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Short communication

Application of a novel ultra-low elution volume 96-well solid-phase extraction method to the LC/MS/MS determination of simvastatin and simvastatin acid in human plasma

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Abstract

A novel extraction method has been utilized in the LC/MS/MS determination of simvastatin and simvastatin acid in human plasma. In this method, 300 μl of plasma sample was loaded onto a Waters Oasis 96-well HLB μElution plate, the stationary phase was washed using 2 × 400 μl of 5% methanol in water, and the analytes were eluted using 35 μl of 95/5 acetonitrile/H_2O twice. The sample extracts were diluted with 40 μl of methyl ammonium acetate (1 mM, pH 4.5). Chromatography was performed on a Phenomenex Synergi Max-RP column (2.0 mm × 50 mm, 4 μm). A PE Sciex API 3000 tandem mass spectrometer interfaced with a turbo ionspray source was used for mass detection. Compared to solid-phase extraction, liquid–liquid extraction and solid-supported liquid–liquid extraction methods that were developed and previously used in our laboratory, this method reduced the labor cost and was less time consuming in sample preparation, due to the fact that post-extraction solvent evaporation and reconstitution steps were avoided using this μElution solid-phase extraction plate. The method has been proved to be fast, reliable and reproducible.

Keywords: Simvastatin; Simvastatin acid; SPE; μElution plate; LC/MS/MS; Human plasma

1. Introduction

In recent years, LC/MS/MS has been widely used for the quantitative determination of drugs and their metabolites in biological matrices. With the large amount of samples generated from clinical studies, sample preparation has become a major bottleneck during high-throughput analysis. The recent introduction of the Waters Oasis μElution solid-phase extraction (SPE) plate provides a step forward for sample preparation technology. This technology allows for the rapid isolation of analytes from complex matrices using an ultra-low elution volume thus eliminating the need for post-extraction solvent evaporation and reconstitution steps which can be costly in terms of time.

Simvastatin (Fig. 1) is a highly effective agent for the treatment of hypercholesterolemia [1]. Following oral administration, simvastatin, an inactive lactone, is hydrolyzed in vivo rapidly to its corresponding β-hydroxy acid, simvastatin acid (SVA) (Fig. 1). The latter is a potent inhibitor of HMG-CoA reductase. LC/MS/MS methods for the quantitative determination of SV and SVA in human plasma have been previously reported using various extraction procedures [2–5], including solid-phase extraction [2], liquid–liquid extraction [3] and solid-supported liquid–liquid extraction [4] used in our laboratory and an on-line extraction [5] by Jemel et al. The on-line extraction provided a simple and labor-saving sample clean-up, however, its relatively high lower limit of quantitation (LLOQ) of 0.5 ng ml^{-1} and higher interconversion between SV and SVA (≤1.0%) do not meet our requirement. Among the off-line extraction methods, the solid-supported liquid–liquid extraction on Chem Elut cartridges has been extensively used: it was sensitive (LLOQ was 0.05 ng ml^{-1}), reproducible, and showed excellent extraction efficiency with no or negligi-

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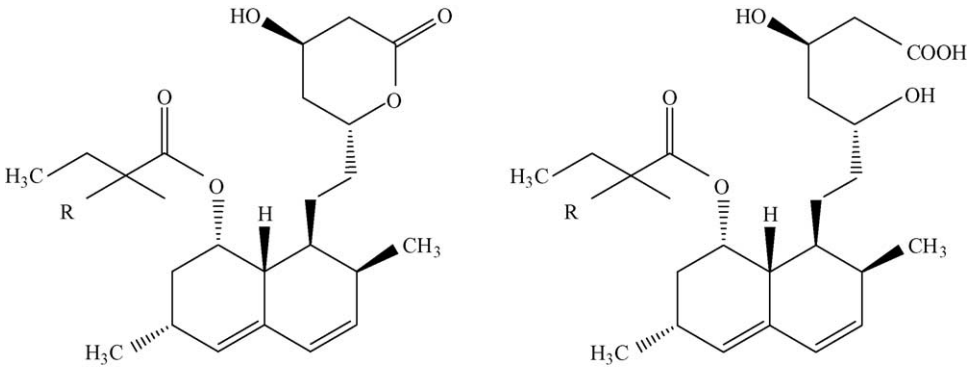

Simvastatin (SV): R = CH_3, MW = 418
$^{13}CD_3$-SV: R = $^{13}CD_3$, MW = 422

Simvastatin Acid (SVA): R = CH_3, MW = 436
$^{13}CD_3$-SVA: R = $^{13}CD_3$, MW = 440

Fig. 1. Structures of analytes and internal standards.

ble interconversion between SV and SVA. However, the automated version of the solid-supported liquid–liquid extraction [6,7] was not very straightforward. The solvent evaporation step was very tedious due to the fact that a large volume of elution solvent (methyl *t*-butyl ether, MTBE) is required to achieve optimum recovery while the capacity (loading volume) of 96-well plate is limited. This resulted in the necessity of multiple elution and evaporation steps.

In this work, we report the development and validation of a new method using the Oasis HLB μElution SPE for the quantitative LC/MS/MS determination of SV and SVA in human plasma, which requires no solvent evaporation and reconstitution and greatly reduces sample preparation time and improves assay efficiency.

2. Experimental

2.1. Materials, 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. The isotopic purities for both internal standards were >98.5%.

The μElution plates packed with Oasis HLB SPE sorbent were obtained from Waters (Milford, MA, USA). Ammonium acetate (HPLC grade), methylamine (40% solution in water), acetonitrile and methanol were purchased from Fisher Scientific (Fairlawn, NJ, USA). Pooled human control plasma (heparinized) was purchased from Biological Specialty (Lansdale, PA, USA). De-ionized water was prepared using a Milli-Q Plus Ultra-Pure water system (Millipore, Bedford, MA, USA).

2.2. 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 of SVA and $^{13}CD_3$-SVA in acetonitrile–water (60:40, v/v), to yield for each compound a concentration of 1.0 mg ml^{-1}. All concentrations were calculated based on the free acid or neutral form. Diluted solutions at 10 μg ml^{-1} for each compound were prepared by mixing 200 μl of the appropriate stock solutions with 1.8 ml of acetonitrile–water (60/40, v/v). 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).

Standard working solutions of SV and SVA at 250, 50, 10, 5, 1 and 0.5 ng ml^{-1} were prepared by serial dilutions 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 50 ng ml^{-1} was prepared by mixing 100 μl of 10 μg ml^{-1} diluted stock solution of each with 19.8 ml of acetonitrile–water (60/40, v/v).

QC working solutions for both SV and SVA at concentrations of 400, 200 and 1 ng ml^{-1} were prepared by 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 were prepared fresh daily by spiking 50 μl of the appropriate standard working solutions into 450 μl of human control plasma to yield calibration concentrations of 50, 25, 5, 1, 0.5, 0.1 and 0.05 ng ml^{-1}. Fifty microliters of acetonitrile–water solution (60/40, v/v) was added to plasma double blank and blank samples to make up the volume.

Plasma QC samples were prepared by adding 5 ml of the appropriate QC working solutions to 45 ml of human control plasma to yield QC concentrations of 40, 20 and 0.1 ng ml^{-1}. After preparation, the bulk QC plasma samples

were aliquoted in 3.6 ml nunc tubes and stored at −70 °C until needed.

2.3. Plasma sample extraction

Sample and reagent transfer were performed on an 8-probe Packard Multiprobe-II HT EX liquid handling system and a Tomtec Quadra 96 workstation. For analyte stability concern, 100 μl of 100 mM ammonium acetate at pH 4.5 was first added to each well of the 2.2 ml 96-well plate using the Tomtec workstation. A 300 μl aliquot of plasma sample was transferred to each well, followed by the addition of 30 μl of internal standard to each well (except for the double blank). The 2.2 ml 96-well plate was sealed with a cap mat, vortexed briefly and then centrifuged at 3000 rpm, 4 °C, for 5 min. The contents were then transferred to an Oasis HLB μElution 96-well SPE plate conditioned with 200 μl of methanol and water consequently on the Tomtec Quardra-96 system. The loaded plate was then washed with 400 μl of 5% methanol in water twice to remove matrix interferences. Finally samples were eluted into a 1.2 ml 96-well plate with 35 μl of acetonitrile–water (95/5, v/v) 2X. The eluent was simply diluted with 40 μl of methyl ammonium acetate buffer (1 mM, pH 4.5) and the samples were ready for LC/MS/MS analysis.

2.4. Chromatographic conditions

The liquid chromatograph system consisted of a Perkin-Elmer serial 200 pump and a Leap CTC autosampler. The analytes were separated through a Phenomenex Synergi Max-RP column (50 mm × 2.0 mm, 4 μm) at a flow rate of 200 μl min^{-1} under ambient condition. Mobile phase was optimized at 80/20 acetonitrile/methyl ammonium acetate (1 mM, pH 4.5). The retention times were about 1.1 min for SVA and 2.0 min for SV. Representative chromatograms of double blank plasma, blank plasma and a real plasma sample are shown in Fig. 2a–c.

2.5. MS/MS conditions

A PE Sciex API 3000 triple quadrupole mass spectrometer interfaced with the liquid chromatograph via a turbo ionspray source was used for mass analysis and detection. The turbo ionspray temperature was at 350 °C. Quantitation was performed using selected reaction monitoring (SRM)

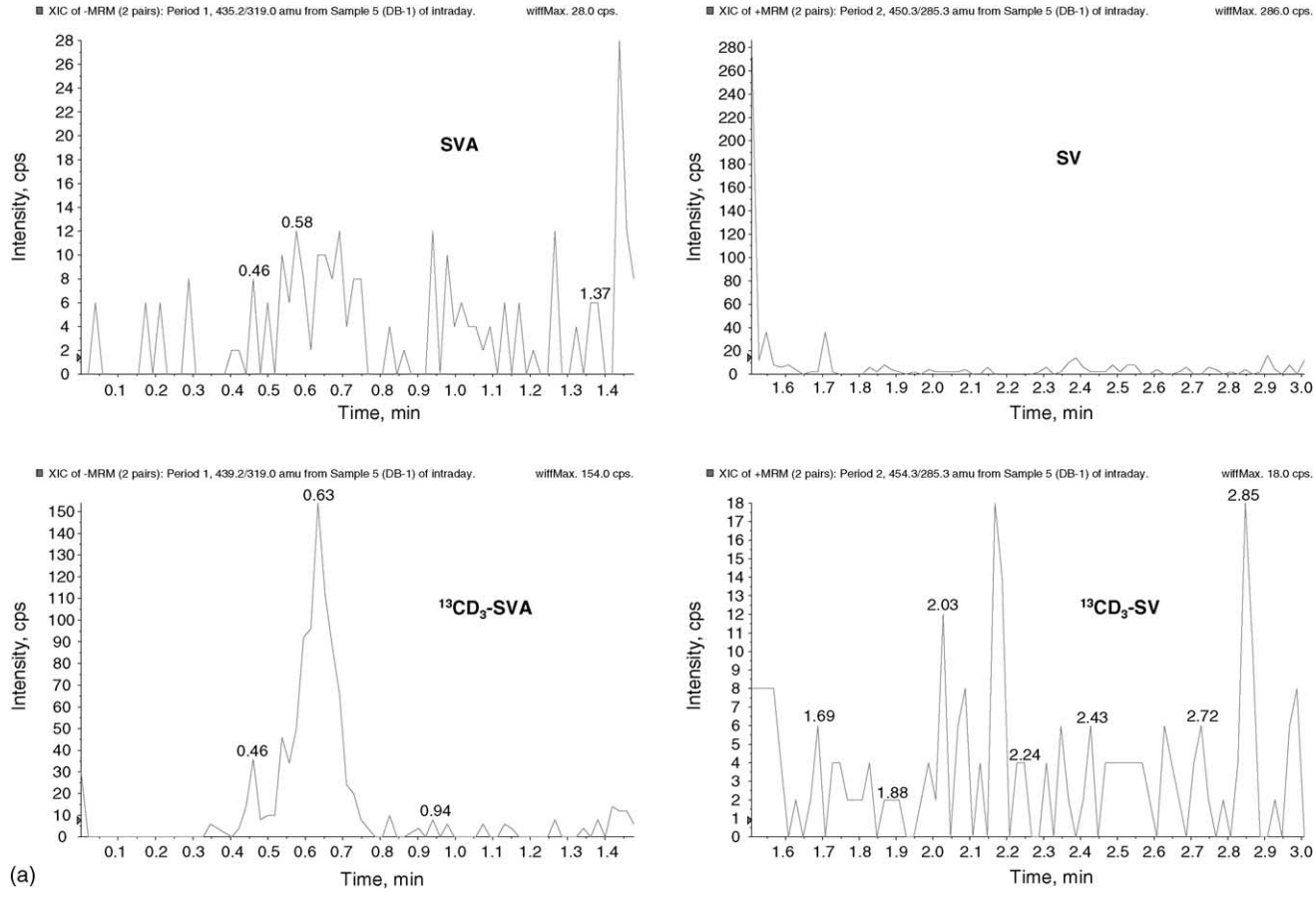

Fig. 2. (a) The extracted ion current chromatograms of a double blank in human plasma matrix. (b) The extracted ion current chromatograms of a human pre-dose plasma sample. (c) The extracted ion current chromatograms of a human plasma sample at 2 h post-dose with 80 mg SV (concentrations of SV and SVA are 0.993 and 4.785 ng ml^{-1}, respectively).

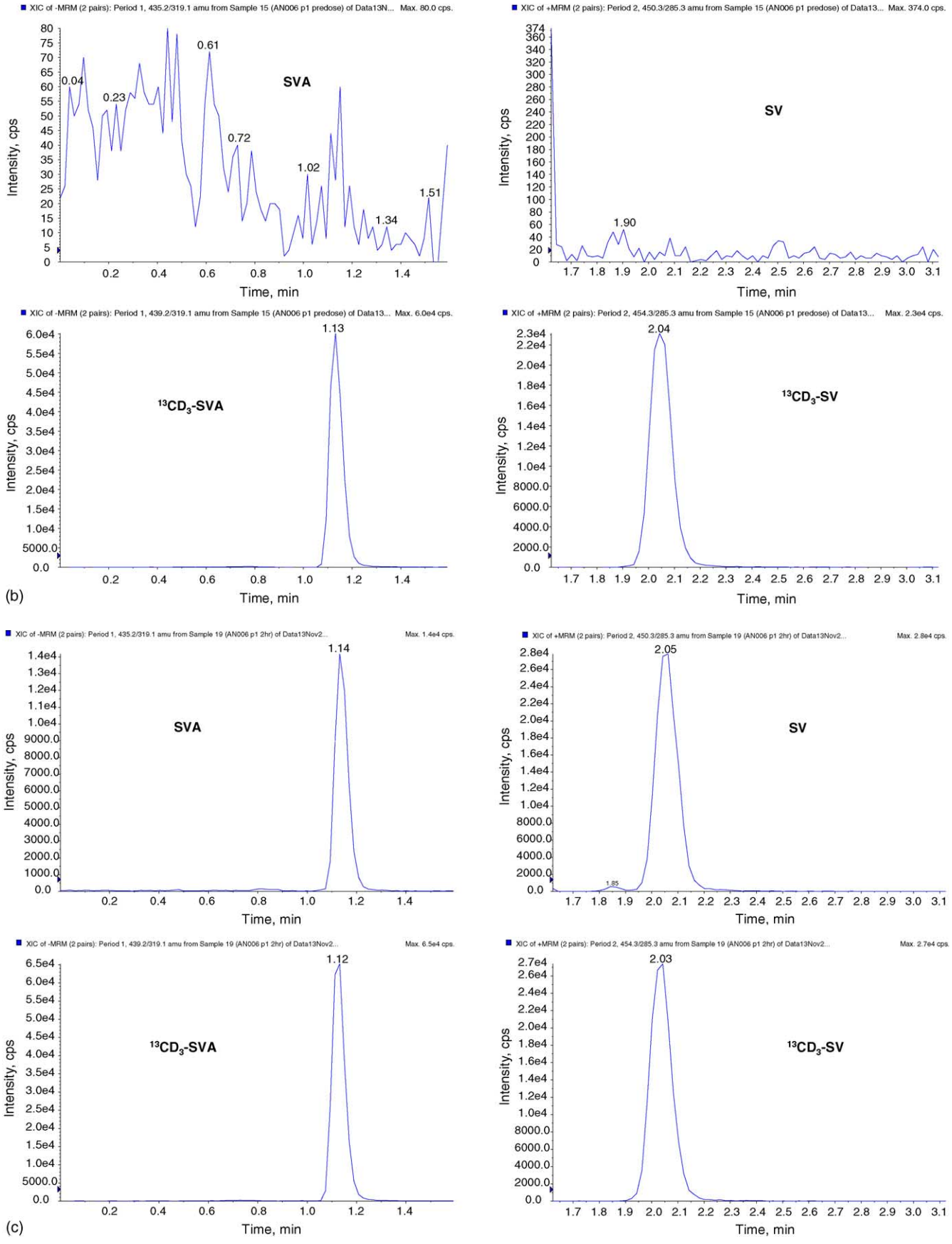

Fig. 2. (*Continued*).

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* 454.3 $[M+CH_3NH_3]^+ \rightarrow$ 285.1 (for $^{13}CD_3$-SV) and *m/z* 450.3 $[M+CH_3NH_3]^+ \rightarrow$ 285.1 (for SV). The mass spectrometer was operated in the negative ion mode for about first 2 min with a dwell time of 500 ms, and then in the positive mode for the rest of the analytical run with a dwell time of 500 ms. Methyl ammonium acetate buffer was used in the mobile phase to improve the ionization efficiency and sensitivity for SV [10].

3. Results and discussion

3.1. Optimization of sample preparation conditions

Sample transfer, internal standard and buffer additions were performed on the Packard Multiprobe II liquid handling system and the Tomtec Qudra-96 station. Sample mixture was then loaded onto the Waters Oasis HLB μElution plate for extraction. The novel 96-well HLB μElution SPE plate consists of a 2 mg high-capacity SPE sorbent and a focusing tip which allows loading of up to 750 μl of plasma and the use of ultra-low elution volume [8,11]. The sorbent incorporates both a lipophilic divinylbenzene and a hydrophilic *N*-vinylpyrrolidone to give maximum retention ability for different polarity compounds from biological matrix and also excellent wetting properties. The unique design of the focusing tip provides the most efficient use of the sorbent particles, and gives the ability to efficiently elute target compounds using as little as 25 μl of elution solvent. Due to the great concentrating ability of the plate, the evaporation and reconstitution steps are not necessary.

Plasma samples (300 μl aliquot plus internal standard) were treated with 100 mM ammonium acetate at pH 4.5. Experiments were performed to evaluate the impact of the different pHs on the interconversions between SV and SVA. Fig. 3 clearly demonstrates that a higher pH facilitated the conversion from SV to SVA, while a lower pH induced the conversion from SVA to SV and from SV to SVA as well. The buffer pH chosen was 4.5 because at this pH minimum conversion (<0.08%) for both SV and SVA was observed.

After the sample mixture was loaded on a 96-well μElution SPE plate, it was washed with 400 μl 5% MeOH in water twice. In order to optimize washing condition to achieve the best clean-up results without loss of analytes, washing solvents with different percentage (5%, 10%, 20% and 30%) of methanol in water were evaluated. The results indicated that a wash solvent with 5% of methanol achieved the minimum loss of analytes. In order to clean up the matrix extract more thoroughly, 2 × 400 μl wash solvent volume was used on the SPE.

The composition of the elution solution was also investigated with different combinations of acetonitrile/H_2O and acetonitrile/methanol. An elution solution of 95/5 acetonitrile/H_2O was found to give the optimal extraction recovery for both SV and SVA. Although the addition of methanol in the elution solution improved the recovery of SVA, it was found not good for the chromatography of SVA, bad peak shapes were observed. The eluted extracts were then diluted with 40 μl of methyl ammonium acetate buffer (1 mM, pH 4.5); the plate was capped and ready for injection. The application of μElution SPE plate eliminated the need of evaporation and reconstitution steps, which are typically required in other off-line extraction procedures, such as liquid–liquid extraction, solid-phase extraction and solid-supported liquid–liquid extraction.

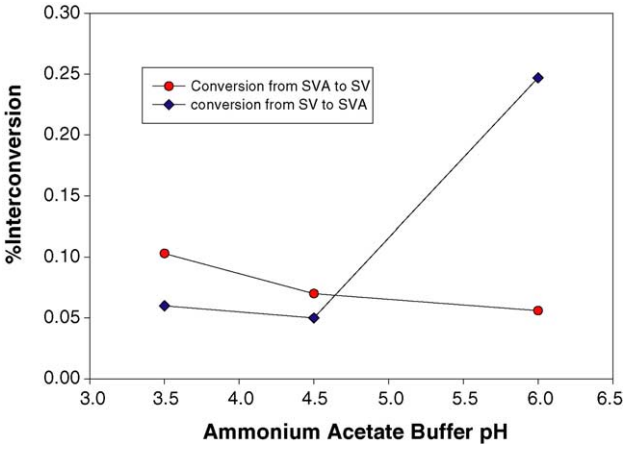

Fig. 3. Buffer pH impact on SV and SVA interconversion.

This μElution plate SPE provides an easy to operate (compared to the traditional solid-phase extraction [2]), and to automate (compared to the liquid–liquid cartridge extraction [6,7]) sample extraction tool for SV and SVA in plasma matrix. The interconversion between SV and SVA was negligible (<0.08%), which was an advantage to the liquid–liquid extraction [3] and the on-line extraction methods [5].

3.2. Assay validation

3.2.1. Intra-assay precision and accuracy

The intra-assay precision and accuracy were evaluated by analyzing within the same run five sets of standard samples using five different lots of plasma. The intra-assay precision ranged from 1.2% to 7.0%, and the accuracy, expressed as percentage of nominal value, ranged from 94.4% to 105.7% for both analytes (Table 1).

3.2.2. Inter-assay precision and accuracy

The inter-assay precision and accuracy of the method were assessed by analyzing the QC samples (five replicates of each level) through three analytical runs. The results showed that the inter-assay precision ranged from 0.4% to 4.8% RSD and the accuracy ranged from 91.2% to 99.0% of nominal values (Table 2).

Table 1
Intra-assay precision and accuracy 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	0.053	105.7	7.0
	0.1	0.094	94.4	5.4
	0.5	0.491	98.2	3.2
	1	0.990	99.0	3.1
	5	5.096	101.9	2.6
	25	25.400	101.6	1.6
	50	49.520	99.0	3.0
SVA	0.05	0.050	99.7	2.4
	0.1	0.099	98.7	2.1
	0.5	0.505	101.1	4.0
	1	0.994	99.4	2.3
	5	5.036	100.7	3.8
	25	25.220	100.9	1.4
	50	49.700	99.4	1.2

[a] $n = 5$.
[b] Accuracy (%) = [(mean calculated value)/(nominal value)] × 100.
[c] Calculated using peak area ratios.

Table 2
Inter-assay QC precision and accuracy of SV and SVA in human plasma

Analyte	Nominal concentration (ng ml^{-1})	Mean calculated concentration[a] (ng ml^{-1})	Accuracy[b] (%)	Precision (%)
SV	0.1	0.098	98.0	2.3
	20	18.500	92.5	0.5
	40	36.487	91.2	0.4
SVA	0.1	0.099	99.0	4.8
	20	18.887	94.4	1.3
	40	37.687	94.2	0.5

[a] $n = 5$.
[b] Accuracy (%) = [(mean calculated value)/(nominal value)] × 100.

3.2.3. Extraction recovery

The extraction recovery of the analyte was determined by comparing the mean peak area ratios of the pre-spiked with that of the post-spiked QC samples. The internal standard was spiked after the extraction in both cases. The extraction recoveries for SV and SVA were 66% and 73%, respectively. Although this μElution plate SPE showed slightly lower extraction recoveries than those obtained in other methods, the volume of the elution solvent used (70 μl) was only a very small fraction of those used in other methods (12 ml of MTBE used in solid-supported liquid–liquid extraction [4], 1.2 ml of MTBE used in liquid–liquid extraction [9]).

3.2.4. Specificity and matrix effect

Specificity of the method was assessed by analyzing human plasma double blanks from five individual subjects. Interference peaks were not observed at the retention times of either analytes or the internal standards.

The relative matrix effect, or the variation due to the use of different source of matrix was evaluated by analyzing five sets of standard samples using five different lots of plasma within the same run. The precision ranged from 1.2% to 7.0%, and the accuracy ranged from 94.4% to 105.7% for both analytes. This result indicated that no relative matrix effect was observed.

The absolute matrix effect, or matrix suppression or enhancement of ionization, of the analyte was determined by comparing the mean peak area of the post-spiked with that of the neat QC samples. There was about 16% ion suppression observed for SV and no ion suppression or enhancement was observed for SVA.

3.2.5. Analyte stabilities

Stability of the analytes under various process and storage conditions, such as undergoing three freeze (−70 °C)-thaw (4 °C) and in an ice-bath (4 °C) benchtop have been previously evaluated and reported [4]. Since those procedures do not change with the sample extraction steps, they were not repeated during this method evaluation. However, the 24-h autosampler (4 °C) storage stability of the analytes was assessed due to the different composition in the ready-to-inject samples. The results indicated that the analytes were stable under the tested autosampler storage conditions.

4. Conclusion

A novel μElution SPE technique has been successfully applied to LC/MS/MS determination of SV and SVA

in human plasma. In this method, SV and SVA were successfully extracted on an Oasis μElution SPE plate. The analytes and internal standards were separated on a Phenomenex Synergi Max-RP column and detected on a PE Sciex API 3000 tandem mass spectrometer. The method had an LLOQ of 0.05 ng ml^{-1} with a linear calibration range of 0.05–50 ng ml^{-1} using 0.3 ml of sample. The extraction efficiency was 66% for SV and 73% for SVA. Compared to the previously reported methods, this method had no evaporation and reconstitution steps which greatly improved assay efficiency, and showed to give the same excellent precision and accuracy. The use of the Oasis μElution SPE plate provides a fast, easy to automate, sensitive and reliable method for the analysis of SV and SVA in plasma samples.

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