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Method development for quantitative determination of seven statins including four active metabolites by means of high-resolution tandem mass spectrometry applicable for adherence testing and therapeutic drug monitoring

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Abstract

Background: Statins are used to treat and prevent cardiovascular diseases (CVDs) by reducing the total serum cholesterol concentration. Unfortunately, dose-related side effects and sub-optimal response, attributed to non-adherence amongst others, were described. Therefore, a fast and sensitive liquid chromatography-high-resolution tandem mass spectrometry (LC-HRMS/MS) method for adherence testing and therapeutic drug monitoring of all currently marketed statins and their active metabolites in human blood plasma should be developed, validated and tested for applicability.
Methods: Atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin, as well as ortho- and para-hydroxy-atorvastatin, lovastatin hydroxy acid and simvastatin hydroxy acid were included and several internal standards (IS) tested. Validation was performed according to the guideline of the European Medicines Agency including selectivity, carry-over, accuracy, precision, matrix effects, dilution integrity and analyte stability. Finally, applicability was tested using 14 patient samples submitted for regular toxicological analysis.
Results: Due to an analytical interference of atorvastatin-d5, diazepam-d5 and pentobarbital-d5 were chosen as IS for positive and negative ionization mode, respectively. All statins and metabolites fulfilled the validation acceptance criteria except for fluvastatin, which could not be quantified reliably and reproducibly, most probably due to instability. Analyses of human plasma samples revealed concentrations of statins and metabolites below the reference plasma concentrations in the case of eight patients. However, nothing was known concerning patients' adherence and time between intake and sampling.
Conclusions: An LC-HRMS/MS method for identification and quantification of atorvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin and four active metabolites was successfully developed and applicability demonstrated.

Keywords: adherence monitoring; cardiovascular diseases; HMG-CoA reductase inhibitors; statins; TDM.

Introduction

Cardiovascular diseases (CVDs) are the most common cause of death worldwide [1]. Their incidence can be directly correlated to elevated low-density lipoprotein cholesterol (LDL-C) concentrations in blood [2]. Statins, inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, are the most important cholesterol-lowering agents, which were also shown to have pleiotropic effects such as improved endothelial function, enhanced stability of atherosclerotic plaques, decreased oxidative stress, and inhibition of thrombogenic responses [3, 4].

Standard statin regimens have been well-established to reduce the risk of occlusive vascular events and international guidelines recommended the use of statins for a broad range of patients to prevent and treat CVD [2, 5, 6]. However, dose-dependent side effects such as myalgia and liver toxicity were frequently described [7, 8].

Recently, Akyea et al. reported a sub-optimal LDL-C response to statin therapy. The authors found that over half of patients started on statin therapy for primary prevention of CVD did not experience an optimal therapeutic reduction of their LDL-C levels 24 months after therapy initiation [9]. Individual biological and genetic variability, but also variations in adherence have been identified as main reasons

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[10, 11]. Both, the patients' non-adherence and the dose-dependent side effects underline the importance of measuring blood plasma concentrations of statins [7, 8]. Several methods for quantification of often one single statin were published in the past but a comprehensive method covering all relevant compounds is still missing [4, 12–24].

A liquid chromatography (LC)-high-resolution tandem mass spectrometry (HRMS/MS) method for identification and quantification of all currently marketed statins (atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin) in human blood plasma should be developed and validated [8]. Furthermore, the active metabolites ortho (o)-hydroxy-atorvastatin, para (p)-hydroxy-atorvastatin, lovastatin hydroxy acid and simvastatin hydroxy acid were included. Chemical structures of the parent compounds and the metabolites can be found in Figure 1. Standard daily doses, elimination half-life, and reference plasma concentrations are summarized in Table 1 [25]. The developed method should be applicable for adherence monitoring as well as therapeutic drug monitoring, demonstrated by analyses of authentic patient blood plasma samples.

Materials and methods

Chemicals

Simvastatin hydroxy acid ammonium salt, pitavastatin hemicalcium salt, p-hydroxy-atorvastatin calcium salt,

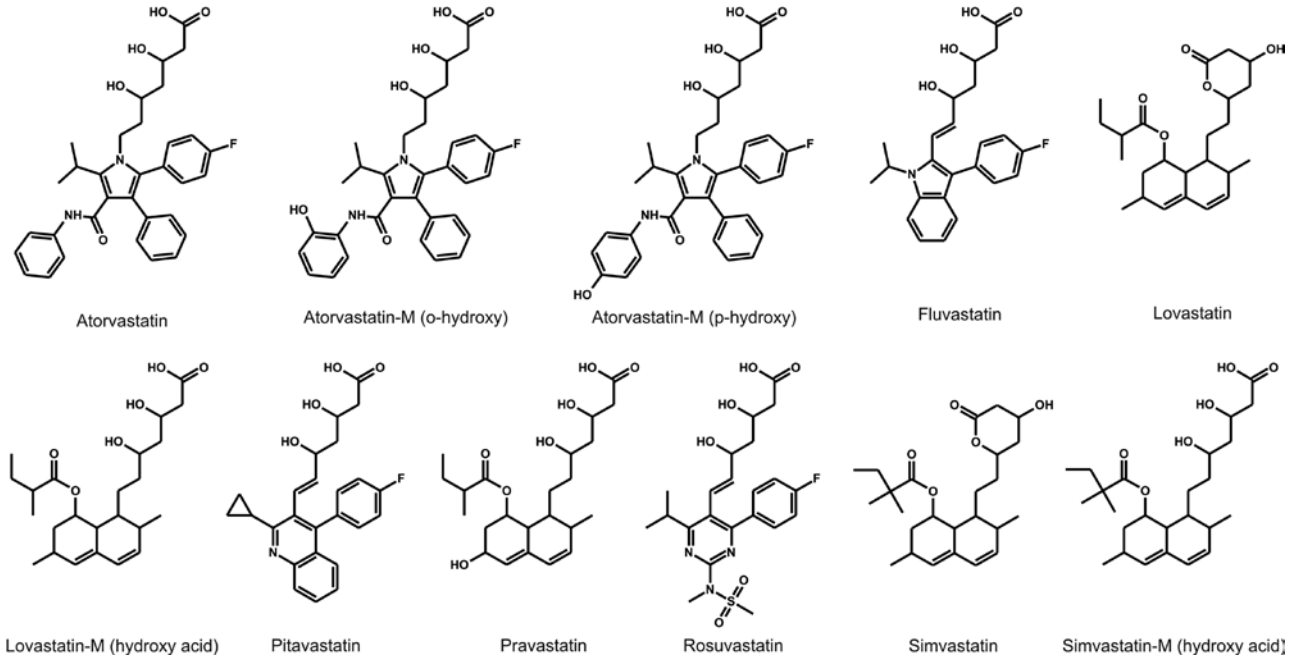

Figure 1: Chemical structures of the investigated seven statins and their four active metabolites.
M, metabolite.

Table 1: Standard daily dose, elimination half-life, and reference plasma concentration of seven statins according to [25].

Analyte	Standard daily dose, mg	Elimination half-life, h	Reference plasma concentration, ng/mL	
Atorvastatin	10–80	11–24	7–252	(Atorvastatin),
			13–43	(o-Hydroxy-atorvastatin),
			1–5	(p-Hydroxy-atorvastatin)
Fluvastatin	20–80	1–3	54–438	
Lovastatin	10–80	2–3	3–7	(Lovastatin),
			3–18	(Lovastatin hydroxy acid)
Pitavastatin	1–4	5–13	31–81	
Pravastatin	10–40	2–3	4–69	
Rosuvastatin	5–40	12–32	8–20	
Simvastatin	5–80	2–4	3–6	(Simvastatin),
			1–9	(Simvastatin hydroxy acid)

o-hydroxy-atorvastatin calcium salt, and atorvastatin-d5 calcium salt (internal standard, IS) were purchased from Alsachim (Shimadzu Group Company, France), rosuvastatin hemicalcium salt, ammonium formate, dimethylsulfoxid (DMSO), and formic acid from Sigma–Aldrich (Taufkirchen, Germany), lovastatin, lovastatin hydroxy acid, simvastatin and atorvastatin from LGC Standards (Wesel, Germany), pravastatin from Daiichi-Sankyo Europe GmbH (Munich, Germany), fluvastatin from Sandoz Pharma AG (Basel, Switzerland), acetonitrile (ACN), diethyl ether, ethyl acetate and methanol from VWR International GmbH (Darmstadt, Germany), and diazepam-d5 (IS) and pentobarbital-d5 (IS) from LGC Standards (Wesel, Germany). Drug free pooled human blank plasma was obtained from a local blood bank.

Preparation of stock and working solutions

Stock solutions (1 g/L) of each compound were prepared separately in DMSO. The working solution containing all substances (10 mg/L each) was prepared by dilution of stock solutions with ACN. Solutions were stored at –20 °C.

Sample preparation

Based on a previously published extraction procedure [21], 100 µL blood plasma, 10 µL IS mix (100 ng/mL atorvastatin-d5 or 250 ng/mL diazepam-d5 and 1000 ng/mL pentobarbital-d5 in methanol), and 10 µL formic acid (100%) were mixed in an 1.5 mL reaction tube. One milliliter of a diethyl ether-ethyl acetate mixture (1:1, v/v) was added. Afterwards, the mixture was shaken (10 min) and centrifuged (2 min, 18,407×*g*). Eight hundred µL of the clear organic layer were evaporated to dryness at 45 °C under a gentle stream of nitrogen, reconstituted with 40 µL of a mixture of eluents A and B (1:1, v/v, see LC-HRMS/MS conditions), and analyzed by the LC-HRMS/MS system described below.

LC-HRMS/MS apparatus

Samples were analyzed using a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 Rapid Separation LC system consisting of a degasser, a quaternary pump, and a HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), coupled to a TF Q-Exactive mass spectrometer (TF, Dreieich, Germany) with a heated electrospray ionization (HESI)-II source. Gradient elution was performed on a TF Hypersil GOLD C18 column (1.9 µm, 2.1×100 mm) at 40 °C. Eluent A (millipore water containing 1% ACN, 0.1% formic acid, and 2 mM ammonium formate) and eluent B (ACN containing 1% mL Millipore water, 0.1% mL formic acid, and 2 mM ammonium formate) were used as mobile phases. The gradient was programmed as follows: 0–3 min from 35% to 50% eluent B, 3–5 min hold 50% eluent B, 5–8 min to 80% eluent B, 8–9 min hold 80% eluent B, 9.01–11 min hold 35% eluent B, with a constant flow rate of 0.7 mL/min. The injection volume was set to 5.0 µL.

The HESI-II source conditions were as follows: ionization mode, pos/neg switching; sheath gas flow rate, 60.0 arbitrary units (AU); auxiliary gas flow rate, 10.0 AU; spray gas voltage 3.0 kV (positive polarity) and –4.0 kV (negative polarity); auxiliary gas heater temperature, 320 °C; ion transfer capillary temperature, 320 °C; and S-lens RF level, 60.0. Full scan analysis (scan range mass-to-charge ratio value, *m/z*, 130–1000) was used to determine the retention times of all analytes. All other analyses were performed using targeted selected ion monitoring (t-SIM) mode with an inclusion list containing the masses of interest and expected retention times. The settings for t-SIM were as follows: resolution 35,000; microscans 1; automatic gain control target 5e4; maximum injection time 250 ms; isolation window 1.0 *m/z*. Xcalibur Qual Browser software version 2.2 was used for data evaluation.

Method validation

Method validation was performed according to the Guideline on Bioanalytical Method Validation published by the European Medicines Agency [26]. For data evaluation, peak area ratios of analyte (accurate masses are given in Table 2) and IS (atorvastatin-d5 for positive and negative or diazepam-d5 for positive and pentobarbital-d5 for negative ionization mode) were used. Statistical evaluation was performed using Microsoft Excel for Mac Version 16.18 (Microsoft, Redmond, WI, USA) and GraphPad Prism 5.00 (GraphPad Software, La Jolla, CA, USA).

To test selectivity, 12 human plasma samples from different donors were tested for peaks interfering with the detection of the analytes or IS in t-SIM mode. These samples did not contain statins, but other frequently prescribed drugs such as antidepressants (e.g. mirtazapine, sertraline), neuroleptics (e.g. pipamperone), sedatives (e.g. midazolam), and cardiovascular drugs (e.g. metoprolol, torasemide), or other substances for example, ethanol, nicotine, and tetrahydrocannabinol (THC). For carry-over testing, a blank sample was injected after analysis of a high concentration sample (all analytes 100 ng/mL).

Table 2: Precursor ion masses, used ionization modes (+, positive; –, negative), retention order, and retention times of the statins, their active metabolites, and internal standards (marked as IS).

Analyte	Ionization mode and precursor ion mass, *m/z*	Retention order	Retention time, min
Atorvastatin	+559.2603	9	4.5
Atorvastatin-M (o-hydroxy)	–573.2406	7	3.9
Atorvastatin-M (p-hydroxy)	–573.2406	5	2.1
Diazepam-d5 (IS)	+290.1103	6	2.6
Fluvastatin	+412.1919	8	4.3
Lovastatin	+405.2634	12	6.6
Lovastatin-M	–421.2596	10	5.1
Pentobarbital-d5 (IS)	–230.1558	2	1.1
Pitavastatin	+422.1762	3	1.3
Pravastatin	–423.2388	1	0.9 (and artifact at 1.2 min)
Rosuvastatin	+482.1756	4	1.9
Simvastatin	+419.2792	13	7.6
Simvastatin-M	–435.2752	11	6.0

M, metabolite.

Two calibration ranges were defined, both consisting of six calibration points. Calibrators were prepared by spiking blank plasma with working solution (final plasma concentrations see Table 3) and were extracted as described in the section "sample preparation". The lower limit of quantification (LLOQ) was defined to be 1 ng/mL for all analytes and the upper limit of quantification (ULOQ) 20 or 100 ng/mL depending on the analyte. Additionally, blank sample (blank plasma without analytes and IS) and zero sample (blank plasma without analytes, but with IS) were extracted. For all calibration curves, a linear calibration model with different weighting factors (equal, 1/x, or $1/x^2$) was tested by back calculating calibration standards. Back calculated concentrations should be within ±15% of the nominal value (±20% for LLOQ).

Accuracy and precision of the quantification results were determined using four different levels as quality controls (QC), listed in Table 3: LLOQ QC, low QC, medium QC, and high QC. The within-run accuracy and precision were determined by analyzing five samples per level in a single run. The between-run accuracy and precision were determined by analyzing five samples per level in three runs analyzed on three different days. For positive assessment of accuracy, the mean concentration should be within ±15% of the nominal values for the QC samples, except for the LLOQ, which should be within ±20% of the nominal value. For positive assessment of precision, the coefficient of variation (CV) value should not exceed ±15% for the QC samples, except for the LLOQ, which should not exceed ±20%.

Dilution integrity was determined by spiking five blank blood plasma samples to final plasma concentrations 5-times above the high QC (90 or 425 ng/mL, respectively, depending on the analyte). Afterwards, they were diluted with blank blood plasma (1:10, v/v, final plasma concentration 9 or 42.5 ng/mL, respectively), analyzed, and accuracy and precision determined.

Matrix effects should be investigated using the matrix factor (MF) of each analyte and IS determined by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked after extraction with analyte) to the peak area in absence of matrix (pure solution of the analyte) in six lots of matrix. Determined MFs were afterwards used for calculation of the IS-normalized MF by dividing the MF of the analyte by the MF of the IS. This should be done at a low and a high level of concentration and the CV of the IS-normalized MF calculated from the six lots of matrix should not be greater than ±15%.

Stability of analytes should be determined during storage before and after sample preparation at two concentration levels (low and high QC, n = 3, respectively). QC samples were analyzed immediately after preparation and after the evaluated storage conditions using a freshly prepared calibration curve. Afterwards, the obtained concentrations were compared to the nominal concentrations. The mean concentration at each level should be within ±15% of the nominal concentration. For short-term stability of the analytes in the matrix, blank blood plasma was spiked and stored for 20 h at room temperature or +4 °C. For long term stability of the analytes in the matrix, storage took 3 weeks at –20 °C. Stability of processed samples in the autosampler was evaluated by reanalyzing processed samples stored for 20 h in the autosampler (+10 °C). For freeze and thaw stability, spiked plasma samples were stored in a freezer at –20 °C for 20 h, thawed at room temperature, extracted, analyzed (freeze/thaw cycle 1), and the remaining spiked plasma was refrozen. Overall, three freeze/thaw cycles were conducted.

Application to authentic samples

Human blood plasma samples of 14 different individuals with prescribed statin therapy were used for applicability testing. Samples were submitted to the authors' laboratory for regular toxicological screening purposes. Medication plans were thus also provided. All specimens used for applicability testing were submitted to the authors' laboratory for regular toxicological screening purposes including statin determination.

Results

Method development and validation

As shown in Figure 2, chromatographic separation was performed within 8 min. Identification of a compound was based on two criteria. First, the accurate mass of the protonated or deprotonated precursor ion and second, the corresponding retention time in t-SIM mode. Peak area ratios of analyte and IS were used for quantification. If pravastatin

Table 3: Final plasma concentrations (ng/mL) of the analytes in calibrator samples (Cal 1–6) and quality control samples (QC) as well as weighting factors used in a linear calibration model.

Analyte	Cal 1 (=LLOQ)	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Low QC	Medium QC	High QC	Weighting
Atorvastatin	1	20	40	60	80	100	3	50	85	$1/x^2$
Atorvastatin-M (o-hydroxy)	1	20	40	60	80	100	3	50	85	$1/x^2$
Atorvastatin-M (p-hydroxy)	1	4	8	12	16	20	3	10	18	Equal
Fluvastatin	1	20	40	60	80	100	3	50	85	–
Lovastatin	1	4	8	12	16	20	3	10	18	Equal
Lovastatin-M	1	4	8	12	16	20	3	10	18	1/x
Pitavastatin	1	20	40	60	80	100	3	50	85	$1/x^2$
Pravastatin	1	20	40	60	80	100	3	50	85	1/x
Rosuvastatin	1	20	40	60	80	100	3	50	85	$1/x^2$
Simvastatin	1	4	8	12	16	20	3	10	18	$1/x^2$
Simvastatin-M	1	4	8	12	16	20	3	10	18	1/x

M, metabolite.

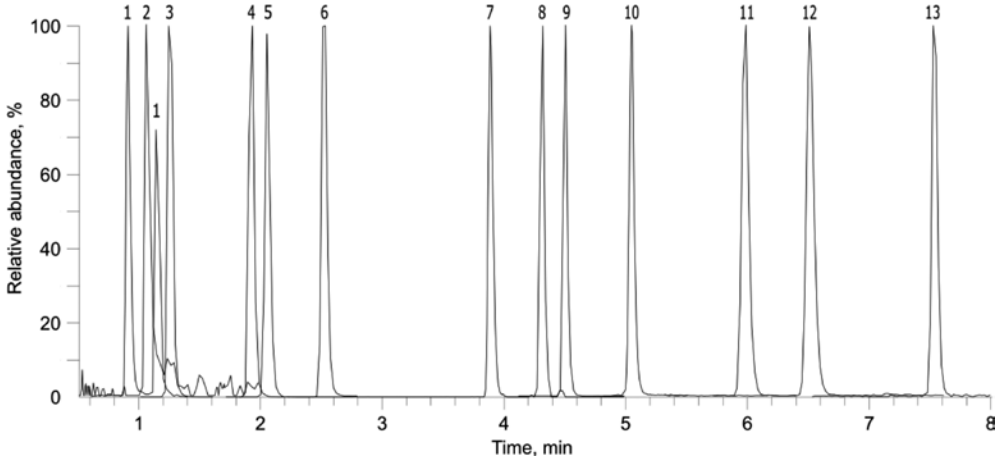

Figure 2: Reconstructed chromatogram of the *m/z* of all analytes in blood plasma (100 ng/mL) measured in t-SIM mode.
1, Pravastatin; 2, pentobarbital-d5 (internal standard); 3, pitavastatin; 4, rosuvastatin; 5, p-hydroxy atorvastatin; 6, diazepam-d5 (internal standard); 7, o-hydroxy atorvastatin; 8, fluvastatin; 9, atorvastatin; 10, lovastatin hydroxy acid; 11, simvastatin hydroxy acid; 12, lovastatin; 13, simvastatin.

was present, an additional peak with the same *m/z* as pravastatin (*m/z* 423.2388 in negative ionization mode) was detected in some analytical runs (see Figure 2). The second peak eluted later (1.2 min) and had a lower abundance and smaller peak area in comparison to pravastatin (0.9 min). The second peak was most probably a pravastatin isomer formed during sample preparation and was quantified together with the pravastatin if present [27]. During method validation, atorvastatin-d5 (final plasma concentration 10 ng/mL) provided a lower peak area in zero samples containing only the IS, than in samples containing additional atorvastatin. Therefore, the influence of the co-eluting analytes atorvastatin and atorvastatin-d5 on each other's signal intensity was tested by comparing their peak areas in combination (both 100 ng/mL) to those after single analysis (n = 3, respectively). Co-eluting atorvastatin led to an ion enhancement >50% of the atorvastatin-d5 peak area. The signal intensities of diazepam-d5 and pentobarbital-d5, used as alternative to atorvastatin-d5, were not influenced by the analytes.

Twelve blank blood plasma samples obtained from individual sources were analyzed and evaluated for interferences. No interfering signals or false positive results were observed during analysis of the 12 selectivity test samples. No analyte carry-over was detectable in the blank sample injected after analysis of the high concentration sample containing an analyte concentration of 100 ng/mL, respectively. For all analytes except of fluvastatin a linear calibration model could be fitted using the weighting factors given in Table 3. In case of fluvastatin, back-calculation of the calibrator concentrations exceeded the limits (±15% and ±20% for LLOQ of the nominal value) using a linear calibration model and the aforementioned weighting factors. Therefore, fluvastatin was not considered further for validation due to reasons discussed later.

Within-run and between-run accuracy were found to be acceptable with mean concentrations within ±15% of the nominal values for the low, medium, and high QC samples (0–15%) and within ±20% for the LLOQ QC samples (2–20%) for all analytes. Within-run and between-run precision were expressed as the CV and also found to be acceptable with CV values within ±15% for the low, medium, and high QC samples (3–15%) and within ±20% for the LLOQ QC samples (2–20%) for all analytes. After dilution of samples spiked above the ULOQ, determined concentrations were also found to be accurate (1–14% variation) and precise (CVs of 4–14%) for all analytes.

Determined IS-normalized MFs and CVs of all compounds are given in Table 4. The presence of matrix had the highest influence on pravastatin and p-hydroxy-atorvastatin signals at low concentrations (–75%, and +72%, respectively), as well as at high concentrations (–41% and +75%, respectively). However, these effects were found to be reproducible with CVs within 15%. This was also true for all other analytes at both concentration levels (CVs of 6–15%).

Results for short- and long-term stability testing of the analytes in the matrix are given in Table 5. Lovastatin and simvastatin were unstable in matrix if stored for 20 h at room temperature (22 °C). After storage, the concentration of lovastatin decreased by more than 90% at low and high concentration levels and of simvastatin by more than 70%, while the concentrations of their hydroxy acid metabolites increased in a comparable extent. This was not observed after storage for 20 h at +4 °C or for 3 weeks at –20 °C. Mean concentrations in low and high QC were

Table 4: Internal standard (IS)-normalized matrix factors (MF) of the analytes and coefficients of variation (CV) derived from measurements of six lots of matrix at a low (low QC) and a high level concentration (high QC, see Table 3).

Analyte	Low QC		High QC	
	IS-normalized MF	CV, %	IS-normalized MF	CV, %
Atorvastatin	1.70	±15	1.28	±14
Atorvastatin-M (o-hydroxy)	1.48	±14	1.04	±14
Atorvastatin-M (p-hydroxy)	1.72	±14	1.75	±15
Lovastatin	1.33	±13	1.09	±11
Lovastatin-M	0.94	±13	0.99	±6
Pitavastatin	0.95	±9	0.96	±10
Pravastatin[a]	0.25	±13	0.59	±15
Rosuvastatin	0.92	±14	0.90	±6
Simvastatin	1.44	±12	1.22	±8
Simvastatin-M	0.91	±13	0.99	±6

[a]Summarized peak areas of pravastatin and pravastatin artifact were used for quantification. M, metabolite.

Table 5: Stability of analytes in matrix after short or long term storage or three freeze and thaw cycles (3FT) given as relative mean concentration ± coefficient of variation (CV) of low and high quality control (QC) samples (n = 3) determined before and after application of the following matrix storage conditions: 20 h at room temperature (RT), 20 h at +4 °C, and 3 weeks (w) at −20 °C, three freeze and thaw cycles including 20 h at −20 °C and thawing at RT, each.

Analyte	Relative mean concentration ± CV, %									
	Low QC					High QC				
	20 h, RT	20 h, +4 °C	3 w, −20 °C	3FT	AS	20 h, RT	20 h, +4 °C	3 w, −20 °C	3FT	AS
Atorvastatin	102 ± 9	110 ± 12	101 ± 7	108 ± 16	102 ± 6	114 ± 11	111 ± 12	112 ± 8	104 ± 15	99 ± 5
Atorvastatin-M (o-hydroxy)	97 ± 14	114 ± 12	112 ± 19	96 ± 16	107 ± 15	97 ± 11	111 ± 11	106 ± 14	90 ± 10	100 ± 7
Atorvastatin-M (p-hydroxy)	94 ± 5	105 ± 4	86 ± 10	109 ± 8	100 ± 12	100 ± 11	103 ± 11	86 ± 10	88 ± 10	95 ± 12
Lovastatin	2[b] ± 61	103 ± 5	99 ± 10	105 ± 20	97 ± 15	9[b] ± 69	92 ± 9	115 ± 13	114 ± 21	105 ± 13
Lovastatin-M	204[b] ± 40	110 ± 12	111 ± 7	109 ± 13	97 ± 15	269[b] ± 57	107 ± 7	101 ± 8	102 ± 16	105 ± 13
Pitavastatin	110 ± 8	102 ± 4	106 ± 10	111 ± 17	114 ± 8	112 ± 9	104 ± 7	113 ± 9	108 ± 10	115 ± 9
Pravastatin[a]	115 ± 19	112 ± 23	100 ± 20	102 ± 11	105 ± 9	100 ± 21	97 ± 18	108 ± 18	91 ± 18	109 ± 9
Rosuvastatin	103 ± 6	114 ± 10	103 ± 6	109 ± 11	102 ± 6	111 ± 10	110 ± 11	104 ± 7	110 ± 9	99 ± 7
Simvastatin	21[b] ± 72	101 ± 10	104 ± 8	108 ± 21	102 ± 13	22[b] ± 63	93 ± 8	97 ± 13	107 ± 12	95 ± 8
Simvastatin-M	199[b] ± 38	111 ± 10	105 ± 12	114 ± 16	101 ± 19	199[b] ± 37	100 ± 7	112 ± 10	93 ± 18	97 ± 8

Stability of processed samples in the autosampler (AS, 20 h at +10 °C) is also given; M, metabolite. [a]Summarized peak areas of pravastatin and pravastatin artifact were used for quantification. [b]Concentration changes >15% are marked.

within ±15% of the nominal concentration for all other analytes.

After storage of processed samples in the autosampler for 20 h, mean concentrations of all analytes in low and high QC were within ±15% of the nominal concentration as given in Table 5. The same was true for the mean concentrations of all analytes in low and high QC after three freeze/thaw cycles of the matrix (only results of the third cycle given in Table 5).

Application to human plasma samples

The concentrations, which were determined in 14 plasma samples, are listed in Table 6.

Discussion

LC in combination with MS/MS is the technique of choice for the quantification of statins in biological matrices due to its sensitivity, selectivity, and simplicity [12]. The review articles by Nirogi et al. and Patel and Kothari provide an overview on published statin bioanalysis [4, 12]. Multiple LC-MS/MS methods focusing on quantification of only one statin, sometimes plus metabolite(s), in human blood plasma were published recently [13–20]. Only a few numbers of methods including more than one statin were published in the past [21–24]. However, to the best of our knowledge, this is the first LC-HRMS/MS method aiming to include all seven currently marketed statins plus their active metabolites for adherence

Table 6: Results of applicability testing using 14 human samples.

Sample no.	Analyte	Daily dose, mg	Measured plasma concentration, ng/mL
1	Atorvastatin	10	2
	Atorvastatin-M (o-hydroxy)		2
	Atorvastatin-M (p-hydroxy)		<1
2	Atorvastatin	10	<1
	Atorvastatin-M (o-hydroxy)		<1
	Atorvastatin-M (p-hydroxy)		n.d.
3	Atorvastatin	40	4
	Atorvastatin-M (o-hydroxy)		7
	Atorvastatin-M (p-hydroxy)		1
4	Atorvastatin	40	15
	Atorvastatin-M (o-hydroxy)		8
	Atorvastatin-M (p-hydroxy)		n.d.
5	Atorvastatin	20	<1
	Atorvastatin-M (o-hydroxy)		2
	Atorvastatin-M (p-hydroxy)		<1
6	Atorvastatin	20	<1
	Atorvastatin-M (o-hydroxy)		1
	Atorvastatin-M (p-hydroxy)		<1
7	Atorvastatin	20	<1
	Atorvastatin-M (o-hydroxy)		n.d.
	Atorvastatin-M (p-hydroxy)		<1
8	Pravastatin[a]	40	18
9	Simvastatin	40	4
	Simvastatin-M		9
10	Simvastatin	10	3
	Simvastatin-M		1
11	Simvastatin	40	2
	Simvastatin-M		<1
12	Simvastatin	40	1
	Simvastatin-M		<1
13	Simvastatin	40	3
	Simvastatin-M		<1
14	Simvastatin	40	<1
	Simvastatin-M		<1

Prescribed statin, daily dose, and measures plasma concentration of parent compound and metabolites are given. n.d., not detected; M, metabolite. [a]Summarized peak areas of pravastatin and pravastatin artifact were used for quantification.

monitoring, as well as therapeutic drug monitoring purposes.

Sample preparation by liquid-liquid extraction was based on the recommendation by Pilli et al. [21] with minor modifications. Only 100 µL of blood plasma was mixed with diethyl ether and ethyl acetate instead of pure ethyl acetate to achieve a better separation of aqueous and organic phases. Chromatographic separation of all analytes was achieved within 8 min and a single analysis was performed in a total run time of 11 min. Such a quick sample preparation and short run time is expected to save resources and will facilitate the integration in daily routine work of toxicological laboratories. During analysis of authentic samples, each analytical batch consisted of a blank sample, a zero sample, the calibration standards, three levels of QC samples (low, medium, high) in duplicate, and the authentic samples. In case of pravastatin, two peaks were detected in some analytical runs (see Figure 2). Pravastatin was described to be prone to isomerization under acidic conditions [27]. Therefore, the second peak was expected to be a pravastatin artifact formed during sample preparation, which was not detectable during analysis of neat standard solution. Both peaks had to be used for correct quantification.

Atorvastatin-d5, the only commercially available deuterated statin at that time, was intended to be used as IS due to the structural similarity with the analytes and the possibility of analysis in positive and negative ionization mode. However, co-eluting atorvastatin caused ion enhancement of the atorvastatin-d5. As the signal intensities of diazepam-d5 and pentobarbital-d5 were not influenced by co-eluting compounds mainly due to successful chromatographic separation (see Figure 2), they were used as IS for positive and negative ionization mode, respectively.

The developed method was considered as selective and no analyte carry-over was observed in the blank sample after injection of a high concentration sample. However, a washing run with blank plasma is recommended if authentic samples with a concentration above 100 ng/mL are analyzed.

Six statins and four active metabolites can be quantified reliable and reproducible even after dilution. In summary, the present method allows plasma quantification of p-hydroxy-atorvastatin, lovastatin, lovastatin hydroxy acid, simvastatin, and simvastatin hydroxy acid in the range of 1–20 ng/mL and of atorvastatin, o-hydroxy-atorvastatin, pitavastatin, pravastatin and rosuvastatin in the range of 1–100 ng/mL. These ranges cover their reference plasma concentrations, which are summarized in Table 1. Only for atorvastatin, plasma concentrations above 100 ng/mL were described [25]. However, as dilution integrity was tested during validation, samples as high as 5-times above the high QC can be quantified reliably after dilution with blank plasma.

Only fluvastatin failed the validation acceptance criteria most probably due to its photosensitivity [28–30]. Nevertheless, light protection of blood plasma by wrapping the flask with aluminum foil and use of amber glass vials did not improve the results. Fluvastatin was also described to be unstable under acidic conditions leading to lactonization [30].

Further evaluation of stability of all other analytes was carried out to ensure that the storage conditions used before and after sample preparation do not affect the analytes [26]. Conditions were the same that were expected to occur during analysis of authentic samples. Short-term stability testing clearly indicated, that plasma samples containing lovastatin or simvastatin should be stored at 4 °C until analysis. No stability issues after storage of plasma samples at –20 °C for 3 weeks and three freeze and thaw cycles were observed. Stability of processed samples in the autosampler for at least 20 h is of advantage if long analytical series are planned. However, the duration of an analytical run should not exceed 20 h, as stability of processed samples in the autosampler can only be guaranteed for 20 h, based on these experiments.

Applicability of the current method was tested using 14 human plasma samples of patients under statin therapy. Atorvastatin was prescribed to seven patients, simvastatin to six patients and pravastatin to one patient. These statins represent the most widely prescribed statins in Germany [31]. Unfortunately, samples containing the other statins were not available during the development of the study to further evaluate the applicability. Nevertheless, other statins may be predominately prescribed in other parts of the world and the current method is expected to be also applicable for their plasma quantification. It was remarkable that most of the determined concentrations (in the case of eight patients for parent compound and metabolite/metabolites and three patients only for metabolites) were below the reference plasma concentrations (see Table 1) [25]. However, it should be considered that the reference plasma concentrations were peak concentrations.

In the case of the authentic samples analyzed within the current study, sampling was not expected to be performed at the time of peak concentration, as intake was prescribed once daily in the evening and sampling was usually performed in the morning. Comparably low plasma concentrations were determined in previous studies [14, 24, 32]. Nirogi et al. described plasma concentrations of atorvastatin and its metabolites below 1 ng/mL 3 h after the administration of an oral single dose of 10 mg of atorvastatin [14], while Polagani et al. reported atorvastatin plasma concentrations below 10 ng/mL approximately 10 h after administration of 40 mg [32]. The short elimination half-lives of pravastatin and simvastatin (see Table 1) should also be considered. However, the exact time between intake and sampling was not documented, as well as storage conditions before arrival at the laboratory. Furthermore, nothing was known about the adherence of those patients. For future application of the current method, sample storage at +4 °C until analysis is recommended.

Conclusions

An LC-HRMS/MS method for simultaneous quantification of six statins and four active metabolites was successfully developed and validated. Only 100 µL of human plasma was needed and a quick and easy sample preparation was followed by fast chromatographic separation. Applicability was demonstrated by analysis of 14 human plasma samples. These samples contained the three most widely prescribed statins in Germany plus three active metabolites. Determined plasma concentrations were below the reference peak plasma concentrations most probably due to elevated time span between sampling and intake. In the case of application of the current method for therapeutic drug monitoring or adherence testing time between intake and sampling has to be documented carefully and sampling is recommended to be performed at the time point of the maximum plasma concentration.

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