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Liquid chromatography-tandem mass spectrometry assay for the simultaneous quantification of simvastatin, lovastatin, atorvastatin, and their major metabolites in human plasma

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Abstract

Millions of individuals are treated with a variety of statins that are metabolized to a variety of active metabolites. A single assay capable of simultaneously quantifying commonly used statins and their major metabolites has not been previously reported. Herein we describe the development and validation of a novel and robust liquid chromatography-tandem mass spectrometry assay for simultaneously quantifying simvastatin, lovastatin, atorvastatin, and their metabolites, simvastatin acid, lovastatin acid, para-hydroxy atorvastatin, and ortho-hydroxy atorvastatin in human plasma. Plasma samples were processed with a simple protein precipitation technique using acetonitrile, followed by chromatographic separation using an Agilent Zorbax Extend C₁₈ column. A 12.0 minute linear gradient elution was used at a flow rate of 400 µL/minute with a mobile phase of water and methanol, both modified with 2 mM ammonium formate and 0.2% formic acid. The analytes and internal standard, hesperetin, were detected using the selected reaction monitoring mode on a TSQ Quantum Discovery mass spectrometer with positive electrospray ionization. The assay exhibited a linear range of 1–1000 nM for simvastatin acid and lovastatin acid, and a linear range of 0.1–100 nM for the other analytes in human plasma. The accuracy and the within- and between-day precisions of the assay were within acceptable ranges, and the method was successfully utilized to quantify the statins and their metabolites in human plasma samples collected from an ongoing pharmacokinetic study.

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Keywords

Statin; LC-MS/MS; Validation; Metabolite; Human plasma

1. Introduction

Statins (3-hydroxy-3-methylglutaryl-coenzyme A [HMG CoA] reductase inhibitors) are widely used and highly effective in the treatment and prevention of cardiovascular disease. Twenty-five percent of Americans over the age of 45 years use a statin [1]. Despite average benefit in large clinical trials, the efficacy and toxicity of statins varies widely among individual patients. For example, many patients experience cardiovascular events despite statin therapy, and adverse effects such as muscle pain are common [2]. Much of the inter-patient variability in statin efficacy and toxicity can be attributed to wide inter-patient variability in statin pharmacokinetics [3]. Characterization of the factors affecting statin pharmacokinetics could potentially prevent unnecessary toxicity and ineffectiveness in patients treated with statins.

A variety of statins are used clinically, and several distinct metabolites are associated with each statin type. A single assay that could simultaneously quantify several statins and metabolites would play a critical role in advancing statin pharmacokinetic research. Various approaches have been developed for the quantification of statin concentrations in biological matrices. These include LC-UV detection [4–6], LC-fluorescence detection [7], GC/MS [8], LC/MS [9] and LC-MS/MS [10,11], and of these, LC-MS/MS has superior sensitivity and specificity. A single, robust LC-MS/MS assay for simultaneous quantification of simvastatin (Sim), lovastatin (Lov), and atorvastatin (Ator) and their respective major metabolites, simvastatin acid (SimA), lovastatin acid (LovA), para-hydroxy-atorvastatin (*p*-OH-Ator) and ortho-hydroxy-atorvastatin (*o*-OH-Ator) has not been previously reported. Measurement of the major metabolites is critical because Sim and Lov are administered as inactive lactone pro-drugs, which are metabolized *in vivo* to their respective active acid forms [12]. Ator is administered in the active acid form, but its two major metabolites, *p*-OH-Ator and *o*-OH-Ator, are also active [13]. Therefore our goal was to develop and validate a novel, sensitive, and robust LC-MS/MS assay to simultaneously quantify Sim, Lov, Ator and their active metabolites SimA, LovA, *p*-OH-Ator and *o*-OH-Ator in human plasma.

2. Materials and Methods

2.1 Chemicals and reagents

Sim, Lov, Ator and hesperetin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). SimA, LovA, *p*-OH-Ator and *o*-OH-Ator were purchased from Clearsynth Labs Ltd (Mumbai, India). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Reagent-grade formic acid (FA) and ammonium formate (AF) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All chemicals and reagents were used without further purification. A Thermo Scientific Barnstead ultrapure water purification system was used to obtain HPLC-grade water (>18 mΩ).

2.2 Preparation of standard and quality control solutions

Starting solutions (10 mM) for all analytes were prepared separately in DMSO. The stock solution was prepared by diluting the starting solutions with acetonitrile to achieve a concentration of 100 μ M for Sim, Lov, Ator, *p*-OH-Ator, and *o*-OH-Ator and of 1 mM for SimA and LovA. Stock solutions were subsequently stored at -80°C . Series of standard solutions (10–10000 nM for SimA and LovA and 1–1000 nM for the other analytes) were prepared by diluting aliquots of the stock solutions with 50% acetonitrile. Working internal standard (IS) and quality control (QC) solutions were prepared separately in a similar fashion.

2.3 LC-MS/MS conditions

A Thermo Dionex rapid-separation liquid-chromatography system comprising a degasser, ternary pump, column oven, and auto-sampler was used for solvent and sample delivery. Chromatographic separation was performed on an Agilent ZORBAX Extend C₁₈ column (50 $\times$ 2.1 mm I.D, 3.5 μ m particle size) coupled with an Agilent Extend C₁₈ guard cartridge (2.1 mm $\times$ 12.5 mm, 5 μ m particle size) at 40°C with a total run time per sample of 12 min. A wash cycle with 500 μ L 50% methanol/50% water was performed after each injection to limit potential carryover, and a 2 hr column washing with 80% acetonitrile/20% water followed each sample run to maximize assay reproducibility. The mobile phases consisted of methanol and water, both with 0.2% FA and 2 mM AF, pumping at a flow rate of 400 μ L/min. A linear gradient elution was used with the following program: 0.0 min (70% A and 30% B), 1.0 min (70% A and 30% B), 1.5 min (40% A and 60% B), 8.0 min (25% A and 75% B), 11.5 min (25% A and 75% B), 11.51 min (70% A and 30% B) and 12.0 min (70% A and 30% B).

A TSQ Quantum Ultra mass spectrometer equipped with electrospray ionization (ESI) source was used for mass analysis and detection. ESI was performed in the positive ion mode with argon as the collision gas and nitrogen as the sheath and auxiliary gas. The detector was operated at unit resolution (FWHM at 0.7 for both Q1 and Q3) in selected reaction monitoring (SRM) mode using the transitions from the precursor ions to product ions at m/z 405.21>199.13, 285.17, and 303.19 for Lov; m/z 419.23>199.13, 285.17, and 303.19 for Sim; m/z 423.23 >199.13, 285.17, and 303.19 for LovA; m/z 437.24>199.13, 285.17, and 303.19 for SimA; m/z 559.20>440.21 for Ator; m/z 575.20>440.21 for *p*-OH-Ator and *o*-OH-Ator; and m/z 303.06>153.01 for IS. Optimum values for MS parameters were as follows: ion spray voltage, 5000V; skimmer offset, 10V; tube lens offset, 95V; ion transfer tube temperature, 325°C ; collision gas, 1.5 mTorr; sheath and auxiliary gas, 40 and 20 (arbitrary units), respectively. The scan time for each ion transition channel was set to 50 ms and the scan width was set to 0.5 m/z . A switch valve was only used to introduce eluent from 2.0 min to 10.5 min into MS to maintain MS preference.

2.4 Sample Preparation

To 200 μ L patient plasma sample (or blank human plasma), 20 μ L IS solution, 20 μ L 50% acetonitrile (or a standard or QC solution), and 1 mL acetonitrile were combined in a 1.7 mL tube and centrifuged (4°C , 17,110 g, 10 min) after being vortexed (30 sec). The supernatant was then transferred to a glass tube for evaporation under a gentle stream of nitrogen. The

dried residues were reconstituted with 120 μ L of 30% methanol/0.2% FA/2 mM AF, transferred to 1.7mL tubes, and centrifuged (4°C, 17,110 g, 10 min). Supernatant (20 μ L) was subsequently injected into the LC-MS/MS system for analyte quantification.

2.5 Method validation

The assay was fully validated for linearity, accuracy, precision, selectivity, carryover, recovery, matrix effect, and stability in accordance with FDA guidelines [14]. The selectivity of the assay was determined by analyzing six different lots of human plasma with and without analytes at low limit of quantitation (LLOQ) level and internal standard. Carryover was evaluated by injecting double blank sample after the injection of the upper limit of quantitation (ULOQ) sample. Three independent calibration curves, prepared individually on three separate days, based on seven spiked plasma samples (1, 3, 10, 30, 100, 300 and 1000 nM for SimA and LovA, and 0.1, 0.3, 1, 3, 10, 30 and 100 nM for the other analytes) were evaluated to validate the linearity of the method. The calibration curves were analyzed by peak-area ratios of analytes/IS (weighted linear regression, $1/x^2$). Accuracy was determined by comparing the corresponding calculated concentrations to nominal concentrations. Accuracy was required to be between 85–115%, except for the LLOQ, where 80–120% was acceptable. Within and between-day precisions (as CV %) were determined using six replicates of QC samples (1, 3, 30, and 800 nM for simvastatin acid/lovastatin acid and 0.1, 0.3, 3 and 80 nM for the other analytes). Recoveries of analytes and IS were determined by comparing peak areas of QC samples with those of corresponding concentrations of QC solutions dissolved in the supernatant of the processed blank matrix samples. Matrix effects were evaluated by comparing the peak areas of processed blank matrix samples spiked with QC solutions to those of standard solutions. Stability tests were performed by comparing QC samples representing the following conditions: long term storage (one month at -80°C), freeze-thaw (three cycles), bench top (6 hr at 4°C), and autosampler (reconstituted sample at 4°C for 12 hr). Stability testing of the analytes in the stock solution was also performed in samples stored at -80°C for one month.

2.6 Application of the assay

The assay was used to quantify analytes (statins and their metabolites) in plasma samples collected from participants in an ongoing statin pharmacokinetics study ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=NCT01569867) ID NCT01569867) at The Ohio State University (OSU). Patients receiving Sim, Lov, or Ator for treatment of a dyslipidemia can be enrolled in the study. Participants are required to be between 35 and 65 years of age, inclusive, and to have a body mass index between 20 and 30 kg/m^2 . Those taking concomitant medications known to interfere with statin metabolism, those with a history of liver or muscle disease, and those with a history of cardiovascular events are excluded from participation in the study. The study is conducted under the auspices of the OSU internal review board, and informed consent is documented for all study participants. Blood samples are collected in 10 mL EDTA-containing “lavender-top” vacutainers, transported in a thermally-insulated bag with ice, and centrifuged at 1900 rpm for 10 minutes at 4°C . Aliquots of plasma are stored at -80°C (< 1 year) before being analyzed for quantities of statins and metabolites. Based on our previous research and the previously reported pharmacokinetic properties of Sim, Ator, and Lov and

[15], we expected that the concentrations of the statins and their metabolites would be detectable within the linear calibration range of the assay.

3. Results and Discussion

3.1 Method development

All analytes produced higher signals by ESI than by atmospheric pressure chemical ionization. Full-scan product ion-spectra and fragmentation pathways of analytes and IS are shown in Figure 1. All analytes and IS exhibited dominant protonated molecular ions. To achieve optimal sensitivity for quantification, the three most abundant product ions were chosen for ion transition monitoring for Sim, Lov, and their metabolites. Likewise, the most abundant product ions were chosen for monitoring Ator and its metabolites. For tuning the instrument response and optimizing the MS parameters, concentrated standard solutions of each analyte were infused separately through a syringe pump (5 $\mu\text{L}/\text{min}$) into the stream (400 $\mu\text{L}/\text{min}$) of mobile phases eluting from the column. All parameters, except the collision energy and lens offset voltage, were optimized for LovA because since it had the lowest MS response of the analytes being quantified.

Among the several commercial columns tested, an Agilent Zorbax Extend C_{18} column was selected because it provided optimal chromatography, minimal matrix effects, and minimal run time. Methanol was selected as the organic component for the mobile phases because it provided 20% higher MS response than acetonitrile. The column oven temperature was set to 40°C to reduce elevated backpressure. A weakly acidic (pH 3.5) aqueous mobile phase with formic acid (FA) and ammonium formate (AF) was selected to minimize the inter-conversion of acid and lactone forms [16–18]. The FA concentration was set to 0.2% (v/v) to reduce peak tailing and improve peak shape without decreasing response, and the AF concentration was set to 2 mM to provide sufficient buffer strength without jeopardizing the dominance of $[\text{M}+\text{H}]^+$ signal. A linear gradient program was used to achieve better separation between *p*-OH-Ator and *o*-OHAator and to allow fast elution of the other analytes. A cleanup section was also added, improving analysis reproducibility. The entire assay required 200 μL of biosamples and allowed for a relatively high sample throughput (120 samples/24 hr). The single step protein precipitation was selected because of its simplicity, high recovery, and because it can accommodate the wide range of physical and chemical properties among analytes. A switching valve was used after column elution in order to attenuate hydrophilic interference.

In general, the crosstalk between SRM channels from the analytes with similar product spectra is a potential problem, which could be caused by source-induced collision and insufficient collision cell cleanup. By separately injecting each analyte to MS, we have determined 50 ms scan time is sufficient for the collision cell cleanup, and the crosstalk of our assay was mainly contributed by the source-induced collision of LovA and SimA to Lov and Sim, respectively. Because of the different retention times, no significant impact was found in quantitation of Lov and Sim from LovA and SimA.

Selecting an appropriate IS and sample preparation method are critical for an accurate and robust LC-MS/MS assay. Although an isotopic IS would be ideal, it was not practical for our

assay because of varied retention times among the analytes. Hesperetin was chosen as the IS because it had a satisfactory peak shape, relatively high recovery, and stable MS response.

3.2 Assay validation

The assay presents great selectivity, as no significant interference peak was observed at the retention times of the analytes and IS from six different lots of human blank plasma (Figure 2). A summary of the three separated calibration curves is shown in Table 1. The calibration curves were linear in the range of 1–1000 nM with an LLOQ of 1 nM for SimA and LovA. For all other analytes, the calibration curves were linear in the range of 0.1–100 nM with an LLOQ of 0.1 nM. The correlation coefficients (r^2) were > 0.995 and the accuracy within 86.19–109.76%. In addition, the precisions (CV %) of three calibration curves were less than 13.32%, indicating good assay reproducibility.

The accuracy and precision results are listed in Table 2. Based on six replicates of QC samples at LLOQ, low, mid, and high levels, the within-run and between-run precisions (CV %) were 10.66%, and the mean assay accuracies were between 91.37–109.94%. The percentage of carryover was calculated by comparing the responses of all the analytes in the double blank sample injected immediately after the ULOQ sample to the responses of the analytes in the ULOQ sample. The carryovers of statins and their metabolites in the ULOQ sample were less than 0.042 %, and thus carryover is not expected to have a significant impact. The carryover of IS was also evaluated similarly, and no significant carryover was found.

Recovery and matrix effect were evaluated with triplicate samples at QC concentrations of 1, 3, 30, and 800 nM for SimA and LovA and 0.1, 0.3, 3, and 80 nM for the remaining analytes. As demonstrated by the values presented in Table 3, this single step protein precipitation provided consistently high recovery for all analytes throughout the linearity range. Although matrix effect is a common barrier in LC-MS/MS bioassay development, no significant matrix effect was found for the analytes in this assay (Table 3). All analytes were stable under the experimental conditions described above (Table 4). Mean recoveries of triplicate samples of four QC concentrations were measured to be 90.80–110.67% of the nominal concentrations (Table 4), indicating no significant lactone-carboxylic acid conversion occurred during sample analyses. The stock solution stability was tested with the ULOQ standard solution, and the range of accuracy was 94.93 to 104.48%.

3.3 Application and limitations of the assay

Measured concentrations of statins and metabolites in plasma collected from a representative subset of study participants are presented in Table 5. Measured concentrations were consistent with the statin type reported in the patients' medical records. Non-zero quantification of Lov and LovA in participant samples could not be demonstrated because all participants enrolled thus far ($n \approx 100$) had been prescribed only Ator or Sim. The accuracy and precision for quantifying Lov and LovA, however, were demonstrated in the analyses of QC samples (Table 2). Our assay was not developed to detect atorvastatin lactone (implicated in adverse effects of atorvastatin treatment [3]), or other statins such as rosuvastatin, fluvastatin, or pravastatin. Our assay was only validated to quantify the

analytes in plasma and no other potentially relevant biologic matrices, such as skeletal muscle tissue.

4. Conclusion

We developed and validated a novel, sensitive, specific, and robust assay to simultaneously quantify simvastatin, lovastatin, atorvastatin, and their major metabolites in human plasma. Millions of patients are treated with statins, and the assay we have developed has significant utility for a variety of statin research applications involving Sim, Ator, and Lov.

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Abbreviations

Statins	3-hydroxy-3-methylglutaryl-coenzyme A [HMG CoA] reductase inhibitors
Sim	Simvastatin
Lov	Lovastatin
Ator	Atorvastatin
SimA	Simvastatin acid
LovA	Lovastatin acid
p-OH-Ator	Para-hydroxy atorvastatin
o-OH-Ator	Ortho-hydroxy atorvastatin
FA	formic acid
AF	ammonium formate

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Highlights

- A novel and robust LC-MS/MS assay was developed for multiple statins & metabolites.
- Simple protein precipitation provided high recovery for all analytes.
- The linear quantifiable ranges were validated for 0.1–100nM and 1–1000nM.
- The accuracy and precision of the assay met FDA standards.
- The assay was successfully applied to human plasma samples from a clinical study.

Fig. 1.
Full-scan product ion spectra of $[M-H]^+$ for lovastatin (B), simvastatin (C), lovastatin acid (D), simvastatin acid (E), atorvastatin (F), para-hydroxy atorvastatin/ortho-hydroxy atorvastatin (G/H) and the internal standard, hesperetin (A).

Fig. 2.

Representative SRM chromatograms of blank human plasma (I) and blank human plasma spiked with analytes at LLOQ (II): IS, hesperetin (A), lovastatin (B), Simvastatin (C), lovastatin acid (D), Simvastatin acid (E), atorvastatin (F) and para-hydroxy atorvastatin/ortho-hydroxy atorvastatin (G/H).

Table 1

Calibration curves (n=3) of analytes in human plasma

Analyte	Nominal concentrations (nM)	0.1/1	0.3/3	1/10	3/30	10/100	30/300	100/1000
AtorOH-P	Calculated concentrations (nM, Mean)	0.10	0.31	1.02	2.89	9.92	28.74	94.91
	Accuracy%	98.80	102.86	101.96	96.34	99.24	95.81	94.91
	CV%	6.51	7.44	11.24	7.01	5.19	5.06	3.97
AtorOH-O	Calculated concentrations (nM, Mean)	0.10	0.31	0.97	3.25	9.16	28.93	96.60
	Accuracy%	104.16	102.55	96.51	108.47	91.59	96.42	96.60
	CV%	4.88	7.05	3.39	3.68	4.39	5.63	4.52
Ator	Calculated concentrations (nM, Mean)	0.10	0.31	0.93	2.97	9.95	31.50	104.02
	Accuracy%	100.32	102.34	93.03	98.98	99.52	104.99	104.02
	CV%	2.73	7.63	1.88	5.69	4.59	8.28	4.59
LovA	Calculated concentrations (nM, Mean)	1.02	2.91	9.31	29.44	107.14	286.97	953.37
	Accuracy%	101.93	97.10	93.14	98.12	107.14	95.66	95.34
	CV%	2.83	3.92	5.85	4.23	4.48	3.59	3.89
Lov	Calculated concentrations (nM, Mean)	0.10	0.28	0.95	2.96	10.40	30.33	103.35
	Accuracy%	103.13	93.10	95.14	98.78	104.05	101.10	103.35
	CV%	4.49	4.36	6.32	6.31	2.51	4.11	5.68
SimA	Calculated concentrations (nM, Mean)	0.98	2.92	10.98	29.81	99.43	296.14	1070.26
	Accuracy%	98.03	97.28	109.76	99.38	99.43	98.71	107.03
	CV%	10.60	4.31	3.13	4.64	5.86	3.97	10.50
Sim	Calculated concentrations (nM, Mean)	0.10	0.30	0.86	2.67	10.50	30.48	96.01
	Accuracy%	104.87	98.45	86.19	89.01	105.03	101.61	96.01
	CV%	4.63	5.74	4.40	13.32	1.89	3.18	1.99

AtorOH-P = para-hydroxy atorvastatin; AtorOH-O = ortho-hydroxy atorvastatin; Ator = atorvastatin; LovA = lovastatin; SimA = simvastatin acid; Lov = lovastatin; Sim = simvastatin

Table 2

Within- and between-day precision and accuracy of QC samples in human plasma

Analyte	Nominal Conc. (nM)	Within-day (n=6)			Between-day (n=6)		
		Mean Conc. (nM)	Accuracy %	CV%	Mean Conc. (nM)	Accuracy %	CV%
AtorOH-P	0.10	0.10	102.00	3.04	0.10	101.94	4.58
	0.30	0.30	100.65	3.32	0.30	99.08	4.29
	3.00	3.01	100.47	5.55	2.92	97.43	3.55
	80.00	80.52	100.65	3.32	77.67	97.08	4.47
AtorOH-O	0.10	0.10	97.48	4.59	0.10	97.53	3.83
	0.30	0.30	100.85	2.59	0.30	98.38	2.72
	3.00	3.03	100.88	3.01	2.99	99.75	2.43
	80.00	80.68	100.85	2.59	79.60	99.49	4.11
Ator	0.10	0.10	95.15	1.84	0.09	92.15	2.46
	0.30	0.29	97.02	2.89	0.28	92.80	3.04
	3.00	2.92	97.48	1.65	2.84	94.78	2.34
	80.00	77.62	97.02	2.89	75.04	93.80	2.66
LovA	1.00	1.02	101.88	4.44	0.99	99.22	4.74
	3.00	3.07	102.47	5.21	3.00	99.94	3.69
	30.00	29.48	98.25	4.95	30.51	101.69	5.44
	800.00	782.00	97.75	3.53	797.56	99.69	4.18
Lov	0.10	0.10	96.17	3.03	0.10	97.33	1.68
	0.30	0.29	96.95	2.22	0.30	100.09	5.03
	3.00	2.87	95.75	2.64	2.94	97.83	3.27
	80.00	79.73	99.67	4.08	77.61	97.01	7.11
SimA	1.00	0.97	97.48	1.00	0.94	94.49	3.22
	3.00	2.88	95.98	1.32	2.81	93.83	1.67
	30.00	29.97	99.90	1.74	28.10	93.67	7.22
	800.00	776.67	97.08	1.51	779.00	97.38	1.93

Analyte	Nominal Conc. (nM)	Within-day (n=6)			Between-day (n=6)		
		Mean Conc. (nM)	Accuracy%	CV%	Mean Conc. (nM)	Accuracy%	CV%
Sim	0.10	0.10	95.33	3.96	0.10	95.56	4.24
	0.30	0.29	95.75	4.55	0.31	103.47	5.71
	3.00	2.95	98.22	3.76	2.93	97.52	8.60
	80.00	78.13	97.67	4.56	72.20	90.25	3.92

AtorOH-P = para-hydroxy atorvastatin; AtorOH-O = ortho-hydroxy atorvastatin; Ator = atorvastatin; LovA = lovastatin acid; Lov = lovastatin; SimA = simvastatin acid; Sim = simvastatin

Table 3

Recovery and matrix effect of analytes in human plasma

Analyte	Nominal Conc. (nM)	Recovery (n=3)		Matrix effect (n=3)	
		Accuracy%	CV%	Accuracy%	CV%
AtorOH-P	0.10	96.40	8.03	97.45	6.54
	0.30	96.52	4.14	100.15	8.20
	3.00	95.96	8.69	107.04	5.18
	80.00	98.41	4.88	109.98	3.01
AtorOH-O	0.10	91.42	5.76	100.05	0.36
	0.30	95.33	2.99	107.47	0.80
	3.00	102.67	2.06	103.95	7.35
	80.00	101.74	2.88	107.94	8.74
Ator	0.10	91.20	4.58	106.44	2.52
	0.30	101.91	9.31	107.03	1.79
	3.00	109.94	2.85	102.21	11.95
	80.00	105.30	2.60	89.66	3.12
LovA	1.00	100.12	7.13	105.66	8.26
	3.00	104.29	10.66	102.35	0.73
	30.00	101.07	4.71	102.79	5.80
	800.00	101.96	8.73	97.92	4.81
Lov	0.10	91.37	1.37	95.01	2.96
	0.30	91.90	5.00	110.08	2.36
	3.00	92.64	1.78	114.07	5.93
	80.00	97.01	4.11	94.46	5.72
SimA	1.00	92.14	1.29	103.44	1.51
	3.00	95.96	4.00	102.13	3.27
	30.00	100.32	1.83	103.30	8.14
	800.00	101.03	4.28	99.86	3.78

Analyte	Nominal Conc. (mM)	Recovery (n=3)		Matrix effect (n=3)	
		Accuracy%	CV%	Accuracy%	CV%
Sim	0.10	98.75	8.63	96.57	2.72
	0.30	100.44	7.38	100.01	1.77
	3.00	99.14	2.85	99.01	5.75
	80.00	97.19	2.21	99.95	4.54

AtorOH-P = para-hydroxy atorvastatin; AtorOH-O = ortho-hydroxy atorvastatin; Ator = atorvastatin; LovA = lovastatin acid; Lov = lovastatin; SimA = simvastatin acid; Sim = simvastatin

Table 4

Stability of analytes in human plasma samples (n=3)

Analyte	Nominal Conc. (nM)	Benchtop, 4°C		Autosampler, 4°C		Three freeze-thaw cycles		One month storage, -80°C	
		Accuracy%	CV%	Accuracy%	CV%	Accuracy%	CV%	Accuracy%	CV%
AtorOH-P	0.10	96.69	2.30	101.44	3.50	96.07	5.35	98.47	11.17
	0.30	101.54	7.60	105.34	2.04	97.23	2.91	90.80	3.18
	3.00	103.02	6.51	101.00	2.62	102.40	4.74	104.80	9.40
	80.00	95.81	2.56	99.00	4.40	105.20	3.71	106.60	3.02
AtorOH-O	0.10	99.52	4.34	99.23	2.17	100.67	0.57	106.60	3.02
	0.30	100.80	1.40	96.62	1.01	95.23	2.97	95.67	5.06
	3.00	109.42	0.56	100.30	9.05	106.07	4.14	96.47	3.17
	80.00	98.25	6.85	102.63	6.05	102.13	7.15	102.07	6.07
Ator	0.10	97.63	3.84	102.02	3.35	103.13	4.99	100.13	5.74
	0.30	104.64	4.42	103.78	2.56	98.10	2.70	94.47	4.40
	3.00	102.62	5.92	96.10	7.37	99.60	5.39	100.47	7.14
	80.00	95.81	2.56	95.83	6.48	104.20	7.01	98.60	11.10
LovA	1.00	95.34	3.33	98.25	3.17	100.30	2.34	96.47	4.00
	3.00	105.11	1.16	95.58	6.52	98.73	2.03	99.33	1.11
	30.00	106.66	2.94	101.67	0.57	101.93	2.16	93.40	7.49
	800.00	99.81	4.53	94.77	5.82	105.47	4.50	109.40	2.87
Lov	0.10	104.93	0.96	109.17	2.35	102.03	3.34	98.53	3.68
	0.30	106.00	0.47	106.17	4.06	97.70	9.67	108.80	5.12
	3.00	101.33	6.51	95.43	6.08	100.20	2.30	100.07	1.20
	80.00	102.50	6.56	94.27	2.65	103.13	5.37	105.00	5.24
SimA	1.00	99.40	2.66	100.10	3.56	101.33	6.57	96.20	9.21
	3.00	105.33	3.63	102.33	2.69	97.17	3.43	98.80	7.35
	30.00	104.67	5.70	102.33	3.14	103.40	1.96	99.87	4.99
	800.00	99.67	1.26	95.20	7.11	104.33	4.57	101.43	2.62
Sim	0.10	107.07	3.40	99.03	3.99	102.67	2.98	100.20	7.97
	0.30	110.67	2.14	104.00	4.74	98.17	2.56	98.47	11.17
	3.00	106.83	0.97	92.23	1.42	104.53	3.35	102.77	5.25
	80.00	96.43	3.35	104.77	7.79	106.33	2.56	109.03	1.97

AtorOH-P = para-hydroxy atorvastatin; AtorOH-O = ortho-hydroxy atorvastatin; Ator = atorvastatin; LovA = lovastatin acid; Lov = lovastatin; SimA = simvastatin acid; Sim = simvastatin

Table 5

Measured concentrations of statins and metabolites in plasma samples collected from a preliminary subset of study participants.

Sample Identifier	Statin Type	Plasma concentrations (nM)						
		AtorOH-P	AtorOH-O	Ator	LovA	Lov	SimA	Sim
PS A1	Ator	0.27	0.33	0.11	-	-	-	-
PS A2	Ator	0.78	2.69	2.70	-	-	-	-
PS A3	Ator	1.99	5.86	3.89	-	-	-	-
PS A4	Ator	4.63	10.93	2.82	-	-	-	-
PS A5	Ator	1.05	2.67	1.44	-	-	-	-
PS A6	Ator	0.95	3.45	2.25	-	-	-	-
PS A7	Ator	1.72	3.43	6.27	-	-	-	-
PS A8	Ator	2.31	9.23	4.16	-	-	-	-
PS A9	Ator	7.36	28.11	9.75	-	-	-	-
PS A10	Ator	9.45	21.39	17.78	-	-	-	-
PS A11	Ator	2.78	5.18	1.85	-	-	-	-
PS A12	Ator	6.17	35.09	44.74	-	-	-	-
PS A13	Ator	1.23	4.87	2.07	-	-	-	-
PS S1	Sim	-	-	-	-	-	-	0.55
PS S2	Sim	-	-	-	-	-	-	0.33
PS S3	Sim	-	-	-	-	-	-	1.05
PS S4	Sim	-	-	-	-	-	-	0.40
PS S5	Sim	-	-	-	-	-	1.34	1.19
PS S6	Sim	-	-	-	-	-	6.58	6.85
PS S7	Sim	-	-	-	-	-	-	0.59
PS S8	Sim	-	-	-	-	-	1.42	0.18
PS S10	Sim	-	-	-	-	-	2.61	0.29

AtorOH-P = para-hydroxy atorvastatin; AtorOH-O = ortho-hydroxy atorvastatin; Ator = atorvastatin; LovA = lovastatin acid; Lov = lovastatin; SimA = simvastatin acid; Sim = simvastatin