A method for direct monitoring of atorvastatin adherence in cardiovascular disease prevention: Quantification of the total exposure to parent drug and major metabolites using two-channel chromatography and tandem mass spectrometry

Running title: Atorvastatin adherence assay

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Abstract

BACKGROUND: Low adherence to statin therapy remains a public health concern associated with poor prognosis in cardiovascular disease patients. A feasible method for statin adherence monitoring in clinical practice has yet to be developed. Herein, we describe a novel method designed for the direct monitoring of atorvastatin adherence based on the sum of parent drug and major metabolites in blood samples.

METHODS: Acid and lactone forms of atorvastatin, 2-OH-atorvastatin and 4-OH-atorvastatin were assayed. Plasma proteins were precipitated with an acidified mixture of methanol, acetonitrile and aqueous zinc sulphate, and the supernatant was analyzed with two-channel reversed-phase chromatography coupled to tandem mass spectrometry. Assay validation was performed according to the guidelines provided by the European Medicines Agency and the US Food and Drug Administration.

RESULTS: The effective run-time was 1 minute and 45 seconds per sample. Mean accuracy ranged from 92 to 110 %, and coefficients of variation were ≤ 8.1 % over the measurement ranges for individual compounds. The sum of acids and corresponding lactones was stable in clinical plasma samples kept at ambient temperature for up to six days after blood sampling (mean sum within 96.6 to 101 % of baseline).

CONCLUSIONS: A fast and reliable assay for the quantification of atorvastatin and its five major metabolites in clinical blood samples is reported. Limitations of pre-analytical stability were solved using the sum of the acid and lactone forms. The assay is feasible for implementation in clinical practice and the sum of parent drug and metabolites may be used for direct monitoring of atorvastatin adherence.

KEYWORDS: Atorvastatin, adherence, cardiovascular disease (CVD), liquid chromatography, mass spectrometry

Introduction

The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (i.e. statins) have a pivotal role in preventing cardiovascular disease (CVD). Statin treatment is cost-effective and recommended by European and US guidelines to a large number of people with elevated cardiovascular risk and to most patients with established CVD.[1, 2] The beneficial effects of statins on cardiac prognosis are mainly mediated by lowering low-density lipoprotein-cholesterol (LDL-C) and other apolipoprotein B-containing lipoproteins, and by reducing arterial inflammation.[3-5] For every 1.0 mmol/L LDL-C reduction by statins, a 20-25 % reduction in five-year incidence of CVD events has been reported.[5]

Even though the prescription of statins has increased over the past decades, observational studies have revealed substantial challenges in lowering blood lipids after CVD events in clinical practice.[6] In a recent study of 1095 Norwegian CVD patients, the most comprehensive evaluation of predictors of unfavorable LDL-C control in CVD patients yet, we reported that 57 % had LDL-C above the recommended target of 1.8 mmol/L. Self-reported low statin adherence, low statin doses, and perceived statin side-effects were the major factors associated with unfavorable LDL-C control.[7] Low statin adherence is an independent risk factor for mortality after cardiovascular events.[8]

Adherence to prescribed drug therapy has traditionally been monitored by indirect methods such as self-report questionnaires, pill counts, electronic pillboxes and prescription refill rates.[9] Such methods may overestimate drug intake due to recall bias or patient manipulation.[10] Prescription refill rates obtained from pharmacy registries provide the most accurate estimates of adherence,[9] and US data indicate that only 30 to 60 % of patients remain adherent to statins within the first two years of initial prescription.[11] However, the assessment of prescription refill rates from registries is laborious and not feasible in daily

clinical practice.[9, 12] The lack of a direct method for monitoring adherence is recognized as a major barrier,[12] concerning clinicians, health care providers and authorities.

Direct assessment of drug levels in blood is an objective method, [9] allowing adherence to be monitored during patient follow-up. Measurement of antihypertensive drugs in blood has shown potential to improve the blood pressure control in patients with resistant hypertension.[13] Several analytical methods for the measurement of systemic statin levels have been reported, [14, 15] generally designed for clinical studies. Methods that are feasible for health care providers and routine clinical laboratories with respect to technical implementation and interpretation of statin adherence are yet to be described. General prerequisites for the implementation of an assay in the routine clinical setting include a simple work-flow, equipment and reagents that harmonize with other common assays, and low time occupancy on the analytical instrumentation.

Atorvastatin is the most commonly prescribed statin in CVD prevention[16] with doses ranging from 10 to 80 mg/day. We aimed to develop and validate pre-analytical and analytical methodologies for the measurement of atorvastatin in clinical blood samples, specifically in the context of drug adherence monitoring and feasibility for the routine clinical laboratory.

Atorvastatin is converted to hydroxy and lactone metabolites *in vivo*. The hydroxylations are catalyzed primarily by CYP3A4[17] which demonstrates highly variable enzyme activity between individuals.[18] UDP-glucuronosyltransferases are likely to be involved in the lactone formation through intermediate acyl glucuronides, and the lactones are proposed to be both chemically and enzymatically (esterases) hydrolyzed to corresponding open acid forms.[19] The metabolism of atorvastatin is illustrated in Figure 1. Our strategy was to determine the sum of parent drug and major hydroxylated metabolites to level out within- and between-individual variability of drug metabolism. A major concern for the accurate determination of atorvastatin plasma concentrations is the instability of the lactone metabolites. If not

stabilized, the lactones will be gradually converted to corresponding acid forms after blood sampling. Stabilization can be achieved at cold temperatures, lowered pH and with proper anticoagulants.[20, 21] Nevertheless, consistent implementation of temperature- or pH-dependent sample handling and shipment in clinical practice would be highly recommended. In the context of atorvastatin adherence monitoring, we aimed to reveal whether the sum of the acid and corresponding lactone forms could be used to balance the impact of pre-analytical lactone instability. Moreover, a wide range of drug concentrations must be measured due to the large atorvastatin dose range and the variable pharmacokinetics between patients, adding to the variability of drug adherence. Together, these prerequisites demand that the parent drug and the major metabolites in both acid and lactone forms are included in an assay with wide measuring ranges and feasible operational procedures. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) would be the preferable technique for this purpose.

Recently published atorvastatin assays are generally designed for application in clinical studies on pharmacokinetics and bioequivalence, focusing on the atorvastatin acid forms.[15, 21-25] Practical pre-analytical handling of samples in the routine setting has not been adequately addressed. We aimed to develop an atorvastatin adherence assay with pre-analytical and analytical conditions that are feasible for the outpatient clinics, the general practitioners and the laboratories.

In the present study, we describe a novel assay satisfying the requirements for clinical routine application and designed for direct monitoring of atorvastatin adherence in CVD patients.

Materials and Methods

REAGENTS

HPLC-grade acetonitrile and methanol were obtained from Rathburn Chemicals, Walkerburn, Scotland. Atorvastatin acid, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide,

LC-MS grade ammonium acetate, HPLC-grade water and zinc sulfate 0.10 mol/L were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mobile phases were prepared with UHPLC-MS grade water and methanol with 0.10 % formic acid from Honeywell, NJ, USA. Autonorm drug-free animal serum was a product from Sero AS (Billingstad, Norway). Atorvastatin lactone, 2-OH-atorvastatin acid and lactone, 4-OH-atorvastatin acid and lactone, as well as D_5-atorvastatin acid and lactone, D_5-2-OH-atorvastatin acid and lactone, D_5-4-OH-atorvastatin acid and lactone were purchased from Toronto Research Chemicals (Toronto, Canada).

Stock solutions of calibrators (1 mg/mL) and deuterated internal standards (IS; 1 mg/mL) were prepared in dimethyl sulfoxide. Analyte working solutions (acid and lactone forms separately) were prepared by diluting stock solutions in acetonitrile. The IS stock solutions were diluted in acetonitrile to prepare a common IS working solution. For the preparation of calibrators and quality controls (QCs) at the upper limit of quantification (ULOQ), 100 µL analyte working solution was mixed with 100 µL cold water and then added cold Autonorm to a final volume of 5.00 mL, acids and lactones were kept separate. Lower levels were prepared by dilution in cold Autonorm. Stock solutions, working solutions, calibrators and QCs were stored at -70 °C.

The IS working solution was diluted 100-fold in the precipitation solution which consisted of methanol with 0.10 % formic acid and 2.0 mmol/L ammonium acetate (mobile phase B):acetonitrile:water:zinc sulfate 0.10 mol/L 2:2:1:1. In this reagent, IS concentrations of 0.90 nmol/L were obtained. The precipitation solution with IS was kept at 2 to 8 °C.

SAMPLE HANDLING AND PREPARATION

Venous blood was sampled in EDTA vacutainers without gel. Two sample handling procedures were followed during assay development and validation. 1) Low temperature

procedure: Blood was collected in pre-cooled tubes, immediately placed on ice and thereafter centrifuged at 4 °C (2000 g, 10 minutes). Then, plasma was immediately transferred to polypropylene tubes and frozen at -70 °C. The frozen plasma was shipped on dry ice from the outpatient clinic to the hospital laboratory. 2) Ambient temperature procedure: All steps including blood collection, sample handling, centrifugation (2000 g, 10 minutes), transfer of plasma to polypropylene tubes, intermediate plasma storage and shipment to the laboratory were performed at ambient temperature. The plasma samples were frozen at -70 °C upon arrival in the laboratory, or they were frozen after specific storage intervals at room temperature (RT) during validation of pre-analytical stability.

High level calibrators, QCs and patient samples were thawed at RT and immediately placed on ice. The calibrators were serially diluted (4-fold steps) with cold Autonorm to generate six calibrator levels for acids and lactones, separately. One hundred microliters cold calibrator, QC or patient sample was pipetted to a deep-well plate (2.2 mL 96 deep-well polypropylene plate with square wells; Hamilton, Bonaduz, Switzerland) kept on ice. Two parallels were used for calibrators at the lower limit of quantification (LLOQ). Then, 400 µL cold precipitation solution with IS was added to each well, and the plate was sealed (Slit Seal, BioChromato, Kanagawa-ken, Japan) and shaked for six minutes (1400 rpm, 3 mm orbit; High-Speed Multi Plate Shaker, BioSan, Riga, Latvia). The plate was centrifuged for 10 minutes (2000 g, 4 °C; Rotanta, Hettich, Tuttlingen, Germany), and transferred to the autosampler with temperature set at 10 °C.

ANALYSIS

The analytical instrumentation was a Transcend II LX-2 UHPLC-system coupled to a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Using this system, two LC-channels with separate injection ports and exact length tubing (Viper Fingertight Fittings, Thermo, Waltham, MA, USA) were alternately directed to the mass

spectrometer, thereby allowing overlapping chromatographic runs and reduced batch analysis time. Ten microliters supernatant from the upper part of the well was injected. The needle and injection port were washed with acetonitrile:methanol:isopropanol 50:25:25 and water:acetonitrile 95:5 between injections. Chromatographic separation was performed at 25 °C on a Raptor Biphenyl column (30 x ID 3.0 mm, particle size 5 µm; Restek, Bellefonte, PA, USA). The conditions for chromatography are specified in Table 1. Electrospray was used for evaporation and positive ions entered the mass spectrometer using voltage 3400 V. Nitrogen gas flow: Sheath gas 60 AU, auxiliary gas 8 AU and sweep gas 4 AU. Vaporizer and ion transfer tube temperatures were 470 °C and 270 °C, respectively. Settings for selective reaction monitoring (SRM) are listed in Table 2. The ion chromatograms were smoothed and peaks were integrated with software algorithms (TraceFinder, Thermo, Waltham, MA, USA). The peak area ratio between analyte and internal standard was used as signal response. A quadratic calibration model with origo excluded and $1/x^2$ weighting was applied for all analytes. The mean concentration of the specific acid form (i.e. measured) in lactone calibrators was used to correct the nominal concentration of corresponding lactone.

METHOD VALIDATION

The assay was validated in accordance with the guidelines on bioanalytical method validation provided by the European Medicines Agency and the US Food and Drug Administration.[26, 27]

Analytical selectivity was assessed with samples from CVD patients not using atorvastatin. The IS selectivity was additionally investigated with samples from CVD patients receiving atorvastatin treatment. Selectivity was also investigated by analysis of a commercial QC containing 22 therapeutic drugs (amikacin, caffeine, carbamazepine, chloramphenicol, clonazepam, cyclosporine, desipramine, diazepam, digoxin, disopyramide, ethosuximid, flecainide, gentamicin, haloperidol, imipramine, lidocaine, lithium, methotrexate, nortriptyline, paracetamol, phenobarbitone, phenytoin, primidone, procainamide, quinidine,

salicylate, theophylline, tobramycin, valproic acid, vancomycin; Seronorm Pharmaca L-2, Sero AS, Billingstad, Norway).

We assessed carry-over between subsequent injections by analyzing the blanks after QCs spiked with analyte concentrations at ULOQ and IS at regular concentration. Potential analyte and IS responses in the blanks were calculated relative to the responses in QCs at LLOQ added regular IS concentration. The presence of potential late-eluting peaks was examined by sequential analysis of 10 blank samples following an atorvastatin patient sample, and with sequential analysis of 10 blank samples following injection of calibrators at ULOQ.

Calibration curves were examined with respect to the accuracy of back-calculated concentrations vs. nominal concentrations at each level.

Within- and between-series accuracy and imprecision were determined with spiked QCs. The nominal concentrations of lactone QCs were corrected according to the content of corresponding acid, similar to the lactone calibrators. We used five concentration levels due to the wide measurement ranges. Signal to noise was calculated at the LLOQ. The coefficient of variation (CV) was used as measure of imprecision. Between-series validations were performed with QCs just after the calibrators, as well as in the end of the sequence. All experiments were performed with full batches (i.e. injection from all 96 wells).

Inclusion of the IS in the precipitation solution could potentially cause a matrix-dependent recovery bias compared with addition of the IS to the sample prior to precipitation. This was examined by processing two pre-dose samples and four post-dose samples (at t_{1h} and t_{3h}) from six CVD patients using atorvastatin. The IS mixture was added directly to the clinical samples and calibrators in 20 µL methanol:water 1:1. Thereafter, these samples were mixed

for 5 minutes at 1000 rpm, kept at the bench for 5 minutes, and then added precipitation solution without IS. A parallel batch was prepared with IS in the precipitation solution.

Dilution integrity was assessed by 4-fold dilution of QCs spiked with 2-fold ULOQ concentrations. One hundred microliters cold QC was added to 300 µL cold blank matrix (Autonorm) in 2 mL polypropylene tubes, and then vortexed for 5 seconds.

Matrix effects were assessed with samples from CVD patients not using atorvastatin. The post-precipitation addition method was used; with analytes at twofold LLOQ and ULOQ, and IS at regular concentrations. The matrix factor (MF) was calculated vs. non-matrix (water), and the IS-normalized MF was calculated as the ratio between the analyte MF and the IS MF.

Stability of acid and lactone forms of atorvastatin, separately spiked at twofold LLOQ and at ULOQ in calibrator/QC matrix, was investigated at RT and at 2 to 8 °C (3 hours, 1-3-7 days), and at -70 °C (1 month). The stabilizing effect of EDTA was also assessed in spiked matrix at RT. Furthermore, the pre-analytical stability of samples from CVD patients on atorvastatin was investigated at ambient temperature (4 hours, 1-2-3-4-5-6 days). With regard to patient samples, the low temperature procedure for sample handling was used for the measurement of baseline levels (and plasma was frozen within 1 hour after sampling). One and two pre-analytical freeze/thaw cycles was examined with QCs at twofold LLOQ and at ULOQ. Examination of post-preparative stability was performed with calibrators and QCs at 10 °C in the autosampler the first night and subsequent storage at 2 to 8 °C (peak areas within ± 30 % as underlying criteria). The stability of stock and working solutions was assessed at -70 °C, and the stability of the precipitation solution with IS was assessed at 2 to 8 °C.

PILOT STUDY FOR PROOF-OF-CONCEPT

Quantification of atorvastatin and metabolites were performed with samples from two CVD patients using lowest (10 mg) and highest (80 mg) atorvastatin doses. This was part of an

ongoing pharmacokinetic study aiming to define cut-off levels for non-adherence. After reaching steady-state with once daily dosing, the patients omitted administration of the statin for three days to simulate non-adherence under controlled conditions. The study protocol was reviewed by the regional committee for medical research ethics without remarks, and written informed consent was obtained from the participants.

Results

The chromatography run-time per injection was 3 minutes and 28 seconds, allowing an effective run-time of approximately 1 minute and 45 seconds per sample when using two alternating LC-channels. The isobaric 2/4-OH-metabolites and the acid/lactone pairs were chromatographically separated. Representative ion chromatograms are shown in Figure 2A and 2B. Also, isobaric impurities in the internal standards were separated from the compounds of interest, and glycerophosphocholines were eluted to waste towards the end of each sample run (data not shown).

No analyte or IS interferences were observed in atorvastatin-free samples from CVD patients (n = 6) or from commercial QC with therapeutic drugs. No IS interferences were observed in samples from CVD patients on atorvastatin (n = 3). A baseline-separated peak was observed in the 4-OH-atorvastatin lactone ion chromatogram in three out of six atorvastatin-free patient samples and in the drug-containing QC. Also, a baseline-separated peak was observed in the D_5-4-OH-atorvastatin lactone ion chromatogram in one of the atorvastatin-free patient samples. The carry-over between subsequent injections was equal to zero for all analytes and IS (n = 5), and no late-eluting peaks were detected.

The mean accuracy ranged from 92 to 110 %, and CVs were ≤ 8.1 % over the entire measurement ranges for all analytes (details in Table 3). Four-fold dilution of QCs (at 2-fold ULOQ) was performed with CVs ranging from 0.9 to 1.5 % and mean accuracy within 100 to

106 % for all analytes (n = 6). Individual signal-to-noise ratios at LLOQ were ≥ 15 for all analytes (n = 6 in three series). The accuracy of individual back-calculated calibrator concentrations ranged from 91 to 109 % above LLOQ and from 85 to 117 % at LLOQ (n = 3 curves per analyte).

The mean deviations for results obtained with IS in the precipitation solution compared to addition of IS to the sample prior to precipitation ranged from + 0.12 to +5.3 %. Individual deviations ranged from -3.2 to +13 % for all six analytes (n = 6 patient sources).

Significant ion suppression was demonstrated for atorvastatin acid, atorvastatin lactone and 2-OH-atorvastatin acid. Mean suppression ranged from 13 to 24 %. The CVs for the IS-normalized MFs ranged from 2.8 to 14.7 % (n = 6 patient sources) when examined at low and high analyte concentrations.

Pre-analytical stability was examined with plasma from CVD patients on atorvastatin 20 mg, 40 mg and 80 mg (n = 2+2+2). Samples were collected 11 to 24 hours after dose. Following 6 days at ambient temperature and protected from light, the mean ± SD analyte levels relative to baseline were as follows: atorvastatin acid 182 ± 28 %, atorvastatin lactone 15.4 ± 3.3 %, 2-OH-atorvastatin acid 241 ± 33 %, 2-OH-atorvastatin lactone 31.5 ± 4.2 %, 4-OH-atorvastatin acid 227 ± 73 %, 4-OH-atorvastatin lactone 30.1 ± 7.9 %; sum of atorvastatin acid and lactone 91.8 ± 5.3 %, sum of 2-OH atorvastatin acid and lactone 99.7 ± 4.5 %, sum of 4-OH-atorvastatin acid and lactone 115 ± 7.4 %; sum of parent drug and five metabolites 101 ± 3.4 %. With respect to all tested intervals up to 6 days, the mean sum of parent drug and five metabolites was within 96.6 to 101 %, and all individual sums were within 91.2 to 105 % of baseline. In absolute terms, the individual sums of parent drug and metabolites ranged from 25.3 to 199 nmol/L. The profiles of pre-analytical alterations are shown in Figure 3 and in Figure 4.

The acid forms in calibrator/QC matrix were stable for 7 days at RT and at 2 to 8 °C (mean deviation -5.8 to 0.0 %, triplicates). The lactone forms in calibrator/QC matrix were stable for 3 hours at 2 to 8 °C (mean deviation 2.3 to 14 %, triplicates), but not for 3 hours at RT (mean deviation -73 to -28 %, triplicates). When EDTA 5.0 mmol/L was added, atorvastatin lactone and 4-OH-atorvastatin lactone were stable (mean deviation -1.4 to 13 %, duplicates) and 2-OH-atorvastatin lactone was not stable (mean deviation -20 to -16 %, duplicates) for 3 hours at RT. Acids and lactones in calibrator/QC matrix were stable for 1 month at -70 °C (mean deviation +0.3 to +15 %, n = 7). Corrections of the lactone calibrators due to acid content ranged from -28 to -12 %. Mean deviations following two freeze/thaw cycles were within -6.5 to +8.9 %. Stock and working solutions were within -14 to +2.0 % after 3.5 months at -70 °C. The precipitation solution with IS was stable for 8 days at 2 to 8 °C (mean deviation for the IS responses ranged from -1.2 to +3.2 %, n = 6).

Post-preparative storage under cool conditions was acceptable for 48 hours: Mean deviations for analyte and IS peak areas ranged from -10 to +9.7 % and -11 to +8.2 %, respectively. Mean deviations for measured analyte concentrations ranged from -0.8 to +2.6 %.

In the pilot investigations, the sum of atorvastatin and metabolites was quantified up to 96 hours after the last dose (10 mg and 80 mg) in CVD patients. The dose-adjusted sum decreased 10 to 21-fold after two days off statin and 18 to 96-fold after three days off statin, compared with baseline steady-state level (Figure 5).

Discussion

The presented assay for monitoring atorvastatin and its five major metabolites in clinical blood samples demonstrated to be accurate and precise over wide concentration ranges, and the lactone instability was controlled during the pre-analytical and analytical phases. The calculated sum of atorvastatin acid and lactone forms counteracted the effect of lactone-to-

acid conversion. Straightforward sample preparation with protein precipitation was used, and the analytical requirements harmonized with common LC-MS/MS configurations and reagents. The effective run-time per sample on the instrument was less than 2 minutes when using two-channel chromatography, and the total turnaround time for a 30-sample batch would be less than three hours, including preparation and analysis. The assay will be valuable for studying atorvastatin adherence during CVD prevention, and it will be feasible to implement for routine analyses in the clinical laboratory. The assay runs seamlessly on two identical LC-MS/MS instruments with two-channel configuration in our laboratory, and we are currently performing pharmacokinetic studies to provide algorithms for the categorization of atorvastatin adherence in CVD patients.

The instability of the lactone forms of atorvastatin and metabolites is the Achilles heel for reproducible measurements in clinical samples. Acidification of samples or low temperature handling will improve the stability.[21] Although such sample handling has been applied in clinical studies, [15, 21, 24, 25, 28] it would be challenging for outpatient clinics and general practitioners to comply with such principles in clinical practice. The present assay was designed for adherence monitoring and the lactone instability was taken into consideration for both pre-analytical and analytical aspects. Our results showed that the sum of acids and corresponding lactones was stable in EDTA plasma kept at ambient temperature for up to six days after blood sampling. This is of major importance for the handling of samples in a clinical setting. In parallel, a better correlation between drug dose and exposure may be achieved using the sum of parent drug and major metabolites in order to level out metabolic variations within and between individuals. The lactone instability was controlled during the analytical operations: The sample preparation was fast and continuously performed under cool conditions, acids and lactones were kept separate in calibrators and in spiked QCs, and the lactone calibrators were corrected for the content of corresponding acid. EDTA may be

added to calibrators and QCs to enhance the lactone stability. The assay conditions allowed reanalysis with repeated thawing if necessary and post-preparative storage for two days.

We aimed to develop a highly sensitive assay based on simple protein precipitation and fast chromatography. A large-particle biphenyl column was selected for robustness and for proper separation, allowing a moderate gradient and elution of the compounds with high organic content in the mobile phase to facilitate the instrument response. Moreover, these conditions allowed fast clean-up, wash-out and equilibration. Quantification of low concentrations (< 0.10 nmol/L) was possible without any up-concentration step. Precise measurements over 1000-fold concentration ranges were facilitated with the inclusion of an isotope-labeled IS for each analyte (demonstrating adequate corrections for matrix effects), as well as applying a robust calibration model.

Non-human serum was used in the calibrator and QCs for practical reasons, although compromising the principle of similarity between calibrator and sample matrices. Such a lyophilized matrix can be stored and reconstituted in suitable volumes when needed, and it includes technical documentation that should be appropriate with respect to compliance with evolving regulations for *in vitro* diagnostic testing.

The performance criteria given in the validation guidelines were fulfilled for atorvastatin and the five metabolites, although certain precautions must be exercised due to lactone instability. Also, one should be aware of potential isobaric interferences with 4-OH-atorvastatin lactone and its D_5-labeled IS (caused by concomitant drugs). Baseline-separation of these interferences was achieved as well as separation of the isobaric hydroxylated atorvastatin metabolites. Apparent interference between lactones and the acid mass transitions has been reported, [20] and chromatographic separation of corresponding acids and lactones was also ensured.

The preliminary pharmacokinetic results indicated a more than 10-fold reduction of the dose-adjusted sum of parent drug and major metabolites when patients omitted two and three atorvastatin once-daily doses. Cut-off values that may discriminate non-adherent from adherent patients will be influenced by the between-individual variability of total atorvastatin exposure per dose, as well as the variability of post-dose exposure reduction rate.

Conclusion

The authors have developed a fast and reliable method for the precise quantification of atorvastatin and its five major metabolites in clinical blood samples. Limitations of pre-analytical stability were minimized using the sum of the acid and lactone forms. The assay is technically feasible for implementation in clinical practice. It may be used to determine the total systemic atorvastatin exposure, thereby providing a method for the direct monitoring of statin therapy adherence.

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Figure legends

Figure 1. *In vivo* metabolism of atorvastatin. Hydroxylation at position 2 (ortho) and position 4 (para) is catalyzed by cytochrome P450 3A (CYP3A), predominately by the CYP3A4 isoform. The lactonization is likely to be dependent on UDP-glucuronosyltransferases catalyzing the formation of intermediate acyl glucuronide products. Chemical hydrolysis and esterase-dependent hydrolysis are proposed to be involved in the conversion of lactones to open acid forms.

Figure 2A. Ion chromatograms of the acid forms of atorvastatin and major hydroxylated metabolites. Blank: A blank sample prepared with the addition of internal standards. The identified peaks are related to impurities in the internal standard mixture. LLOQ: A blank sample spiked with analytes at the lower limit of quantification and prepared with the addition of internal standards. Clinical sample: A pre-dose sample obtained from a patient using 40 mg atorvastatin once daily, prepared with the addition of internal standards. Vertical axis (instrument response, counts per second). Horizontal axis (elution time, minutes): Time zero depicts the starting point for the mass spectrometry window, i.e. switch from waste to inline.

Figure 2B. Ion chromatograms of the lactone forms of atorvastatin and major hydroxylated metabolites. Blank: A blank sample prepared with the addition of internal standards. The identified peaks are related to impurities in the internal standard mixture. LLOQ: A blank sample spiked with analytes at the lower limit of quantification and prepared with the addition of internal standards. Clinical sample: A pre-dose sample obtained from a patient using 40 mg atorvastatin once daily, prepared with the addition of internal standards. Vertical axis (instrument response, counts per second). Horizontal axis (elution time, minutes): Time

zero depicts the starting point for the mass spectrometry window, i.e. switch from waste to inline.

Figure 3. Pre-analytical stability of acid and lactone forms of atorvastatin. Samples were obtained from cardiovascular disease patients on atorvastatin treatment (n = 6). EDTA-plasma was shipped and stored at ambient temperature (protected from light). Baseline level represents plasma kept cool and frozen within 1 hour after sampling. Mean (range).

Figure 4. Pre-analytical stability of the sum of parent drug and major metabolites: Atorvastatin acid and lactone, 2-OH-atorvastatin acid and lactone, 4-OH-atorvastatin and lactone. Samples were obtained from cardiovascular disease patients on atorvastatin treatment (n = 6). EDTA-plasma was shipped and stored at ambient temperature (protected from light). Baseline level represents plasma kept cool and frozen within 1 hour after sampling. Mean (range).

Figure 5. Time profile of the sum of parent drug and major metabolites. Two cardiovascular disease patients were at steady-state with atorvastatin once-daily dosing and their doses were withheld for three days under controlled conditions.