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Quantitative analysis of simvastatin and its β-hydroxy acid in human plasma using automated liquid–liquid extraction based on 96-well plate format and liquid chromatography-tandem mass spectrometry

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Abstract

An assay based on automated liquid–liquid extraction (LLE) and liquid chromatography-tandem mass spectrometry (LC/MS/MS) has been developed and validated for the quantitative analysis of simvastatin (SV) and its β-hydroxy acid (SVA) in human plasma. A Packard MultiProbe II workstation was used to convert human plasma samples collected following administration of simvastatin and quality control (QC) samples from individual tubes into 96-well plate format. The workstation was also used to prepare calibration standards and spike internal standards. A Tomtec Quadra 96-channel liquid handling workstation was used to perform LLE based on 96-well plates including adding solvents, separating organic from aqueous layer and reconstitution. SV and SVA were separated through a Kromasil C18 column (50 mm × 2 mm i.d., 5 μm) and detected by tandem mass spectrometry with a TurboIonspray interface. Stable isotope-labeled SV and SVA, $^{13}CD_3$-SV and $^{13}CD_3$-SVA, were used as the internal standards for SV and SVA, respectively. The automated procedures reduced the overall analytical time (96 samples) to 1/3 of that of manual LLE. Most importantly, an analyst spent only a fraction of time on the 96-well LLE. A limit of quantitation of 50 pg/ml was achieved for both SV and SVA. The interconversion between SV and SVA during the 96-well LLE was found to be negligible. The assay showed very good reproducibility, with intra- and inter-assay precision (%R.S.D.) of less than 7.5%, and accuracy of 98.7–102.3% of nominal values for both analytes. By using this method, sample throughput should be enhanced at least three-fold compared to that of the manual procedure.

Keywords: Simvastatin; Liquid chromatography-tandem mass spectrometry; Automated liquid–liquid extraction; Human plasma

1. Introduction

Simvastatin is an inactive lactone precursor (SV, Fig. 1) that is quickly hydrolyzed following oral administration to its corresponding β-hydroxy acid metabolite (SVA, Fig. 1), an effective cholesterol lowering agent. SVA is a potent 3-hydroxy-3-methylglu-

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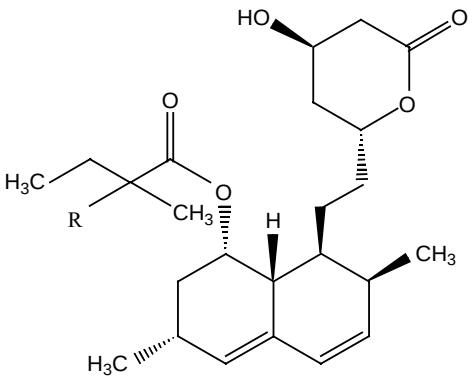
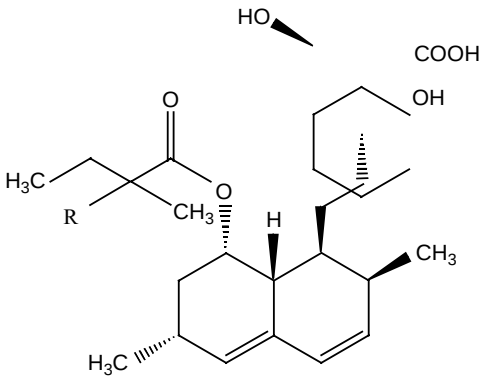


R=$^{13}CD_3$, [$^{13}CD_3$] Simvastatin, MW =422

R = CH_3, Simvastatin, MW = 418



R=$^{13}CD_3$, [$^{13}CD_3$] Simvastatin Acid, MW =440

R = CH_3, Simvastatin Acid, MW = 436

Fig. 1. Chemical structures, names and molecular weights of the analytes and internal standards.

taryl-coenzyme A (HMG-CoA) reductase inhibitor, the main enzyme that regulates in vivo synthesis of cholesterol. Due to the high first-pass hepatic extraction, SV and SVA concentrations in systemic circulation (e.g. plasma) are low [1,2]. Also, it is known that SV undergoes hydrolysis under both acidic and basic conditions while SVA is subject to lactonization at low pH. These issues have brought challenges to the quantification of SV and SVA in plasma such as assay specificity, sensitivity and stability. Since clinical phase I type studies usually involve large quantity of samples, sample throughput also becomes significantly important.

High performance liquid chromatography (HPLC) determination of SV and SVA in plasma with UV or fluorescence detection were previously reported. In these methods lower limit of quantitation (LOQ) of 20 and 0.1 ng/ml were achieved for the two analytes (analyzed in separate runs) with UV and fluorescence (derivatization required) detection, respectively [3,4]. Earlier, gas chromatography-mass spectrometry (GC/MS) procedures were used in analyzing SV/SVA in plasma and identifying SV metabolites in biological matrices [5–8]. Liquid chromatography coupled with atmospheric pressure ionization tandem mass spectrometry (LC-API-tandem MS) which provides high sensitivity, short analysis time and the possibility of monitoring multiple ions simultaneously, has become the most effective analytical method for quantitative analysis of SV and SVA in biological samples [8–13]. Development of sample preparation methods that are compatible to LC/MS/MS has become more demanding at this stage. Solid phase extraction (SPE) methods were employed initially to extract SV and SVA separately from animal and human plasma, following which SV was converted to SVA by hydrolysis. Each analyte was analyzed in separate runs [3,4,8]. Methods of simultaneous determination of SV and SVA concentrations in animal and human plasma by LC/MS/MS utilizing also SPE [13], liquid–liquid cartridge extraction [10] and liquid–liquid extraction (LLE) [14] were reported later and became well used. These methods achieved the best sensitivity with a LOQ of 0.05 ng/ml for both SV and SVA [10,14]. Interconversion between SV and SVA was carefully controlled to negligible levels [10]. However, no automated procedures were adapted for the purpose of high throughput in these assays. A method using direct-injection LC/MS/MS to assess SV and SVA concentration was recently developed with a run time of only 2.5 min for each sample [3]. However, the interconversion rate (1.0%) and LOQ (0.5 ng/ml) value were not acceptable to profile pharmacokinetics of SV/SVA in human samples.

Liquid–liquid extraction is a traditional but effective method to extract drugs from biological samples. It can be quickly developed and applied to most categories of drug compounds. It is compatible with the electrospray ionization source because it desalts samples well. However, only a few automated LLE assays based on 96-well plate format have been reported [15–17]. Some technical problems exist when 96-well plate is

adapted for LLE. Cross-well leaking easily occurs during mixing. Lack of sensitivity is also a potential problem since low volumes of sample and solvent have to be used. In order to implement fully automated liquid transferring to all steps, it may require more than one workstation. Because of the instability of SV and SVA at room temperature, sample racks and 96-well plates have to be kept at low temperature during the whole assay. Strict precision and accuracy criteria of clinical assays also brings challenges to automated LLE assays.

Here, we report the development and validation of a sensitive and reliable automated LLE for the quantitation of SV and SVA in human plasma based on 96-well plate format. The extracted samples were analyzed by LC/MS/MS under negative and positive ion mode, via within-run polarity switching, for SVA and SV, respectively.

2. Experimental

2.1. Chemical and reagents

Standard compounds of SV and SVA were obtained from Merck Research Laboratories. Stable isotope labeled SV and SVA, $^{13}CD_3$-SV and $^{13}CD_3$-SVA were synthesized by Drug Metabolism at Merck and were used as the internal standards for SV and SVA, respectively. Both SVA and $^{13}CD_3$-SVA were in ammonium salt form. Ammonium acetate (HPLC grade) and acetonitrile (optima grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Acetic acid (glacial) was purchased from Aldrich (Milwaukee, MI, USA). Pooled human control plasma (heparinized) was purchased from Biological Specialty Corporation (Lansdale, PA, USA). Nitrogen (refrigerated liquid) was obtained from Praxair, Inc. (Danbury, CT, USA). De-ionized water was prepared through the Milli-Q Plus Ultra-Pure water system (Millipore Corporation, Bedford, MA, USA).

2.2. LC/MS/MS method

The HPLC system consisted of a PE series 200 micro pump (Perkin-Elmer, Foster City, CA) with an on-line degaser and a Leap autosampler (model CTC PAL) equipped with cooling stack with 6-plate capacity (Leap Technology, Carrboro, NC). The injection syringe was a 100 μl Hamilton syringe (Microliter Analytical Supplies, Suwanee, GA). The liquid chromatography was performed on a Kromasil C18 column (2.0 mm × 50 mm, 5 μm) (Keystone Scientific, Inc., Bellefonte, PA, USA) with a liquid flow of 200 μl/min under ambient condition. The mobile phase was 75% acetonitrile and with 25% 1 mM ammonium acetate with pH adjusted to 4.5. The temperature of the autosampler was maintained at 4 °C.

A PE Sciex API 3000 triple quadrupole mass spectrometer (MDS-Sciex, Concord, Ont., Canada) interfaced with the HPLC via a turbo ionspray source was used for the mass analysis and detection. The turbo spray temperature was optimized at 300 °C. The precursor/product ion transitions were monitored at *m/z* 439.2 → *m/z* 319.1 for $(^{13}CD_3\text{-SVA}-H)^-$, *m/z* 435.2 → *m/z* 319.1 for $(SVA-H)^-$, *m/z* 423.1 → *m/z* 199.1 for $(^{13}CD_3\text{-SV}+H)^+$ and *m/z* 419.1 → *m/z* 199.1 for $(SV+H)^+$. The mass spectrometer was operated in the negative ion detection mode for the first ∼2 min with dwell time of 500 ms, then positive ion mode for the rest of the analytical run with dwell time of 600 ms. The collision energy (Q0-RO2) setting was optimized at 22.2 V for negative detection mode and 31.9 V for positive ion mode.

The data acquisition software was MassChrom (version 1.4) installed on a Power Macintosh G3. Peak integration and linear regression were performed using the MacQuan Software (version 1.6) residing on a Power Macintosh G3. Calibration curves were constructed by weighted (1/*x*) least square regression of peak area ratios (analyte over internal standard) versus concentrations of the calibration standards. Concentrations of samples were determined by interpolation from the calibration curve.

2.3. Automated LLE process

2.3.1. Instruments and materials

A Packard MultiProbe II workstation (Packard Instrument Company, Meridon, CT) equipped with a 4-tip robotic arm coordinated *x*-, *y*- and *z*-axis. The programmable WinPrep Software controlled the workstation. A custom designed sample rack (dimension: 6 in. width × 16.0 in. length × 4.8 in. height) was made which could hold total 192 cryogenic vials (43049, Corning Science Products, Acton, MA) with 24 vials in each column and eight vials in each row.

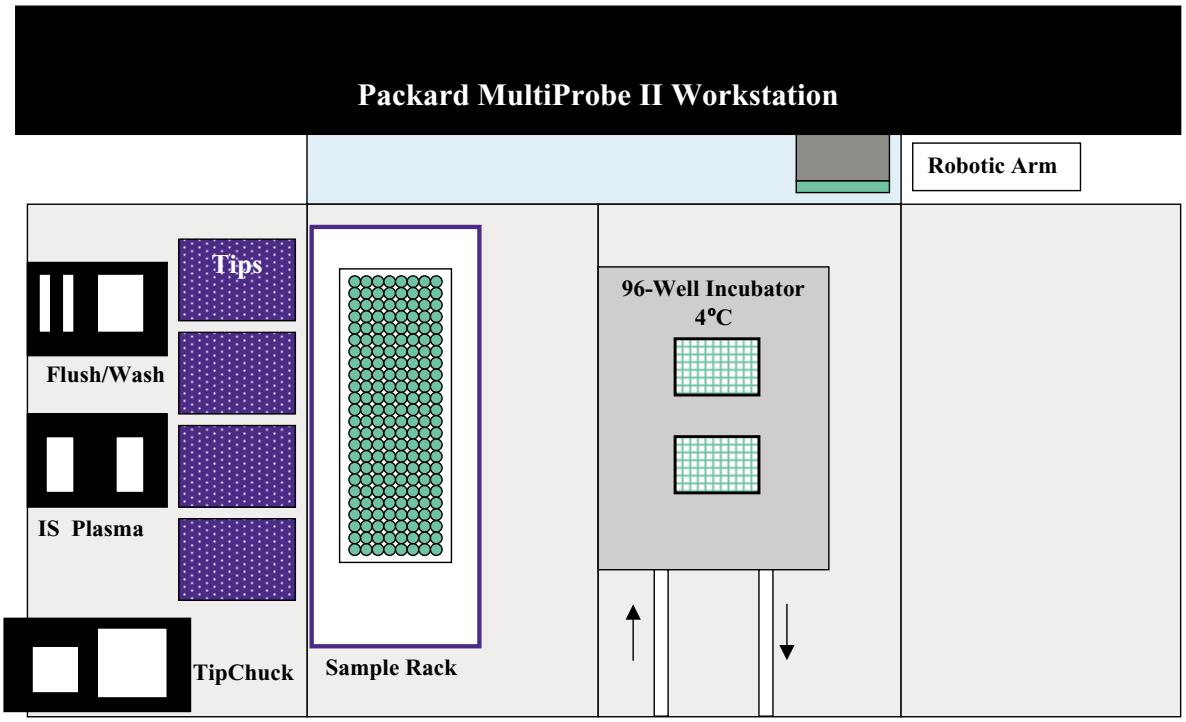


Fig. 2. Schematic setup of the Packard MultiProbe II workstation.

It served as the rack for sample tubes. The rack was immersed in a container (7 in. width × 18 in. length × 4.3 in. height) filled with ice/water mixture for the purpose of keeping samples at 4 °C. Another custom made lab item was a 96-well plate incubator for two 96-well plates (Matrix Technologies, Hudson, NH) enclosed in a 9 in. × 12 in. × 3.5 in. container with circulating ice/water mixture. Small conductive disposable tip-boxes, a tipchute and a tip flush/wash station were purchased from Packard Instrument Company. The schematic deckview of the MultiProbe workstation is shown in Fig. 2. A Tomtec Quadra 96 model 320 workstation equipped with a 96-tip pipetting head was purchased from Tomtec Company (Hamden, CT). It could be programmed to transfer reagents among six movable stations.

The 1.2-ml 96-well plate and the 1.2 and 2.2-ml 96-well piercable plate mats were purchased from Orochem Technologies (Gurnee, IL). An orbital Mixer (Model 099A RD50) (Glas-Col) was used for mixing aqueous and organic phases in 96-well plate during extraction. A SPE DRY-96 solvent evaporator with temperature control was purchased from Jones Chromatography (Lakewood, CL). A refrigerated benchtop Eppendorf centrifuge (model 5810R, Hamburg, Germany) that could accommodate 96-well plates was used. During centrifugation, the plates were sealed by 96-well plate mat sealers (Corning, Acton, MA). A Beckman Model GS-6 centrifuge (Beckman, Columbia, MD) was used to centrifuge samples and quality control samples at 3000 rpm.

2.3.2. Preparation of standards and quality control samples

Stock solutions of SV and $^{13}CD_3$-SV were prepared by dissolving the accurately weighed standard compounds in acetonitrile, and those of SVA and $^{13}CD_3$-SVA in acetonitrile–water (60:40, v/v), to yield for each compound a concentration of 1.00 mg/ml. Working standard solutions containing SV and SVA at concentrations of 500, 250, 50, 10, 5, 1 and 0.5 ng/ml were prepared by serial dilutions using acetonitrile–water. An internal standard working solution containing $^{13}CD_3$-SV and $^{13}CD_3$-SVA at

250 ng/ml each was prepared by diluting and mixing stock solutions of $^{13}CD_3$-SV and $^{13}CD_3$-SVA with acetonitrile–water. Quality control (QC) working solutions at concentrations of 4000, 2000, and 80 ng/ml were prepared using SV and SVA stock solutions which were separately prepared. The stock and working solutions were stored at −20 °C. Plasma QC samples were prepared by adding 400 μl of the appropriate QC working solutions into 45-ml polypropylene screw-cap tubes containing 39.6 ml of human plasma. The bulk QC plasma was vortexed, and then 2 ml aliquots were transferred into labeled cryogenic tubes (3.6 ml), capped and stored in a −70 °C freezer.

2.3.3. Assay procedure

Cryogenic tubes containing plasma samples, QCs, and working solutions were thawed in the ice/water bath. The tubes were vortexed thoroughly and centrifuged for 5 min after being thawed. Calibration standards were freshly made for every analytical run by spiking working solutions into human control plasma using the MultiProbe II workstation.

One hundred microliters of 100 mM ammonium acetate buffer was added into a 2.2-ml 96-well plate using the Tomtec. The 96-well plate was then clamped tightly and placed in an ice/water incubator on the MultiProbe II workstation. The following procedures were performed:

(A) Transfer 250 μl of human control plasma from trough to nine wells in the 96-well plate.
(B) Spike 25 μl of standard working solutions into the wells containing the control plasma.
(C) Transfer 250 μl of both QC and samples to the 96-well plate.
(D) Add 25 μl of internal standard solution into each well containing samples and calibration standards (except double blank).

Following the above steps, 1.2 ml of methyl *tert*-butyl ether (MTBE) was added to each well in the plate using the Tomtec workstation. The plate was then sealed and mixed at medium speed for 10 min. After being centrifuged for 5 min (at 4 °C), the sealing mat was carefully peeled off. About 90% of the MTBE upper layer was aspirated and dispensed into a 1.2-ml 96-well plate by the Tomtec, and subsequently dried using the SPE DRY-96 solvent evaporator at ambient temperature. One hundred microliters of reconstitution solution (70:30 acetonitrile:ammonium acetate buffer, 1 mM, pH 4.5) was added to the plate by the Tomtec workstation. The plate was sealed with a pierceable cap and vortexed for 1 min. It was finally loaded to the Leap autosampler to be introduced to the LC/MS/MS system.

2.4. Validation procedures

Individual plasma from five subjects were extracted (in five replicates) to check for peaks interfering with the analytes. SV-only and SVA-only working solutions (500 ng/ml), as well as $^{13}CD_3$-SV-only and $^{13}CD_3$-SVA-only working solutions (250 ng/ml) were injected separately to check potential interference due to cross-talk, isotopic effect, and/or impurities in the standards.

Cross-well contamination and assay carryover was tested by transferring plasma containing the analytes (at the highest concentration of the calibration range, 50 ng/ml) and pure water alternatively five times so that plasma containing analytes and water were in adjacent wells in the 96-well plate. The plate was processed following the described extraction and analysis procedure.

The extraction recoveries for each analyte were determined by comparing the peak area ratios of analyte over internal standard obtained from plasma samples with the analytes spiked before extraction to those spiked after the extraction. The internal standards were spiked after extraction in each case.

To test for matrix effect, two sets of testing samples were prepared by direct spiking of the analytes into reconstitution solution with and without the presence of residue extracted from pooled control plasma. The matrix effect was evaluated for SV and SVA at three QC concentrations by comparison of mean analyte peak areas obtained from these two sets of testing samples. For further comparison, an additional extraction step was added in a separate experiment: after the upper MTBE layer was transferred to the 1.2-ml plate following LLE, 100 μl of an ammonium acetate buffer (50 mM, pH 5) and 800 μl of MTBE were added to the same 96-well plate. The plate was vortexed briefly, the upper layer was transferred to another 1.2-ml 96-well plate and MTBE evaporated. Seventy microliters of reconstituting solution was added into each well. QC working solutions were spiked into the wells with

and without extracted plasma residue, respectively. Three replicates were tested at each concentration level.

Assessment of interconversion between SV and SVA during sample extraction was evaluated by analyzing plasma samples ($n = 3$) spiked with SV-only or SVA-only at 50 ng/ml following the described procedure. The percentage of lactonization (SVA → SV) was determined by comparing the peak area ratios of (SV/$^{13}CD_3$-SV) obtained from SVA-only samples over those from SV-only samples. The percentage of hydrolysis (SV → SVA) was determined by comparing the peak area ratio of (SVA/$^{13}CD_3$-SVA) obtained from SV-only samples over those from SVA-only samples. The mean interconversion was measured and compared using buffers with different pH during extraction.

Intra-assay precision and accuracy were assessed by analyzing five replicates of plasma standards ($n = 5$) at all concentrations used to construct the calibration curve. The initial inter-assay precision and accuracy were determined by analyzing five replicates of the quality control samples at concentrations of 0.8, 20 and 40 ng/ml for both analytes through three assay runs. The accuracy was expressed by (mean observed concentration)/(nominal concentration) × 100%. The precision was expressed as percentage relative standard deviation (%R.S.D.).

3. Results and discussion

In the drug development process, clinical studies throughout phases I–V usually involve many subjects, large quantity of samples collected over long periods of time and multiple clinical sites. Although modern analytical instrumentation, especially LC/MS/MS, has greatly facilitated bioanalytical work, the most labor intensive and time consuming part of the job is still sample processing. Operational errors can easily occur and it is difficult to trace back if everything is done manually. The attempt to collect samples directly into 96-well plate or 96-tube cluster format is very attractive to simplify handling procedures [18], but it has practical problems when the samples are collected for complicated studies. The Packard MultiProbe II workstation provided the flexibility of converting various sample formats (different sizes of tubes and racks) into 96-well plate format automatically in a parallel fashion. Plasma samples covering a wide range of drug concentrations are very typical in clinical pharmacokinetic studies and the MultiProbe II workstation eased the job of multiple sample dilutions. Then the Tomtec workstation equipped with 96 manifold pipetting head would process the plate in high speed. This is the first assay reported using LLE based on 96-well plate format with full automation of all the liquid transfer steps in a parallel sample processing mode to support clinical pharmacokinetic studies. It also should be easily adaptable to other related applications in drug discovery and development.

Due to the instability of SV and SVA, both the 24 × 8 sample rack and 96-well plates were placed in ice/water incubators located on the deck of the MultiProbe II workstation, so that the samples were kept at 4 °C during the whole sample processing. A refrigerated centrifuge and autosampler are also necessary in maintaining sample stability during and after sample extraction.

For the LC/MS/MS part of the method, a similar procedure was already reported [10]. Briefly, the selected reaction monitoring (SRM) mode was used for the mass spectrometric detection. SVA and its internal standard were analyzed under negative ion mode, the precursor ions were the deprotonated SVA and $^{13}CD_3$-SVA, respectively. The fragment ion of *m/z* 319.0 formed by loss of 2,2-dimethyl-butyrate from parent ions was chosen as the daughter ion for both SVA and $^{13}CD_3$-SVA. SV and $^{13}CD_3$-SV were analyzed under positive ion mode. The precursor ions were the protonated SV and $^{13}CD_3$-SV. The daughter ions were the *m/z* 199.1 ion for both SV and $^{13}CD_3$-SV which formed after several steps of fragmentation from the parent ions as proposed in the previous work [10]. The retention time was about 1.4 min for SVA and about 2.8 min for SV. The polarity switching occurred at about 2 min after the start of the run. The representative chromatograms of plasma samples spiked with 5 and 25 ng/ml of both internal standards are shown in Fig. 3.

3.1. Assay specificity

No interfering peaks were observed in the extracted ion current (XIC) chromatograms for all the individual and pooled blank plasmas at the reten-

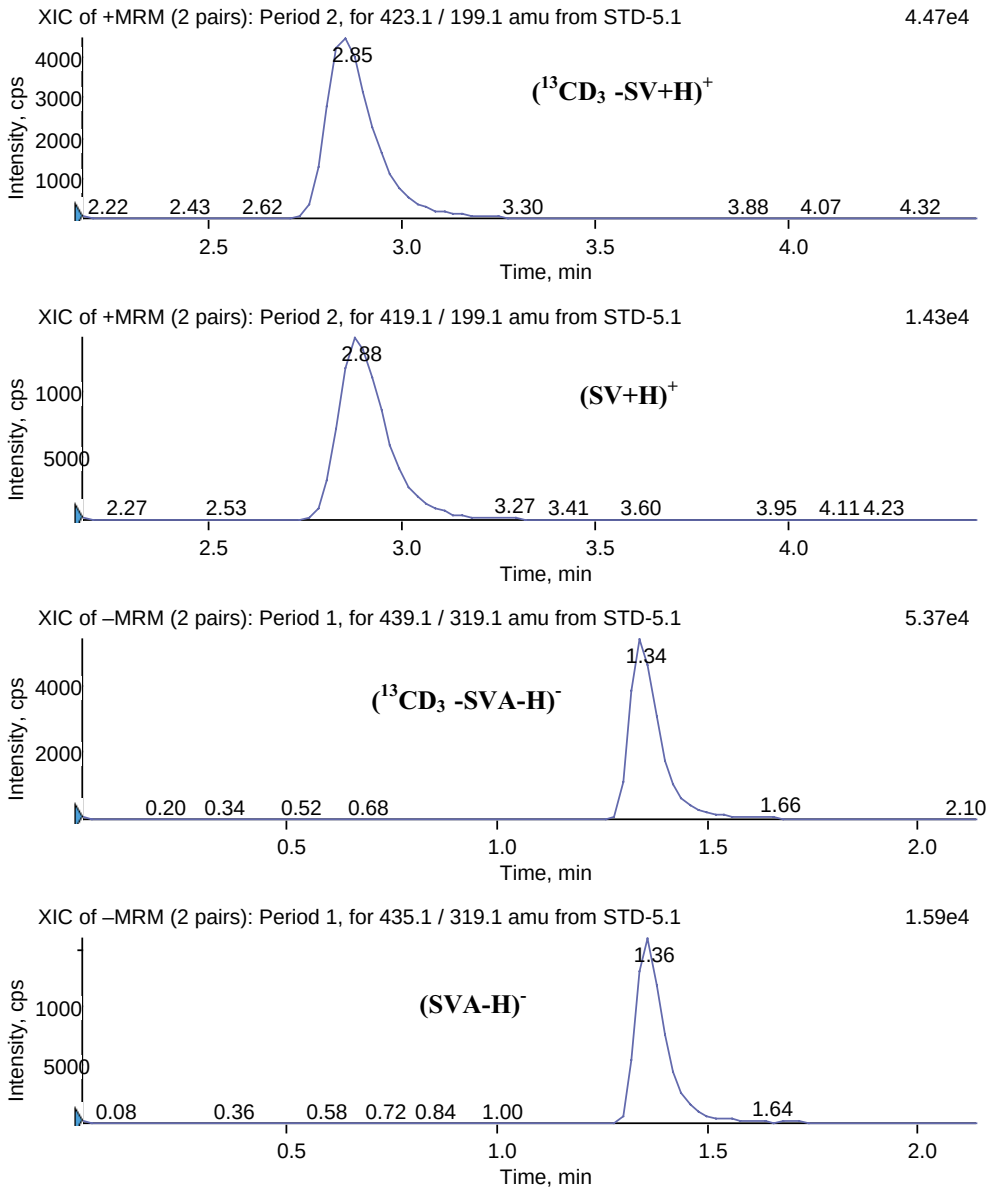


Fig. 3. Representative extracted ion current (XIC) chromatograms of SV and SVA in human plasma spiked with SV and SVA at 5 ng/ml each and with $^{13}CD_3$-SV and $^{13}CD_3$-SVA at 25 ng/ml each.

tion times of the analytes and internal standards. Less than 0.013% (peak area of $^{13}CD_3$-SV/SV*100) cross-talk was observed from SV (419.1 → 199.1) to $^{13}CD_3$-SV (423.1 → 199.1) when 500 ng/ml of SV-only neat solution was analyzed. No ion transition of *m/z* 439.1 → 319.1 was detected in $^{13}CD_3$-SVA channel of the XIC chromatogram when 500 ng/ml of SVA neat solution was tested. No SV and SVA signals were detected for all the blank samples (spiked with IS and control plasma only).

3.2. Cross-well contamination and assay carryover investigation

Since a relatively large calibration range (three orders of magnitude) was used, carryover of the assay was tested to ensure the limit of quantitation (0.05 ng/ml) was applicable and the accuracy was not affected by the carryover from high concentration samples. For the LLE based on 96-well plate, there are several potential steps in the procedure which would cause cross well contamination and carryover. First, the samples were directly transferred by the fixed tips of MultiProbe II workstation so that careful washing was necessary to ensure the cleanness of the tips after each transfer. Second, the sealing of the 96-well plate was challenging when the volatile solvent MTBE was used. Several sealing mats were tested and no leaking was observed when the right plate was chosen. Third, the carryover test on the Leap autosampler was needed. The described experiment was designed to test all the potential carryover since blank and highest standard samples were transferred into adjacent wells. No detectable carryover peaks were observed among those wells containing deionized water.

3.3. Extraction recovery

The most important parameter affecting the SV and SVA recovery is the pH value used in the extraction. Extraction buffer concentration and solvent type did not play a significant role in improving SV and SVA recoveries. Previous work (manual LLE) also agreed with this [14]. One hundred microliters of 100 mM ammonium acetate buffer at pH 4, 4.5, 5, 5.5 and 6, respectively, were added to separate 250 μl of plasma samples to adjust the pH for extraction. The recoveries of SV and SVA at these pH values are listed and compared in Table 1. The extraction recovery for SV was not affected significantly in the pH range of 4–6. The recovery for SVA decreased as the pH increased due to the ionization of SVA in water.

Table 1
Extraction recoveries (%) of SV and SVA (40 ng/ml) at different pH values ($n = 3$)

	pH				
	4.0	4.5	5.0	5.5	6.0
SV ($n = 5$)	83.4	85.2	87.1	91.2	83.7
SVA ($n = 5$)	82.3	83.5	72.7	48.0	35.8

3.4. Ion suppression effect for the assay

Since stable isotope labeled internal standards were used in this assay, ion suppression which affects the variability of individual (matrix effect during electrospray ionization) peak area ratios of analytes to internal standards is not a factor [19–21]. However, to evaluate the quality of sample cleanup for the established sample preparation methods, ion suppression or enhancement caused purely by biological matrix should be compared with the signals of neat solutions at different concentrations. This is especially important if electrospray ionization is used in LC/MS/MS analysis. When the described extraction procedure was performed, the signal (peak area) decreased by 22% (SV) and 16% (SVA) compared with those on average for neat solutions. More ion suppression was observed at lower concentrations. With an extra extraction step in the procedure (described in Section 2.4), signal enhancement was observed for SVA, and the ion suppression was reduced to 10% for SV. The results are summarized in Table 2. In conclusion, the ion suppression caused by matrix could be eliminated or kept to minimum using this automated LLE method based on 96-well plate format.

3.5. Evaluation of interconversion between SV and SVA

Interconversion between SV and SVA easily occurs and is very sensitive to several parameters such as temperature, pH, storage condition, and sample extraction method. Improper mass spectrometric methods especially the conditions of the ESI source also contribute to the interconversion to form so-called in source ionization [22,23]. With within-run polarity-switching, in source ionization was not observed. A relatively large extent of interconversion was observed when using solid-phase extraction methods [24]. Although based on the same sample extraction mechanism, manual LLE methods also caused interconversion that was not negligible [14]. The assay with lowest percent interconversion between SV and SVA was the Chem-Elut cartridge extraction method [4]. However,

Table 2
Summary of matrix effects[a] on SV and SVA measurements using the automated LLE method at three concentrations and comparison of matrix effects by extracting once[b] and extracting twice[c]

Concentration (ng/ml)	Experiment 1			Experiment 2		
	Mean peak area once extracted ($n = 3$)[b]	Mean peak area of neat solution ($n = 3$)	Matrix effect[a]	Mean peak area twice extracted ($n = 3$)[c]	Mean peak area of neat solution ($n = 3$)	Matrix effect[a]
SVA						
0.8	52713	67832	77.7	45791	45188	101.3
20	1349915	1647936	81.9	1251991	1178966	106.2
40	2761127	3020031	91.4	2541285	2383548	106.6
Average			83.7			104.7
SV						
0.8	23175	32057	72.3	15646	18437	84.9
20	699023	888791	78.6	523344	572489	91.4
40	1462883	1737388	84.2	1062273	1176477	90.3
Average			78.4			88.9

[a] Matrix effect = (analyte peak area with extracted plasma residue)/(analyte peak area of neat solution) × 100.
[b] The extraction was performed as described in Section 2.3.3.
[c] An extra extraction (back extraction) was performed as described in Section 2.4.

no automated procedure was involved. The interconversion issue was also evaluated in this automated LLE method. Parallel studies for comparing the interconversion among different pHs were conducted by adding ammonium acetate buffer (100 μl and 100 mM) at pH 4, 4.5, 5, 5.5 and 6. The conversion between SV and SVA was clearly dependent on the pH as predicted from the structures of SV and SVA. In combination of recovery results, pH 5 was chosen for the final condition for LLE. The results and comparison are summarized in Table 3.

3.6. Assay sensitivity, precision and accuracy

3.6.1. Sensitivity

Although only half the sample size (250 μl) was used in this method compared with the manual LLE procedure (0.5 ml), similar sensitivity was observed.

Table 3
Evaluation of interconversion between SV and SVA at different pH value ($n = 5$) and comparison among different methods[a]

pH	Ratio of SV/$^{13}CD_3$-SV		Ratio of SVA/$^{13}CD_3$-SVA		Interconversion (%)[b]	
	SV only	SVA only	SV only	SVA only	SV to SVA (%)	SVA to SV (%)
Automated LLE						
4.0	14.445	0.095	0.002	7.995	0.025	0.658
4.5	13.282	0.032	0.003	8.072	0.037	0.241
5.0	14.597	0.009	0.005	7.078	0.071	0.062
5.5	14.562	0.002	0.009	6.366	0.141	0.014
6.0	14.953	0.002	0.020	7.541	0.265	0.013
Solid phase extraction method [24]					0.60	3.00
Manual liquid–liquid extraction [14]					0.12	0.49
Chem-Elut cartridge extraction [10]					0.06	0

[a] SV-only and SVA-only plasma samples at highest concentration level (50 ng/ml) in the calibration range were tested.
[b] Percentage was calculated as (taking measuring SV → SVA as example)% = (peak area ratio SVA/$^{13}CD_3$-SVA from SV-only solution)/(peak area ratio SVA/$^{13}CD_3$-SVA from SVA-only solution) × 100.

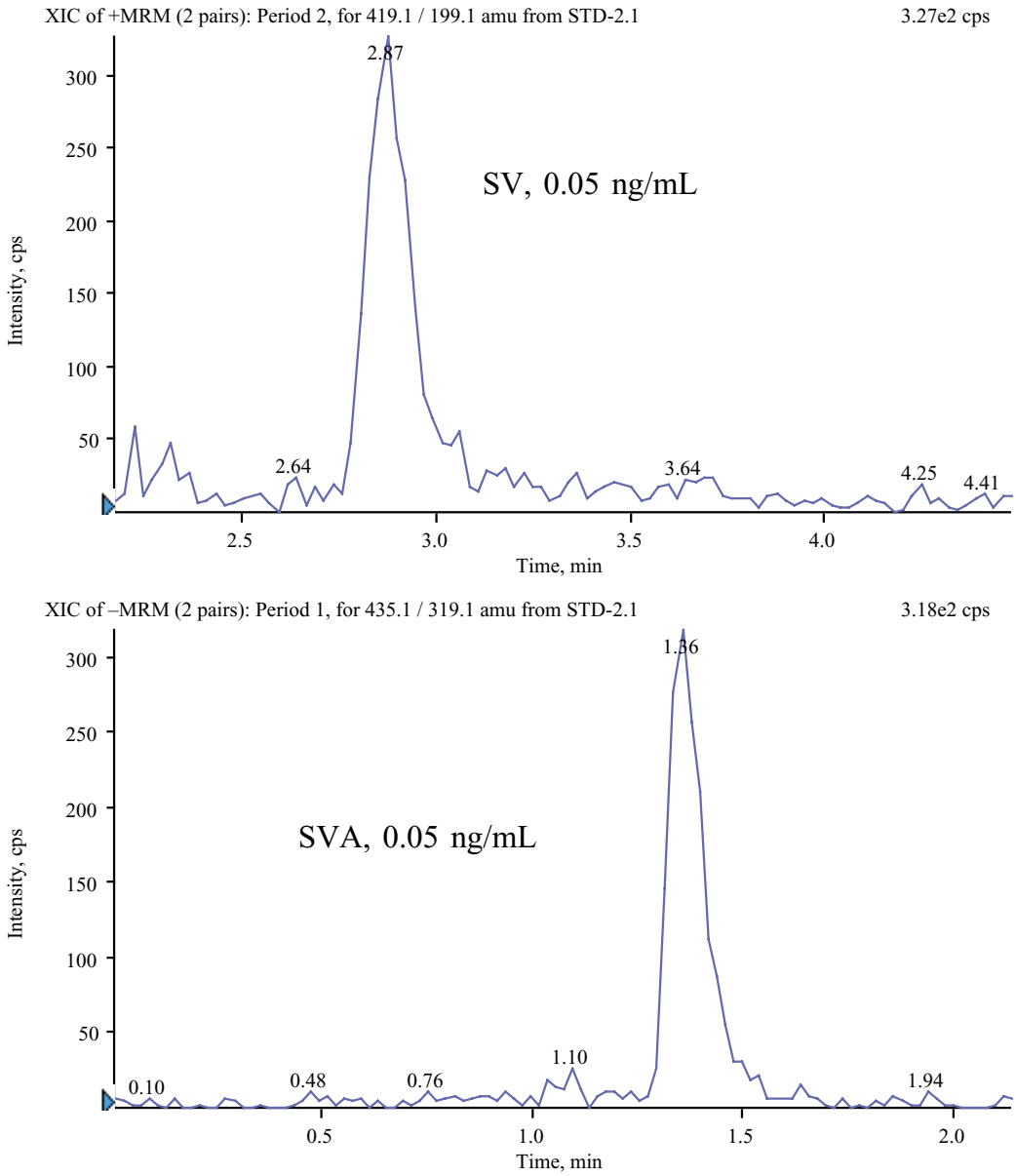


Fig. 4. Selected reaction monitoring (SRM) chromatograms of a plasma sample spiked with SV and SVA at 0.05 ng/ml (limit of quantification, LOQ).

Better sensitivity was achieved compared with that of Chem-Elut cartridge extraction method [10] using the same sample volume (250 μl). Fig. 4 shows the extracted ion current chromatograms of the analytes at 0.05 ng/ml which is the LOQ.

3.6.2. Assay precision and accuracy

The Packard MultiProbe II liquid handling workstation was calibrated for pipetting precision and accuracy using a gravimetric method with deionized water as the testing liquid. The actual volume being trans-

ferred was calculated by the ratio of the weight over the density of water at the recorded temperature. Volumes of 20-, 50-, 100-, 250- and 500-μl were calibrated with five replicates for each of the four tips. The precision (%R.S.D.) was less than 2% and accuracy was 97–103% of nominal volume for each tip at each volume. The tip-to-tip variation was less than 1.53% R.S.D. The workstation's performance was evaluated periodically.

The assay procedure proved to be very precise and accurate. The intra-day precision ranged from 1.67 to 7.47% R.S.D. for SV and 0.99 to 4.44% R.S.D. for SVA, for all seven standard concentrations ($n = 6$). The intra-assay accuracy, expressed as a percentage of nominal values, ranged from 98.7 to 101.2% for SV and 97.3 to 102.3% for SVA, for all seven standard concentrations (Table 4). The initial inter-assay precision (%R.S.D.) ranged from 1.85 to 4.07% for SV and 2.29 to 3.32% for SVA, for all three QC concentrations ($n = 3$ assays, five replicates per assay). The initial inter-assay accuracy ranged from 95.3 to 98.0% for SV and from 97.1 to 100.9% for SVA, for all three QC concentrations (Table 5).

Table 4
Intra-assay precision and accuracy of measurement of SV and SVA in human plasma ($n = 6$)

Nominal concentration (ng/ml)	Mean	Precision[a]	Accuracy (%)[b]
SV			
0.05	0.050	5.28	100.67
0.1	0.099	7.47	98.83
0.5	0.494	3.94	98.73
1	1.006	1.67	100.60
5	5.057	2.15	101.15
25	24.946	2.40	99.79
50	49.997	2.87	99.99
SVA			
0.05	0.049	4.44	97.33
0.1	0.100	2.67	99.67
0.5	0.497	2.85	99.30
1	1.023	3.55	102.27
5	5.055	0.99	101.10
25	24.978	2.23	99.91
50	49.949	1.69	99.90

[a] Expressed as %R.S.D.
[b] Calculated as (mean calculated concentration)/(nominal concentration) × 100.

Table 5
Inter-assay precision and accuracy of measurement of SV and SVA in human plasma ($n = 5$)

	Nominal concentration (ng/ml)		
	0.8	20	40
SV			
Day 1	0.756	19.553	38.699
Day 2	0.796	19.983	39.726
Day 3	0.735	19.263	37.857
Mean	0.762	19.599	38.760
Precision[a]	4.07	1.85	2.41
%Accuracy[b]	95.3	98.0	96.9
SVA			
Day 1	0.791	20.605	40.900
Day 2	0.796	20.248	40.696
Day 3	0.749	19.689	38.848
Mean	0.779	20.181	40.148
Precision[a]	3.32	2.29	2.82
%Accuracy[b]	97.3	100.9	97.1

[a] Expressed as %R.S.D.
[b] Calculated as (mean calculated concentration)/(nominal concentration) × 100.

3.7. Comparison with manual L–L extraction procedures

Compared with manual LLE, the described automated LLE based on 96-well plate improved sample handling efficiency. If 96 samples are to be analyzed, the actual time an analyst spends on the automated LLE reduced to about 1 h instead of 6 h or more which is typical in manual procedure. The speed-limiting steps in the automated LLE are sample thawing, capping and de-capping vials. Using pierceable (by workstation probe) caps for sample vials provided a practical solution for sample transfer directly from uncapped tubes [16]. Overall, by using the automated LLE procedure described in this report, the sample preparation throughput can be improved to at least three-fold compared to that of manual procedure. Much less organic solvent is consumed in LLE procedure based on the 96-well plate format. Plasma samples from one subject following single oral dose of SV were analyzed using both the procedure in this paper and Chem-Elut cartridge extraction [10]. The samples collected at predose, 0.5, 1, 2, 4, 6, 8, 12, 16 and 24 h were analyzed. The excellent match of the results obtained by the two methods is shown in Fig. 5.

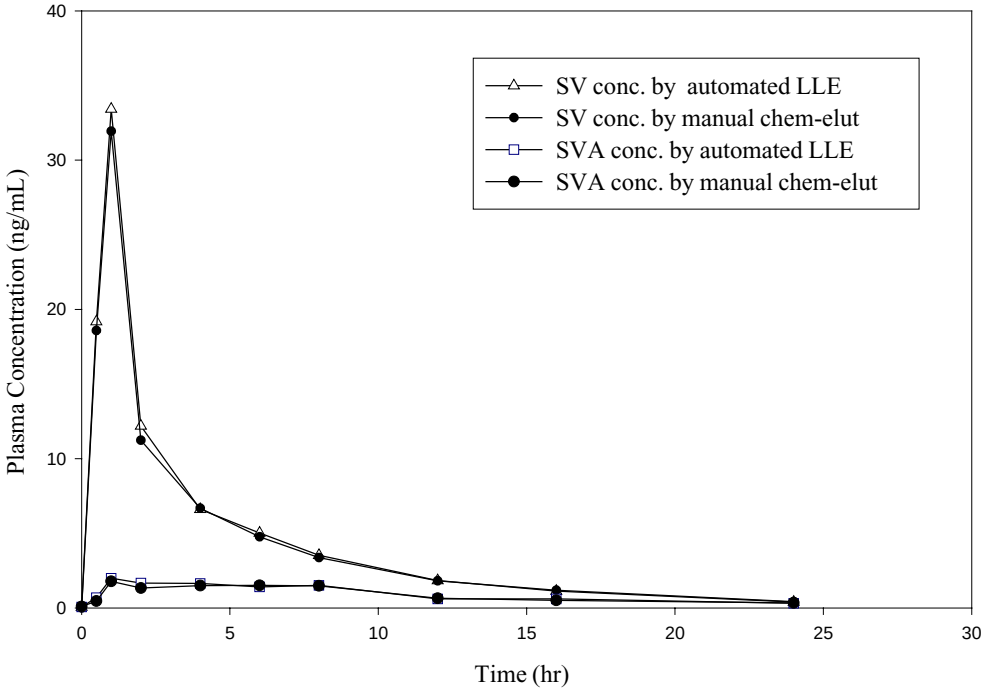


Fig. 5. Quantitative comparison of SV and SVA using Chem-Elut cartridge extraction and automated LLE based on 96-well plate in a subject dosed with SV.

4. Conclusion

Liquid–liquid extraction, a frequently used method for extraction of drugs from biological fluids, was automated into a 96-well plate format for the LC/MS/MS analysis of SV and SVA in human plasma. A 4-probe liquid handling workstation was used to pipette and convert samples to 96-well plates and then a 96-channel robotic liquid handler processed the samples in parallel. The sample preparation efficiency was improved by approximately three-fold compared to that of manual procedure. The automated LLE should also be readily adaptable to drug analysis in other biological fluids and related drug discovery and development areas. SV and SVA were extracted by MTBE from plasma and the extraction recovery was optimized at pH 5 using 100 mM ammonium acetate buffer. Interconversion between SV and SVA during sample extraction, which is a common concern for analytes existing in both lactone and acid forms, was shown to be very low or negligible ($\leq$0.06% for lactonization and $\leq$0.07% for hydrolysis). The assay showed good intra and inter-assay precision and accuracy. Application of the automated LLE to clinical plasma samples proved SV and SVA concentration data that were an excellent match to those generated by a manual procedure.

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