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ORIGINAL ARTICLE

Validated LC–MS/MS method for simultaneous determination of SIM and its acid form in human plasma and cell lysate: Pharmacokinetic application

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Abstract Simvastatin (SIM) is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor widely used in hyperlipidemia therapy. SIM has recently been studied for its anticancer activity at doses higher than those used for the hyperlipidemia therapy. This prompted us to study the pharmacokinetics of high-dose SIM in cancer patients. For this purpose, an LC–MS/MS method was developed to measure SIM and its acid form (SIMA) in plasma and peripheral blood mononuclear cells (PBMCs) obtained from patients. Chromatographic analyte separation was carried out on a reverse-phase column using 75:25 (% v/v) acetonitrile:ammonium acetate (0.1 M, pH 5.0) mobile phase. Detection was performed on a triple quadrupole mass spectrometer, equipped with a turbo ion spray source and operated in positive ionization mode. The assay was linear over a range 2.5–500 ng/mL for SIM and 5–500 ng/mL for SIMA in plasma and 2.5–250 ng/mL for SIM and 5–250 ng/mL for SIMA in cell lysate. Recovery was >58% for SIM and >75% for SIMA in both plasma and cell lysate. SIM and SIMA were stable in plasma, cell lysate and the reconstitution solution. This method was successfully applied for the determination of SIM and SIMA in plasma and PBMCs samples collected in the pharmacokinetic study of high-dose SIM in cancer patients.

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1. Introduction

Simvastatin (SIM) is a well-established drug for the treatment of hyperlipidemia. SIM is a prodrug administered in the lactone form, which is converted in the liver into the active acid form (Fig. 1). It is this active carboxylate form that reduces cholesterol biosynthesis by competitively inhibiting the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)

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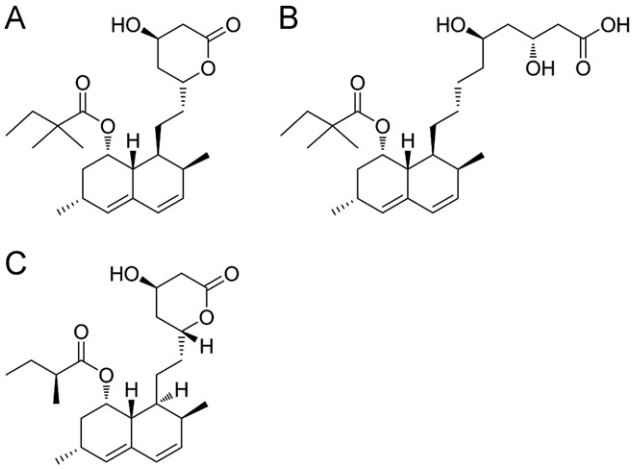

Figure 1 Chemical structures of (A) simvastatin, (B) simvastatin acid and (C) lovastatin.

reductase, the rate limiting enzyme in the mevalonate pathway [1,2]. Additionally, statins inhibit the synthesis of other downstream products in the mevalonate pathway, such as the isoprenoids [1,2]. Isoprenoids, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are known to be involved in important cellular processes such as proliferation and apoptosis [3]. Thus, statins have recently been tested for their potential use as anticancer agents. As with all agents in this class, in vitro studies have shown that SIM displays anticancer activity, but only at concentrations that are higher than those observed in plasma of patients being administered typical doses associated with the hyperlipidemia therapy [4].

Several clinical trials were subsequently conducted to study the safety and tolerability of high dose statin analogs, including simvastatin, in cancer patients [5–7]. Oral statins were found to be well tolerated at high doses with minor side effects. In a phase I study, lovastatin (LOV) given orally at a dose of 25 mg/kg daily was well tolerated and safe in patients with solid tumor [6]. In the case of SIM, a phase I study in patients with myeloma or lymphoma has shown that the maximal tolerated dose (MTD) of SIM, given orally, is 7.5 mg/kg twice a day, which is 25-fold higher than typical dose. The most common side effects of high dose SIM were nausea, diarrhea, muscle weakness and myalgia [7]. However, pharmacokinetics (PK) was not defined and it is not known if SIM plasma concentrations can reach the levels necessary for the antitumor activity observed in vitro. In this context, we initiated a clinical study to characterize the pharmacokinetics of simvastatin lactone and its acid form (simvastatin acid, SIMA) in plasma and peripheral blood mononuclear cells (PBMCs) after oral administration of SIM at 7.5 mg/kg twice daily in patients with recurrent and refractory chronic lymphocytic leukemia (CLL).

SIM has low systemic bioavailability which is attributed to the high extraction by the liver, the main site of action for treating hyperlipidemia. Therefore, sensitive analytical methods have previously been developed to assay both SIM and SIMA in plasma [8–11]. The first analytical method developed was an LC coupled with UV detection (238 nm); nonetheless, low sensitivity for quantitation of SIM and SIMA in biological fluids was reported [12]. Better sensitivity using UV detection was achieved later with an LOQ of 0.5 ng/mL but with run time >28.7 min [13]. A more sensitive HPLC-FD method using 1-bromoacetylpyrene for derivatization has been reported with an LOQ of 0.1 ng/mL for both analytes [14]. Although this LC-FD method is highly sensitive, sample preparation using solid phase extraction and analyte derivatization is inconvenient and time consuming. On the other hand, several LC–MS/MS methods have been developed for the determination of SIM and SIMA in biological fluids which are more sensitive and specific [8–11]. These methods are coupled with either solid phase extraction (SPE) or liquid–liquid extraction (LLE) procedures. Solid phase extraction has yielded good recoveries for SIM but SIMA recovery was low [15]. LLE showed better recoveries for both SIM and SIMA compared to SPE [8,10,11]. Current analytical methods have not been validated for the analyses of SIM and SIMA in cell lysates. Moreover, few assays have been validated to measure plasma concentration of SIM and SIMA at higher levels [16–18]. Here we report the development and validation of an LC–MS/MS method for the analysis of SIM and SIMA human plasma and PBMCs.

2. Experimental

2.1. Chemicals and reagents

SIM was purchased from Toronto Research Chemicals Inc. (North York, Canada). Ammonium acetate (Mallinckrodt Baker, Philipsburg, NJ, USA) and sodium hydroxide (EM Science, Gibbstown, NJ, USA) were purchased from VWR (West Chester, PA, USA). HPLC grade acetonitrile and diethyl ether were obtained from Sigma-Aldrich (St Louis, MO, USA). LOV (Alexis Biochemicals, San Diego, CA, USA), hydrochloric acid and glacial acetic acid were from Fisher Scientific (Fair Lawn, NJ, USA). Anhydrous ethanol was obtained from IBI Scientific (Peosta, IA, USA). K562, a chronic myelogenous leukemia (CML) cell line, was purchased from ATCC (Manassas, VA, USA).

2.2. LC–MS/MS instrumentation and conditions

All analyses were performed using an HPLC system consisting of a Shimadzu LC-20AD pump and a Shimadzu SIL-20AC VP autosampler (Shimadzu, Columbia, MD, USA). The LC system was interfaced to an API 2000 ESI-MS/MS (Applied Biosystems, Foster City, CA, USA). The analytical column used was a Phenomenex Luna C_{18} (2.0 mm $\times$ 100 mm i.d.; 2.5 µm particle size), connected to a C_{18} guard column (Phenomenex C_{18}, 2.0 mm $\times$ 4 mm; 5 µm particle size). An isocratic mobile phase was used consisting of 75:25 (% v/v) acetonitrile:ammonium acetate (0.1 M, pH 5.0 adjusted with acetic acid). The flow rate was 0.15 mL/min under ambient temperature. The autosampler temperature was maintained at 4 °C and the injection volume was 20 µL. The run time was 10 min. All analytes and internal standard were detected on a triple quadrupole mass spectrometer (API 2000), equipped with a turbo ion spray source (MDS SCIEX, Toronto, Canada) and operating in the positive ion mode. LOV was used as an internal standard (IS). Quantitation was performed using multiple reaction monitoring (MRM) of precursor/

product ion transitions at m/z 419.3/199.3 for SIM; 437.3/303.3 for SIMA; and 405.2/199.3 for LOV.

The optimized source parameters for SIM, SIMA and LOV were as follows: the nebulizer gas pressure was set at 30 psi, the heater gas at 90 psi, the ion spray voltage was 5500 V and the turbo heater temperature was 500 °C. The curtain gas pressure was set at 40 psi and the collision activation dissociation (CAD) gas at 10 psi. Lastly the entrance potential, declustering potential, collision energy and cell exit potential applied were set at 8.27, 14, 17 and 5.25 V for SIM, 7, 3.8, 14 and 8.5 V for SIMA and 8.7, 12.5, 21.2 and 5.4 V for LOV, respectively. All the parameters were controlled by the Analyst software version 1.4.2 (Applied Biosystems, Foster City, CA, USA).

2.3. Preparation of standard and quality control samples

Stock solutions of SIM, SIMA and LOV (1 mg/mL) were prepared in ethanol. SIMA was prepared by alkaline hydrolysis of SIM [19]. Standard working solutions of SIM and SIMA were prepared by serial dilution of the appropriate stock solutions with mobile phase. Standards were prepared fresh for each run by spiking 25 μL of the appropriate working solutions of both analytes and internal standard into 425 μL of drug free human plasma to obtain calibration concentrations of 2.5, 5, 10, 50, 100, 250, 500 ng/mL SIM, 5, 10, 50, 100, 250, 500 ng/mL SIMA and 50 ng/mL LOV. Similar to plasma calibration standards, cell lysate calibration standards were prepared at calibration concentrations of 2.5, 5, 10, 50, 80, 100, 250 ng/mL SIM, 5, 10, 50, 80, 100, 250 ng/mL SIMA and 50 ng/mL LOV. Cell lysate matrix was prepared by lysing K562 cells in deionized water (3×10^7 cells/mL) via sonication.

Plasma quality control (QC) sample concentrations were 7.5, 150 and 400 ng/mL for SIM and 15, 150 and 400 ng/mL for SIMA. Cell lysate QC sample concentrations were 7.5, 90 and 200 ng/mL for SIM and 15, 90 and 200 ng/mL for SIMA. QC samples were prepared using stock solutions other than those used for calibration standards preparation. Both calibration standards and QC samples were prepared at 4 °C in an ice bath.

2.4. Processing of plasma and cell lysate samples

All plasma and cell lysate samples were stored at −80 °C and thawed at room temperature. A 25 μL aliquot of LOV was added to 475 μL of plasma or cell lysate sample in 16 mm × 100 mm glass test tube. The tubes then were vortexed for 10 s. After the addition of 500 μL of ammonium acetate buffer (0.1 M, pH 5.0), tubes were vortexed again for 1 min. Diethyl ether (3 mL) was then added to each tube and samples were placed on a shaker at 200 rpm for 15 min at 4 °C. Extracted samples were centrifuged at 3000 rpm for 10 min at 4 °C. After centrifugation, the aqueous layer was frozen by placing the tubes in dry ice for 1 min. The organic layer was decanted into a new 16 mm × 100 mm test tube and was evaporated till dryness at room temperature using a gentle stream of nitrogen. The residue was reconstituted in 50 μL of mobile phase and 20 μL was injected into the HPLC column.

2.5. Method validation

The method validation of SIM and SIMA in human plasma and cell lysate was performed according to the FDA guidelines [20]. The assay was validated for specificity and sensitivity, linearity, precision and accuracy, extraction recovery, matrix effect, and stability.

2.5.1. Specificity and sensitivity

Assay specificity and sensitivity were conducted in eight different lots of blank plasma that was either left blank or spiked with both analytes and IS. Analytes were extracted using the previously described extraction procedure and analyzed to determine the extent of interference by endogenous plasma components at the retention time of both analytes and IS. The lowest limit of quantitation (LLOQ) was assessed in the same plasma lots used for specificity. The determination of LLOQ was based on the criteria that the deviation of the measured concentrations should not be more than 20% from the nominal concentration and that the signal to noise ratio be ≥ 5.

2.5.2. Linearity

Linearity was evaluated using plasma samples spiked with both SIM and SIMA at concentration ranges 2.5–500 ng/mL and 5–500 ng/mL, respectively. The internal standard, LOV, concentration was 50 ng/mL in all calibration standards. Three calibration curves were prepared and analyzed by plotting area ratios of analyte to internal standard against the concentration of each calibration standard. The results were fitted into a linear regression model using (1/y) as a weighting factor for both SIM and SIMA. A cell lysate calibration curve was prepared similar to plasma calibration curve, but at concentration ranges 2.5–250 ng/mL and 5–250 ng/mL for SIM and SIMA, respectively.

2.5.3. Precision and accuracy

The intra-day precision and accuracy was evaluated at three different QC levels (low, medium and high) in eight replicates on the same day and in five replicates on three different days for inter-day precision and accuracy determination. Acceptable deviation was set within 15% of the nominal concentration for accuracy and within 15% relative standard deviation for precision.

2.5.4. Extraction recovery and matrix effect

The recovery efficiency of the extraction procedure was performed at low and high QCs using the extraction procedure described in Section 2.4. Recovery was evaluated as a percentage of the peak area of analytes that were spiked into a matrix before extraction to the peak area of analytes that had been spiked after extraction of a blank matrix. Endogenous substances present in the biological matrix can possibly enhance or suppress analyte ionization to affect the sensitivity, precision or accuracy of the described method. The matrix effect was assessed as a percentage of the peak areas of control plasma extracted and then spiked with analyte, to neat standards injected directly in the same reconstitution solvent. The matrix effect was carried out on five different lots of blank plasma and at low and high QC levels.

2.5.5. Stability

The short term and long term stability of SIM and SIMA in plasma and cell lysate samples was evaluated under different storage conditions. All stability experiments were performed at low and high QC levels. Both analytes were spiked individually in order to assess the potential for interconversion between the lactone and acid forms.

Short term stability of SIM and SIMA was evaluated in plasma and cell lysate samples at 4 °C (ice-bath) for 6 h. The autosampler storage stability was determined by storing the reconstituted QC samples for 6 h under autosampler conditions (i.e., 4 °C). Samples were stored for a month at −80 °C to evaluate long term stability of SIM and SIMA. Lastly, the stability of SIM and SIMA in plasma and cell lysate samples was assessed after repeated cycles of freeze and thaw (2 cycles). In each cycle the samples were removed from −80 °C storage and allowed to thaw at room temperature.

2.6. Pharmacokinetic study

In a pilot clinical trial, patients received an oral dose of 7.5 mg/kg SIM twice daily for one week. Only patients who signed a written consent form were enrolled in this study. Blood samples (8 mL) were collected after the first oral dose of SIM at pre-dose, 0.25, 1, 2, 3, 6, 8, and 12 h. All samples were collected in heparinized BD Vacutainer Cell Preparation Tubes (CPT) and immediately centrifuged (1800*g* for 30 min at room temperature) to separate plasma and PBMCs. Collected plasma and PBMCs were stored at −80 °C until analysis. At time of analysis, PBMC pellets were thawed and lysed in 1 mL deionized water via sonication then processed as described in Section 2.4.

3. Results and discussion:

3.1. Performance of LC and MS/MS

The Phenomenex Luna C_{18} column, used in this study, gave a symmetric peak shape for all analytes with an acceptable run time (10 min). Mobile phase components were selected based on previous works where ammonium acetate was used to enhance ionic strength of the analytes [9]. Also, different volumetric ratios of acetonitrile and ammonium acetate buffer were tested to obtain the best peak shape for both analytes with reasonable retention time (<10 min). In previously developed methods, SIM and LOV (Fig.1) were detected in positive ion mode whereas negative ion mode was typically favored for simvastatin acid detection [8,9,11]. Few studies have utilized the positive ion mode for detecting SIM acid [16,17]. However, in our studies simvastatin acid gave better fragmentation in positive ion mode with higher product ion signal intensities. Thus, both analytes (SIM and SIMA) and IS (LOV) were detected in positive ion mode without the need to switch polarity during the sample run. MS source parameters, as well as analytes parameters, were optimized to achieve the highest signal intensity.

3.2. Selectivity and sensitivity (LLOQ)

Plasma samples from eight different sources were tested for the presence of endogenous substances that might interfere at the retention times of peaks of interest as evaluated by chromatograms of blank plasma and cell lysate, plasma and cell lysate spiked with SIM and SIMA at QC1 level or LOV at 50 ng/mL, plasma and PBMCs collected from patients at predose and 12 h after receiving SIM at 7.5 mg/kg twice daily (Fig.2). Both SIMA and SIM were well separated with retention times of 2.65 and 7.1 min, respectively. LOV was detected at 5.6 min. The chromatograms show no interfering peaks at the retention times of both analytes and IS in the blank plasma. However, in-source lactonization of SIMA into SIM was recognized as shown in Fig.2B, where a small peak (Peak 1) can be seen on the simvastatin MRM channel (m/z 419.3/199.3) at the retention time of SIMA. A similar peak (Peak 2) occurs on the simvastatin acid MRM channel (m/z 437.3/303.3) at the retention time of SIM, this peak was explained as the interference of A+1 isotope from $[M+NH_4]^+$ of the SIM lactone form but not by in-source hydrolysis [21]. Therefore, chromatographic separation between SIM and SIMA is needed to eliminate the contribution of the post column in-source lactonization and the interference of $[M+NH_4]^+$ isotope of simvastatin lactone.

The LLOQ was tested at different levels ranging from 1 to 10 ng/mL and it was found to be 2.5 ng/mL for SIM with an accuracy of 97% and 8% precision while SIMA showed an LLOQ of 5 ng/mL with 105% accuracy and 7% precision. Previous analytical methods have proven to be highly sensitive with a limit of quantitation ranging from 0.05 to 0.1 ng/mL [8,9,11,16]. These methods developed for the determination of low SIM and SIMA plasma levels achieved by typical doses (40 mg). However, our method is developed for PK study of high dose SIM that is 25-fold higher than typical doses, thus LLOQ achieved was sufficient for the purpose of this study.

3.3. Linearity, precision and accuracy

The calibration curves of SIM (2.5–500 ng/mL) and SIMA (5–500 ng/mL) in human plasma and SIM (2.5–250 ng/mL) and SIMA (5–250 ng/mL) in cell lysate showed acceptable linearity. These ranges encompassed the concentrations observed in human plasma and PBMCs collected in a pharmacokinetic study following the oral administration of high dose simvastatin. Calibration curves ($n=3$) prepared in human plasma yielded the following regression equations $y=0.005\ (\pm0.001)+0.61(\pm0.03)x$ with $R^2=0.997$ and $y=0.002\ (\pm0.002)+0.23\ (\pm0.02)x$ with $R^2=0.997$ for SIM and SIMA, respectively. Similarly, calibration curves ($n=3$) prepared in cell lysate yielded the following regression equations $y=0.003\ (\pm0.002)+0.65\ (\pm0.11)x$ with $R^2=0.997$ and $y=0.001\ (\pm0.002)+0.31\ (\pm0.09)x$ with $R^2=0.992$ for SIM and SIMA, respectively.

Inter- and intra-day precision and accuracy were determined at three concentration levels (7.5, 200 and 400 ng/mL for SIM and 15, 90 and 150 ng/mL for SIMA). As shown in Table 1, inter- and intra-day precision values of SIM and SIMA, expressed as % relative standard deviation (RSD), ranged from 1.1% to 5.3%, whereas accuracy values ranged between 88.6% and 110.2%. The results from intra- and inter-day precision and accuracy indicate that the method reproducibility is acceptable within the same day and on different days.

3.4. Recovery and matrix effect

Analytes were extracted from biological samples using a liquid-liquid extraction procedure; several organic solvents were tested for their extraction efficiencies such as methyl tertiary butyl ether (MTBE), diethyl ether, ethyl acetate, acetonitrile and methanol. Ethyl acetate showed fair recovery for SIM, but extraction efficiency for SIMA was poor. MTBE

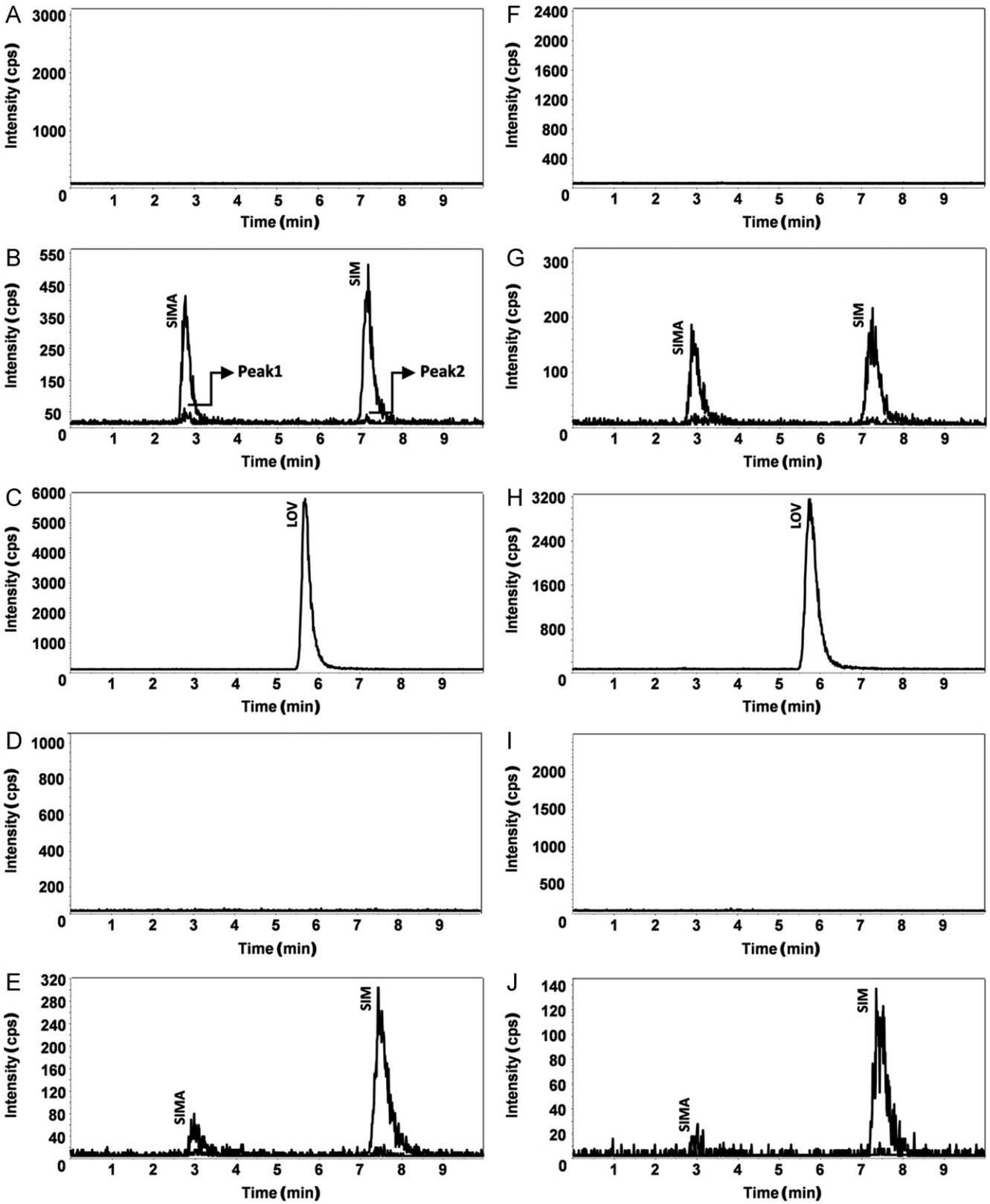

Figure 2 Representative chromatograms of: blank plasma (A) and cell lysate (F), plasma (B) and cell lysate (G) spiked with both SIM and SIMA at QC1 level, plasma (C) and cell lysate (H) spiked with LOV at 50 ng/mL, patient plasma (D) and PBMCs (I) samples collected at predose and patient plasma (E) and PBMCs (J) samples collected 12 h after oral administration of simvastatin (7.5 mg/kg).

Table 1 Intra- and inter-day precision and accuracy.

Analyte	Nominal concentration (ng/mL)	Intra-day ($n=8$)		Inter-day ($n=5$)	
		Accuracy (mean±SD, %)	Precision (% RSD)	Accuracy (mean±SD, %)	Precision (% RSD)
Simvastatin					
QC1	7.5	110.2±5.7	5.2	96.5±2.5	2.6
QC2	150	105.2±1.4	1.4	97.1±2.4	2.4
QC3	400	99.6±2.1	2.1	94.7±2.3	2.4
Simvastatin acid					
QC1	15	95.3±3.0	3.2	92.9±4.9	5.3
QC2	150	89.4±1.0	1.1	90.3±2.3	2.5
QC3	400	86.8±2.1	2.4	91.1±1.4	1.5

SD, standard deviation and RSD, relative standard deviation.

Table 2 Recovery and matrix effect.

Analyte	Recovery (mean±SD, %)($n=3$)		Absolute matrix effect (mean±SD, %) ($n=5$)
	Human plasma	Cell lysate	
Simvastatin			
QC1	75.3±5.8	95.7±4.1	98.9±3.3
QC3	68.6±5.4	67.5±7.6	99.0±5.6
Simvastatin acid			
QC1	73.2±4.4	98.1±5.8	96.4±3.9
QC3	58.9±4.1	63.8±10.1	98.7±1.0

SD, standard deviation.

and diethyl ether were found to have comparable extraction efficiencies for both SIM and SIMA and were higher than those obtained by the other organic solvents used. Although MTBE was commonly used in previous methods, diethyl ether was chosen for LLE procedure in this study. Mean recovery values of SIM and SIMA in human plasma were found to be 75.3% and 73.2% at QC1 level whereas at QC3 level they were 68.6% and 58.9%, respectively. In cell lysate, mean recovery of SIM and SIMA were higher at QC1 levels compared to plasma recovery with 95.7% and 98.1%, respectively. Recovery values of both SIM and SIMA in cell lysate at QC3 level were similar to those in human plasma. Furthermore, the mean matrix effect values are within the acceptable range for both SIM and SIMA, indicating that the matrix effect has no impact on the analytes quantification. The results of the recovery and matrix effect are summarized in Table 2.

3.5. *Stability*

The interconversion between SIM and SIMA is a result of hydrolysis of SIM and lactonization of SIMA. It has been found that the interconversion can be reduced either at low temperature or when pH is adjusted between pH 4 and pH 5 [15]. Acidified samples stored under low temperature conditions display very low interconversion (<1% at 4 °C and 0.05% at −20 °C for four weeks) [15]. Thus, during method validation, the plasma and cell lysate samples were kept at 4 °C at all stages of analysis and the reconstitution solution was buffered at pH 5. As shown in Table 3, SIM and SIMA were found to be stable in human plasma, cell lysate and the buffered reconstitution solution for at least 6 h at 4 °C. For long term stability, both analytes were stable in human plasma and cell lysate for at least one month at −80 °C (Table 4). Over two freeze–thaw cycles of human plasma and cell lysate, SIM and SIMA were also found to be stable (Table 5).

Stability of SIM and SIMA in stock and working solutions has been tested in several previous works. Over different solutions compositions both SIM and SIMA were found to be stable for at least one month [8,11,15,16]. However, we have tested the stability of both SIM and SIMA in working solution kept at −80 °C, and they were found to be stable for at least one year. Lastly, no stability studies were carried out for LOV as it has previously been shown to be stable under similar storage conditions [22].

3.6. *Pharmacokinetic study*

This method was successfully applied for the determination of SIM and SIMA in human plasma and PBMCs samples collected from leukemia patients following the oral administration of high dose simvastatin. Fig.2 shows the MRM chromatograms of both plasma and PBMCs samples collected from a patient 12 h after receiving SIM at 7.5 mg/kg twice daily. Fig.3 depicts a typical pharmacokinetic profile of SIM and SIMA in plasma and SIM in PBMCs from a CLL patient who received high dose simvastatin. Unlike SIM, SIMA concentrations in PBMCs were below the detection limit of

Table 3 Short term stability of the analytes in mobile phase extract, human plasma and cell lysate stored at 4 °C ($n=3$).

Analyte	Analyte concentrations at different time points (mean ± SD)[a]								
	Mobile phase extract			Human plasma			Cell lysate		
	1 h	3 h	6 h	1 h	3 h	6 h	1 h	3 h	6 h
Simvastatin									
QC1	99.7 ± 6.7	106.7 ± 11.8	102.7 ± 10.2	96.6 ± 4.0	99.8 ± 14.4	101.9 ± 8.8	118.9 ± 11.7	121.9 ± 21.6	109.9 ± 10.7
QC3	100.3 ± 2.4	101.0 ± 2.9	97.9 ± 2.8	94.0 ± 4.7	113.5 ± 3.0	115.7 ± 0.6	97.1 ± 12.0	110.4 ± 11.8	97.3 ± 8.6
Simvastatin acid									
QC1	101.2 ± 4.6	89.6 ± 3.2	92.8 ± 3.7	85.5 ± 24.6	94.5 ± 8.5	102.8 ± 13.5	121.0 ± 16.8	111.9 ± 8.6	101.1 ± 9.2
QC3	100.0 ± 3.9	98.3 ± 1.8	96.8 ± 3.1	95.0 ± 10.4	97.4 ± 10.1	97.5 ± 2.9	89.9 ± 1.2	81.4 ± 3.4	90.3 ± 3.0

[a]Analyte concentrations are expressed as the mean percentage of time zero concentrations ± standard deviation (SD) .

Table 4 Long term stability in human plasma and cell lysate ($n=3$).

Analyte	Analyte concentrations at different time points (mean ± SD)[a]								
	Human plasma					Cell lysate			
	1 day	3 days	1 week	2 weeks	4 weeks	1 day	1 week	2 weeks	4 weeks
Simvastatin									
QC1	105.5 ± 7.6	95.6 ± 9.4	102.5 ± 10.9	99.6 ± 5.2	97.0 ± 6.5	99.7 ± 7.8	90.2 ± 9.2	98.3 ± 4.8	90.5 ± 5.7
QC3	112.1 ± 9.4	97.3 ± 3.6	102.3 ± 2.6	101.4 ± 18.3	95.4 ± 11.9	87.4 ± 4.4	106.5 ± 8.4	99.9 ± 5.2	101.6 ± 9.2
Simvastatin acid									
QC1	91.3 ± 7.7	106.1 ± 13.4	105.9 ± 11.0	106.8 ± 6.5	91.3 ± 16.4	97.1 ± 5.6	104.3 ± 8.7	104.5 ± 3.2	114.7 ± 7.1
QC3	99.1 ± 7.3	106.5 ± 3.6	96.7 ± 2.2	106.9 ± 17.3	108.3 ± 7.2	90.4 ± 14.0	96.2 ± 9.4	83.0 ± 13.2	96.2 ± 12.5

[a]Analyte concentrations are expressed as the mean percentage of time zero concentrations ± standard deviation (SD).

Table 5 Freeze and thaw stability in human plasma and cell lysate ($n=3$).

Analyte	Analyte concentrations at given cycle (mean ± SD)[a]			
	Human plasma		Cell lysate	
	1st cycle	2nd cycle	1st cycle	2nd cycle
Simvastatin				
QC1	113.9 ± 11.8	103.7 ± 4.7	101.5 ± 10.0	95.6 ± 5.1
QC3	102.9 ± 5.1	104.5 ± 5.1	88.7 ± 6.5	100.3 ± 10.1
Simvastatin acid				
QC1	85.4 ± 4.9	95.1 ± 16.5	91.4 ± 5.3	109.9 ± 6.8
QC3	96.5 ± 11.7	90.3 ± 3.3	86.1 ± 6.9	105.3 ± 3.3

[a]Analyte concentrations are expressed as the mean percentage of time zero concentrations ± standard deviation (SD).

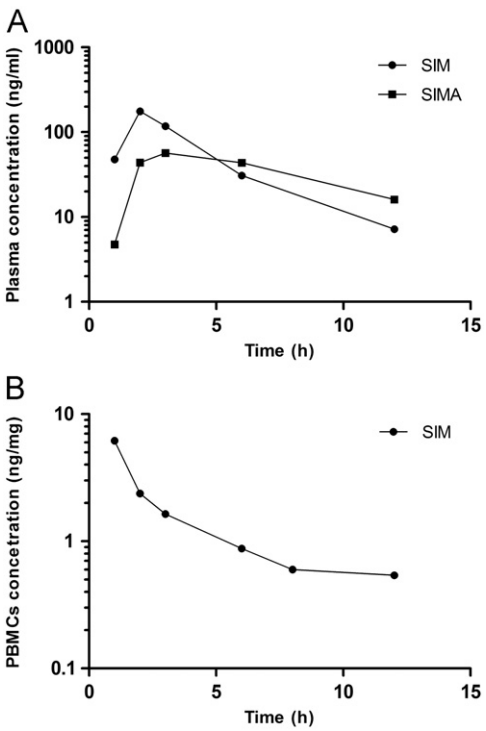

Figure 3 Pharmacokinetic profiles of (A) simvastatin lactone and carboxylate in plasma and (B) simvastatin lactone in PBMCs after oral administration of high dose simvastatin in a CLL patient. simvastatin concentration in PBMCs is normalized to the protein concentration of each PBMCs sample.

the assay at all the time points of the PK study. This could be attributed to the hydrophilicity of the carboxylate form which may limit its accessibility to the PBMCs. Alternatively, the carboxylate may be subject to efflux by an ATP-binding cassette transporter.

4. Conclusions

In conclusion, an LC–MS/MS was developed and validated for the determination of simvastatin and its acid form in human plasma and cell lysate. This assay is the first method developed for the analysis of SIM and SIMA in cell lysate. Moreover, this assay spans the concentration range of quantification of both SIM and SIMA that is applied for high dose SIM administration. Overall, this analytical method has proved to be successful for the analysis of SIM and SIMA in plasma and PBMCs samples collected from a high dose simvastatin pharmacokinetic study.

References

[1] J.L. Goldstein, M.S. Brown, Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol, J. Lipid Res. 25 (1984) 1450–1461.
[2] J.L. Goldstein, M.S. Brown, Regulation of the mevalonate pathway, Nature 343 (1990) 425–430.
[3] P.A. Konstantinopoulos, M.V. Karamouzis, A.G. Papavassiliou, Post-translational modifications and regulation of the RAS superfamily of GTPases as anticancer targets, Nat. Rev. Drug Discovery 6 (2007) 541–555.
[4] S. Sleijfer, A. van der Gaast, A.S. Planting, et al., The potential of statins as part of anti-cancer treatment, Eur. J. Cancer 41 (2005) 516–522.
[5] W.S. Kim, M.M. Kim, H.J. Choi, et al., Phase II study of high-dose lovastatin in patients with advanced gastric adenocarcinoma, Invest. N. Drugs 19 (2001) 81–83.
[6] A. Thibault, D. Samid, A.C. Tompkins, et al., Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer, Clin. Cancer Res. 2 (1996) 483–491.
[7] E. van der Spek, A.C. Bloem, N.W. van de Donk, et al., Dose-finding study of high-dose simvastatin combined with standard chemotherapy in patients with relapsed or refractory myeloma or lymphoma, Haematologica 91 (2006) 542–545.
[8] C. Apostolou, C. Kousoulos, Y. Dotsikas, et al., An improved and fully validated LC–MS/MS method for the simultaneous quantification of simvastatin and simvastatin acid in human plasma, J. Pharm. Biomed. Anal. 46 (2008) 771–779.
[9] B. Barrett, J. Huclova, V. Borek-Dohalsky, et al., Validated HPLC–MS/MS method for simultaneous determination of simvastatin and simvastatin hydroxy acid in human plasma, J. Pharm. Biomed. Anal. 41 (2006) 517–526.
[10] H. Yang, Y. Feng, Y. Luan, Determination of Simvastatin in human plasma by liquid chromatography-mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 785 (2003) 369–375.
[11] J.J. Zhao, I.H. Xie, A.Y. Yang, et al., Quantitation of simvastatin and its beta-hydroxy acid in human plasma by liquid-liquid cartridge extraction and liquid chromatography/tandem mass spectrometry, J. Mass. Spectrom. 35 (2000) 1133–1143.

[12] G. Carlucci, P. Mazzeo, L. Biordi, et al., Simultaneous determination of simvastatin and its hydroxy acid form in human plasma by high-performance liquid chromatography with UV detection, J. Pharm. Biomed. Anal. 10 (1992) 693–697.
[13] B.C. Kim, E. Ban, J.S. Park, et al., Determination of simvastatin in human plasma by columnswitching HPLC with UV detection, J. Liq. Chromatogr. Relat. Technol. 27 (2005) 3089–3102.
[14] H. Ochiai, N. Uchiyama, K. Imagaki, et al., Determination of simvastatin and its active metabolite in human plasma by column-switching high-performance liquid chromatography with fluorescence detection after derivatization with 1-bromoacetylpyrene, J. Chromatogr. B Biomed. Sci. Appl. 694 (1997) 211–217.
[15] M. Jemal, Z. Ouyang, M.L. Powell, Direct-injection LC–MS–MS method for high-throughput simultaneous quantitation of simvastatin and simvastatin acid in human plasma, J. Pharm. Biomed. Anal. 23 (2000) 323–340.
[16] B.N. Patel, N. Sharma, M. Sanyal, et al., Simultaneous determination of simvastatin and simvastatin acid in human plasma by LC–MS/MS without polarity switch: application to a bioequivalence study, J. Sep. Sci. 31 (2008) 301–313.
[17] A.V. Ramani, P. Sengupta, R. Mullangi, Development and validation of a highly sensitive and robust LC-ESI-MS/MS method for simultaneous quantitation of simvastatin acid, amlodipine and valsartan in human plasma: application to a clinical pharmacokinetic study, Biomed. Chromatogr. 23 (2009) 615–622.
[18] N.R. Pilli, R. Mullangi, J.K. Inamadugu, et al., Simultaneous determination of simvastatin, lovastatin and niacin in human plasma by LC–MS/MS and its application to a human pharmacokinetic study, Biomed. Chromatogr. 26 (2012) 476–484.
[19] S.M. Ostrowski, B.L. Wilkinson, T.E. Golde, et al., Statins reduce amyloid-beta production through inhibition of protein isoprenylation, J. Biol. Chem. 282 (2007) 26832–26844.
[20] FDA. Guidance for industry Bioanalytical Method Validation. US Food and Drug Administration Center for Evaluation and Research, 2001.
[21] M. Jemal, Z. Ouyang, The need for chromatographic and mass resolution in liquid chromatography/tandem mass spectrometric methods used for quantitation of lactones and corresponding hydroxy acids in biological samples, Rapid Commun. Mass Spectrom. 14 (2000) 1757–1765.
[22] Y. Wu, J. Zhao, J. Henion, et al., Microsample determination of lovastatin and its hydroxy acid metabolite in mouse and rat plasma by liquid chromatography/ionspray tandem mass spectrometry, J. Mass Spectrom. 32 (1997) 379–387.