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Quantitation of simvastatin and its β-hydroxy acid in human plasma by liquid–liquid cartridge extraction and liquid chromatography/tandem mass spectrometry

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A sensitive and reliable procedure for the simultaneous determination of simvastatin (SV) and its active β-hydroxy acid metabolite (SVA) in human plasma was developed and validated. The analytes were extracted simultaneously from 0.5 ml aliquots of human plasma samples by methyl *tert*-butyl ether (MTBE) via Chem Elut cartridge extraction [also called liquid–solid extraction (LSE) or liquid–liquid cartridge extraction (LLCE)], separated through a Kromasil C_{18} column (50 × 2 mm i.d. 5 μm) and detected by tandem mass spectrometry with a turbo ionspray interface. Stable isotope-labeled SV and SVA, $^{13}CD_3$-SV and $^{13}CD_3$-SVA, were used as internal standards. SV and SVA were detected in positive and negative ion modes, respectively, via within-run polarity switching. The use of Chem Elut cartridges not only provided a simple and efficient means of plasma sample extraction but also successfully reduced the interconversion between SV and SVA to an undetectable (for lactonization of SVA) or negligible (<0.07%, for hydrolysis of SV) level. The method showed excellent reproducibility, with intra- and inter-assay precisions <4.5% (RSD), and intra- and inter-assay accuracy between 94% and 107% of nominal values, for both analytes. The extraction recoveries were 78% and 87% on average for SV and SVA, respectively. The analyte was found to be stable in plasma through three freeze (−70 °C)–thaw (4 °C) cycles and for at least 3 h under bench-top storage condition in an ice-bath (4 °C), and also in the reconstitution solution at 4 °C for at least 24 h. The method has a lower limit of quantitation (LOQ) of 50 pg ml^{-1} with a linear calibration range of 0.05–50 ng ml^{-1} for both analytes, and has proved to be very reliable for the analysis of clinical samples.

KEYWORDS: simvastatin; liquid chromotography/tandem mass spectrometry; liquid–liquid cartridge extraction via Chem Elut cartridges; turbo ionspray; human plasma

INTRODUCTION

Simvastatin (SV, Fig. 1), (+)-(1*S*,3*R*,7*S*,8*S*,8*aR*)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2*R*,4*R*)-tetrahydro-4-hydroxy-6-oxo-2*H*-pyran-2-yl]-1-naphthyl-2,2-dimethylbutanoate], is a cholesterol-lowering agent.[1] Following oral administration, SV is hydrolyzed *in vivo* rapidly to its corresponding β-hydroxy acid (SVA, Fig. 1). The latter is a potent inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an essential enzyme involved in the *in vivo* synthesis of cholesterol. SV and SVA are present at low concentrations in plasma, probably owing to high first-pass hepatic extraction resulting in low oral availability (∼5%).[2] Very sensitive, efficient and reliable analytical methods for the determination of SV and SVA are therefore required for assessing plasma drug concentrations.

SV and SVA were previously determined in plasma by high-performance liquid chromatography (HPLC),[3–5] gas chromatography/mass spectrometry[6,7] and atmospheric pressure chemical ionisation liquid chromatography/tandem mass spectrometry (LC/MS/MS).[8] In these methods, SV and SVA were separately extracted from plasma samples by solid-phase extraction (SPE). SV was converted into SVA by base hydrolysis and both then were derivatized and analyzed in separate analytical runs. With some of these methods the lower limit of quantitation (LOQ) was as low as 0.25 ng ml^{-1} (using a 0.5 ml sample size)[7] and 0.1 ng ml^{-1} (using a 1.0 ml sample size).[5] However, the sample preparation and derivatization steps are time consuming, and two analytical runs are needed for each plasma sample.

The simultaneous determination of SV and SVA in biological samples, which could save a considerable amount of sample preparation and analytical run time, was considered to be difficult initially owing to the different polarities of the two analytes. Such methods were later developed for lovastatin (LV) and its β-hydroxy acid (LVA) in animal plasma[9] and for SV and SVA in human plasma (Ref. 10 and also analytical protocol and validation report for SPE-LC/MS/MS determination of SV and SVA in human plasma; Merck internal document) using SPE and LC/MS/MS procedures with within-run polarity switching between positive (for

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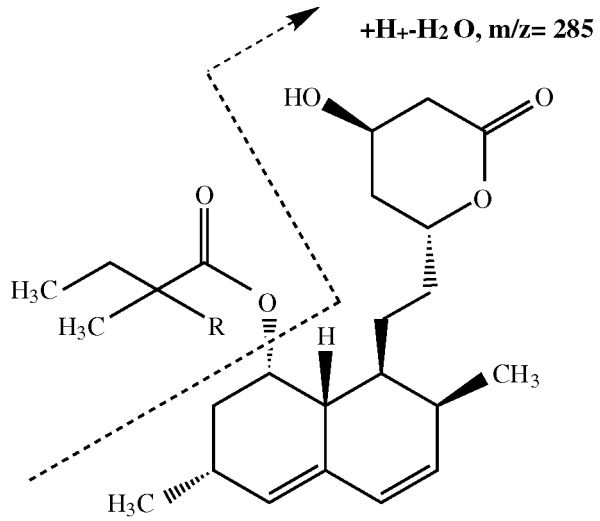
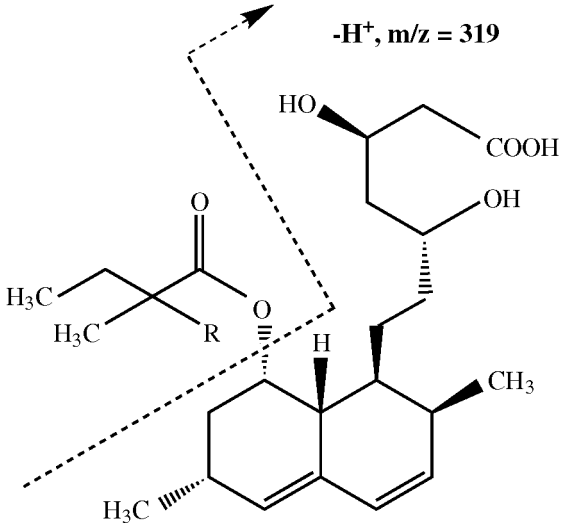


R = CH_3: Simvastatin (SV), MW = 418

R = $^{13}CD_3$: $^{13}CD_3$-SV, MW = 422



R = CH_3: Simvastatin Acid (SVA), MW = 436

R = $^{13}CD_3$: $^{13}CD_3$-SVA, MW = 440

Figure 1. Structures of SV, $^{13}CD_3$-SV, SVA and $^{13}CD_3$-SVA.

lactones) and negative (for acids) ion modes. In these methods sodium adduct ions $[M + Na]^+$ of LV and SV were used in MS/MS detection, and, because of the use of a low-pH solution during extraction, interconversion between the lactone and acid forms was a potential problem.

More recently, an LC/MS/MS method with liquid–liquid extraction (LLE) was developed in our laboratories for the simultaneous determination of SV and SVA in human plasma with an LOQ of 0.05 ng ml^{-1} for both analytes.[11] A similar method with an LOQ of 0.5 ng ml^{-1} for the lactone and acid forms of atorvastatin was also reported.[12] In this atorvastatin work, unlike ours, both forms of atorvastatin were detected in the positive ion mode. The LLE LC/MS/MS method for SV and SVA provided further simplified sample preparation steps and better extraction efficiency for both SV and SVA. However, there was still some interconversion (<0.5%) between SV and SVA during sample extraction which, although at a small percentage, was still not satisfactory to us. In this paper, we report an alternative and improved LC/MS/MS procedure employing Chem Elut (a trade mark of Varian) cartridge extraction (also called liquid–liquid cartridge extraction (LLCE) or liquid–solid extraction (LSE)) for more accurate and precise quantitation of both SV and SVA in human plasma.

EXPERIMENTAL

Chemicals and reagents

Standard compounds of SV and SVA were synthesized by Merck Research Laboratories. Stable isotope-labeled SV and SVA, $^{13}CD_3$-SV and $^{13}CD_3$-SVA, were synthesized by Drug Metabolism of Merck Research Laboratories and were used as the internal standards for SV and SVA, respectively. For both internal standards, the isotopic composition was >98.5% M + 4, <1% M + 3, <0.5% M + 2 and 0% M + 1 and M + 0. 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), formic acid (purity 99%) and acetic acid (glacial) from Aldrich (Milwaukee, WI, USA) and methyl *tert*-butyl ether (MTBE) (HPLC grade) from Burdick & Jackson (Muskegon, MI, USA). Regular pooled human control plasma (heparinized) and different individual lots of heparinized human plasma were purchased from Biological Specialty Corporation (Lansdale, PA, USA). Nitrogen (refrigerated liquid) was obtained from West Point Supply (West Point, PA, USA). De-ionized water was prepared using a Milli-Q Plus Ultra-Pure water system (Millipore, Bedford, MA, USA).

LC/MS/MS instrumentation and analytical conditions

The liquid chromatograph system consisted of an LC pump (Model LC-10AD), an autosampler (Model SIL-10A), an on-line solvent degasser (Model GT-104) and a system controller (Model SCL-10A), all made by Shimadzu Scientific Instruments (Columbia, MD, USA). Liquid chromatography was performed on a Kromasil C_{18} column (2.0 × 50 mm i.d., 5 μm particle size) (Keystone Scientific, Bellefonte, PA, USA) with a liquid flow-rate of 200 μl min^{-1} under ambient conditions. The mobile phase was prepared by mixing 750 ml of acetonitrile with 250 ml of 1 mM ammonium acetate with the pH adjusted to 4.5 using formic acid. The temperature of the sample tray in the autosampler was maintained at 4 °C.

A PE SCIEX API 365 triple-quadrupole mass spectrometer (Perkin-Elmer SCIEX Instruments, Concord, ON, Canada) interfaced with the liquid chromatograph via a turbo ionspray source was used for mass analysis and detection. The turbo ion-spray temperature was optimized and maintained at 300 °C. Quantitation was performed using selected reaction monitoring (SRM) of

precursor–product ion transitions at m/z 439.2 $[M-H]^- \rightarrow$ 319.1 (for $^{13}CD_3$-SVA), m/z 435.2 $[M-H]^- \rightarrow$ 319.1 (for SVA), m/z 423.1 $[M+H]^+ \rightarrow$ 285.1 (for $^{13}CD_3$-SV) and m/z 419.1 $[M+H]^+ \rightarrow$ 285.1 (for SV). The mass spectrometer was operated in the negative ion mode for the first ~2 min with a dwell time of 500 ms, and then in the positive ion mode for the rest of the analytical run with a dwell time of 600 ms. The collision energy was optimized at 22.2 V (Q0–RO2) for the negative ion detection mode and 31.9 V for the positive ion mode. The nitrogen collision gas thickness in the Q2 collision cell was maintained at 1.9×10^{15} atoms cm^{-2} for both negative and positive ion modes. Mass calibration was performed by the infusion of a 10^{-4} M polypropylene glycols (PPG) solution into the ionspray source. The peak widths of precursor and product ions were maintained at ~0.7 u at half-height in the SRM mode.

Data acquisition, peak integration and calculation were performed using PE Sciex MacQuan software residing on Macintosh 9500 computers. Peak area ratios of analytes to internal standards were utilized for the construction of calibration curves, using $1/x$ weighted linear least-squares regression of plasma concentrations and the measured peak area ratios. Concentrations of analytes in quality control (QC) or unknown samples were calculated by interpolation from the calibration curves.

Preparation of standard and quality control samples

Stock solutions of SV and $^{13}CD_3$-SV were prepared by dissolving 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^{-1}. All concentrations were calculated based on the free acid or neutral molecule form. Diluted solutions of SV or SVA at a concentration of 10 μg ml^{-1} were prepared by mixing 200 μl of the appropriate stock solutions with 19.8 ml of acetonitrile–water (60:40, v/v) solution. A standard working solution of 500 ng ml^{-1} for both SV and SVA was prepared by mixing 1 ml of each of the above 10 μg ml^{-1} solutions with 18 ml of acetonitrile–water (60:40, v/v). Working standard solutions at 250, 50, 10, 5, 1 and 0.5 ng ml^{-1} were prepared by successive dilution of the 500 ng ml^{-1} solution with acetonitrile–water (60:40, v/v). An internal standard working solution containing $^{13}CD_3$-SV and $^{13}CD_3$-SVA at a concentration of 250 ng ml^{-1} each was prepared by diluting and mixing the 1.00 mg ml^{-1} stock solutions of $^{13}CD_3$-SV and $^{13}CD_3$-SVA with acetonitrile–water (60:40, v/v).

QC working solutions at concentrations of 4000, 2000 and 80 ng ml^{-1} for each analyte were prepared by mixing and successive dilution of 10 μg ml^{-1} SV and SVA solutions which were made from separately prepared 1.00 mg ml^{-1} stock solutions of SV and SVA.

Plasma standards of SV and SVA were prepared fresh daily by spiking 50 μl of the appropriate standard working solutions into 500 μl human control plasma to yield calibration concentrations of 50, 25, 5, 1, 0.5, 0.1 and 0.05 ng ml^{-1}. Acetonitrile–water solution (60:40, v/v) was added to blank plasma, at 100 and 50 μl, to make up plasma double blank and blank samples, respectively.

Plasma QC samples were prepared by adding 400 μl of the appropriate QC working solutions to 45 ml polypropylene tubes containing 39.6 ml of human control plasma to yield QC concentrations of 40, 20 and 0.8 ng ml^{-1}. The bulk QC plasma samples were vortex mixed and then 0.5 ml aliquots were transferred into individual labeled polypropylene tubes (16 × 100 mm), capped and stored at −70 °C.

For analyte stability reasons which will be discussed later, all the standard and QC stock and working solutions were stored at −20 °C. Standard and QC working solutions were brought to room temperature before use. And all the standard and QC samples were prepared at ~4 °C in an ice-bath.

Plasma sample extraction

To each labeled polypropylene tube (16 × 100 mm) placed in an ice-bath and containing 0.5 ml of blank sample, plasma standard, thawed QC or unknown plasma sample, 50 μl of internal standard working solution were added (except for the double blank), followed by the addition of 300 μl of ammonium acetate solution (100 mM, pH 4.5). The contents were immediately vortex mixed and loaded on 1.0 ml Chem Elut extraction cartridges (Varian Sample Preparation Products, Harbor City, CA, USA). After ~5 min to allow the plasma to dissipate into the cartridge packing materials, the analytes and internal standards were eluted with 3 × 4 ml of MTBE. The eluents were collected in another set of labeled polypropylene tubes (16 × 100 mm) and the solvent was evaporated in a TurboVap evaporator (Zymark, Hopkinton, MA, USA) at 40 °C with nitrogen. The residues were reconstituted with 100 μl of 70:30 acetonitrile–ammonium acetate (1 mM, pH 4.5) and transferred into 0.2 ml polypropylene autosampler vials and sealed with snap caps. Volumes of 20 μl of each reconstituted sample were injected into the LC/MS/MS system for analysis.

Validation procedures

The intra-day precision and accuracy of the method were assessed by analyzing five replicates of plasma standards at all concentrations used to construct the calibration curve. The initial inter-day precision and accuracy were determined by analyzing five replicates of the quality control samples at concentrations of 0.8, 20 and 40 ng ml^{-1} for both analytes through three assay runs. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision as relative standard deviation (RSD).

The extraction recoveries for each analyte at three QC concentration levels were determined by comparing the peak area ratios of analyte to 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. Assay specificity was assessed by analyzing human plasma double blanks from 10 individual subjects and pre-dose samples and checking for interfering peaks with the analytes.

The stability of SV and SVA in human plasma was determined under a variety of storage and process conditions. The freeze–thaw stability was evaluated by analyzing QC samples at three concentrations after undergoing

three freeze (−70 °C)–thaw (4 °C) cycles. The bench-top storage stability was assessed by placing QC samples at three concentrations at 4 °C (in an ice-bath) for a fixed period of time before being extracted and analyzed. The autosampler storage stability was determined by storing the reconstituted QC samples at three concentrations for 24 h under autosampler condition (maintained at 4 °C) before being analyzed. The −20 and −70 °C freezer storage stability of the analytes was determined by extracting and analyzing QC samples at three concentrations after being stored at −20 or −70 °C for fixed periods of time.

RESULTS AND DISCUSSION

Under turbo ionspray ionization condition, SV and SVA exhibit favorable sensitivity in positive and negative ion detection modes, respectively.[9–11] Figure 2 shows the

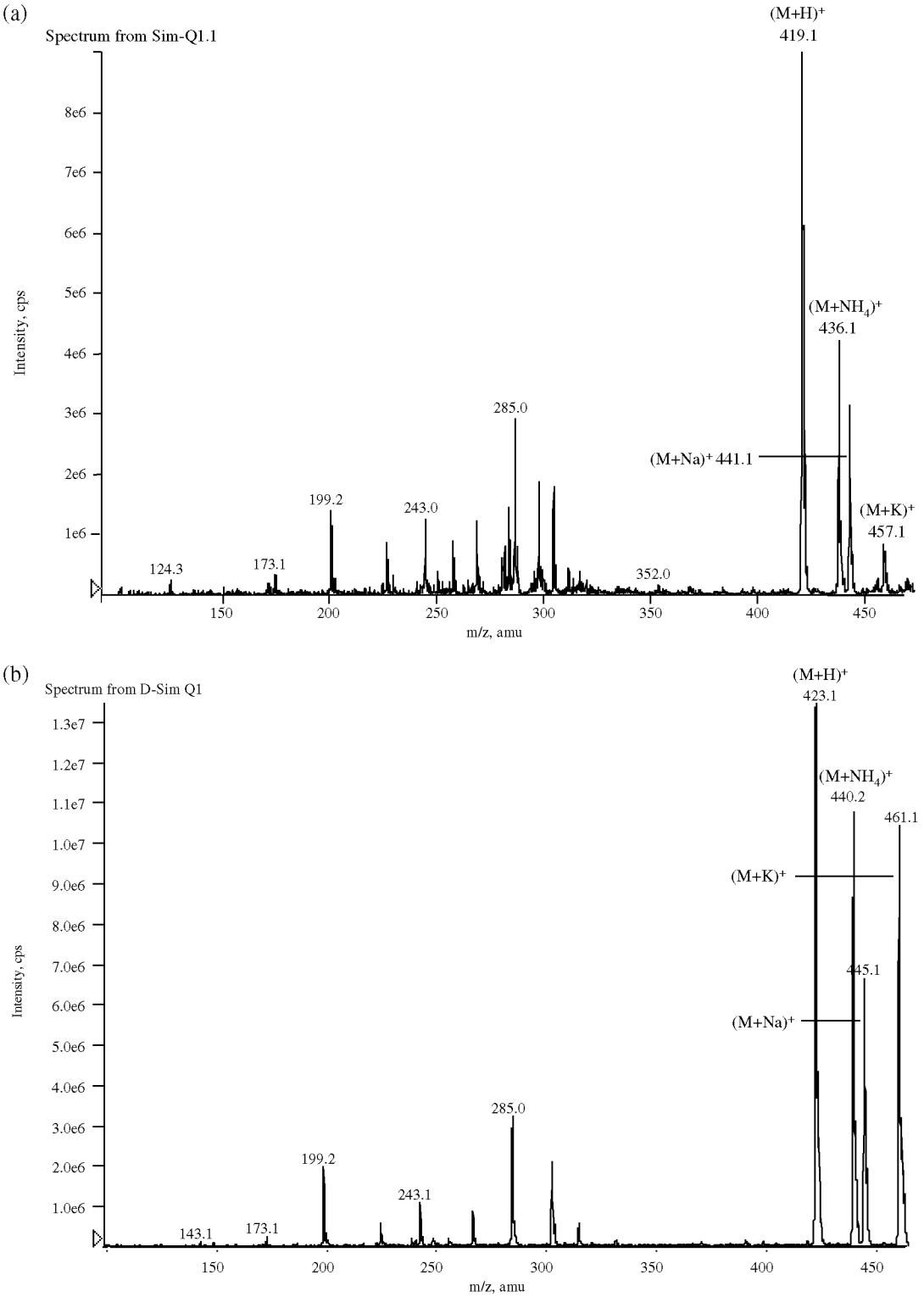


Figure 2. Full-scan Q1 spectra of (A) SV and (B) $^{13}CD_3$-SV.

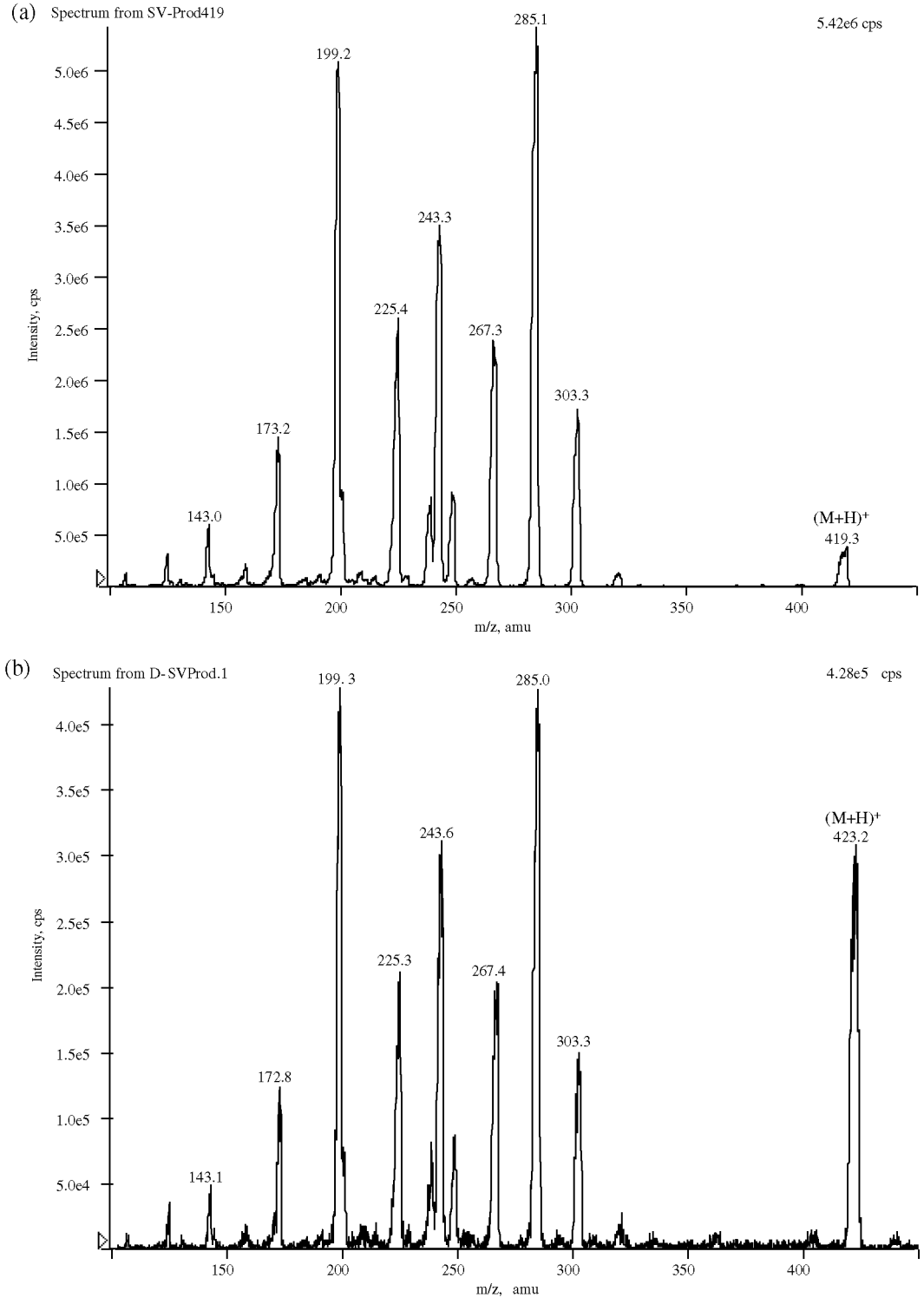


Figure 3. Product ion scan spectra of (A) SV and (B) $^{13}CD_3$-SV.

full-scan Q1 positive ion spectra of SV and $^{13}CD_3$-SV. Both formed protonated molecules $[M + H]^+$ as major ion peaks. Adduct ions of SV and $^{13}CD_3$-SV with sodium, potassium or ammonium were also observed. The ammonium content was from the ammonium acetate buffer used in the mobile phase. The sodium and potassium are believed to be originated from the biological matrix or from the glass containers used during sample preparation and analysis. The sodium-adducted SV showed higher sensitivity than the protonated SV when both were obtained under optimized conditions, since the former forms single major parent and product ions whereas the latter forms several major parent and product ions. Primarily for this reason, the sodium adduct ion of SV was used as the precursor ion in earlier LC/MS/MS methods.[9,10] The sodium content, however, was found not to be totally controllable during sample preparation and analysis, which caused variations in the sodium adduct ion abundance.[10] To avoid the variations caused by the sodium content, protonated SV was then used as the precursor ion

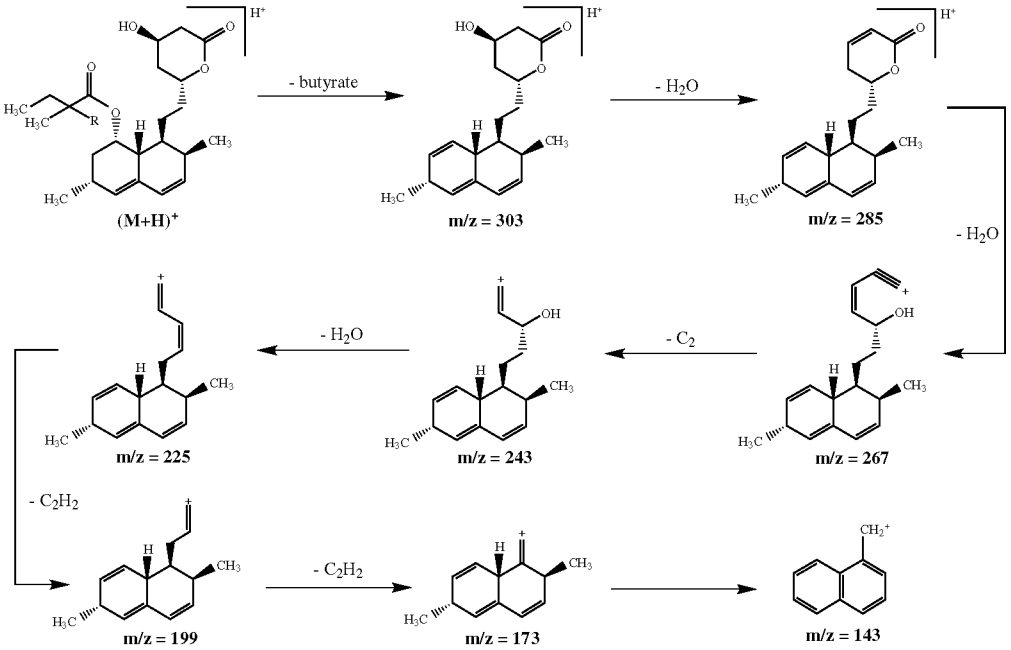


Figure 4. Proposed fragmentation pathway of SV and $^{13}CD_3$-SV.

in the SRM detection. The ammonium adduct ion of SV, which forms the same fragment ions as for protonated SV plus a large $[M + H]^+$ ion peak, was not chosen as the precursor ion. The factors affecting the relative abundance of the sodium-adducted SV and protonated SV include not only general experimental conditions such as mobile phase buffer content and pH, solvents and mass spectrometer parameters, but also the materials of the sample and solution containers. Generally, to reduce the sodium content and favor the formation of $[M + H]^+$ ions, containers made of glass and solutions or solvents containing an alcohol such as methanol should be avoided throughout the sample preparation and analysis process.

The product ion scan spectra of $[M + H]^+$ for SV and $^{13}CD_3$-SV showed several major fragment ions at m/z 143, 173, 199, 225, 243, 267, 285 and 303 (Fig. 3). Figure 4 shows a proposed fragmentation pathway of SV and $^{13}CD_3$-SV. The fragment ion at m/z 303 was formed by losing the 2,2-dimethylbutyrate moiety of the molecule. The fragment ion at m/z 285, formed by losing a neutral water from the m/z 303 ion and present in the highest abundance, was selected as the product ion for both SV and $^{13}CD_3$-SV. Further experimental verification of the structures of the fragments and the fragmentation mechanism is beyond the scope of this paper.

Full-scan Q1 negative ion spectra of SVA and $^{13}CD_3$-SVA are shown in Fig. 5. In contract to the SV case, the molecular ion $[M - H]^-$ is the only dominant ion for SVA. The product ion spectra of $[M - H]^-$ for SVA and $^{13}CD_3$-SVA showed only two fragment ion peaks, located at m/z 319 and 115 (Fig. 6), respectively. The fragment ion at m/z 319, formed by losing butyrate from the molecular ion, was chosen as the product ion for both SVA and $^{13}CD_3$-SVA. The fragments found with SV were basically not observed here owing to the stability of the open-ring acid form.

The pH of the ammonium acetate buffer in the mobile phase was optimized at 4.5. The retention time was ~1.3 min for SVA in period 1 of the two-period acquisition and ~2.7 min for SV in period 2. Representative SRM ion chromatograms of SV and SVA in human plasma double blank and in plasma samples spiked with 5 ng ml^{-1} of SV and SVA are displayed in Fig. 7. The assay specificity was checked for any interference from endogenous species in the plasma. This was done via analysis of plasma double blank and pre-dose plasma samples. No interfering peaks were found at the retention times of the analytes. The use of stable isotope-labeled SV and SVA seemed to eliminate the matrix or ion suppression effects, as was examined using a procedure in which the peak area ratios of SV and SVA were compared in standard and QC samples prepared in five different sources of human control plasma.[13]

Interference between the internal standards and the analytes and between the acids and lactones due to the isotopic effect, 'cross-talk' effect and/or impurities in standard compounds were examined by injecting a neat solution containing only one of the four compounds (SV, SVA, $^{13}CD_3$-SV or $^{13}CD_3$-SVA) at the upper limit of quantitation (ULQ) concentration (for analytes) or working concentration (for internal standards) and monitoring the other three compounds on the extracted ion chromatograms (chromatograms not shown). No 'cross-talk' effect was found. No isotopic effect on the analytes from the internal standards was observed. The isotopic effect on the internal standards from the M + 4 ions of SV and SVA was <0.1%. The interference between the acids and lactones was <0.01%.

Interconversion between SV and SVA during sample preparation

During plasma sample preparation, the analytes can easily undergo lactonization (SVA → SV) and/or hydrolysis (SV → SVA) under conditions with high or low pH combined with an organic extraction environment. Such interconversions make the quantitation less accurate.

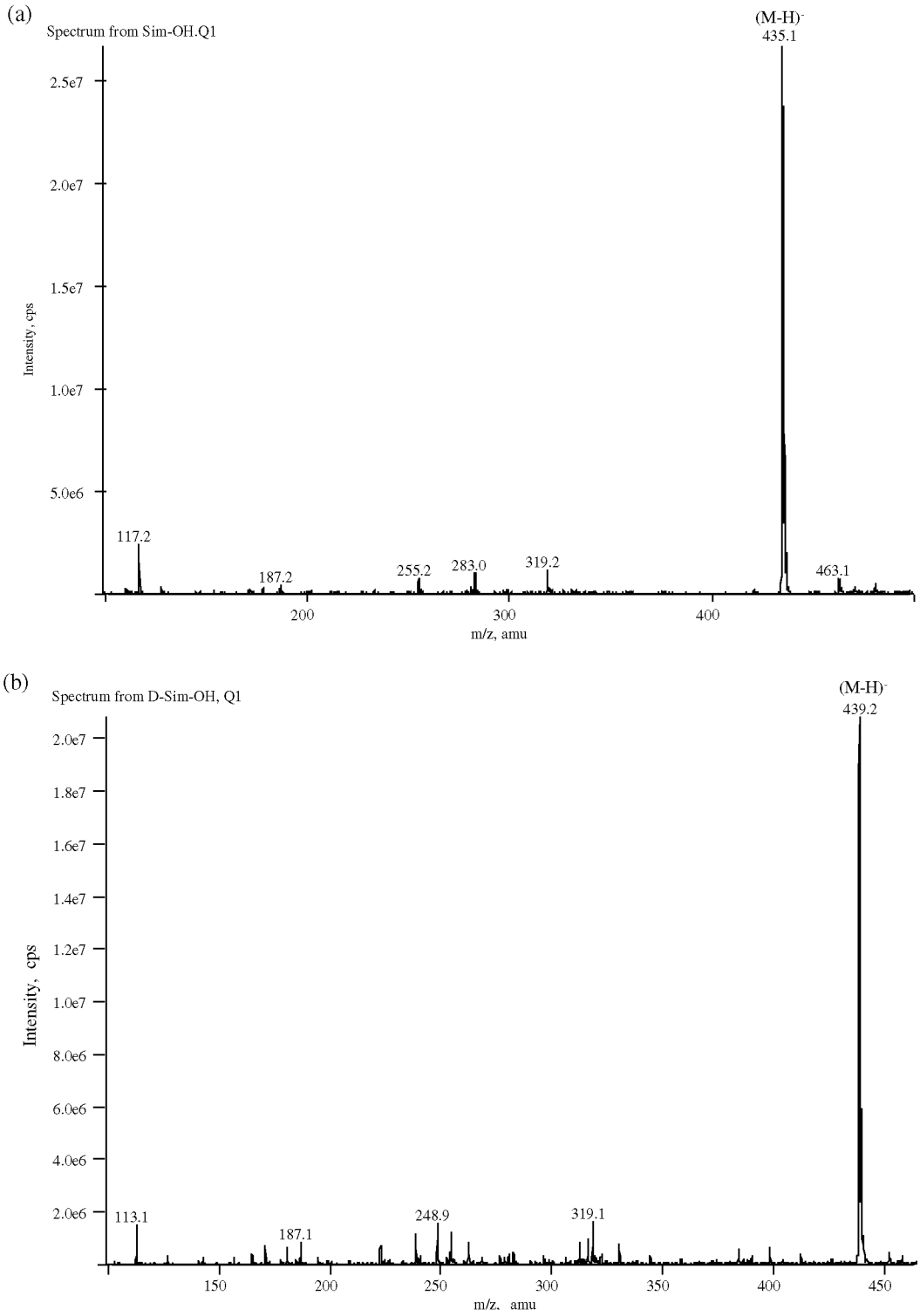


Figure 5. Full-scan Q1 spectra of (A) SVA and (B) $^{13}CD_3$-SVA.

Previous methods with SPE or LLE procedures all showed some degree of interconversion. With the SPE procedure, the interconversion was as high as 3% owing to the use of 5% formic acid during extraction. The interconversion was reduced to <0.5% with the LLE procedure. These extents of interconversion would produce little effect in cases when the plasma concentrations of SV and SVA are similar or comparable. In extreme cases, when the plasma concentration of one analyte is approaching the ULQ while the other is near the LOQ, the effect on the lower concentration analyte would be significant. For example, 0.5% lactonization of SVA at a concentration of 50 ng ml^{-1} (ULQ concentration) would produce 0.25 ng ml^{-1} of SV, which is five times higher than the LOQ concentration (0.05 ng ml^{-1}) of SV.

Further reduction or elimination of the interconversion, as a necessary step towards a more accurate and reliable method, was achieved by performing the extraction on Varian Chem Elut cartridges. These hydrophilic matrix (trade marked as Hydromatrix)-filled cartridges

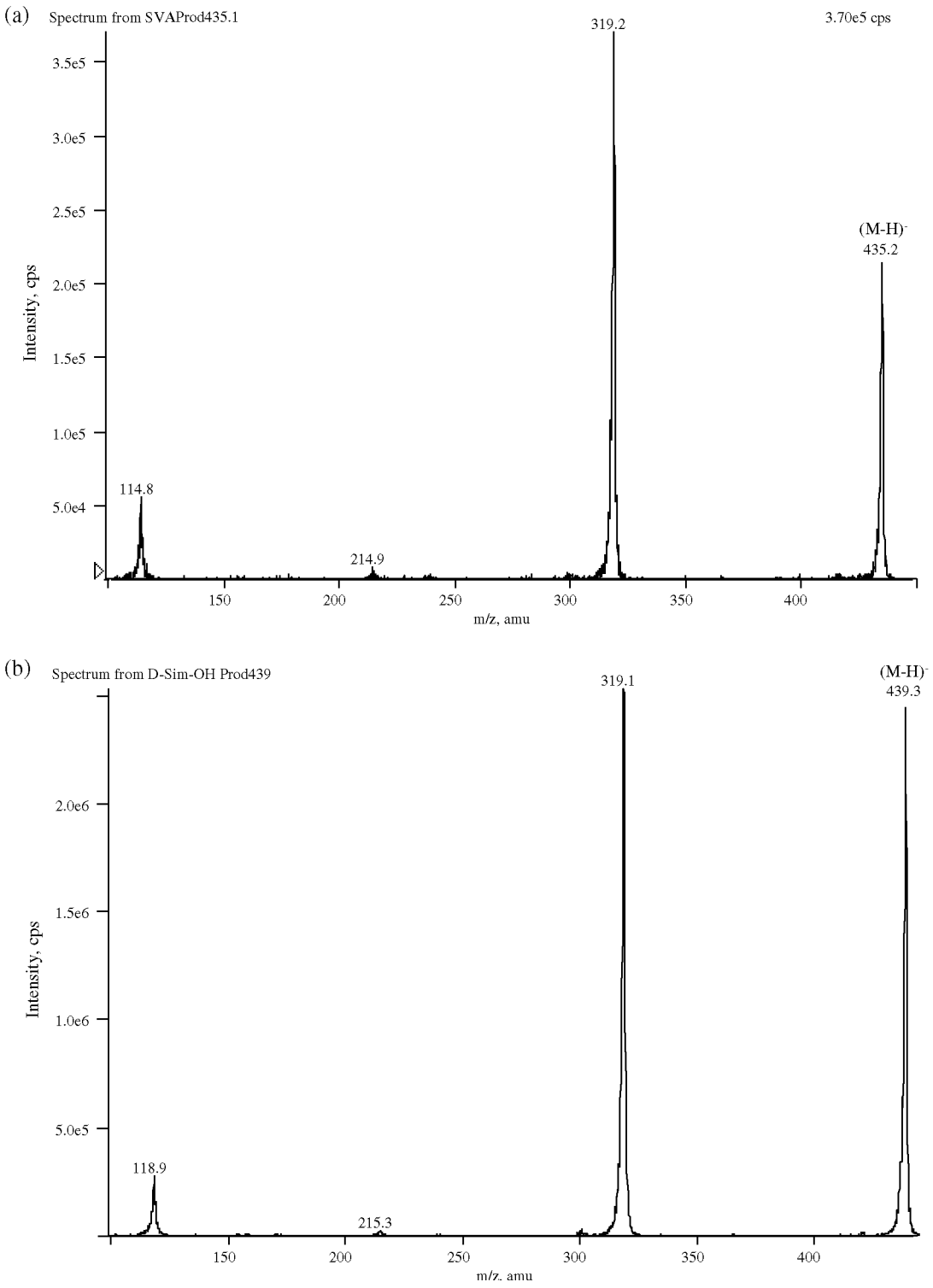


Figure 6. Product ion scan spectra of (A) SVA and (B) $^{13}CD_3$-SVA.

contain a special grade of flux-calcined, high-purity, inert diatomaceous earth. During extraction, the aqueous matrix was adsorbed on the packing material while the organic solvent passed through cartridge and eluted the compounds of interest. A phase-separation filtering material is incorporated into the cartridge as a safeguard to ensure that organic eluents remain uncontaminated by the aqueous matrix. With the Chem Elut 6cartridge extraction, lactonization was not observed and hydrolysis was reduced to a negligible level (<0.07%). The percentage hydrolysis of SV was measured by comparing the peak area ratio of SVA to $^{13}CD_3$-SVA obtained from SV-only samples with those from SVA-only samples (SV and SVA were spiked into plasma at the same concentration). A similar procedure was used for the measurement of lactonization of SVA. The interconversions obtained from the three different extraction procedures employed are summarized in Table 1.

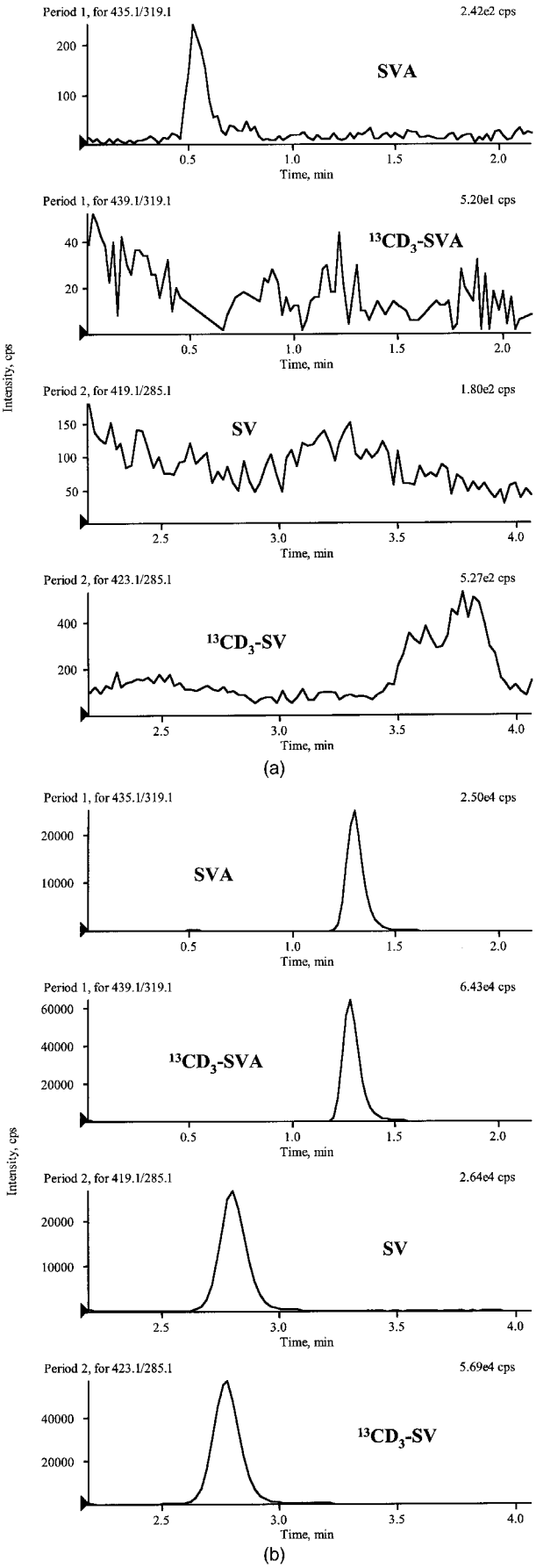


Figure 7. Representative SRM chromatograms of (A) human plasma double blank and (B) plasma sample spiked with SV and SVA at 5 ng ml^{-1} each.

Table 1. Extraction recovery of SV and SVA and interconversions between SV and SVA during sample extraction with different extraction procedures

Extraction procedure	Extraction recovery (%)[a] SV	SVA	Interconversion (%)[b] SV → SVA	SVA → SV
Solid-phase (previous work[10])	75.4	68.3	0.60	3.00
Liquid–liquid (previous work[11])	80.6	95.6	0.12	0.49
Chem Elut cartridge (this work)	78.1	87.2	0.06	0

[a] Mean extraction recovery was obtained by averaging recovery values measured at high, medium and low QC concentrations, with five replicates at each concentration level.
[b] With two sets of plasma samples, one set spiked with SV only and another with SVA only, both at the same concentration (high QC), the interconversion (%) was calculated as follows (taking SV → SVA as example): % = (peak area ratio SVA/$^{13}CD_3$-SVA without SVA spiked)/(peak area ratio SVA/$^{13}CD_3$-SVA with SVA spiked) × 100%.

Analyte stability

The stability of the analytes under various process and storage conditions has been evaluated mostly in previous work[10,11] using SV-only, and SVA-only, and the combination samples or solutions. Since the pH of human plasma with heparin added as an anticoagulant is commonly ~8, SV is easily hydrolyzed to SVA when plasma samples are thawed or stored under room temperature. For this reason, unless pH control was used, sample preparation was performed at 4 °C in an ice-bath; at this temperature SV and SVA were shown to be stable in plasma for at least 3 h. Both SV and SVA were stable after undergoing three freeze (−70 °C)–thaw (4 °C) cycles. The analytes were also found to be stable under autosampler storage conditions for at least 24 h at 4 °C and in a reconstitution solution of 70:30 acetonitrile–ammonium acetate (1 mM, pH 4.5). The pH of reconstitution solution did not have any effect on analyte stability between pH 4.0 and 5.0. SV was found to be degraded (hydrolyzed) after storage for 1 month at −20 °C in plasma, whereas no sign of degradation was found for SVA during the same storage period. Both analytes were stable in plasma at −70 °C for at least 6 months.

The storage stability of SV and SVA in stock and working solutions was evaluated by measuring the lactonization of SVA in SVA-only solutions and hydrolysis of SV in SV-only solutions. The SV-only and SVA-only working solutions were made by dilution of SV or SVA stock solutions with 60:40 acetonitrile–water to a concentration of 200 ng ml^{-1}, both with internal standards added. Table 2 shows the hydrolysis and lactonization of SV and SVA in working solution stored at 4 and −20 °C for 4 weeks. Both SV and SVA showed <1% interconversion at 4 °C after 4 weeks, with the percentage of SV hydrolysis much higher than that of SVA lactonization. At −20 °C, the interconversion was <0.05% after 4 weeks. For long-term storage of standard and QC working solutions, storage at −20 °C is much safer.

Extraction recovery

The extraction conditions were evaluated to achieve maximum extraction efficiency for both SV and SVA and,

Table 2. Percentage[a] of SV and SVA generated from lactonization or hydrolysis during storage of SVA and SV working solutions (in 60:40 acetonitrile–water): measurement of stability of SV and SVA in working solutions under refrigerator (4 °C) and freezer (−20 °C) storage conditions

Storage temperature (°C)	Length of storage 0	1 week	2 weeks	3 weeks	4 weeks
Measuring SV in SVA-only solutions[b] —					
4	0	0	0.073	0.078	0.105
−20	0	0	0	0	0
Measuring SVA in SV-only solutions[b] —					
4	0	0.268	0.457	0.627	0.892
−20	0	0.015	0.013	0.023	0.046

[a] Percentage was calculated as (taking measuring 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%.
[b] $n = 4$. Measured with QC working solutions at 200 ng ml^{-1}.

at the same time, minimum interconversion between SV and SVA. The optimum pH for the extraction buffer was between 4 and 5. Any pH >5.0 would produce a lower extraction efficiency for SVA since more SVA would be present in the ionized form, which is less extractable by organic solvents. A pH <4.0 showed increased interconversion between SV and SVA. A pH of 4.5 was finally chosen after evaluation of three pH values between 4 and 5. The extraction buffer concentration was also evaluated using 10, 20, 50 and 100 mM ammonium acetate. No significant differences in extraction recovery and interconversion were observed among these concentrations. MTBE, among all the extraction solvents tested, showed the best extraction recovery, which was measured to be 78.1% for SV and 87.2% for SVA averaged over three QC levels ($n = 5$ for each level). The extraction recoveries measured from the SPE, LLE and Chem Elut extraction procedures are given in Table 1. Although the Chem Elut cartridge extraction showed slightly lower extraction recoveries than those obtained from the LLE procedure, the interconversion situation was certainly an advantage for the former.

Table 3. Intra-assay precision and accuracy of measurement of SV and SVA in human plasma

Analyte	Nominal concentration (ng ml^{-1})	Mean calculated concentration[a] (ng ml^{-1})	Accuracy[b] (%)	Precision[c] (%)
SV	0.05	0.052	104.6	4.3
	0.1	0.101	101.0	1.8
	0.5	0.488	97.7	3.3
	1	0.978	97.8	2.7
	5	4.934	98.7	0.8
	25	25.00	100.0	1.9
	50	50.10	100.2	1.5
SVA	0.05	0.053	106.6	2.7
	0.1	0.103	102.6	2.2
	0.5	0.484	96.9	1.2
	1	0.962	96.2	1.7
	5	4.883	97.7	1.7
	25	24.89	99.6	1.1
	50	50.27	100.5	0.9

[a] From the linear least-squares regression of the standard line using all points ($n = 5$) at all concentrations.
[b] Calculated as (mean calculated concentration)/(nominal concentration) × 100%.
[c] Expressed as RSD.

Assay precision and accuracy

This method showed very good precision and accuracy. The intra-assay precision, expressed as RSD, was measured to be from 0.8 to 4.3% for SV and from 0.9 to 2.7% for SVA, for all seven standard concentrations

Table 4. Inter-assay precision and accuracy of measurement of SV and SVA in human plasma

Analyte	Nominal concentration (ng ml^{-1})	Mean calculated concentration[a] (ng ml^{-1})	Accuracy[b] (%)	Precision[c] (%)
SV	0.8	0.756	94.6	0.9
	20	19.60	98.0	0.2
	40	39.63	99.1	1.7
SVA	0.8	0.749	93.6	2.3
	20	19.33	96.6	0.5
	40	39.48	98.7	1.7

[a] $n = 3$ days (five replicates per day).
[b] Calculated as (mean calculated concentration)/(nominal concentration) × 100%.
[c] Expressed as RSD.

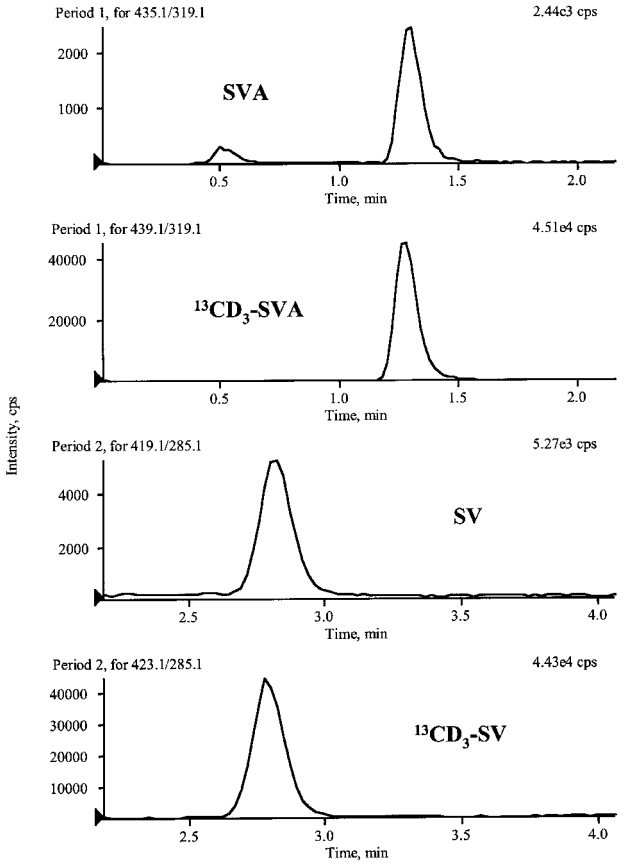


Figure 8. SRM chromatograms of a plasma sample collected from a subject 2 h after a single oral dose of 20 mg of SV.

($n = 5$). The intra-assay accuracy, expressed as a percentage of nominal values, was measured to be in the range 97.7–104.6% for SV and 96.2–106.6% for SVA, for all seven standard concentrations (Table 3). The initial inter-assay precision (RSD) ranged from 0.2 to 1.7% for SV and from 0.5 to 2.3% for SVA, for all three QC concentrations ($n = 3$ assays, five replicates per assay). The initial inter-assay accuracy ranged from 94.6 to 99.1% for SV and from 93.6 to 98.7% for SVA, for all three QC concentrations (Table 4). At the LOQ concentration (50 pg ml^{-1}), a precision (RSD) of 4.3% and an accuracy of 104.6% were obtained for SV and RSD 2.7% and accuracy 106.6% for SVA (Table 3).

Linearity

Good linearity was always obtained with this method. The linear regression parameters for a typical calibration curve (fitted by first-order polynomial model $y = ax + b$, where $a =$ slope and $b =$ intercept) are $a = 0.042$ and $b = 0.00002$. Typical values for the correlation coefficient (r^2) were between 0.9998 and 1.0000.

Applications

The method was successfully applied to the determination of SV and SVA concentrations in human plasma samples. Figure 8 shows the SRM chromatograms of a clinical sample collected from a healthy subject 2 h after an oral administration of 20 mg of SV.

CONCLUSION

A turbo ionspray LC/MS/MS method was developed and validated for the simultaneous determination of SV and SVA in human plasma. The method has an LOQ of 50 pg ml^{-1} for both analytes using a 0.5 ml plasma sample size and is very sensitive, selective, and reliable. The sample treatment and extraction conditions were chosen so that the interconversions between SV and SVA were reduced to an undetectable or negligible (<0.07%) level. The Chem Elut cartridge extraction was shown to be a procedure that is simple, efficient and easy to automate. The method was used successfully to determine plasma drug concentrations in human plasma samples.

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