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An improved and fully validated LC–MS/MS method for the simultaneous quantification of simvastatin and simvastatin acid in human plasma

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Abstract

A fully automated high-throughput liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous quantification of simvastatin (SV) and simvastatin acid (SVA) in human plasma. Plasma samples were treated by acetonitrile (ACN) addition for protein precipitation (PP) and subsequent two-step liquid–liquid extraction (LLE) in 96-deepwell plates, using methyl *t*-butyl ether (MTBE) as the organic solvent. ACN addition step was proven to enhance method sensitivity, as well as producing cleaner samples for injection. Lovastatin (LV) and lovastatin acid (LVA) were used as internal standards (IS) for SV and SVA quantification respectively. A relatively small plasma volume (300 μL) was employed and all procedure liquid transfer steps were performed automatically, by the use of robotic liquid handling workstations. Both electrospray (ESI) and atmospheric pressure chemical ionization (APCI) sources were applied and compared for LC–MS/MS sample analysis, with ESI proven to be more sensitive for the specific analytes. Polarity switch (from negative to positive ionization mode) was performed during the same analytical run, so as for the simultaneous SV and SVA determination to be possible. The method had a short sample preparation time, as well as a chromatographic run time of just 1.9 min, the shortest so far reported for SV determination. It was validated and fulfilled all preset criteria for sensitivity, specificity, linearity (0.100–40.0 ng/mL), inter- and intra-accuracy and precision for both molecules. The proposed method was applied to the rapid and reliable simultaneous determination of SV and SVA in a bioequivalence study, after *per os* administration of a SV tablet (80 mg).
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1. Introduction

Simvastatin is one of the major representatives of 3-hydroxy-3-methyl-glutaril coenzyme A (HMG-CoA) reductase (HMGR) inhibitors, also known as “statins”. Simvastatin is administered as a cholesterol-lowering agent in order to control the in vivo synthesis of cholesterol and treat hypercholesterolemia [1]. SV is known to be rapidly hydrolyzed *in vivo* to its corresponding β-hydroxy acid (SVA). Low concentrations of both SV and SVA are found in systemic circulation because of the high first-pass hepatic extraction [2,3]. Therefore, highly sensitive analytical methods are needed in order to determine both SV and its active metabolite SVA in body fluids.

Several analytical methods for SV determination in human plasma have been reported, including gas chromatography–mass spectrometric (GC–MS) [4,5] and liquid chromatographic coupled to UV detector [6,7]. However, GC–MS methods include analyte derivitization with a generally complicated sample preparation procedure. On the other hand, LC/UV methods are rarely sensitive enough to determine very low SV and SVA concentration levels. LC–MS methods are usually employed to SV determination in biological fluids, as they are sensitive, specific and simple. An LC–MS method has been reported for determination of SV without taking SVA into account [8], while several tandem mass spectrometric methods have been proposed for simultaneous determination of SV and SVA employing solid phase extraction (SPE) [9], liquid–solid extraction [10] and direct injection [11]. Some of the previous methods did not have a suitable concentration range for pharmacokinetic studies. In two other publications it was proven that automation along with

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LLE resulted in lower interconversion rates between SV and SVA [12,13].

In the present study we report the development and validation of a fully automated high throughput 96-well format based LLE LC–MS/MS method for the simultaneous determination of SV and SVA in human plasma. The method involved a PP step, by ACN, followed by a two-step LLE with MTBE. The combination of PP and two-step extraction LLE has never been employed before for SV analysis in human plasma and it was proven to be very effective, in terms of method sensitivity increase for all analytes and matrix interferences reduction. Preparation of calibration standards and quality control samples (QCs), transfer of study samples, addition of IS, addition of the organic solvent, as well as supernatant organic layer transfer after LLE were performed automatically using robotic workstations. Both SV and SVA were determined in the same analytical run of 1.9 min, the shortest so far reported, by employing a polarity switch within the run. This specific protocol could be applied for LV/LVA determination as well, with SV/SVA being the internal standards. Furthermore, this general approach for sample preparation can be suggested for employment in other bioanalytical protocols that require both ionization modes and low quantitation limits.

The current method enabled the automated high throughput and reliable simultaneous determination of SV and SVA in a bioequivalence study after *per os* administration of a SV tablet (80 mg) to 43 healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Simvastatin ammonium salt and simvastatin acid were purchased from Biocon (Bangalore, India). Lovastatin was obtained from Sigma–Aldrich (Chemilab, Athens, Greece) while lovastatin hydroxy acid ammonium salt was obtained from Synfine Research (Ontario, Canada). Acetonitrile (HPLC grade) and ammonium acetate (analysis grade) were purchased from Chemilab (Athens, Greece). MeOH (HPLC grade) was obtained from Neohimiki (Athens, Greece) while MTBE (HPLC grade) from Techline (Athens, Greece). All aqueous solutions and buffers were prepared using de-ionized and doubly distilled water (Resistivity > 18 MΩ) from a Millipore Milli-Q Plus System (Malva, Athens, Greece). Pooled human control plasma was kindly donated from Ippokrateio hospital (Athens, Greece).

2.2. Instrumentation

A PerkinElmer Multiprobe II HT-EX workstation (PerkinElmer, Downers Grove, IL, USA) equipped with an 8-tip robotic arm and controlled by WinPrep Software was employed for all liquid transfers, including buffer, IS addition, as well as plasma samples transferring from 2 mL eppendorf microfuge tubes (Lab Supplies, Athens, Greece) into 2.2 mL square 96-deepwell plates (Sigma–Aldrich, Athens, Greece). 1000 μL conductive disposable tip-boxes were purchased from E&K Scientific Products (Cambell, CA, USA). A tipchute, reagent troughs and a tip flush/wash station were purchased from PerkinElmer. ACN and MTBE addition as well as supernatant organic layer transferring after extraction into a new 2.2 mL 96-deepwell plates and reconstitution of the samples after evaporation were performed employing a Tomtec Quadra 96 model 320 robotic liquid handling system equipped with a 96-tip pipetting head (Bidservice, NJ, USA). Evaporation was performed into a Zymark TurboVap 96-well format plate evaporator (Malva, Athens, Greece) by nitrogen application, produced by an Agilent Nitrogen Generator (Duratec, Hockenheim, Germany), that receives air from a SF4 Air Compressor (Atlas Copco, Athens, Greece). An Eppendorf 5810 R (Bacakos, Athens, Greece) centrifuge that could accommodate 96-well plates as well as Eppendorf microfuge tubes was also utilized during sample preparation. Eppendorf deepwell mats for covering the 96-well plates were obtained from Sigma–Aldrich. Two 96-well plate vortex-mixers (MS1 Minishaker) were purchased from Metrolab (Athens, Greece). The CTC PAL autosampler (Hellamco, Athens, Greece) could accommodate six 96-deepwell plates stored before analysis at a fixed temperature inside the autosampler drawers. The HPLC system included one Agilent 1100 series binary pump, a degasser as well as a column oven/cooler (Hellamco, Athens, Greece). Finally, a PE Sciex API 3000 triple quadrupole mass spectrometer (Biosolutions, Athens, Greece) interfaced with the HPLC via an electrospray ionization (ESI) or an atmospheric pressure chemical ionization (APCI) source was used for the mass analysis and detection, operating under Analyst 1.4.2 software.

2.3. Chromatographic conditions

An isocratic HPLC elution mobile phase was used, consisting of 82% acetonitrile and 18% ammonium acetate 5 mM (v/v), adjusted to pH 4.5 with acetic acid. A flow rate of 0.7 mL/min was used for sample analysis on a YMC ODS-A (C18) (Schermbeck, Germany) analytical column (50 mm × 4.0 mm i.d.). The column was maintained at ambient temperature (∼23 °C), while the autosampler temperature was set at 10 °C. The pressure of the system during the analysis was ∼600 psi. The injection volume was 60 μL and the total run time was set for 1.9 min.

2.4. Mass spectrometric conditions

A PE Sciex API 3000 triple quadrupole mass spectrometer interfaced with the HPLC via a turbo ionspray source was used for the mass analysis and detection. The tuning parameters were optimized for SV, SVA and LV, LVA by infusing a 200 ng/mL standard solution containing all four compounds in mobile phase at 30 μL/min, via an external syringe pump (Harvard 11 plus) directly connected to the mass spectrometer. The turbo ionspray source temperature was optimized at 425 °C. The analytes were detected by monitoring the precursor → product ion transition using multiple reaction monitoring (MRM) scan mode. The MRM was performed at *m/z* 435.3 → 319.0 for SVA, 421.1 → 319.0 for LVA and 419.1 → 199.3 for SV, 405.4 → 199.3 for LV. The current MS method consisted of

two periods combining both negative and positive ionization mode. Specifically, the mass spectrometer operated in the negative detection mode for 1.21 min with a dwell time of 150 ms followed by a period of 0.69 min in the positive mode with the same dwell time. The curtain gas was set at 9 (arbitrary units), the declustering potential (DP) at −56 V for SVA and LVA, and 56 V for SV and LV. The nebulizer gas (GS1) was set at 12 (arbitrary units) during period 1, and at 9 during period 2 while the turbo ionspray gas (GS2) at 7 L/min. The collision-induced dissociation (CID) gas setting was optimized at 12 and 9 (arbitrary units) for periods 1 and 2 respectively, the collision energy at −24 V, for SVA and LVA while for SV and LV the parameter values were 19 and 17 V respectively. Data was acquired using the Analyst 1.4.2 software.

2.5. Standards and quality control/method validation samples preparation

Stock solutions of SV and SVA {100 μg/mL both (SS_1) and (SS'_1)} were prepared in ACN. LV stock solution {100 μg/mL (IS_1)} was also prepared in ACN, while LVA stock solution {50 μg/mL (IS'_1)} was prepared in a mixture of ACN/H_2O (75:25, v/v). A mixed SV and SVA stock solution {2 μg/mL (SS_2)} was prepared by diluting each of the initial stock solutions by ACN. Working solutions of 800, 400, 200, 100, 40.0, 20.0, 10.0, 4.00 and 2.00 ng/mL for both SV and SVA were prepared by diluting SS_2 with ACN/ H_2O 50/50 (v/v). Two quality control/method validation (QC/MV) stock solutions (100 μg/mL each) were prepared from separate weighing of SV and SVA, as well as a quality control/method validation (QC/MV) mixed diluted stock solution (2 μg/mL each). Dilutions were used to prepare four levels of QC working solutions, 600, 60.0, 6.00 and 2.00 ng/mL. All working solutions were prepared in 2 mL eppendorf tubes. A mixed, diluted IS solution (1 μg/mL each, IS_2) was prepared daily by diluting the IS stock solutions in MeOH. SV and SVA stock solutions, as well as the diluted stock solutions were stored at −75 °C. LV and LVA (IS_1 and IS_2) stock solutions were stored at 4 °C, while all working solutions were stored at −30 °C.

Nine calibration standards were prepared by spiking 100 μL of each working solution to 1900 μL of blank human plasma (20 times dilution). A blank sample (matrix sample processed without IS), a zero sample (matrix sample processed with IS) completed the calibration curve covering the expected range of concentrations to be quantified. Final standard concentrations were 40.0, 20.0, 10.0, 5.00, 2.00, 1.00, 0.500, 0.200, and 0.100 ng/mL. The following concentration levels of QC/MV samples were prepared: MV_L (0.100 ng /mL), MV_1/QC_1 (0.300 ng/mL), MV_2/QC_2 (3.00 ng/mL) and MV_3/QC_3 (30.0 ng/mL). Standards were prepared in bulk and dispensed in 2.0 mL aliquots into properly labeled eppendorf tubes and stored at −30 °C.

2.6. Protein precipitation and two-step LLE procedure

Two evaluation tests were performed so as to assess the importance of ACN PP step, as well as the two-step LLE procedure followed in the current method. Initially, two separate sample preparation procedures both of them including a two-step LLE were conducted, while only one of them involved a PP step. Matrix effects, as well as peak areas, were compared so as to evaluate the influence of the PP step in extracts cleanness and in method sensitivity, too. A second test was performed to evaluate the importance of the two-step LLE procedure versus single LLE. Two sets of plasma samples were treated with 150 μL of ACN prior to LLE with the same MTBE volume. In the one set of samples a second step of LLE was performed. Peak areas resulting from the two procedures were compared, so as for the sensitivity increase to be assessed.

Both evaluation tests were performed in three concentration levels for both SV and SVA (0.300, 3.00 and 30.0 ng/mL) in triplicate. Matrix effect profiles investigation for the whole chromatographic run time was performed by the post-column infusion protocol [14,15]. Briefly, blank sample extracts were injected into the LC–MS/MS system by the simultaneous post-column infusion of a mixture of all analytes and IS at 10 ng/mL in mobile phase via the Harvard syringe pump. The flow rate was set at 30 μL/min while the syringe pump was connected in parallel via a PEEK tee.

2.7. Sample preparation

All plasma samples were stored at −30 °C, thawed at room temperature, vortex mixed and centrifuged at 3500 rpm for 5 min at 4 °C prior to analysis. Eppendorf tubes were decapped and placed in 24-position microfuge racks on the deck of the Multiprobe, along with 2.2 mL 96-deepwell plates and two reagent troughs containing buffer reagent and IS solution. Multiprobe transferred 50 μL of ammonium acetate buffer 100 mM adjusted at pH 4.5 as well as 50 μL of IS_2 solution into the appropriate wells of a 96-well plate with fixed tips. Then, 300 μL of each of the calibration, QC and subject samples were transferred from the eppendorf tubes into the appropriate wells of a 96-well plate employing 1000 μL disposable conductive tips. The 96-well plates were removed from the Multiprobe and vortex mixed for 5 min. Next, 150 μL of ACN were added from a reservoir on the Tomtec into all 96-wells of each plate, so as for PP to take place and once again plates were vortex mixed for 5 min. Then, 1100 μL of MTBE were transferred from a reservoir on the Tomtec containing the organic solvent into all 96-wells of each plate and the plates after being covered with a mat, were vortex mixed for 20 min, centrifuged for 15 min, at 3500 rpm and 4 °C and then frozen for 10 min at −30 °C. After removing the mats carefully 900 μL of the supernatant organic layer, in 3 aliquots of 300 μL, were transferred from the original sample plates, into the respective positions of new 2.2 mL 96-deepwell plates. Next, a second extraction was performed by adding another 1000 μL of MTBE to the plates containing the plasma samples by Tomtec and the same procedure of vortex mixing and centrifugation was followed as before. The plates were then frozen for 30 min at −30 °C and another 900 μL of organic solvent were transferred from the plates to the same new plates used for organic extracts placement before. A total volume of about 1800 μL was placed at each well of the new 96-well

plates when the latter were placed into the Zymark Nitrogen evaporator, so as for the organic extracts to be evaporated to dryness by applying an increasing flow rate, beginning from approximately 15 and ending up to 60 (arbitrary units). All dry residues were reconstituted by the addition of 150 μL of mobile phase and the plates after vortex mixing for 5 min were placed into the appropriate autosampler drawer for direct injection.

3. Results and discussion

3.1. Sample preparation optimization

LLE, SPE and PP are the most common preparation techniques for plasma samples analysis. LLE is generally considered to be providing cleaner extracts than the other two as it is proven by evidenced lower matrix effect. However, lower recovery due to the transfer of a fraction of the organic extract after the extraction may be a disadvantage of the current technique. In addition, when automated 96-well format LLE is the case, not satisfactory mixing between plasma sample and organic solvent may also occur because of the small well volume, which further reduces analytes recovery. As a result, the option of automated 96-well LLE may be rejected at the step of method development, when low concentrations have to be detected, especially when cross-well contamination is observed to take place. An alternative choice is the utilization of larger volume test tubes where detection of lower concentrations could be an easier task to achieve. However, the drawbacks of the current choice are numerous and the method will become time consuming and labor intensive. The problem of human error can also easily arise, because of the high number of samples involved. Especially when multi-sample studies are the case, automated, rapid as well as reliable determination is of major importance.

The present analytical method proposes a sample pretreatment protocol, which results in sensitivity increase, as well as higher sample cleanness. At the same time automation is maintained during both sample preparation and chromatographic analysis, because of the employment of a 96-well format. It requires a small plasma volume for analysis (300 μL) and allows the automated, high throughput simultaneous monitoring of SV and SVA in human plasma. MTBE as an organic solvent was chosen among several others tested, such as ethyl acetate, mixture of diethyl ether/hexane, etc. Prior to LLE, PP by the addition of ACN was performed, while after the first extraction step, another MTBE volume was added to plasma samples resulting in increased quantities for all analytes at the final extracts.

3.2. ACN protein precipitation

Matrix effect comparison between the two procedures previously described, shows a decrease of matrix effect ranging from 13.0 to 22.4% for SV and from 3.90 to 14.7% for SVA (Table 1) for the three concentration levels, when PP was performed. Investigation of the matrix effect profile for the whole chromatographic run further supports the previous allegation (Fig. 1). Endogenous plasma ingredients usually remain in extracts after LLE, causing signal suppression (at 0.85 and 1.55 min, respectively) by competing analyte ions inside the ion source. The number of charged ions in the gas phase, as well as the number of ions reaching the detector, is thus affected [16,17]. The use of ACN as a precipitating reagent results in decrease of matrix effect, due to the elimination of these matrix components from the resulting extracts.

Moreover, ACN precipitation resulted in a significant peak area increase for all analytes quantified, as can be seen in Fig. 2. Peak area increase attributed to PP was similar for both SV and

Table 1
Ion suppression decrease as a result of plasma PP with ACN

MV sample ($n = 3$)	% Ion suppression decrease	
	SV	SVA
MV_1 (0.300 ng/mL)	19.4	14.7
MV_2 (3.00 ng/mL)	13.0	10.7
MV_3 (30.0 ng/mL)	22.4	3.90

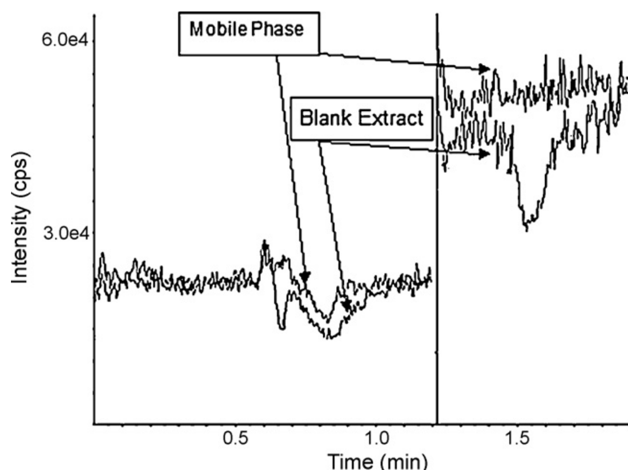

Fig. 1. Matrix effect profile for the whole chromatographic run.

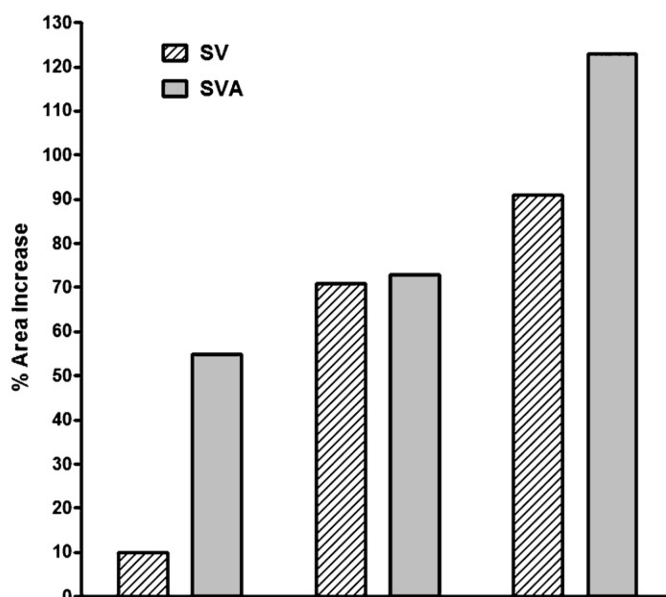

Fig. 2. Peak area increase, due to addition of ACN PP step.

SVA, increasing for higher concentration samples and reaching as much as 91% for SV and 123% for SVA. This increase in sensitivity is primarily due to analytes displacement from plasma proteins and thus facilitating the contact between analyte molecules with the organic solvent. In conclusion, ion suppression decrease, due to lower matrix components concentration in final extracts, is accounted for the sensitivity increase. Several ACN volumes (120, 150, 200, 250 μL) were tested in order to achieve the highest peak area, with 150 μL volume providing to be the best. In addition, the specific ACN volume eliminated the irregular emulsion observed after organic extracts evaporation, when MTBE is employed for LLE of analytes from plasma. Effective mixing of ACN with MTBE was achieved as MTBE/ACN ratio remained > 3 for both extraction steps [18].

PP reagent is often used as a dilution solvent for IS, so as to reduce the number of reagent additions. However, in the present method ACN addition was preferred to take place as an extra step within the sample preparation procedure after IS addition. The reason for this choice lies at the very precipitating nature of ACN. IS addition prior to protein precipitation ensures a more satisfying binding of IS molecules with plasma proteins, simulating the binding of proteins with analytes in real human plasma.

3.3. Two-step extraction

Plasma samples at three concentration levels were treated with 150 μL of ACN in triplicate in two parallel procedures: in the first one a single LLE step took place, while in the second one, an additional LLE step was performed with MTBE being the organic extraction solvent. SV and SVA peak areas increased in all three concentration levels, as can be seen in Fig. 3. Peak area increase was similar for both analytes and varied from 44.0 to 95.6% for SV and from 45.6 to 92.9% for SVA for the three concentration levels. The phenomenon of more effective extraction of the analytes from plasma after adding the second extraction step is mainly responsible for the increase in peak areas.

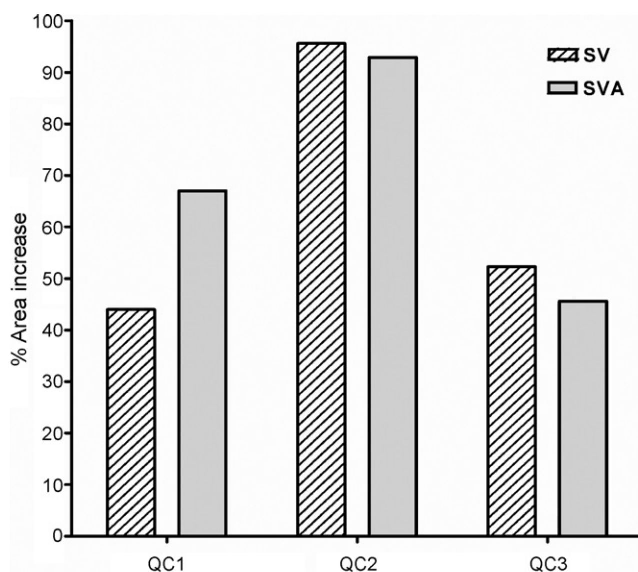

Fig. 3. Peak area increase due to two-step LLE as opposed to single LLE.

3.4. Automation and high throughput of procedure

The employment of a 96-well format was the most important step to achieve a high degree of automation. All liquid transfers including (i) working and standards preparation, (ii) reagents and plasma addition, (iii) organic solvent addition, (iv) organic extracts transfer and (v) evaporated extracts reconstitution were performed by the use of two robotic liquid handling workstations, Multiprobe and Tomtec. Moreover, the simultaneous evaporation of 192 samples (2 plates) further reduced the sample preparation time. As a result, 2000 plasma samples could be analyzed in only 5 days, with chromatographic analysis being the most time-consuming step, despite the short chromatographic run time of the method. In addition, automation of the procedure reduced the human agent involvement, minimizing this way the possibility of human error. Therefore, the automated 96-well format is extremely important when multi samples analyses are the case. On the contrary, the use of 96-well plates could result in deficient method sensitivity, especially when low limits of quantitation are involved, due to volume limitations. For this reason, test tubes are some times preferred instead of 96-well plates.

The current method enabled the increase of method sensitivity, while maintaining at the same time the 96-well format that significantly reduced sample preparation time. Despite the performance of the two subsequent LLE steps, the overall sample preparation time is estimated to be half of the respective for the same plasma and organic solvent volumes in a one step LLE procedure in test tubes (2 instead of 4 h for preparation of 96 samples). The approach of ACN PP combined with the two-step LLE was proven to result in both cleaner samples and significantly increased method sensitivity for SV and SVA. This sample preparation approach could generally serve as a model in method development, when low analyte concentrations are to be quantified and procedure automation is desired as well. It could also be applied to LV and LVA determination, with SV and SVA being the ISs, since area increase was also noticed in LV and LVA chromatographic peaks.

As for the chromatographic profile of the method, it was optimized among several columns (CN, C18, C8), buffers (formic acid, ammonium acetate), as well as organic solvent/buffer proportions tested. Retention times for SVA, LVA, SV and LV were about 0.98, 0.86, 1.61 and 1.39 min respectively, with a total run time of 1.9 min (Figs. 4 and 5). To the best of our knowledge, this is the shortest chromatographic run time so far reported for SV and SVA simultaneous quantification, allowing the chromatographic analysis of 96 samples in only 3 h.

3.5. ESI–APCI comparison

Both ESI and APCI sources were utilized for SV and SVA determination during method development phase. APCI was reported to have similar sensitivity to ESI for the current analytes [9]. However, ESI was proven to be significantly more sensitive for both SV and SVA, compared to APCI. Fig. 6 displays the Q1

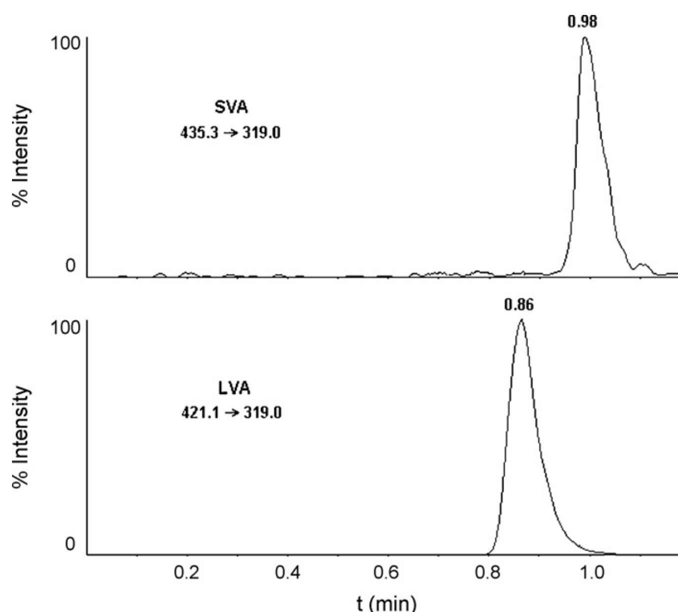

Fig. 4. Representative MRM SVA–LVA chromatogram obtained from a MV_L sample.

scans for SV and LV with both ion sources. Parent SV and LV ions as well as main ion peaks appear in both ion spectra. However, ESI sensitivity for the specific analytes was 3fold higher. Increased ESI as opposed to APCI sensitivity was further confirmed by daughter ions intensity optimization procedure. ESI was proven to be equally more sensitive than APCI for SVA and LVA determination as well.

As far as ESI source optimization is concerned, several factors such as collision energy (CE), declustering potential, collision gas, curtain gas, ion source temperature and others were optimized so as to maximize method sensitivity. CE optimization was proven to be the critical step for both signal maximization, as well as daughter ions selection. Figs. 7 and 8 display the dependence of the three principal daughter ions intensity for each analyte, from CE value. The current optimization step was critical for the selection of the final MRM transitions for SV and SVA.

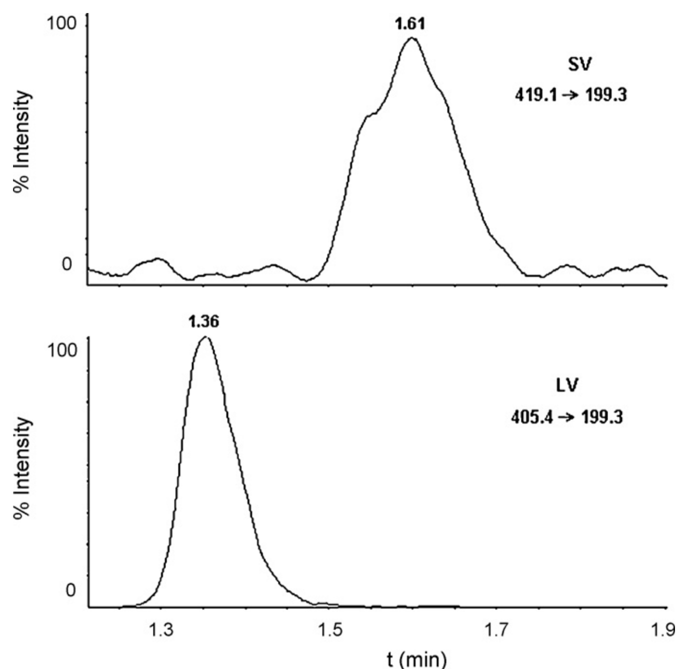

Fig. 5. Representative MRM SV–LV chromatogram obtained from a MV_L sample.

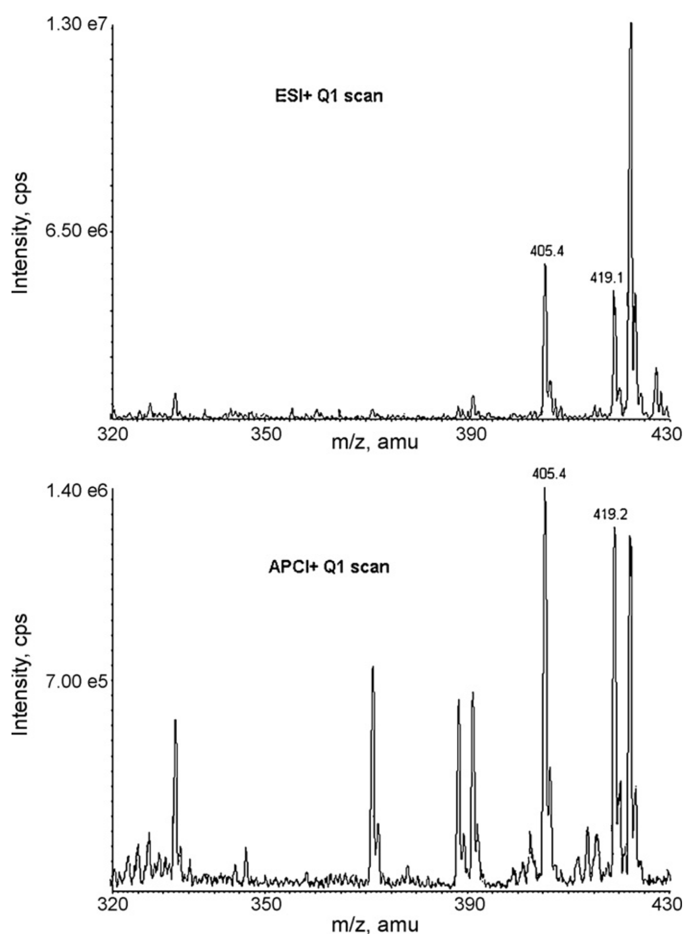

Fig. 6. SV–LV Q1 scans with ESI and APCI sources.

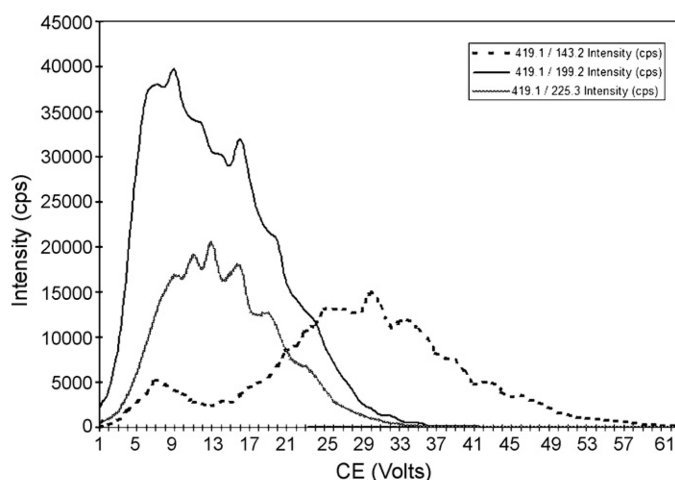

Fig. 7. ESI collision energy optimization for main SV daughter ions.

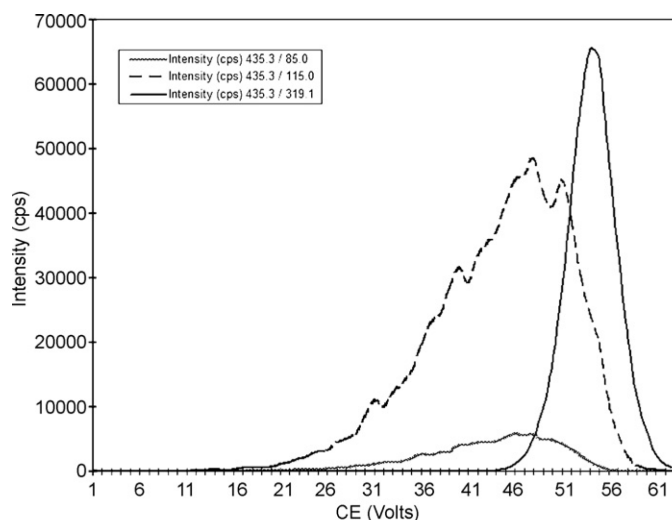

Fig. 8. ESI collision energy optimization for main SVA daughter ions.

3.6. Standard curve and method validation

The method was proven to be highly sensitive with a limit of quantification (LOQ) of 0.100 ng/mL for both SV and SVA. Full validation of the current analytical method was performed in our laboratory, according to US Food and Drug Administration (FDA) bioanalytical method validaton guidance (CDER, 2001) [19]. A calibration curve, containing 9 non-zero standards ranging from 0.100 to 40.0 ng/mL for each analyte (SV and SVA) was prepared for each analytical run. This range was suitable for a pharmacokinetic study after *per os* administration of a 40 or 80 mg tablet of SV. Peak area ratios of SV and SVA to the respective IS were used for regression analysis. The calculated concentrations were determined from linear regression using $1/x^2$ weighting. Individual standard curve data from 5 analytical runs met all of the preset criteria.

The absence of chromatographic interferences was certified by the analysis of a zero and a blank plasma samples. The mean regression coefficient (R-squared) for the five runs was 0.99994 for SV and 0.9996 for SVA, average linear slope was 3.47×10^{-3} ($S_a = 1.00 \times 10^{-5}$) for SV and 9.48×10^{-3} ($S_a = 6.70 \times 10^{-5}$) for SVA, while average intercept was 1.44×10^{-3} ($S_b = 1.50 \times 10^{-4}$) and 3.76×10^{-3} ($S_b = 1.04 \times 10^{-3}$) for SV and SVA, respectively.

However, before applying a statistical hypothesis test to the regression line coefficients it is essential to check whether "lack of fit" exists. This test is based on the analysis of the variance of the residuals from the regression line [20,21]. The ANOVA table (Table 2) divides the total variability of the residuals in y ($y = ax + b$) into two pieces: (i) a pure error piece, which measures the variability between replicate values of y at the same x. Since the variability among these replicates has nothing to do with the fitted model, it is a "pure" estimate of the noise in the data; (ii) a lack-of-fit piece, which estimates groups of replicates variation from the fitted line.

The table also shows the results of an F test comparing the estimated lack of fit to pure error through F ratio = lack-of-fit mean square/pure error mean square. These mean squares values result from dividing the sum of squares due to lack of fit and the sum of squares due to pure error by the corresponding degrees of freedom. Of primary interest is the P-value associated with the test. Small values of P (<0.05) indicate significant lack of fit at the 5% significance level. From P values listed in the table, it is obvious that there is no significant lack of fit and that the current method was capable of producing satisfactory concentration data for SV and SVA standard samples.

As far as accuracy and precision are concerned, all values were within the acceptable range. Data for accuracy and both intra and inter run precision (expressed as CV %) are presented in Table 3. Extraction recovery was examined at 3 concentration levels for SV and SVA and was estimated to be >85% in all cases.

3.7. Stability data

As part of the method validation, data were also generated to ensure that SV and SVA were stable at distinct timing and temperature conditions, as well as the stability of the analytes in the stock and working solutions. Also, interconversion between SV and SVA was evaluated for the current protocol. Plasma samples containing two concentration levels of SV and SVA were used for the stability experiments, low-medium (S_l) 1 ng/mL and medium-high (S_h) 10 ng/mL.

Initially, in order to assess autosampler stability, three samples of each of the two concentration levels (S_l and S_h) remained at autosampler temperature (10 °C) for 30 h, a period of time which was 6 h longer than the respective required for the completion of the analysis of a 4-plates batch. Another set of samples

Table 2
ANOVA results for lack of fit

	Degrees of freedom	Sum of squares	Mean square	F ratio	Probability level
SV					
Error	40	4.73×10^{-5}	1.18×10^{-6}		
Lack of fit	7	1.43×10^{-6}	2.04×10^{-7}	0.15	0.99
Pure error	33	4.59×10^{-5}	1.39×10^{-6}		
SVA					
Error	43	2.91×10^{-4}	6.78×10^{-6}		
Lack of fit	7	1.68×10^{-6}	2.40×10^{-7}	0.030	1.00
Pure error	36	2.90×10^{-4}	8.05×10^{-6}		

Table 3
Intra- and inter-assay accuracy and precision results

MV sample	% Intra-run accuracy[a]		% Inter-run accuracy[b]		Intra-run precision[c] (%CV)		Inter-run precision[b] (%CV)	
	SV	SVA	SV	SVA	SV	SVA	SV	SVA
MV_L (0.100 ng/mL)	105	91.8	103	98.1	16	14	3.3	15
MV_1 (0.300 ng/mL)	95.1	105	104	10	10	13	11	2.6
MV_2 (3.00 ng/mL)	105	104	102	101.8	7.9	5.4	3.7	5.5
MV_3 (30.0 ng/mL)	112	109	103	104.4	4.6	4.4	5.1	5.7

[a] ($n = 5$), expressed as 100 × (mean calculated concentration)/(nominal concentration).
[b] Values obtained from all 5 runs ($n = 30$).
[c] ($n = 5$).

at the two concentration levels was freshly prepared. Mean variance % between the results for the two sets of samples was less than 2.5 for both analytes.

To evaluate freeze/thaw stability, a freeze/thaw cycle was defined as the storage of S_l and S_h samples at −30 °C followed by thawing at room temperature. Samples were analyzed after the fourth cycle, along with fresh reference samples of the same concentration. The results (back-calculated concentrations) of four freeze-thaw cycles as well as fresh ones varied less than 2%, from their nominal values.

Short-term stability was assessed by the following procedure: six aliquots of S_l and S_h were prepared and maintained at room temperature for 1 h, period of time which exceeds the normal time that samples remain at room temperature, before analysis, without the addition of ammonium acetate buffer. To evaluate long-term stability, aliquots of the two sample-types were initially frozen at −30 °C for 90 days, thawed and analyzed. Both short and long term stability proved that the mean variation was below 10% (data not shown). Stock and working solutions stability (stored at 4 °C) was estimated by comparing fresh and old dilutions in mobile phase. The results (data not shown) proved that all working and stock solution remained stable for the time period being stored.

Finally, interconversion between SV and SVA is known to be taking place by hydrolysis of SV and lactonization of SVA. This phenomenon may cause increased variance in accuracy and precision of the method and depends by several parameters such as temperature, pH, storage condition and sample extraction method. As Yang et al. reported, buffer pH highly influences the current interconversion procedure, with pH 4.5 being the optimal value for minimum interconversion rate [12]. The specific pH is applied to both sample preparation and chromatographic conditions. After thawing and being vortexed, samples were transferred to plate wells were buffer reagent was already placed. Interconversion rate between SV and SVA was investigated by allowing two sets of samples in triplicate and with the same low-medium and medium-high concentration levels as in previous stability tests, to remain at room temperature for 1 and 3 h respectively, after thawing and before being mixed with buffer inside plate wells. The current samples were as well divided in two more sets, one of which contained SV only while the other contained SVA only. All samples described above, along with the equivalent fresh ones were treated according to the method protocol developed. Automated LLE is known to be the less susceptible to interconversion between SV and SVA among all sample preparation techniques [13]. In the current method, interconversion of SV to SVA as well as SVA to SV was found to be less than 0.5 % for both concentration levels.

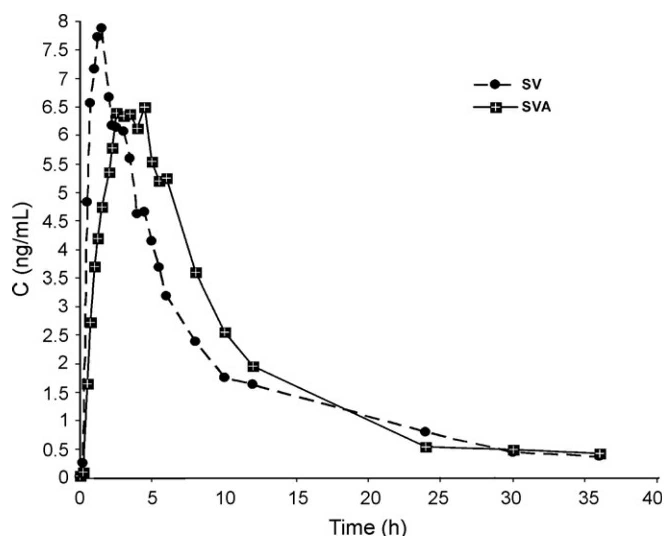

Fig. 9. Mean plasma concentration–time curves from 43 subjects for SV and SVA.

3.8. Application to a pharmacokinetic study

The present method was applied to the analysis of plasma samples obtained from 43 healthy volunteers after the administration of a single dose of 80 mg tablet containing SV, as part of a bioequivalence study. The study was harmonized with the ethical principles that have their origins in the declaration of helsinki and it was in accordance with the good clinical practice (GLP) requirements. The concentration—time profile of SV and SVA in these volunteers displayed in Fig. 9, indicates the suitability of the proposed method for pharmacokinetic studies of SV and SVA in human plasma.

4. Conclusions

We have presented a highly automated 96-well PP two-step LLE, LC–MS/MS method for the quantification of SV and SVA in human plasma. The current method is advantageous compared to the existed ones in terms of sensitivity, extract cleanness

and matrix effect minimization, due to the employment of a protein precipitation and a two-step LLE. The employment of a 96-well format combined with two liquid-handling robotic workstations simplified and minimized the time of sample preparation, automating the whole procedure. Moreover, the current method includes the shortest chromatographic runtime so far proposed for SV and SVA quantification, as well as a relatively small volume of human plasma (300 μL) for analysis. The method was proven to be highly sensitive, accurate, precise and specific and was applied to a bioequivalence study (∼2000 samples), which was completed in a very short period of time. The current sample preparation protocol could serve as a model method development for the analysis of low concentration samples, when at the same time automation and high throughout are highly important, such as bioequivalence or other multi-sample studies.

References

[1] W.F. Hoffman, A.W. Alberts, P.S. Anderson, R.L. Smith, A.K. Willard, J. Med. Chem. 29 (1986) 849–852.

[2] S. Vickers, C.A. Duncan, I.W. Chen, A. Rosegay, D.E. Duggan, Drug Metab. Dispos. 18 (1990) 138–145.

[3] S. Vickers, C.A. Duncan, K.P. Vyas, P.H. Kari, B. Arison, S.R. Prakash, H.G. Ramjit, S.M. Pitzenberger, G. Stokker, D.E. Duggan, Drug Metab. Dispos. 18 (1990) 476–483.

[4] T. Takano, S. Abe, S. Hata, Biomed. Environ. Mass Spectrom. 19 (1990) 577–586.

[5] M.J. Morris, J.D. Gilbert, J.Y.K. Hsieh, B.K. Matuszewski, H.G. Ramjit, W.F. Bayne, Biomed. Environ. Mass Spectrom. 22 (1993) 1–8.

[6] L. Wang, M. Asgharnjard, J. Pharm. Biomed. Anal. 21 (2000) 1243–1248.

[7] G. Carlucci, P. Mazzeo, L. Biordi, M. Bologna, J. Pharm. Biomed. Anal. 10 (1992) 693–697.

[8] H. Yang, Y. Feng, Y. Luan, J. Chromatogr. B 785 (2003) 369–375.

[9] B. Barrett, J. Huclova, V. Borek-Dohalsky, B. Nemec, I. Jelinek, J. Pharm. Biomed. Anal. 41 (2006) 517–526.

[10] J.J. Zhao, I.H. Xie, A.Y. Yang, B.R. Roadcap, J.D. Rogers, J. Mass Spectrom. 35 (2000) 1133–1143.

[11] M. Jemal, Z. Ouyang, M.L. Powell, J. Pharm. Biomed. Anal. 23 (2000) 323–340.

[12] A.Y. Yang, L. Sun, D.G. Musson, J.J. Zhao, J. Pharm. Biomed. Anal. 38 (2005) 521–527.

[13] N. Zhang, A. Yang, J.D. Rogers, J.J. Zhao, J. Pharm. Biomed. Anal. 34 (2004) 175–187.

[14] F.L. Sauvage, J.M. Gaulier, G. Lachâtre, P. Marquet, Ther. Drug Monit. 28 (2006) 123–130.

[15] C. Apostolou, Y. Dotsikas, C. Kousoulos, Y.L. Loukas, J. Chromatogr. B 848 (2007) 239–244.

[16] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.

[17] T.M. Annesley, Clin. Chem. 49 (2003) 1041–1044.

[18] C. Kousoulos, G. Tsatsou, C. Apostolou, Y. Dotsikas, Y.L. Loukas, Anal. Bioanal. Chem. 384 (2006) 199–207.

[19] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Rockville, MD, 2001.

[20] A. Martínez, J. Riu, F.X. Rius, Chemom. Intell. Lab. Syst. 54 (2000) 61–73.

[21] M. Analla, Agric. Syst. 57 (1998) 115–119.