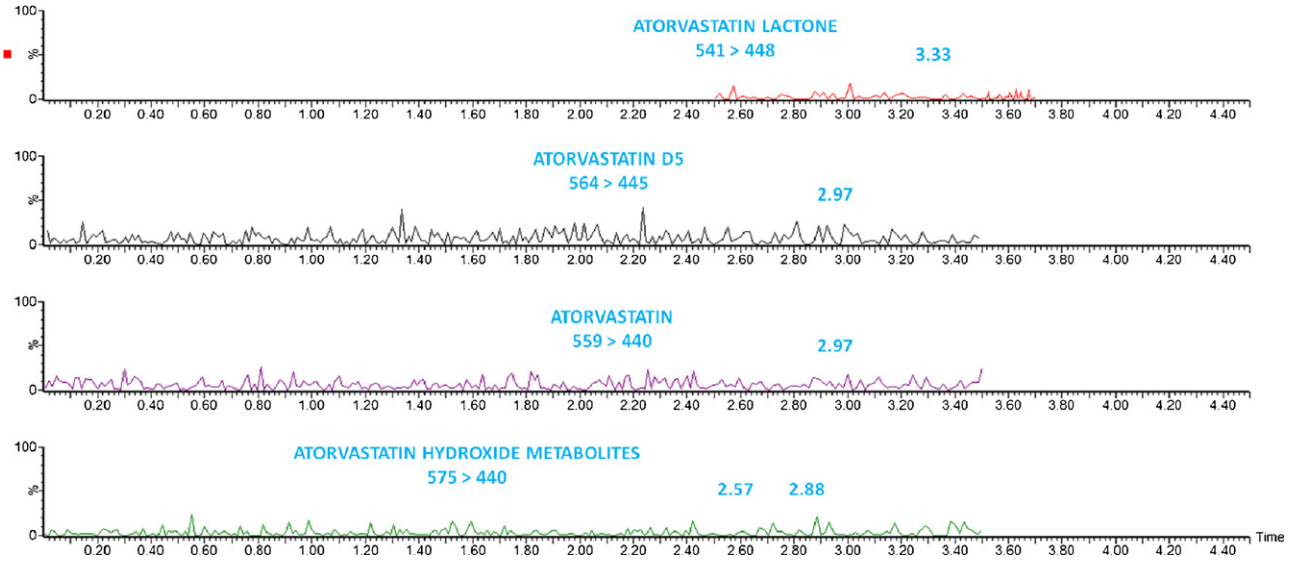


**Fig. 4.** Chromatogram of blank serum after the MEPS extraction: blank was prepared by MEPS procedure after the sample extractions. No carry-over effect was observed.

**Table 3**
Comparison of MEPS and SPE extraction.

| | SPE | MEPS |
|---|---|---|
| Total time of extraction | 25 min | 7 min |
| Type of sorbent | Silica based C18 (100 mg) | Silica based C8 (1–2 mg) |
| Sample volume | 500 μl | 50 μl |
| Solvent volume – conditioning | 2000 μl | 600 μl |
| Solvent volume – washing | 6000 μl | 600 μl |
| Elution volume | 1000 μl | 100 μl |
| Reuse of cartridge | 3–5× | 30× |
| Evaporation | Yes | No |

## 4. Conclusions

A new MEPS sample preparation method for the determination of atorvastatin and its metabolites was developed. MEPS extraction procedure is fast and simple sample preparation method using small volume of sample, washing and elution solvent therefore it is regardful to the patients and environmentally friendly. Because MEPS is less time-consuming, simpler and more regardful than formerly developed SPE method, this technique is predicted to be more suitable for the routine analyses of biological samples in clinical laboratories.

Using lower volume than 50 μl is not suitable for off-line arrangement of MEPS procedure compared to on-line MEPS arrangement which can use volumes even less than 10 μl. The effectiveness of MEPS extraction is highly dependent on the continuous speed of the movement of the plunger. From these reasons on-line connection of MEPS and chromatographic system is more convenient for routine analyses of large number of biological samples. However in case of precise manipulation with the syringe during sampling, using higher volume than 50 μl and the maintenance of continual plunger movement the manual MEPS method demonstrate good recovery, reproducibility within much shorter time needed for extraction (about 7 min). At these conditions manual MEPS method is further more suitable for the routine preparation of biological samples than SPE extraction.

A new MEPS method was validated with good results of linearity precision and accuracy. Analytes could be quantified at nM concen-

trations with typical LOQ 0.08–0.66 nM. MEPS method was used as the sample preparation method for the determination of atorvastatin and its metabolites in serum of two patients with familiar hypercholesterolemia treated by atorvastatin together with extracorporeal elimination procedure and it is planned to be applied to a large number of samples in routine clinical laboratory. MEPS is fast and simple method enabling determination of more serum samples within the same period of time compared to SPE or LLE and using less organic solvent and also less amount of sample thereby lower stress for the patients. For these reasons formerly developed SPE sample preparation method for determination of atorvastatin and its metabolites was replaced by MEPS sample preparation method.

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