A Simple Protein Precipitation-based Simultaneous Quantification of Lovastatin and Its Active Metabolite Lovastatin Acid in Human Plasma by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry using Polarity Switching

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Abstract

Lovastatin is an anti-cholesterol lactone drug indicated for the treatment of hyperlipidemia and to reduce the risk of coronary heart disease. It is converted to the β-hydroxy acid form (lovastatin acid) in vivo, which is the major pharmacologically active metabolite. Here, we describe the development and validation of an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)-based method utilizing polarity switching for the simultaneous quantification of lovastatin and lovastatin acid in human plasma. Simple protein precipitation extraction and direct injection of the extracted samples without drying/reconstitution showed good recoveries of both analytes (~70%). The developed method exhibited satisfactory intra-day and inter-day accuracy and precision. The interconversion between lovastatin and lovastatin acid during sample preparation and storage was minimal (< 1.9%). The lower limits of quantification were 0.5 and 0.2 nM (or 0.2 and 0.084 ng/mL) for lovastatin and lovastatin acid, respectively, using only 50 μL of plasma during extraction. The validated method was successfully applied to analyze plasma samples obtained from a healthy human subject who enrolled in a clinical drug interaction study involving lovastatin.

Keywords: Lovastatin; Lovastatin acid; UPLC-MS/MS; Polarity switching; Protein precipitation extraction; Pharmacokinetics

Abbreviations: Auc: Area Under The Plasma Concentration-Time Curve; Ce: Collision Energy; Cid: Collision-Induced Dissociation; Cmax: Maximum Concentration; Cyp: Cytochrome P450; Cv: Coefficient Of Variation; Dmso: Dimethyl Sulfoxide; Hmgcr: 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase; Hplc-Uv: High-Performance Liquid Chromatography-Ultraviolet Detection; Is: Internal Standard; Lle: Liquid–Liquid Extraction; Loq: Lower Limit Of Quantification; Lv: Lovastatin; Lva: Lovastatin Acid; Mrm: Multiple Reaction Monitoring; Ppe:protein Precipitation Extraction; Qc: Quality Control; S.d.: Standard Deviation; Spe: Solid-Phase Extraction; S/N: Signal-To-Noise Ratio; Sv: Simvastatin; Sva: Simvastatin Acid; T1/2: Terminal Elimination Half-Life; Tmax:time To Reach Cmax; Uplc-Ms/Ms: Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

Introduction

Lovastatin (LV) is a member of the class of cholesterol-lowering agents known as statins and is indicated for the treatment of dyslipidemia and the prevention of coronary heart disease. LV, a lactone, is readily hydrolyzed in vivo to form the pharmacologically active metabolite lovastatin β-hydroxy acid (LVA), which inhibits 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), a key enzyme in the cholesterol biosynthetic pathway [1]. In human liver, LV also is metabolized by cytochrome P450 (CYP) 3A4 to form 6'-β-hydroxy- and 6'-exomethylene-LV, both of which also are HMGCR inhibitors [2,3]. However, neither metabolite is readily detected in vivo. Additional LV metabolites (e.g., 3'-hydroxy-LV and conjugates formed after β-oxidation of the heptanoic acid moiety of LVA) can be detected in vivo, but do not inhibit HMGCR [2]. Therefore, LV and LVA are the major drug-derived molecules that can be used for therapeutic drug monitoring of LV treatment. Sensitive and specific analytical methods for simultaneous measurement of LV and LVA in human plasma are needed.

A number of studies have described the development and validation of analytical methods for LV. Pasha et al. [4] described an HPLC method coupled to UV detection (HPLC-UV), monitoring at a wavelength of 237 nm, to separate LV and four other statins. The lower limit of quantification (LLOQ) for LV was 0.1 μg/mL (~250 nM). This method is useful for pharmaceutical formulation analysis and in vitro metabolism studies, but lacks the sensitivity (sub-nM LLOQ) required for pharmacokinetic studies. A more sensitive HPLC-UV method, which achieved an LLOQ of 1.0 ng/mL (~2.5 nM) for LV, was described more recently [5]. However, it requires a relatively large volume (2 mL) of human plasma. Marked enhancement in sensitivity for LV has been achieved by employing tandem mass spectrometric (MS/MS) detection with the LLOQ ranging from 0.025 to 0.1 ng/mL (~0.06 to 0.25 nM) using 0.2-0.5 mL of human plasma [6-9]. However, all sample preparation methods were complicated and time-consuming, requiring liquid-liquid extraction (LLE) with tert-butyl methyl ethyl, ethyl acetate, ether-methylene chloride or n-hexane-methylene dichloride-isopropanol, followed by evaporation under nitrogen at an elevated temperature (40–50°C). In general, recovery following LLE was approximately 85%, but ethyl acetate gave a low of 55%.

Few reports have described the simultaneous measurement of LV and LVA.
and LVA in human plasma. Sun et al. [10] described a solid-phase extraction (SPE)- and HPLC-MS/MS-based analytical method for LV and LVA in human plasma, applying the method for a comparative pharmacokinetic study of different LV dosage forms. However, the report did not provide details of the method (e.g., plasma volume used, extraction recovery; ions monitored, matrix effect or stability) to allow cross-evaluation. In another report, Wu et al. [11] described an SPE- and HPLC-MS/MS-based analytical method for LV and LVA in mouse and rat plasma. This method, which used 0.1 mL of plasma, had an LLOQ of 0.5 ng/mL (~1.2 nM) and an extraction recovery of ~55% for LV. HPLC-UV methods [12,13] also have been reported for the simultaneous quantification of LV and LVA, exhibiting an LLOQ ≥ 25 ng/mL (~62 nM). However, in general, these methods lack the necessary sensitivity for pharmacokinetic studies. The purpose of the study described herein is to develop and validate a simple protein precipitation extraction (PPE), direct injection, UPLC-MS/MS-based analytical method for the simultaneous quantification of LV and LVA in human plasma.

Materials and Methods

Materials

LV was purchased from U.S. Pharmacopeia (Rockville, MD, USA). LVA, simvastatin (SV) and simvastatin acid (SVA) were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). SV and SVA were used as internal standards (IS) for the quantification of LV and LVA, respectively. Ammonium acetate and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Optima-grade water, acetonitrile, methanol and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA, USA). Blank human plasma (collected in K$_2$-EDTA tubes) was purchased from Innovative Research (Novi, MI, USA).

Instrument and chromatographic conditions

The UPLC–MS/MS system consisted of a Waters Acquity I-Class UPLC and a Xevo TQ-S triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Waters Corporation, Milford, MA, USA). Chromatographic separation was achieved using an ACQUITY UPLC BEH C$_18$ (1.7 µm, 2.1 x 50 mm) column, which was protected by an ACQUITY column in-line filter (0.2 µm). UPLC mobile phase (A) consisted of 100% water with 5 mM ammonium acetate (pH 4.5), while (B) consisted of acetonitrile-ammonium acetate (50 mM; pH 4.5) (90:10, v/v). The pH of each mobile phase (adjusted with acetic acid) was selected to minimize interconversion of LV and LVA, based on previous observations and recommendations for SV and SVA [14,15]. The gradient began with 40% B and was held for 0.5 min. Mobile phase B increased linearly to 70% B at 0.8 min, then to 80% B at 3.5 min. The column was washed with 100% B for 0.5 min before the system was re-equilibrated with 40% B for 1 min prior to the next injection. The total run time was 5 min with a flow rate of 0.4 mL/min. The autosampler was set at 6°C and the UPLC column heated at 50°C. The sample injection volume was 50 µL.

The mass spectrometer was operated under negative ion mode for the first 1.9 min to allow for the detection of LVA and SVA (as IS). Instrumental conditions during this time were: capillary voltage, 3.10 kV; cone voltage, -32 V; source offset, 50 V; desolvation temperature, 500°C; desolvation gas flow, 1000 L/h; cone gas flow, 150 L/h; nebulizer gas flow, 7.0 bar; and collision gas flow, 0.15 mL/min. Analyte-specific instrument parameters (i.e., collision energy, capillary voltage and cone voltage) were optimized prior to analysis using the IntelliStart™ auto-tune with infusion of analyte standards (500 nM in methanol at 0.010 mL/min infusion rate, supplemented with 50:50 (v/v) mobile phase A:mobile phase B at 0.4 mL/min). The specific MRM transitions used for quantification were m/z 421.4 → 319.3 for LVA and 435.4 → 319.3 for SVA. After 1.9 min, the instrument was switched to positive ion mode for the detection of LV and SV (as IS). The instrumental conditions were: capillary voltage, 1.10 kV; cone voltage, 30 V; source offset, 50 V; desolvation temperature, 500°C; desolvation gas flow, 1000 L/h; cone gas flow, 150 L/h; nebulizer gas flow, 7.0 bar; and collision gas flow, 0.15 mL/min. Similar to before, analyte-specific instrument parameters were optimized prior to analysis using the IntelliStart™ auto-tune with infusion of analyte standards (500 nM in methanol at 0.010 mL/min infusion rate, supplemented with 50:50 (v/v) mobile phase A:mobile phase B at 0.4 mL/min). The specific MRM transitions used for quantification were m/z 427.3 → 325.2 for LV as a sodium adduct and m/z 441.3 → 325.2 for SV as a sodium adduct. Peak area ratios of analyte vs. IS were used to generate calibration curves and calculate analyte concentrations in plasma samples.

Preparation of calibration standards and quality controls

Individual stock solutions of LV, LVA, SV, and SVA were prepared by dissolving an appropriate amount of each standard in DMSO to give a final concentration of 10 mM. These stock solutions were stored at -20°C. To prepare working standard stocks, the individual stocks were first diluted to 10 µM with methanol and then further diluted with methanol to yield working concentrations that were 50-fold higher than the intended final concentrations in plasma. Working standard stocks were prepared fresh from the 10 µM stocks and were not stored after being used to prepare calibration standards and QCs. Calibration standards were generated by spiking 1.0 µL of working standard stock into 49 µL blank human plasma, yielding the following final concentrations: 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 nM for LV. Similarly, a separate set of calibration standards was prepared for LVA, LV and LVA quality controls (QCs) also were prepared separately from the individual stock solutions to give final concentrations of 2, 10, 50 and 100 nM LV or LVA in human plasma. Calibration standards were prepared in triplicate and QCs in quadruplicate. All were processed as plasma samples prior to UPLC-MS/MS analysis.

Sample preparation by protein precipitation extraction

Each plasma sample (50 µL), including calibration standards and QCs, was thawed at room temperature and immediately placed on ice. Fifty µL of ice-cold sodium acetate buffer (100 mM; pH 4.5 at room temperature) was added to minimize the interconversion of LV and LVA during sample preparation. Proteins in the mixture were precipitated and extracted with ice-cold methanol (200 µL) containing 0.1% (v/v) acetic acid and 3 nM each of SV and SVA as IS. After vortexing for 10 s, the samples were centrifuged at 2250 g for 15 min at 4°C. The supernatant (180 µL) was transferred to 96-well polystyrene plates, from which an aliquot (50 µL) of sample was injected for UPLC-MS/MS analysis.

Method validation

The specificity of the method was investigated by comparing UPLC-MS/MS extracted ion chromatograms of blank plasma to blank plasma spiked with working standard stocks at the LLOQ. The LLOQ was defined as the lowest concentration of calibration standards that had a signal-to-noise ratio (S/N) greater than 5, an accuracy of 80-120%, and an imprecision of ≤ 20%. The limit of detection (LOD) was defined as the lowest concentration of calibration standards that resulted in an S/N of at least 3.
The extent of interconversion between LV (lactone) and LVA (β-hydroxy acid) that would occur during sample preparation and storage was evaluated by quantifying both LV and LVA present in freshly made 50 and 100 nM LV-only and LVA-only QC samples. These QC samples were analyzed immediately after preparation and again after 24 h of storage in an autosampler at 6°C. Statistical analyses were performed using unpaired, two-tailed Student’s t-tests (GraphPad Prism 5.04; GraphPad Software, Inc., La Jolla, CA, USA).

The intra-day accuracy and precision were determined by replicate analyses of the QCs on the same day. The inter-day accuracy and precision were determined by replicate analyses of the QCs on three separate days. The accuracy (%) was defined as the closeness of the average QC concentration determined by the current method to the true QC concentration. The precision (coefficient of variation [CV]; %) was defined as the spread of individual measures of multiple QC preparations.

The extraction recovery was determined by comparing peak area ratios of an analyte versus IS obtained from QC samples that were spiked with analyte prior to extraction to those obtained from blank plasma extracts that were spiked with known amounts of pure analyte following extraction (represents 100% recovery). The extraction recovery was examined at three QC concentrations (2, 10 and 50 nM for LV and LVA) and in quadruplicate at each concentration.

Matrix effects during the MS/MS analysis were evaluated by comparing peak areas of analyte from blank plasma extracts that were spiked with known amounts of pure analyte to peak areas from samples prepared in the neat solvent (water-100 mM sodium acetate [pH 4.5]-methanol containing 0.1% [v/v] acetic acid [50:50:200, v/v/v]) with known amounts of pure analyte. The matrix effects were examined at three QC concentrations (2, 10 and 50 nM for LV and LVA) and in quadruplicate at each concentration. Results were expressed as % signal remaining relative to standards in the neat solvent (represents 100% signal remaining or no suppression).

Freeze-thaw stability was evaluated by exposing QCs to three freeze (-20°C)-thaw (room temperature) cycles before sample preparation. Long-term storage stability was evaluated by storing QCs and sodium acetate-buffered QCs at -80°C for up to one month. The sodium acetate-buffered QCs were prepared by mixing QCs with an equal volume of 100 mM sodium acetate (pH 4.5). Freeze-thaw stability and long-term

Figure 1: Q1 full-scan mass spectra (MS) and product ion mass spectra (MS/MS) of LV (A) and LVA (B), and the internal standards SV (C) and SVA (D). The precursor and product ion pairs selected for MRM detection are shown in boxes. The postulated structure of each is also depicted. The signal intensity for the most intense peak is in the upper-left corner of each spectrum. CE, collision energy.
storage stability were examined at three QC concentrations (2, 10 and 50 nM for LV and LVA) and in quadruplicate at each concentration. Results were expressed as % signal remaining relative to freshly-made QCs (represents 100% signal remaining).

Analysis of human plasma samples from a clinical study

Blood samples were obtained from a healthy volunteer who participated in a drug-drug interaction clinical study involving lovastatin. Lovastatin was given once daily for 14 days. On the morning of the 7th day following an overnight fast, the volunteer received the 7th dose of lovastatin (40 mg) and a single dose of warfarin (10 mg) by mouth simultaneously. Venous blood (5 mL) was collected in K₂-EDTA Vacutainer® tubes (BD Biosciences, Franklin Lakes, NJ, USA) via an intravenous line at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h post-drug administration. Within 1 h after collection, blood samples were centrifuged (3000 rpm at 4°C for 10 min) and the resulting plasma samples transferred to pre-labeled cryotubes for storage at -80°C until analysis. The clinical study (ClinicalTrials.gov registry number: NCT01250535) was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (Chapel Hill, NC, USA).

The area under the plasma concentration–time curve (AUC), terminal elimination half-life (t½), maximum plasma drug concentration (Cmax), and the time to reach Cmax (Tmax) were calculated using the trapezoidal rule–extrapolation method and non-compartmental analysis (Phoenix WinNonlin Version 6.3; Pharsight, Mountain View, CA, USA).

Results and Discussion

UPLC–MS/MS analysis

Apostolou et al. [16] previously reported that ESI was more sensitive for both SV and SVA than atmospheric pressure chemical ionization (APCI). Due to the structural similarity between LV/LVA and SV/SVA (Figure 1), ESI was selected for method development in this study. Under positive ion mode, LV and SV produced molecular ions at m/z 427.3 and 441.3, indicating the formation of sodium adducts with little protonated ions detected (Figures 1A and 1C). Upon collision-induced dissociation (CID) fragmentation, three major product ions were observed for both LV and SV (m/z 295.2, 310.2 and 325.2). The MRM transitions m/z 427.3→325.2 and 441.3→325.2 were selected for monitoring LV and SV, respectively. Under negative ion mode, LV A and SVA produced molecular ions at m/z 421.4 and 435.3, indicating deprotonated anions (Figures 1B and 1D). Upon CID fragmentation, two major product ions were observed for both LV A (m/z 319.3 and 101.0) and SVA (m/z 319.3 and 115.0). The MRM transitions m/z 421.4→319.3 and 435.3→319.3 were selected for monitoring LVA and SVA, respectively. These precursor and product ions for LV/LVA and SV/SVA also were observed in earlier studies [6,11], although protonated lactone cations and acetonitrile-sodium adducts of the lactones were predominant in other studies [16-18].

Representative extracted ion chromatograms of blank human plasma and calibration standards at LLOQ are shown in Figure 2. Since LV and LVA may undergo interconversion during MS analysis (i.e.,

![Figure 2: Representative extracted ion chromatograms of LV (A) and LVA (B) calibration standards at LLOQ in human plasma (upper panel) and blank human plasma (lower panel)](image_url)

The monitored MRM transitions and signal intensity for the most intense peak are shown in the upper-left and upper-right corners of each chromatogram, respectively.)
in-source conversion), chromatographic resolution of LV from LVA is required to detect and monitor the extent of such interconversion [19]. Chromatographic resolution of SV from SVA also is required for the same reason. Upon chromatographic separation, LV and LVA eluted at 2.06 and 1.56 min, while SV and SVA eluted at 2.34 and 1.69 min (Figures 2 and 3). All are well-resolved from each other. Retention times did not drift after repeat injections (n=3; S.D. ≤ 0.3 s) of the same QC samples separated by 30 injections (data not shown).

**Calibration standards, accuracy and precision**

A linear calibration curve (0.2-100 nM or 0.084-42 ng/mL) with 1/x weighting was obtained for LVA, while a quadratic calibration curve (0.5-100 nM or 0.2-40 ng/mL) with 1/x weighting was obtained for LV. Coefficients of determination ($r^2$) were greater than 0.99 for the calibration curves. Standards with concentrations greater than 100 nM were not tested because LV and LVA plasma concentrations are not expected to exceed 100 nM following a therapeutic dose of LV (40 mg once daily in this study). The on-column sensitivity of the method was 25 and 10 fmol (or 2.8 and 1.2 pg) for LV and LVA, respectively. For QC samples at the low, medium, and high concentration, the intra-day accuracy were range from 97.7-104.9% for LV and 94.5-103.6% for LVA, and the inter-day accuracy were range from 98.2-105.3% for LV and 95.7-99.5% for LVA (Table 1). The precision was determined by calculating CV for QC. The intra-day precision and inter-day precision were all within 10% for LV and LVA. These values are well within the criteria of 15% bias and 15% CV stated in the US FDA bioanalytical method validation guidance [20].

**Method validation**

Interconversion of LV and LVA during sample preparation and storage was examined by monitoring both LV and LVA in single-component QC samples (50 and 100 nM). Immediately after sample preparation, a small amount of LVA was detected in the LV-only QC samples with the LVA concentration representing an average 1.7% of the LV concentration (Table 2). Similarly, a small amount of LV was detected in the LVA-only QC samples with the LV concentration representing an average 1.1% of the LVA concentration (Table 3). Upon storage in

![Image of chromatograms](image-url)
the autosampler at 6°C for 24 h, the same processed QC samples were analyzed again. The extent of LV-LVA conversion (<1.9%; Tables 2 and 3) did not exhibit statistically significant increases after storage.

The extraction recoveries of LV and LVA from human plasma following PPE were 67-74% for LV and 70-75% for LVA (Table 4), which are comparable to recoveries previously reported for PPE extraction with human plasma (52% and 57%, respectively) [18]. Both higher and lower extraction recoveries have been reported for LV using an LLE method (54.8% [8]; ~86% [6,9]) or SPE method (55% [11]). For SV and SVA, higher extraction recoveries have been reported using an SPE method (78-89%) [17,21]. Little matrix effect was observed for LVA, whereas appreciable signal enhancement was observed for LV in the presence of human plasma matrix (Table 4). Since the LV molecular ions monitored in our MRM method were sodium adducts, the presence of plasma matrix could have favored the formation of sodium adduct ions, leading to the observed signal increase compared to the neat solvent.

Both LV and LVA were stable in the QC samples following three freeze-thaw cycles with ≤ 10% reduction in concentration (Table 5), although LVA appeared to be slightly less stable than LV (90-93.2% vs. 97.3-100.5% remaining). In addition, both LV and LVA were stable after long-term storage of plasma QC samples at -80°C (Table 5). No appreciable difference between plasma samples stored as is and sodium acetate-buffered plasma samples was seen (Table 5), indicating that immediate buffering of plasma samples after collection is not necessary.

### Application for pharmacokinetic study in human

The validated simple PPE-based UPLC–MS/MS method was applied for the simultaneous quantification of LV and LVA in human plasma samples obtained from a clinical drug-drug interaction study involving LV. Representative extracted ion chromatograms of LV and LVA for a plasma sample collected 4 h post-lovastatin administration are shown in Figure 3. The plasma concentration–time profiles were plotted for a human subject, who received 40 mg lovastatin orally the morning of the study and was monitored for 24 h post-administration (Figure 4). Pharmacokinetic measurements

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**Table 3:** Conversion of LVA to LV during sample preparation and storage in autosampler.

| Compound | QC Concentration (nM) | LV Relative to QC Concentration (%) | LV after 24-h storage Concentration (nM) | Relative to QC Concentration (%) |
|----------|-----------------------|-------------------------------------|--------------------------------------|----------------------------------|
| LV       | 100                   | 0.8                                 | 1.6                                  | 1.8                              |
| LVA      | 100                   | 0.5                                 | 0.6                                  | 0.6                              |
| LV       | 50                    | 0.4                                 | 0.8                                  | 0.8                              |
| LVA      | 50                    | 1.3                                 | 1.3                                  | 1.3                              |
| LV       | 100                   | 1.0                                 | 1.0                                  | 1.0                              |
| LVA      | 100                   | 1.0                                 | 1.0                                  | 1.0                              |

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**Table 4:** Extraction recovery and matrix effect for LV and LVA.

| Compound | QC Concentration (nM) | PPE Extraction Recovery (%) | S.D. (%) | Matrix Effect Remaining Signal (%) | S.D. (%) |
|----------|-----------------------|-----------------------------|----------|-----------------------------------|----------|
| LV       | 2                     | 73                           | 2.1      | 155                               | 8.1      |
| LVA      | 2                     | 75                           | 9.5      | 102                               | 9.5      |
| LV       | 100                   | 67                           | 4.0      | 134                               | 1.4      |
| LVA      | 100                   | 70                           | 5.1      | 101                               | 5.3      |

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**Table 5:** Freeze-thaw and long-term storage stability of LV and LVA.

| Compound | QC Concentration (nM) | Freeze-Thaw Stability Remaining after one week (%) | Remaining after two week (%) | Remaining after one month (%) |
|----------|-----------------------|---------------------------------------------------|-------------------------------|-------------------------------|
| LV       | 2                     | 97.3                                              | 97                            | 97                            |
| LVA      | 2                     | 90.0                                              | 90                            | 90                            |
| LV       | 100                   | 98.1                                              | 98                            | 98                            |
| LVA      | 100                   | 93.2                                              | 93                            | 93                            |

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**Figure 4:** Plasma concentration-time profiles of LV and LVA for a human subject that received 40 mg of lovastatin orally.
are summarized in (Table 6). These values are in agreement with those previously reported for LV [10], although a greater exposure (i.e., C_max and AUC) of LVA was observed in our study.

Conclusions

A simple PPE-based, direct injection, UPLC-MS/MS analytical method using polarity switching has been developed and validated for the simultaneous quantification of LV and LVA in human plasma samples. The interconversion of LV and LVA during sample preparation and storage was minimized by a lower handling temperature (on ice or ≤ 6°C) and the use of acidified plasma by addition of sodium acetate buffer. The validated method has been successfully applied for the quantification of LV and LVA in human plasma samples obtained from a clinical drug-drug interaction study involving LV.

Acknowledgements

This work was supported by the National Institutes of Health research grant R01-GM089994 (MWZ). MS was supported by the National Institute of General Medical Sciences training grant T32-GM086330.

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Citation: Wujian J, Kuan-wei P, Sihyang Y, Huijing S, Mario S, et al. (2015) A Simple Protein Precipitation-based Simultaneous Quantification of Lovastatin and Its Active Metabolite Lovastatin Acid in Human Plasma by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry using Polarity Switching. J Chromatogr Sep Tech 6: 268. doi:10.4172/2157-7064.1000268