Vol. 9, No. 4, 2018
ISSN 2233-4203/ e-ISSN 2093-8950
LETTER www.msletters.org | Mass Spectrometry Letters

Simultaneous Determination of Statins in Human Urine by Dilute-and-Shoot-Liquid Chromatography-Mass Spectrometry

Haejong Jang[1,#], Xuan-Lan Mai[1,#], Gunhee Lee[1], Jae Hyoung Ahn[1], Jongsook Rhee[2], Quoc-Ky Truong[3], Dinh Vinh[4], Jongki Hong[5], and Kyeong Ho Kim[1,*]

[1]*College of Pharmacy, Kangwon National University, Chuncheon 24341, Korea*
[2]*Forensic Toxicology & Chemistry Division, National Forensic Service Busan Institute, Yangsan 50612, Korea*
[3]*Faculty of Pharmacy, Pham Ngoc Thach University of Medicine, Ho Chi Minh, 700000, Vietnam*
[4]*University of Medicine and Pharmacy Ho Chi Minh City, Ho Chi Minh, 700000, Vietnam*
[5]*College of Pharmacy, Kyung Hee University, Seoul 02447, Korea*

Received October 01, 2018; Revised November 16, 2018; Accepted November 19, 2018
First published on the web December 30, 2018; DOI: 10.5478/MSL.2018.9.4.95
[#]*These authors contributed equally in this study*

Abstract : An innovative, simple, and rapid assay method based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was developed and validated for the simultaneous determination of eight statin drugs in human urine. A simple sample clean-up procedure using the "dilute and shoot" (DAS) approach enabled a fast and reliable analysis. The influence of the dilution factor was investigated to ensure detectability and reduce the matrix effect. Chromatographic separation was performed on a Phenomenex Kinetex C18 column (50 × 3.0 mm i.d., 2.6 μm) using an elution gradient of mobile phase A composed of 0.1% acetic acid, and mobile phase B composed of acetonitrile, at a flow rate of 0.35 mL/min. Quantitation was performed on a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode using electrospray ionization in positive ion mode. The total chromatographic run time was 15 min. The method was validated for selectivity, sensitivity, recovery, linearity, accuracy, precision, and stability. The present method was successfully applied to the analysis of Rosuvastatin in urine samples after oral administration to healthy human subjects.

Keywords : Statins, Urine, Dilute and shoot, Tandem mass spectrometry

Introduction

Statins, a class of lipid-lowering drugs, are now among the most frequently prescribed agents for reducing morbidity and mortality related to cardiovascular diseases. The major therapeutic action of statin drugs is the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; the key enzyme in the metabolic pathway that produces cholesterol.[1]

Statins are grouped into two types: fermentation-derived (lovastatin, mevastatin, simvastatin, and pravastatin) and chemically synthesized (atorvastatin, fluvastatin, pitavastatin, and rosuvastatin). Although all statins share a common action mechanism and structural component that is very similar to the HMG portion of HMG-CoA reductase, they differ in terms of chemical structures (Figure 1). Therefore, the affinities for HMG-CoA reductase and the pharmacokinetic properties are varied among statins.[2]

The development of new analytical methods for statin drugs is necessary due to their importance in clinical use. Because statins are not used in combination with other statin molecules during therapy, analytical methods for the determination of statins were developed individually or simultaneously with their metabolite in plasma. However, the development of a rapid analytical procedure, that is not limited to only one statin, will be very useful for assessment in quality control.[3] There are chromatographic methods for the simultaneous determination of statins in human serum,[4] pharmaceutical formulations,[5,6,7] aqueous samples,[8] and waste water[9] for various applications, such as pharmacokinetic studies, stability studies and quality control of pharmaceutics, and environmental concerns. However, there is only one study about the analytical

*Reprint requests to Kyeong Ho Kim
E-mail: kyeong@kangwon.ac.kr

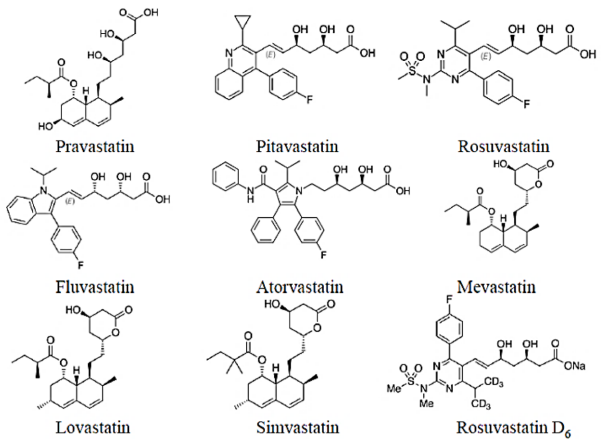

Figure 1. Chemical structures of eight statin drugs and internal standard (IS)

method for pitavastatin in urine.[10] The lack of publications related to the analytical studies of statins in urine is the basis of the current work to develop an innovative method for the quantitation of these drugs in human urine which is the specimen of choice for drug screening in clinical toxicology.[11]

For the analysis of urine samples, a simple sample clean-up procedure, the so called "dilute and shoot" (DAS), has become a trend in the past ten years in both analytical toxicology and doping-control analysis. Its benefits, such as easy sample preparation and omission of time-consuming extractions, lowers the total uncertainty budgets that are the driving forces behind this trend.[12] Therefore, in this study, we applied this approach for the urine sample preparation, and present a convenient high performance liquid chromatography (HPLC) method with tandem mass spectrometric detection for the quantitative analysis of eight drugs in the statin group (pravastatin, pitavastatin, rosuvastatin, atorvastatin, fluvastatin, mevastatin, lovastatin, and simvastatin) in human urine.

Experimental

Material

All chemicals and reagents used in this study were of analytical grade. Pravastatin sodium (≥ 99.0%) was supplied by Yungjin Pharm. (Seoul, Korea). Atorvastatin calcium trihydrate (≥ 99.0%), lovastatin (≥ 99.0%), pitavastatin calcium (≥ 99.0%), rosuvastatin calcium (≥ 99.0%) and simvastatin (≥ 99.0%), were supplied by the Shinpoong Pharmaceutical Co. Ltd (Ansan, Korea). Fluvastatin sodium (≥ 98.0%) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Mevastatin sodium (≥ 98.0%) was purchased from LGC Standard (Teddington, UK). Rosuvastatin-d_6 sodium (98.9%) was purchased from TLC Pharmaceutical Standard. Acetic acid (≥ 99.0%) was purchased from DAEJUNG (Siheung, Korea). Tablets containing 5.0 mg of rosuvastatin were purchased from Samjin Pharm. Co. Ltd (Seoul, Korea). HPLC-grade acetonitrile, ethanol, and methanol were obtained from DAEJUNG (Siheung, Korea). Purified water was prepared in the laboratory using an Aqua Max water purification system from Young Lin Instrument Co., Ltd. (Anyang, Korea).

Instrumentation and LC-MS/MS operation conditions

The LC-MS/MS system consisted of an Agilent 1200 series (Agilent Technologies, Santa Clara, USA) system coupled with an API 3200 Q Trap triple-quadrupole mass spectrometer (ABSCIEX, Foster city, CA, USA) equipped with a Turbo V Ion Spray source. The separation was performed on a Phenomenex Kinetex C18 column (50 × 3.0 mm i.d., 2.6 μm) combined with a C18 guard column (4.0 × 3.0 mm i.d.) from Phenomenex (Torrance, CA, USA) using an elution gradient of 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.35 mL/min; 40% mobile phase B for 0.8 min, 40-90% mobile phase B for 6.7 min, 90% mobile phase B for 0.3 min, and 90-40% mobile phase B for 0.2 min and 40% for 7 min. The temperature of the column was 20°C. The analytical run time was 15.0 min. The positive ESI settings for the analysis of the analytes and IS were as follows: ion source gas 1 (nebulizer gas), 30 (arbitrary units); ion source gas 2 (turbo heater gas), 50 (arbitrary units); curtain gas, 20 (arbitrary units); turbo-gas temperature, 600°C; ion spray voltage, 2000 V. MRM mode was used for quantification (Supplemental Table S1). Analyst 1.6 software was used for LC-MS/MS system control and data processing.

Preparation of calibration standards and quality control samples

Stock solutions of pravastatin, rosuvastatin, fluvastatin, atorvastatin, mevastatin, lovastatin, simvastatin, and rosuvastatin-d_6 (internal standard, IS) were prepared by dissolving the respective accurately weighed compounds in methanol to obtain final concentrations of 1000 μg/mL. The pitavastatin stock solution (1000 μg/mL) was prepared by dissolving the accurately weighed compound in a mixture of methanol and water (1:1, v/v). Working standard mixture solutions of 50, 100, 1000 and 5000 ng/mL were prepared by serial dilutions of the stock solutions in a mixture of acetonitrile and water (3:7, v/v). All solutions were stored at -20°C, and were thawed at room temperature before use. Calibration standards (CS) and quality control (QC) samples were prepared by spiking the diluted standard and internal standard solutions in an aliquot of 500 μL of drug-free human urine, followed by dilution with water to obtain a total volume of 1.5 mL. The resultant mixture was vortex mixed and filtered through a 0.45 μm filter before injection into LC-MS/MS system.

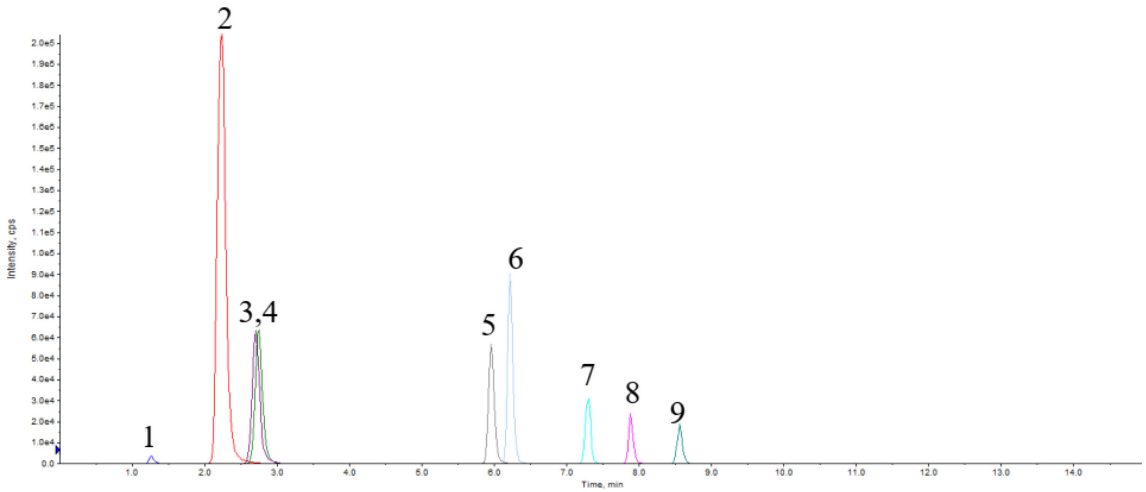

Figure 2. Total chromatogram of eight statins and internal standard. (1) Pravastatin (2) Pitavastatin (3) Rosuvastatin D_6 (4) Rosuvastatin (5) Fluvastatin (6) Atorvastatin (7) Mevastatin (8) Lovastatin (9) Simvastatin

Sample preparation

A quantity of 60 μL of diluted IS solution (5 ng/mL) was spiked into an aliquot containing 500 μL of the urine sample, followed by the dilution with water to obtain a total volume of 1.5 mL. The sample was then vortex mixed and filtered through a 0.45 μm filter and 10 μL of this filtrate was injected into the LC-MS/MS.

Application of the method

This method was applied for the detection and quantitation of rosuvastatin in a urine sample obtained from a healthy male volunteer after oral administration of a tablet containing 5 mg of rosuvastatin. Urine samples were collected after intake of the drug (1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 28, 31, 34 hours) and were stored at - 80°C until further analysis.

Results and discussion

LC-MS/MS

Supplemental Table S1 shows the parent and product ions for the analytes and IS, respectively: pravastatin (447.1→327.3), pitavastatin (422.3→290.2), rosuvastatin (482.2→258.2) fluvasattin (412.2→224.1), atorvastatin (559.3→250.2), mevastatin (391.4→185.3), lovastatin (405.2→199.2), simvastatin (419.2→199.3), rosuvastatin D_6 (488.1→264.2).

Preliminary experiments showed a considerable degradation of the statins (especially lovastatin and simvastatin), therefore, care should be taken during sample preparation and mobile phase selection. According to published articles, statins stability decreases more significantly at basic pH.[13,14,15] Therefore, formic and acetic acids (0.1%) were investigated as the mobile phase components. Methanol and acetonitrile were also investigated at different ratios with acid additives at varying strengths. It was observed that using an elution gradient of 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B) provided the best sensitivity, efficiency, and peak shape.

The elution gradient was as follows: 40% mobile phase B for 0.8 min, 40-90% mobile phase B for 6.7 min, 90% mobile phase B for 0.3 min, and 90-40% mobile phase B for 0.2 min and 40% for 7 min. The total chromatographic run time was 15.0 min for each run (Figure 2).

Sample preparation

In dilute and shoot LC MS (DAS-LC-MS), a fast analysis can be obtained because complex extraction steps are skipped. The common drawbacks are the analyte detectability, matrix effects, and ion suppression/ enhancement. Although dilution is an important step to reduce the matrix effect, multi-fold dilution would hamper the required analyte detectability.[10] In this study, when the samples were five-fold diluted, the intensity of the peaks were relatively low while the one- or two-fold diluted samples caused clogging of the chromatographic system and precipitation on the MS orifice. Thus, three-fold dilution was suitable for the preparation of the urine samples.

Chromatograms of the quantitative ion transitions in the MRM mode for a spiked urine sample with 200 ng/mL of each analyte are shown in Supplemental Figure S1.

Validation

The present method was validated in accordance with the Food and Drug Administration guidelines for the validation of bioanalytical methods (FDA 2013) and the guideline on bioanalytical method validation of the European Medicine Agency (EMA 2011).

Selectivity and lower limit of quantification

No interfering peaks from endogenous compounds were observed at the retention times of the analytes or IS. The total chromatographic run time was 15 min. The LLOQ concentration at which the precision and variance of accuracy were ≤ 20%, and the signal-to-noise ratio (S/N) was ≥ 10. The relative standard deviation (RSD, %) was used to assess the precision. The accuracy was calculated as (measured conc.−nominal conc.)/nominal conc.× 100%.

Qualitative matrix effect and recovery

The matrix factors of the analytes and IS were evaluated by comparing the analyte/IS ratio in human urine and water samples at low, medium, and high concentrations. The recovery results are presented in Supplemental Table S2.

Carryover

The carryover effect was tested by instantly analyzing the blank samples following the samples at the upper limit of quantification (n=3). The carryover of the other compounds was low enough to meet acceptable criteria (less than 20% of the LLOQ) (Supplemental Table S2).

Linearity

The linearity was tested within the concentration range 1-1000 ng/mL for rosuvastatin and fluvastatin; 1-500 ng/mL for atorvastatin; 1-200 ng/mL for pitavastatin; 2-1000 ng/mL for lovastatin; 2-200 ng/mL for simvastatin; and 5-1000 ng/mL for pravastatin and mevastatin. To ensure a high accuracy in such a wide concentration range, a weighting factor of $1/x$ was used in the linear regression analysis. The linearity of the developed method was determined based on their r^2 values, which were higher than 0.9952 for pravastatin, 0.9979 for pitavastatin, 0.9925 for rosuvastatin, 0.9965 for fluvastatin, 0.9946 for atorvastatin, 0.9901 for mevastatin, 0.9917 for lovastatin, and 0.9902 for simvastatin.

Precision and accuracy

The accuracy and intra- and inter-assay precisions were determined by analyzing eleven replicates of the QC samples at four concentrations on three different days. The results are shown in Supplemental Table S3.

Stability

The stabilities of all analytes in human urine under different storage conditions are presented in Supplemental Table S4. No significant degradation was detected under the conditions described in this study, as their concentrations deviated by no more than 15.0% relative to the reference nominal concentrations.

Application

This method was applied to the detection and quantitation of rosuvastatin in a urine sample obtained from a healthy male volunteer after oral administration of a tablet containing 5 mg of rosuvastatin. After 34 h of administration, the concentration of rosuvastatin was 14.8 ng/mL. The maximum concentration in the urine was 371 ng/mL after 6 h of administration. The calculated accumulative amount of rosuvastatin excreted through the urine was 10.8% of the taken dosage (Figure 3) which corresponded to previous literature about rosuvastatin,[16] thus demonstrated the applicability of the method for screening purpose in toxicology and forensic study.

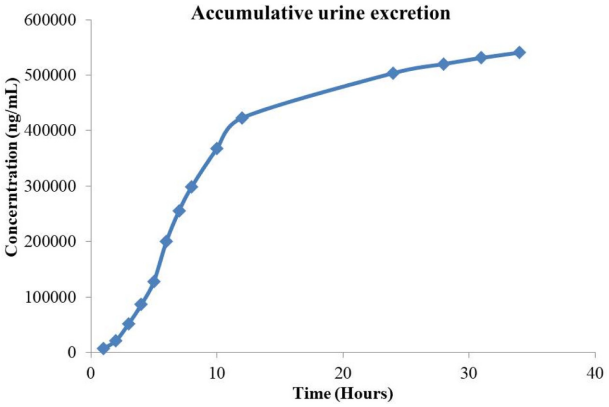

Figure 3. Accumulative excretion of rosuvastatin in urine

Conclusions

A novel dilute and shoot LC-MS/MS method for the quantitation of statins was developed and validated. To the best of our knowledge, this is the first report of a simultaneous analysis of eight statins in human urine. The analytes are effectively quantitated by applying a simple dilution step using a small amount of urine, without complicated collection, storage, or time-consuming sample preparation procedures. Therefore, the method is sensitive, accurate, and precise enough for applications in forensic or toxicology studies.

Acknowledgments

This study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors thank the Institute of New Drug Development Research and Central Laboratory of Kangwon National University for the analytical instrument.

Notes

†Electronic Supplemental Information (ESI) available: https://drive.google.com/open?id=1cwIDlwx3AcS-j4MU-Ex46lYeo3VpGX00

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